Adaptation of Rats to Selenium Intake

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ABSTRACT Changes of liver selenium after intake of organic selenium at different levels and for various lengths of time were studied in 236 young rats. In litters born to mothers fed a diet containing 4.5 ppm selenium (in seleniferous sesame press cake) and kept on this diet after weaning, the liver levels decreased steadily; these levels increased in stock rats fed the same diet. The graphical presentation of these changes resulted in two straight lines, crossing each other. When the selenium diet was fed to dams 5 to 7 days prior to parturition and thereafter, the liver levels of the litters rose rapidly; liver levels increased at a slower rate in rats born to females fed the diet 15 to 19 days prior to birth and decreased in litters from mothers kept on the diet prior to mating. When litters from stock females were nursed by dams fed the seleniferous diet during pregnancy and changed to the stock ration after the birth of their litters, the liver selenium levels rose rapidly for about 3 weeks after weaning and then decreased. On a diet containing 10 ppm selenium, liver values in rats from the stock colony rose for 2 to 3 weeks to high levels and then decreased. Animals bred on the diet containing 4.5 ppm selenium exhibited a slower, more continuous rise of liver selenium when the high selenium diet was fed. The results suggest the existence of an adaptation mechanism which allows rats exposed to chronic selenium ingestion to store less of this element than previously unexposed controls.

In previous experiments on the effect of selenium in growing rats, it was observed that a dietary level of this element which resulted in a small but significant growth depression in young animals from the stock colony was without notable effect in litters born to dams which had been kept on an experimental seleniferous diet prior to mating (1). This was quite unexpected because higher selenium body levels at the start of the experiment would have made the opposite result more likely, and also because young animals are often more severely affected by dietary injury when exposed to it during embryonic life. In another study from this laboratory, it was found that the characteristic drop in hematocrit and hemoglobin levels in rats fed high selenium diets was significantly delayed when the experimental animals had been bred on a seleniferous ration as compared with controls bred on stock rations, (2). These observations pointed to the possible existence of some adaptation to chronic selenium intake in rats. The present experiments were undertaken to investigate whether such a mechanism could be detected through the accumulation of selenium in the liver.

MATERIAL AND METHODS

The experiments were performed with 236 rats descended from the Sprague-Dawley strain, bred in this Institute for about 20 years. They were kept in individual screen-bottom cages. Food and water were offered ad libitum, and the stock diet was a commercial laboratory ration.[1] Each bag was analyzed for selenium content and only those lots with values below 1.0 ppm were used.

Two lots of expeller processed sesame press cake with a selenium content of 0.8 and 23.3 ppm, respectively, were used. They were ground in a hammer mill. By mixing appropriate portions of these lots, diets containing 4.5 and 10 ppm selenium, respectively, were prepared. They contained 16.4% protein. The rest of the diet consisted of the following components: (in percent) corn oil with vitamins A and D added, 5; salt mixture USP XIV, 4; L-lysine·HCl, 0.4; vitamin B mixture (3), 1; and cassava starch to make up 100%.

Animals of experiments 1 and 4 had been weaned at 28 days, those of experiments 2 and 3 at 22 days. They were killed at convenient periods by a blow on the head; the livers were extirpated, weighed, dried at 100° to constant weight, ground in a laboratory mill [2] and duplicate samples of about 100 mg burned in oxygen in a

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[1] Ralston Purina Company, St. Louis, Mo.
[2] Wiley, Intermediate Model, Arthur H. Thomas Company, Philadelphia, Pa.

2-liter Schöninger flask containing 20 ml of bidistilled water. After cooling the content was stirred with a magnetic stirrer for 10 minutes and selenium was determined in a filtered aliquot by the fluorometric method of Dye et al. (4) with 3,3-diaminobenzidine tetrahydrochloride. Two to five animals were killed in each case and the mean liver selenium values were calculated on the fresh weight basis.

Five different experiments were performed. In experiment 1, the selenium contents of the livers of two groups of rats fed a diet containing 4.5 ppm selenium were compared: one group of 34 animals had been bred and nursed on the stock ration and was fed the selenium diet since weaning; the mothers of the second group of 37 animals had been fed the selenium diet 5 days prior to mating and the litters were continued on the same diet after weaning. For mating, one male was kept for 5 days in the cage of one female.

The mothers of the rats of experiment 2 were fed the selenium diet 5 to 7 days (group 1) or 15 to 19 days (group 2) before giving birth, respectively, or 5 days before mating and during the experimental period (group 3). Twenty animals were used in each experimental group.

For experiment 3, two groups of female stock rats were mated simultaneously; one was fed the stock ration and the other was given the seleniferous diet 5 days prior to mating and until birth of the litters. The litters from group 1 were given for nursing to females of group 2 within 24 hours after birth; in turn, the young born of the mothers of group 2 were nursed by group 1 mothers. Both groups received the stock ration after the birth of the litters and for the duration of the experiment. They totaled 22 young rats.

In experiment 4, three groups of rats were fed a diet containing 10 ppm selenium from sesame. Two groups had been bred and kept on the stock ration; the third group was fed the 4.5 ppm seleniferous diet. These animals were 3, 6 and 4 weeks old at the beginning of the experiment and consisted of 14, 18 and 16 animals, respectively.

Fifteen 4-week-old rats, bred on the 4.5-ppm selenium diet and 20 stock rats kept for 4 days on the 10-ppm selenium diet were used in experiment 5. It was expected from the results of the previous experiments that both groups would have similar liver selenium levels. The rate of elimination of liver selenium was compared while both groups were fed the stock ration.

RESULTS

The amounts of selenium detected in liver with the analytical technique involving drying may be smaller than that existing in the fresh organ, because, according to Heinrich and Kelsey (5), volatile selenium compounds may exist. Therefore, the present results refer to the nonvolatile liver selenium as the amount of volatile selenium was not determined.

The liver selenium levels in the two groups of rats of experiment 1 changed in the opposite direction with time (fig. 1). In one group the amount of selenium rose from 1.7 to 3.8 ppm; in the other it fell from 6.3 to 3.2 ppm. The graphic representation of the corresponding values falls on two straight lines which cross each other at the point corresponding to day 87 of the experiment. No gross differences in weight gain or food intake were observed between these groups. The individual selenium values of animals of one group killed at the same age fall generally within the range of ± 0.4 ppm.

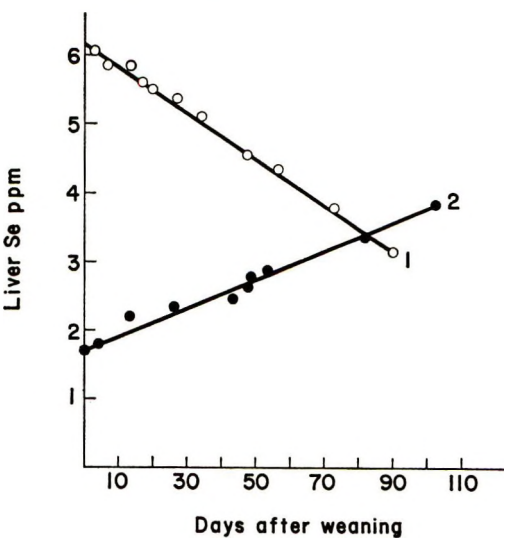

Fig. 1 Experiment 1. Liver selenium levels in rats fed a diet with 4.5 ppm selenium. Group 1: rats bred on the selenium containing diet; group 2: rats bred on the stock diet.

The influence of duration of feeding the seleniferous diet before the birth of the litters, on liver selenium at the time of birth and later, was studied in experiment 2; the results are summarized in figure 2. The young of group 1, born to dams fed the selenium-containing diet 5 to 7 days before giving birth, had at first the lowest liver selenium contents, which rose until they reached higher levels than those in the other series. The mothers of group 2 had been fed the experimental diet for about 2 weeks. The livers of their young had more selenium at birth, the amount of which rose more slowly than in the first series. The last group was comparable to group 1 of experiment 1 and consisted of animals born from mothers which had ingested the experimental ration 5 days prior to breeding. In this case, the liver selenium was highest at birth and decreased steadily.

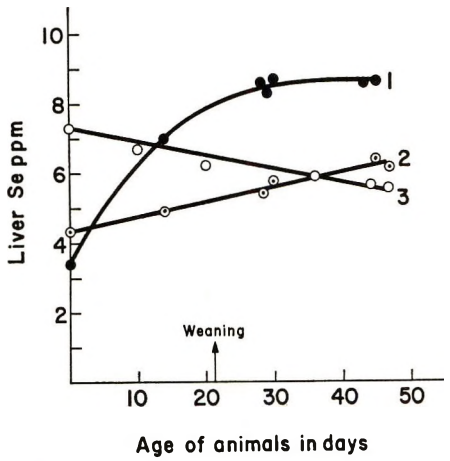

Fig. 2 Experiment 2. Liver selenium levels in young rats reared on a diet containing 4.5 ppm selenium. Group 1: dams put on the seleniferous diet 5 to 7 days before the birth of the litters; group 2: dams put on the diet 15 to 19 days before the birth of the litters; group 3: dams put on the diet 5 days prior to mating.

In experiment 3, litters were changed at birth between mothers fed the stock ration or the selenium diet, respectively. The liver selenium levels observed in figure 3 show a steep rise and subsequent fall in the litters of group 1 (nursed by dams fed selenium). In group 2 (rats born to mothers fed selenium and nursed by stock dams) these levels were high at birth and fell until leveling off at about 1 ppm.

Rats of experiment 4 were fed a diet containing 10 ppm selenium. The changes in liver values are shown in figure 4. In the stock animals these values increased to 14 ppm within 14 or 21 days, respectively, according to the age at which selenium feeding began, and fell later; in group 3, they rose steadily with no tendency to level off during the 52-day experimental period.

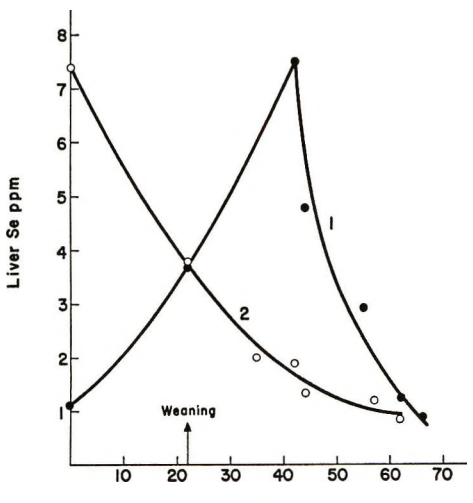

Fig. 3 Experiment 3. Influence of nursing on the liver selenium level of young rats. Group 1: bred on the stock diet, nursed by the mothers of group 2; group 2: bred on the seleniferous diet, nursed by the mothers of group 1.

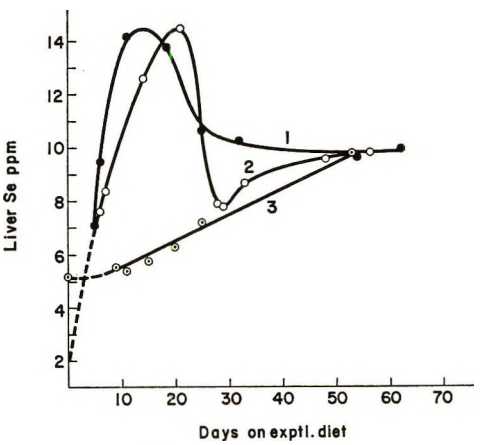

Fig. 4 Experiment 4. Liver selenium levels in rats fed a diet containing 10 ppm selenium. Group 1: stock animals 4 weeks old at the beginning of the experiment; group 2: stock animals 6 weeks old; group 3: animals bred on the 4.5 ppm selenium diet and 4 weeks old.

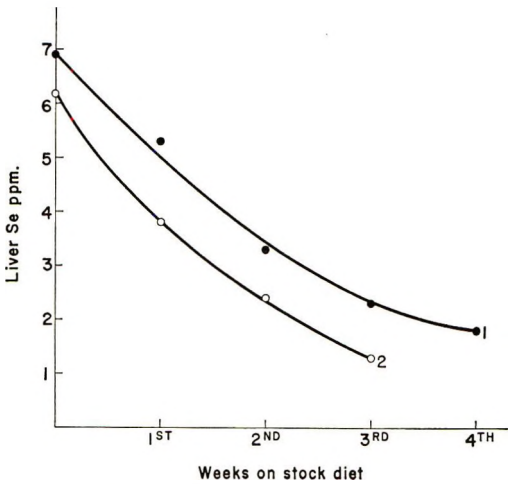

Fig. 5 Experiment 5. Liver selenium in rats fed the stock diet. Group 1: bred on the stock diet, fed for 4 days a diet with 10 ppm selenium; group 2: bred on the 4.5 ppm selenium diet.

The drop in liver selenium after the intake of this element had been discontinued was similar in rats born to mothers fed selenium and in stock animals previously fed a high selenium diet for 4 days (fig. 5, exp. 5). The level dropped from 6.2 to 1.3 ppm in 3 weeks in group 1 whereas it decreased from 6.9 to 2.3 ppm in the stock rats fed selenium.

DISCUSSION

The experimental diets were prepared with seleniferous sesame press cake, a material better suited for this type of investigation than inorganic selenium because it closely simulates the natural condition. The level of 4.5 ppm was chosen because it was the lower limit at which signs of intoxication became apparent in young stock rats under experimental conditions similar to those of the present experiment (1). The diets were supplemented with lysine because fewer symptoms of selenium toxicity developed in rats fed supplemented sesame rations than in those fed unsupplemented sesame rations (2).

The most important result of this study is that of experiment 1. It demonstrated that young rats may eliminate or accumulate selenium in the liver while consuming the same seleniferous diet, depending on the ration fed to their mothers (fig. 1). Those born from dams kept on the selenium-containing diet lost selenium from their livers, whereas rats bred on the stock ration accumulated this element under the same conditions. For the duration of the experiment these changes followed straight lines which crossed each other and showed no tendency for leveling off. This could mean that the animals in the two groups handled selenium metabolism in the livers in a different fashion, possibly because of differences in the intrauterine exposure to this element. Another possibility was that the handling of selenium may be related to the different levels existing at birth or the beginning of the experiment. The existence of an adaptation to selenium intake in rats born to dams fed the seleniferous diet during pregnancy was indicated by these results. It was not investigated whether the different protein levels in the stock diet and the seleniferous diet had any influence on the result.

It would be of interest to investigate how liver selenium will develop further in adapted animals on a seleniferous diet. In a preliminary trial with four second-generation rats, born to mothers which had been bred and kept on the 4.5 ppm selenium diet, the liver levels were much lower at birth than in the first generation, i.e., 4.7 compared with 7.1 ppm.

In experiment 2 the influence of intrauterine exposure on adaptation was studied by following the changes in the selenium contents in the livers of young rats from birth to about 6 weeks of age, and exposure to this element for different lengths of time during embryonic life. The course of change of the selenium levels with time depends on the selenium level at birth, which is, in turn, dependent on the time of feeding the seleniferous diet to the dams prior to the birth of the litters. The fast and discontinuous selenium accumulation in the rats of group 1 (fig. 2, exp. 2) with the lowest initial values was similar to that of the animals of groups 1 and 2 (fig. 4, exp. 4). In both cases the previous exposure to selenium had been less and the rise of the liver levels was faster than in the comparable groups of the respective experiments. Liver selenium increase was faster in rats of group 2 (fig. 2, exp. 2) whose mothers

received the experimental diet for 2 weeks during pregnancy, than in animals of group 1 (exp. 1) which had been bred on the stock ration and were fed the experimental diet after weaning. Perhaps this is related to the results of experiment 3 which gives evidence for a rapid increase of selnium in young rats during nursing. As the animals of this experiment were fed the stock diet, this rise was caused by the selenium excreted in the mother's milk. In experiment 2 this may have enhanced the selenium accumulation within the relatively short experimental period. Comparison with the performance of rats of group 1 (exp. 2) shows that some adaptation had taken place. Possibly liver selenium would level off if the duration of the experiment were extended. The selenium content in livers of the rats of group 3 (fig. 2) decreased at exactly the same rate as in experiment 1. The corresponding curves can be combined into one single, straight line. These results point to the influence of time of feeding the seleniferous diet to the mothers prior to the birth of their litters on the adaptation to selenium.

Experiment 3 was designed to investigate the course of the changes of liver selenium in young rats exposed to different intrauterine and dietetic levels of this element. A very rapid rise was evident in the animals nursed by dams previously fed the selenium diet and kept on the stock ration since birth of the litters, indicating high selenium excretion in the milk. Persistent rise of selenium level after weaning, when selenium intake stopped, points to liver deposition of selenium mobilized from other tissues.

After only 5 days of feeding a diet containing 10 ppm selenium to young stock rats, liver levels rose to over 6 ppm from an initial value of about 1 ppm. The maximum value of 14 ppm was reached earlier in younger animals than in older animals. The subsequent decrease may indicate that an adaptation to selenium intake may develop also under these conditions. The difference in performance between groups 1 and 2 (fig. 4) points to the importance of the age at which exposure to selenium occurred. The initial rise and subsequent fall of selenium in these animals brings to mind an observation of Rosenfeld and Beath (6) that tissue selenium is high in animals dead with blind staggers soon after the disease developed and considerably lower if the disease was prolonged. The rise in liver selenium in rats of this experiment, bred on the 4.5 ppm selenium diet, developed differently from that of the stock animals after the high selenium diet was fed. The corresponding values fell on a straight line in the graphic representation of figure 4 and indicate that these animals had adapted themselves to the selenium intake but could not handle the large intake as they handled that of experiment 1. A slightly lower selenium content (less than 10 ppm) would be more suitable for an experiment of longer duration because signs of intoxication became evident in some of the rats.

The outcome of the last experiment presented in figure 5 shows that rats adapted to selenium could eliminate this element at a slightly faster rate from the liver than the nonadapted controls. The difference, however, was too small to allow any conclusion on the physiological importance of this observation. Ganther et al. (7) observed that volatilization of a single dose of injected selenite was enhanced by prefeeding selenium. According to Hopkins and co-workers (8), urinary excretion also increased progressively with previous selenium intake, indicating an inverse relationship between selenium retention and body selenium under the conditions of a 2-week feeding period of the seleniferous diet. In these cases the experimental conditions were such that different levels of selenium should be expected to exist in the rats which were being compared. The animals of the experiment presented in figure 5 had similar amounts of body selenium as judged by the liver levels. The possibility that adapted rats absorb selenium at a lower rate than unadapted animals, or that differences in volatilization of selenium may exist between adapted and nonadapted animals, was not investigated.

Liver level may or may not reflect the total body selenium content under the present conditions. Hopkins et al. (8) found retention from a single dose of ^{75}Se similar in the livers of rats fed 5 ppm selenium for 2 weeks and in controls which had not been given selenium previously; carcass retention, however, was less in the former than in the latter. If these results, obtained un-

der very different conditions, can be applied to our experiments, it could be expected that carcass selenium between adapted and nonadapted rats differs more widely than liver selenium.

It cannot be concluded from the present experiments that a direct relationship exists between liver selenium and intoxication. The observation of Rosenfeld and Beath (6) on selenium in animals dead from blind staggers does not support the existence of such a relationship under the conditions of lethal intoxication in cattle. A connection between tissue selenium and intoxication may be expected, however, when the selenium intake is near the toxic level. Previous results from this laboratory (1, 2) pointed to the existence of an acquired tolerance to selenium through comparison of reproductive performance, growth, hemoglobin, hematocrit and body water in rats bred on a seleniferous or stock diet and fed a moderately toxic selenium-containing diet. Harr et al. (9) found median survival age greater in rats receiving diets with increasing selenium levels than in those receiving a constant level, but admit that their data are inconclusive concerning selenium tolerance. From observations of Tsuzuki et al. (10) it can be concluded that adaptation to selenium exists in species other than the rat. These authors exposed several generations of mice to selenium intake by inhalation. The animals bred under these conditions were less susceptible to toxic doses of this element than the controls.

The problems of the relationship between liver selenium and resistance to intoxication, the persistence of adaptation after the ingestion of selenium has been discontinued, the selenium balance in the adapted animals and the existence of similar mechanisms in other species, await further investigations and are of considerable interest in relation to chronic selenium ingestion in cattle and man.

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Modification of Lipemic Responses to an Alcohol-Corn Oil Mixture [1]

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ABSTRACT Chronic ingestion of 15% (w/v) alcohol in lieu of drinking water in rats fed ad libitum failed to modify the usual lipemic response to a single dose of corn oil-alcohol mixture. The rise in plasma triglycerides was associated with an increase in their ^{14}C-specific activity indicating that the additional plasma lipids were derived from the ^{14}C-labeled dietary fat. The lipemic response was, however, markedly reduced by restricting the food intake of the animals to 2 hours/day.

It has been shown in previous studies with both human and animal subjects that consumption of a single large dose of alcohol prior to or with a meal enhanced the usual postprandial lipemia (1–7). In animals, the hepatic triglyceride levels were also increased (5–7). Chronic ingestion of relatively large amounts of dilute alcohol, however, failed to affect the plasma and liver triglyceride levels of rats (8, 9), possibly because of the increased activity of alcohol dehydrogenase and faster metabolic breakdown of alcohol reported to occur during long-term administration of alcohol (10, 11).

We decided, therefore, to find out whether previous exposure of animals to dilute alcohol for an extended period of time can modify the hyperlipemic response to a subsequent single large dose of alcohol. In addition, we investigated the effect of a reduced caloric intake on the ethanol-induced rise in plasma and liver lipids.

METHODS

Forty male rats of the Sprague-Dawley strain initially weighing approximately 250 g each were used in the study. Twenty animals received a commercial rat ration[2] ad libitum; the remaining 20 received the same ration restricted to only 2 hours feeding time during each 24-hour period (8 to 10 AM). Each group was further subdivided into two subgroups. One subgroup received drinking water, the other subgroup was offered a 15% ethanol (w/v) solution for 28 days. Weekly weight changes and daily food and liquid intakes were recorded for each animal.

On day 29 of the experiment, food was removed and only water was offered as a liquid to all the animals. One half the animals in each group was given 5 g corn oil and 5 g ethanol/kg body weight by stomach tube. The remaining five animals in each group received in the same manner, 5 g corn oil/kg body weight and an amount of dextrose isocaloric with alcohol. The corn oil contained trace amounts of triolein-1-^{14}C.[3] Analysis by thin-layer chromatography (12) revealed that more than 95% of the radioactivity was associated with the triolein moiety. Sixteen hours later, the animals were bled by heart puncture; their livers were removed, blotted on a filter paper and weighed. The blood samples were immediately centrifugated in a refrigerated centrifuge, and the plasma and tissue samples were kept at $-15°$ until processed. Plasma and liver triglyceride levels were determined by conventional procedures (13, 14) and ^{14}C-activity of the plasma liver extracts lipid was measured in a scintillation spectrometer after absorption of phospholipids (13).

RESULTS

Information on daily weight gain and food and water intake is summarized in table 1. The animals in the group receiving food and water ad libitum consumed an average of 30.4 ± 3.2 g (mean ± standard error) of food and gained 5.3 ± 0.2 g/day

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[1] Supported in part by a grant from the Wisconsin Heart Association.
[2] Purina Rat Chow, Ralston Purina Company, St. Louis, Mo.
[3] Tracerlab, Waltham, Mass.

TABLE 1

Effect of chronic alcohol administration and caloric restriction on weight gain, and food and liquid intakes of rats [1]

Food	Alcohol	No. of rats	Food intake	Wt gain	Water intake
			g/day	*g/day*	*g/day*
Ad libitum	−	10	30.4 ± 3.2	5.3 ± 0.2	46.0 ± 0.5
	+	10	21.9 ± 0.3	3.4 ± 0.3	20.5 ± 0.3
Restricted	−	10	16.0 ± 0.1	1.6 ± 0.3	28.7 ± 0.5
	+	10	12.0 ± 0.2	2.0 ± 0.2	22.0 ± 0.3

[1] Fifteen percent (w/v) alcohol or water was offered for 28 days; animals on caloric restriction received food for 2 hours each day. All values mean ± SE.

during the 4-week experimental period. Replacement of drinking water by 15% ethanol reduced the food intake to 21.9 ± 0.3 g /day and the daily weight gain to 3.4 ± 0.3 g. The liquid intake was also significantly reduced in this group. Animals limited to food for 2 hours/day and offered drinking water consumed 16.0 ± 0.1 g food and gained 1.6 ± 0.3 g/day. Animals in the subgroup with 15% alcohol as the drinking solution ate less food, 12.0 ± 0.2 g, but gained slightly more weight, 2.0 ± 0.2 g/ day, probably reflecting the utilization of alcohol calories. This group drank 22.0 ± 0.3 ml of the 15% alcohol solution per day, slightly more than the animals receiving food ad libitum and 15% alcohol which consumed 20.5 ± 0.3 ml of the alcohol solution per day.

The effect of the dietary regimen and chronic alcohol intake on the lipemic response to the alcohol-corn oil mixture is shown in figure 1. Animals fed ad libitum reacted to the alcohol-corn oil mixture with a pronounced rise in plasma triglyceride levels. This lipid rise did not seem to be modified by a previous 4-week intake of 15% alcohol in lieu of drinking water. The increase in plasma triglycerides was associated with a rise in ^{14}C-specific activity of the lipids, indicating that the additional triglycerides appearing in the blood were derived from the diet. When food intake was restricted to 2 hours/day, the lipemic reaction to the alcohol-corn oil mixture was less marked than in the animal fed ad libitum. Again, the administration of 15% alcohol in place of water for 4 weeks prior to the test did not seem to enhance or reduce to any great extent the response to the alcohol-corn oil mixture.

The changes in hepatic triglyceride levels and ^{14}C-specific activity of hepatic lipids are shown in figure 2. The animals fed ad libitum and offered drinking water had an average liver triglyceride level of 10.7 ± 0.4 mg/g fresh tissue when receiving corn oil with glucose, and 20.1 ± 1.0 mg/g when the corn oil was administered with alcohol. This rise in hepatic triglycerides, however, was not reflected in corresponding changes of ^{14}C-specific activity. The animals fed ad libitum and receiving 15% alcohol for drinking also showed a marked increase of liver triglycerides following administration of corn oil plus alcohol. In this group, the rise in triglyceride level was associated with an increase in ^{14}C-specific activity.

The restriction of food intake to 2 hours/ day was, in general, associated with lower hepatic triglyceride levels and higher ^{14}C-specific activities than seen in the groups fed ad libitum. Regardless of the type of drinking solutions, administration of the corn oil-ethanol mixture was followed by a rise in hepatic triglycerides.

DISCUSSION

Chronic ingestion of 15% alcohol as drinking solution by the rats fed ad libitum failed to modify the lipemic response to the alcohol-corn oil mixture. It would seem, therefore, that an increase in alcohol dehydrogenase activity, observed to occur in animals given alcohol for longer periods of time (10, 11) was not able to prevent the lipemia-enhancing effect of the alcohol-oil combination.

The lipemic response to the alcohol-oil combination was, however, markedly reduced by the restriction of food intake to 2 hours/day. The animals on the restricted food regimen were probably in a state of caloric deficiency. It is possible, therefore, that the administered corn oil was pri-

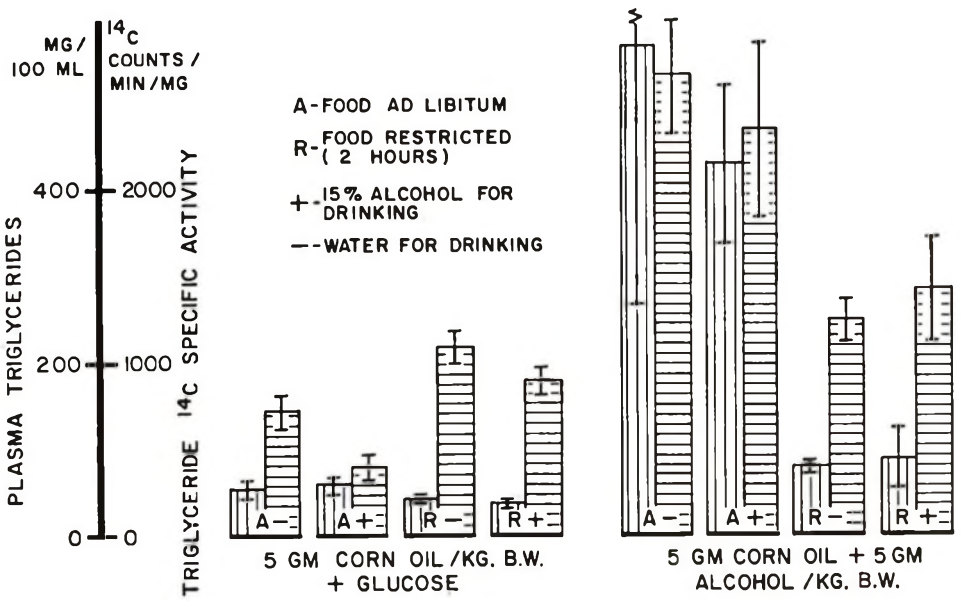

Fig. 1 Effect of chronic alcohol ingestion on plasma triglyceride levels (vertical stippling) and ^{14}C-specific activity of plasma lipids (horizontal stippling) in rats receiving a corn oil-alcohol mixture. The vertical lines represent standard error of the mean.

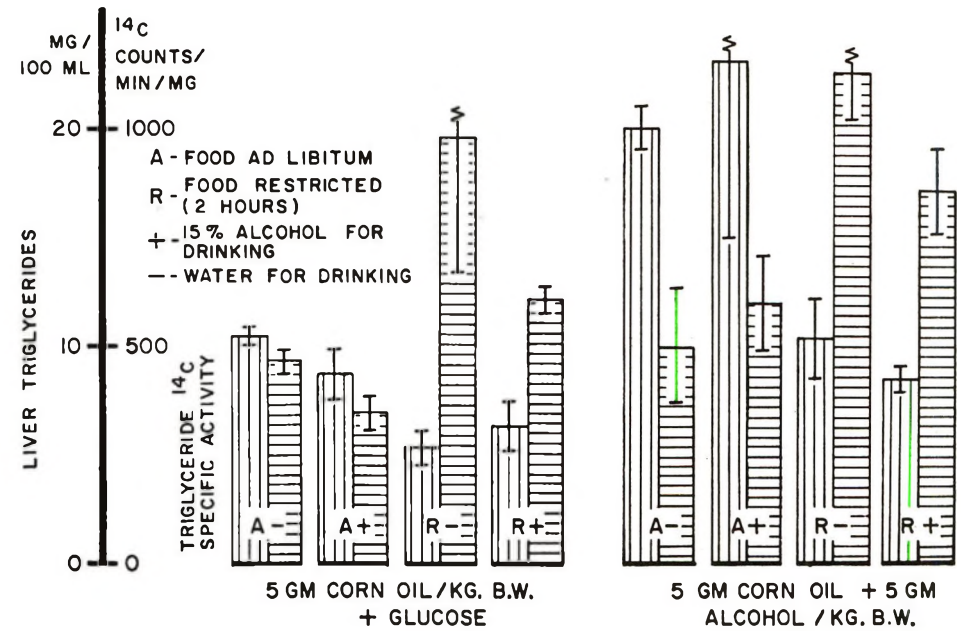

Fig. 2 Effect of chronic alcohol ingestion on hepatic triglyceride levels (vertical stippling) and ^{14}C-specific activity of hepatic lipids (horizontal stippling) in rats receiving a corn oil-alcohol mixture. The vertical lines represent standard error of the mean.

marily utilized as an immediate source of energy and only small portions remained to contribute to the build-up of plasma lipids. A rapid metabolic utilization of the dietary fat by the animals with restricted food intake is also suggested by the higher ^{14}C-activity of their hepatic triglycerides. Furthermore, periodic food restrictions, such as used in the present study have been reported to enhance utilization of dietary substrates (15, 16) and this might have decreased the availability of the administered fat as a source of blood lipids.

The definite mechanism of the alcohol-induced enhancement of postprandial lipemia seen in rats fed ad libitum is not well understood at this time. The increase in ^{14}C-specific activity of plasma lipids indicates that the triglycerides were mainly derived from the diet. The presence of high concentration of the ^{14}C-label in blood lipids as late as 16 hours after administration of the fat suggests a delay in the metabolic processing of the absorbed corn oil (7).

Replacing the drinking water with the 15% alcohol solution in the rats fed ad libitum led to a reduced food intake and lower weight gains. Similar growth-retarding effects of chronic alcohol administration have been observed even in studies in which the energy deficit, due to low food intake was counterbalanced by the caloric content of the ingested alcohol (8, 17). It it not clear whether the observed impairment of growth is due to a direct effect of alcohol (18) or to a reduced intake of dietary factors needed for optimal growth, as suggested by Lucas et al. (19). Animals receiving restricted amounts of food and offered alcohol grew, however, at a slightly faster rate than animals receiving the restricted diet and water. Furthermore, the former group consumed more alcohol than the animals fed ad libitum and given alcohol.

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Magnesium Requirement of the Laying Hen for Reproduction [1]

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ABSTRACT Three experiments were conducted to determine the magnesium (Mg) requirement of the laying hen. Hens fed a semipurified basal ration containing 55 ppm Mg developed anorexia, hypomagnesemia, nervous tremors and seizures within 2 weeks. A severe decrease in egg production, egg weight and hatchability of eggs was also observed. Feeding a ration containing 155 ppm Mg improved egg production, but not egg weight or hatchability. The reduction in hatchability preceded decreases in appetite and egg production, and was closely associated with hypomagnesemia in the hen and lower concentration of magnesium in the egg. Magnesium concentration in the dry matter of livers from hens fed the basal ration was markedly decreased whereas calcium concentration was increased. When expressed on a fat-free basis, however, the magnesium concentration in the livers was not significantly affected by ration treatment. Histological studies of the liver from hens fed the basal ration showed a considerable accumulation of fat globules in the cells. Oxidative phosphorylation, measured as P/O ratio of liver homogenates, from hens fed a magnesium-deficient ration was approximately half that of liver from control hens. The results show that the hen (1.8 to 2.0 kg body weight) requires a minimum of 355 ppm Mg in the diet or an intake of at least 36 mg Mg/day to maintain a high rate of egg production, desirable egg production, desirable egg weight and high rate of hatchability of fertile eggs.

Magnesium (Mg) is one of the elements known to be necessary for normal metabolic functioning of the body. It is an essential nutrient for growth, production and reproduction of animals as has been demonstrated with the rat, guinea pig, dog and other species. Magnesium was first shown to be required for normal growth of mice by Leroy in 1926 (1). Later Kruse et al. (2) demonstrated conclusively that magnesium is an essential nutrient for animals by inducing acute magnesium deficiency in rats. The need for magnesium in the diet of growing chicks was shown by Almquist (3) and Gardiner et al. (4). They reported that the chick requires 250 to 400 ppm Mg for optimum growth. Cox and Sell (5) and Sell et al. (6) have shown that hens require dietary magnesium and described some of the symptoms of magnesium deficiency in the laying hen.

The objectives of the present study were to obtain further information about magnesium deficiency manifestations in the hen and to determine the dietary magnesium requirement of the hen for reproduction.

MATERIALS AND METHODS

Single-comb White Leghorn hens kept in individual wire laying-hen cages were used in three experiments. Prior to the start of the experiments, 3 weeks were allowed for the hens to adjust to the cage environment and become accustomed to a semipurified ration which contained 650 ppm Mg. The composition of the basal ration is presented in table 1. By analysis, it contained 55 ppm Mg. All ration treatments were kept isocaloric and equinitrogenous by appropriate adjustments in Solka Floc [3] and soybean oil. The rations were fed in mash form, except in experiment 1, in which the rations were fed as 3-mm pellets. Feed and distilled water were supplied ad libitum in individual feeders and waterers. Experiments 1 and 2 were of 8 weeks duration; experiment 3 lasted 5 weeks.

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[2] Current address: Mansourieh — Metn, Lebanon.
[3] Solka Floc, BW-40, Brown Company, New York, N. Y.

TABLE 1

Composition of the basal ration

	%
Sucrose	50.00
Isolated soybean protein [1]	21.60
Degummed soybean oil	6.00
Solka Floc [2]	10.47
Vitamin premix [3]	1.35
Mineral premix [4]	0.85
Calcium carbonate, reagent grade	7.39
Calcium phosphate (monobasic), reagent grade	1.83
Choline chloride (99.9%)	0.17
Vitamin E (2200 IU/g)	0.12
DL-Methionine	0.21
Antioxidant [5]	0.01
Laboratory analysis	
Protein, %	18.40
Calcium, %	3.10
Phosphorus, %	0.62
Magnesium, ppm	55.00
Metabolizable energy, kcal/g, calculated	3.04

[1] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2] Solka Floc, BW-40, Brown Company, New York, N. Y.
[3] The vitamin premix supplied the following per kilogram of ration: vitamin A, 7500 IU; vitamin D_3, 1487 ICU; menadione, 500 μg; vitamin B_{12}, 10 μg; riboflavin, 10 mg; folic acid, 4 mg; pantothenic acid, 20 mg; nicotinic acid, 50 mg; thiamine, 10 mg; pyridoxine, 4.5 mg; and biotin, 200 μg.
[4] The mineral premix supplied the following per kilogram of ration: sodium chloride, 3.7 g; potassium chloride, 1.99 g; manganese, 110 mg; zinc, 44 mg; iron, 19.8 mg; copper, 1.98 mg; and iodine, 440 μg.
[5] Santoquin, Monsanto Company, St. Louis, Mo.

The hens were artificially inseminated every 5 days. Feed consumption data were recorded weekly throughout each experiment. Eggs were gathered daily and weighed. The eggs were accumulated and incubated at weekly intervals, and fertility and hatchability of eggs were recorded. In experiments 1 and 2, two infertile eggs (clear at day 19 of incubation) were saved weekly from each ration treatment for magnesium and calcium (Ca) analyses. The "clear" egg contents (albumen plus yolk) were homogenized in a blender,[4] coagulated and stored at − 23° until analyses for magnesium and calcium could be performed.

Calcium and magnesium analyses were performed on rations, egg contents, blood serum and tissues using atomic absorption spectrophotometry. The samples were wet-washed (7) and diluted with lanthanum chloride solution to obtain a final concentration of magnesium and calcium in the range of 0.2 to 2 and 2 to 20 ppm, respectively, in a final solution containing 1% lanthanum.

The ration treatments were assigned to the experimental units according to a completely randomized design with five hens receiving each treatment. In experiment 1, 25 hens about 45 weeks of age were used. The five ration treatments contained the following levels of magnesium: 155, 280, 405, 530, or 655 ppm. Reagent grade $MgSO_4 \cdot 7H_2O$ was the source of supplemental magnesium. Blood was obtained by heart probe from three hens from each treatment at the beginning of week 1 and at the end of weeks 4, 6 and 8 of the experiment. The serum was removed and stored at − 23° until analyses for calcium and magnesium could be performed.

In experiment 2, 30 hens about 30 weeks of age were used. The six rations tested contained the following levels of magnesium; 55, 155, 255, 355, 455 or 555 ppm. At the end of week 2, hens which had been fed the 55-ppm Mg ration were fed a ration containing 555 ppm Mg. The experiment was terminated at the end of 8 weeks.

In experiment 3, 20 hens about 30 weeks of age were used. The four rations tested contained the following levels of magnesium: 55, 155, 255 or 455 ppm. At the end of 5 weeks of the experiment, two hens which had received 55, 155 and 455 ppm Mg were killed and the livers and tibias were removed. Estimates of the rate of oxidative phosphorylation (P/O) by liver homogenates were made (8). The tibias were dried, ashed at 600° for 12 hours and analyzed for magnesium and calcium. Another two birds from the same treatments were killed and histological preparations of their livers and hearts were made, using frozen sections 12 μ in thickness. Liver and heart tissues were stained with a lipid stain (oil red O) using the Lillie and Ashburn method (9). Samples of the livers were saved for magnesium, calcium and lipid analyses.

The data were analyzed by analysis of variance according to Snedecor (10) and multiple range comparisons were made according to Duncan (11).

[4] Waring Blendor, Waring Products Company, Winsted, Conn.

TABLE 2

Effect of dietary magnesium levels on egg production, feed consumption, fertility and egg weight (exp. 1)

Dietary Mg	Egg production	Feed consumption	Egg wt	Fertility
ppm	%	*g*	*g*	%
155	57 [A 1]	103	54.5 [a 1]	88
280	85 [B]	101	58.1 [b]	92
405	79 [B]	100	58.6 [b]	91
530	75 [B]	104	60.9 [b]	92
655	78 [B]	104	60.6 [b]	93

[1] Treatment means within an item showing the same superscript letter are not significantly different (A, B at $P \leq 0.01$ or a, b at $P \leq 0.05$).

RESULTS

In experiment 1, hens fed rations containing 280 ppm Mg or more produced eggs at a high and nearly equal rate during the 8-week experiment (table 2). Average hen-day egg production (average number of eggs produced per hen per day expressed as a percentage) of hens fed the ration containing 155 ppm Mg was significantly ($P \leq 0.01$) less than that of other treatment groups even though feed consumption was not adversely affected. Also, average weight of eggs produced by hens fed 155 ppm Mg was significantly less than that of hens fed 280 ppm Mg or more.

Although fertility of eggs was not affected by ration magnesium level (table 2), hatchability of fertile eggs produced by hens fed 155 ppm Mg was significantly ($P \leq 0.01$) lower than that of eggs from hens fed 280, 405, 530 or 655 ppm Mg (fig. 1). Within 4 weeks of beginning the experiment, hatchability of eggs produced by hens fed 155 ppm Mg decreased from a level of 89 to 64%. This decline continued, reaching 16% on week 8 of the experiment. Hatchability of eggs produced by hens fed 280, 405, 530 or 665 ppm Mg remained high.

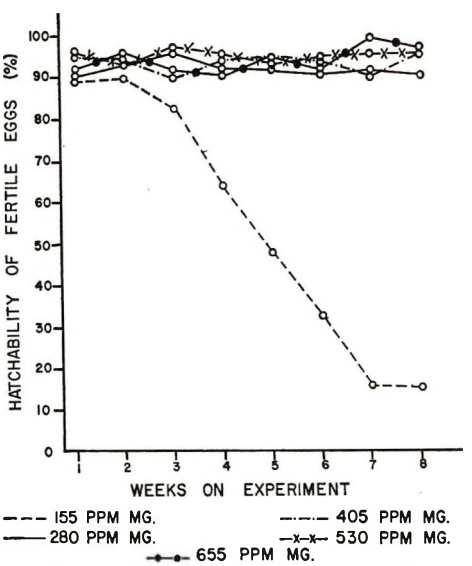

Fig. 1 Influence of varying levels of dietary magnesium on hatchability of fertile eggs (exp. 1).

Most of the embryonic mortality due to feeding hens the ration containing 155 ppm Mg occurred late in incubation (days 19 and 20). Furthermore, many chicks hatched from eggs produced by these hens were morbid and suffered convulsions, followed by coma and death within 2 days after hatching.

Magnesium concentration of egg contents decreased significantly ($P \leq 0.01$) within 3 weeks after feeding the ration containing 155 ppm Mg as compared with the 280, 405, 530 or 655 ppm Mg rations (fig. 2). By week 8 of the experiment, magnesium concentration of egg contents from hens fed 155 ppm Mg had decreased to 69 ppm — less than one-half that observed in week 1.

Within 4 weeks of beginning the experiment, serum magnesium of hens fed 155 ppm Mg decreased from 2.9 to 1.1 mg/100 ml and remained at this low level through week 8 of the experiment (fig. 3). The serum concentration of magnesium of hens fed 280, 405, 530 or 655 ppm Mg also decreased, but only slightly as compared with that at the start of the experiment.

In experiment 2, serum magnesium concentration of hens fed the ration contain-

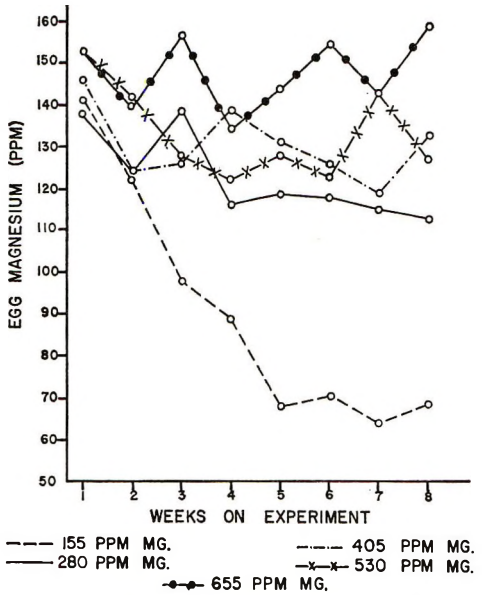

Fig. 2 Influence of varying levels of dietary magnesium on the concentration of magnesium in egg contents (exp. 1).

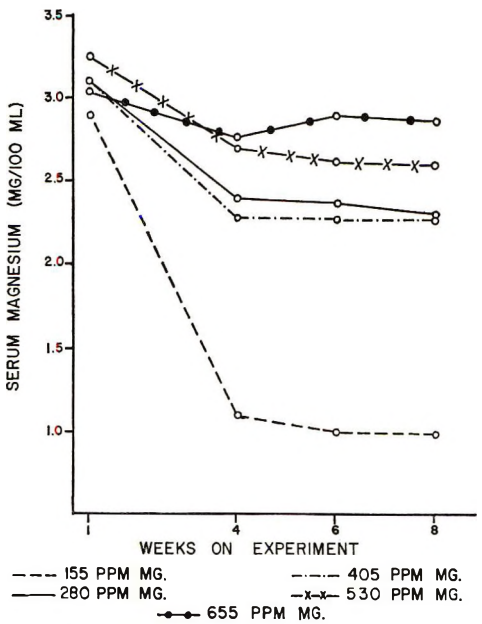

Fig. 3 Influence of varying levels of dietary magnesium on the concentration of magnesium in serum (exp. 1).

ing 55 ppm Mg was extremely low by the end of 2 weeks (table 3). Simultaneously, egg magnesium concentration and average egg weight decreased significantly ($P \leq$

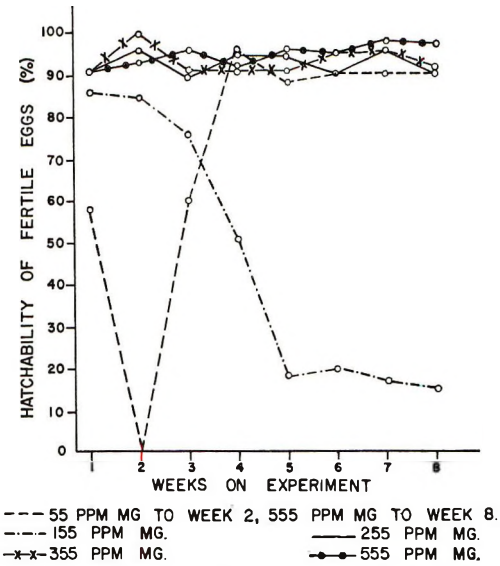

Fig. 4 Influence of varying levels of dietary magnesium on hatchability of fertile eggs (exp. 2).

0.01) as compared with the other treatment groups, and hatchability of fertile eggs decreased to zero (fig. 4). Subsequent feeding of a ration containing 555 ppm Mg to these hens resulted in rapid improvement in hatchability of eggs (fig. 4) as well as in magnesium concentration in serum and egg, and egg weight (table 3).

The results obtained by the end of 8 weeks show that a dietary level of 255 ppm Mg was required to maintain serum magnesium concentration and egg weight in a satisfactory range (table 3). Significantly ($P \leq 0.01$) higher egg magnesium concentrations, however, were not obtained unless 355 ppm Mg or more were fed. Also, the largest relative increases in egg weight with increased time on experiment were observed when hens were fed rations containing 355 ppm Mg or more. These results indicated that a minimum of 355 ppm Mg is required to insure that eggs produced will be of desired size and will contain a high concentration of magnesium.

There were no significant treatment effects on calcium concentration in egg contents (table 3).

During the trial visible signs of magnesium deficiency, anorexia, ruffled feathers, shriveled, pale combs and wattles, emaciation, nervous tremors, gasping and con-

vulsions, were observed in hens fed the diet containing 55 ppm for 2 weeks.

Although not given here, egg production and hatchability data obtained in experiment 3 followed the same pattern of response to ration treatments as observed in previous experiments.

The average weight of fresh liver was not significantly affected by ration treatment in experiment 3 (table 4). The percentage dry matter and the calcium and lipid concentration in the dry matter were significantly higher for hens fed 55 ppm Mg than for hens fed either 155 or 455 ppm Mg. Low magnesium intake also resulted in a significantly lower concentration of magnesium in the dry matter. When liver magnesium was expressed on a fat-free dry matter basis, however, there were no significant differences among treatments. In contrast, the relatively high calcium concentration in liver dry matter of hens fed 55 ppm became more pronounced when expressed on the fat-free basis.

The rate of oxidative phosphorylation, as indicated by P/O ratios of homogenates of

TABLE 3

Effect of dietary magnesium levels on magnesium concentration in serum and egg, egg weight and calcium concentration in egg (exp. 2)

Dietary magnesium	Weeks on experiment							
	2				8			
	Serum magnesium	Egg magnesium	Egg wt	Egg calcium	Serum magnesium	Egg magnesium	Egg wt	Egg calcium
ppm	*mg/100 ml*	*ppm*	*g*	*ppm*	*mg/100 ml*	*ppm*	*g*	*ppm*
55	0.62 [A 1]	61 [A]	46.7 [A]	524	2.84 [A]	145 [A]	57.8 [a]	586
155	2.63 [B]	102 [B]	55.8 [B]	545	1.00 [B]	62 [B]	53.2 [b]	540
255	2.76 [B]	131 [C]	56.0 [B]	571	2.58 [A]	106 [C]	57.1 [a]	541
355	2.91 [B]	136 [C]	56.3 [B]	562	2.72 [A]	134 [A]	58.9 [a]	537
455	2.73 [B]	148 [C]	57.6 [B]	550	2.80 [A]	138 [A]	60.1 [a]	528
555	2.87 [B]	136 [C]	56.4 [B]	567	2.70 [A]	146 [A]	59.1 [a]	568

[1] Treatment means within an item showing the same superscript letter are not significantly different (A, B, C at $P \leq 0.01$) or (a, b at $P \leq 0.05$).

TABLE 4

Effect of dietary magnesium levels on liver magnesium, calcium and lipid concentration, magnesium and calcium in tibia, P/O ratios of liver homogenates and body weight (exp. 3)

	Dietary magnesium		
	55	155	455
	ppm	*ppm*	*ppm*
Liver [1]			
Fresh weight, g	33.7	36.5	36.8
Dry matter, %	38.3 [a 2]	30.6 [b]	31.9 [b]
Magnesium, ppm dry matter	167 [A]	285 [B]	330 [B]
Magnesium, ppm fat-free dry matter	327	424	372
Calcium, ppm dry matter	583 [a]	384 [b]	316 [b]
Calcium, ppm fat-free dry matter	1143 [A]	424 [B]	372 [B]
Lipids, % dry matter	49.0 [A]	9.5 [B]	15.0 [B]
P/O ratio	0.88 [A]	1.91 [B]	1.90 [B]
Bone [3]			
Magnesium, %	0.30	0.33	0.43
Calcium, %	24.8	21.9	24.2
Ash, %	70.3	66.0	68.9
Body wt			
Initial	1654	1592	1681
Final	1112 [A]	1564 [B]	1692 [B]

[1] Each value represents two hens per ration treatment.
[2] Treatment means within an item showing the same superscript letter are not significantly different (A, B at $P \leq 0.01$) or (a, b at $P \leq 0.05$).
[3] Expressed as a percent of dry matter.

livers, was significantly less for hens fed 55 ppm Mg than that of livers from hens receiving 155 or 455 ppm Mg.

Slightly lower bone magnesium concentrations were observed with hens fed the 55 or 155 ppm Mg rations compared with hens fed 455 ppm Mg, but the differences were not significant. There were no significant treatment effects on calcium concentrations or percentage ash in the bone.

Average body weight of hens fed the basal ration decreased markedly during the experiment whereas body weights of hens fed 155 or 455 ppm added Mg remained approximately the same (table 4).

Histological examination of liver tissues from hens fed the ration containing 55 ppm Mg showed a greater accumulation of fat globules in the cells compared with hens fed 155 or 455 ppm Mg. Histological preparation of heart tissues from hens fed the basal ration also showed a slight accumulation of lipids in the muscle cells whereas those prepared from hens fed magnesium-supplemented rations did not.

DISCUSSION

Laying hens fed rations containing 155 ppm Mg or less developed signs of magnesium deficiency within 8 weeks or less. Hypomagnesemia occurred within 2 weeks and 4 weeks when rations containing 55 and 155 ppm Mg, respectively, were fed. Hypomagnesemia due to inadequate dietary magnesium has been observed previously in chickens [5] (6), in rats (12) and in dogs (13).

A ration containing 155 ppm Mg was not sufficient to maintain a high rate of egg production or satisfactory egg weight. Sell et al. (6) found that hens fed 150 ppm Mg produced eggs of small size and at a low rate. In the current study, a minimum of 255 ppm Mg was required for maximum egg production. Hens fed 255 ppm Mg, however, produced smaller eggs than hens fed 355 ppm Mg or more. In addition, the usual rate of increase in egg weight associated with increased time in production was slower in the case of hens fed 255 ppm Mg. These results indicate that a minimum of 355 ppm Mg was required in the ration to obtain a normal pattern of increasing egg weight as the laying cycle progressed.

Although there was little or no effect on fertility, hatchability of fertile eggs was decreased markedly when hens were fed a ration containing 155 ppm Mg or less. This effect on hatchability occurred relatively rapidly and preceded a decline in rate of egg production. The data presented here show that a relative decrease in magnesium concentration of egg contents occurred within 2 weeks of feeding a ration containing 155 ppm Mg. Hatchability was not adversely affected until week 4, however, when magnesium concentration of egg contents decreased to less than 90 ppm. A decrease in magnesium concentration in egg contents of hens fed 255 or 280 ppm Mg was also observed but hatchability did not decrease even though egg magnesium declined to about 100 ppm. This suggests that magnesium concentration in egg contents should be at least 100 ppm to have high hatchability. The severe decline in hatchability coincided with the decline in serum magnesium.

Cox [6] demonstrated that serum magnesium concentrations declined rapidly during a low magnesium regimen, indicating a lag or inadequacy in rate of mobilization of body magnesium stores. He also found that at marginal dietary levels of magnesium, the rate of egg production declined relatively slowly, thereby producing a heavy withdrawal of this element from body stores. Under these conditions, eggs containing abnormally low levels of magnesium in the shell and contents would be produced within a short time after feeding a severely deficient ration. Consequently, the requirement for magnesium to sustain complete embryonic development of the chick could not be met.

In experiment 2, the hatchability of eggs produced by hens fed the low magnesium (55 ppm) ration increased rapidly within 2 weeks after feeding a ration containing 555 ppm Mg. An increase in magnesium intake and absorption should cause a rapid increase in magnesium content of the egg since Edwards et al. (14) showed that a high proportion of ^{28}Mg was detected in

[5] Cox, A. C. 1966 The effects of dietary calcium and phosphorus on the magnesium status of the laying hen. M.S. Thesis, University of Manitoba, Winnipeg, Canada.

[6] See footnote 5.

eggs produced within 48 hours after the radioisotope was injected into hens.

The results of these experiments indicated that the effect of magnesium deficiency on egg production and hatchability was independent of feed intake, but was dependent on magnesium intake per se. This was particularly apparent when rations containing 155 ppm Mg were fed. Under these conditions, rate of egg production, serum and egg magnesium concentrations, and hatchability decreased markedly even though feed consumption was not affected. Sell et al. (6) previously showed that hatchability as related to magnesium deficiency was independent of feed intake and average egg weight.

The majority of embryonic mortality due to magnesium deficiency occurred at 19 to 20 days of incubation and may be indicative of the general role of magnesium in the metabolism of the embryo. As pointed out by Sell et al. (6) it is during this interval of embryonic development that energy metabolism would be nearing a maximum. Since magnesium is involved in a multitude of enzyme systems concerned with energy metabolism, the requirement for this element would be relatively high and perhaps critical.

Considerable accumulation of fat globules in the liver was observed in hens fed magnesium-deficient diets. Approximately 50% of the dry weight of the liver was lipid material. A deficiency of dietary magnesium for the laying hen also decreased the rate of oxidative phosphorylation (P/O ratio) of liver homogenates. After 5 weeks on a deficient ration, the P/O ratio of liver homogenates from hens fed 55 ppm Mg was about half that of livers from hens fed 155 or 455 ppm Mg. Vitale et al. (15) reported that magnesium deficiency in young rats decreased the P/O ratio of heart mitochondria to approximately half that of the control animals within 8 days. Also, a decrease of lesser magnitude was observed in P/O ratio of liver mitochondria from the same animals.

Magnesium serves as an activator in nearly all transphosphorylation reactions involving adenosine triphosphate. Thus, a deficiency of magnesium could cause uncoupling of oxidative phosphorylation, thereby affecting energy metabolism. The decreased P/O ratio of liver homogenates observed in the present study when hens were fed a magnesium-deficient ration may have been due to inadequate magnesium ions in this organ. The relatively low concentration of magnesium in liver dry matter, and a decrease in total liver magnesium indicate that this was the case. The magnesium concentration in the fat-free dry matter, however, was not decreased by feeding the magnesium-deficient ration. If one assumes that magnesium participates in energy transformation reactions primarily in fat-free tissue, the latter observation indicates that adequate magnesium would have been present where needed. The possibility that the reduced rate of oxidative phosphorylation may have been related, directly or indirectly, to the high levels of lipid or calcium, or both, in the liver deserves consideration.

The data from these experiments indicate that the hen requires a minimal dietary magnesium level of 155 ppm (55 ppm in the basal plus 100 ppm added Mg) for prevention of the accumulation of excessive levels of calcium and lipids in the liver (table 5). Although this level of magnesium supported egg production, a minimal dietary magnesium level of 255 ppm was required to maintain a high rate of egg production and hatchability. The data also revealed that the hen required at least 355 ppm Mg in the ration to maintain a high rate of egg production, hatchability and egg weight. This was particularly apparent from the egg magnesium data which suggest that at levels lower than 355 ppm

TABLE 5

Dietary magnesium requirement of the laying hen on the basis of various criteria

Criteria	Minimum dietary requirement for magnesium
	ppm
Absence of excessive calcium and lipid concentrations in liver	155
High rate of egg production and hatchability of eggs	255
High concentration of magnesium in serum and egg contents, and satisfactory egg weight	355

total Mg in the ration, depletion of body stores followed by decreased hatchability of eggs would occur over a prolonged feeding period.

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Role of Calcium in the Nutritional Etiology of a Metabolic Disorder in Ruminants Fed a High Grain Ration [1,2]

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ABSTRACT Four experiments involving 152 wether lambs were conducted to determine the nutritional factor(s) involved in the etiology of a metabolic disorder of cattle caused by feeding a high maize ration containing 0.3% phosphorus and 0.16 to 0.19% calcium. The calcium intake was inadequate to satisfy the needs of the growing-finishing lamb for calcium. Calcium deficiency signs observed were lack of appetite, decreased growth, emaciation, muscular stiffness, decreased plasma serum calcium concentration and rarefaction of bone. Phosphorus levels similar to those encountered in the field ration, in conjunction with the low calcium levels had a detrimental effect; plasma calcium levels were not maintained and plasma magnesium and inorganic phosphorus concentrations were increased. Death resulted from blockage of the urethra by sludge, or calculi, or both. The signs of the disorder were reversed by added calcium carbonate, but not by organic calcium sources or extra sodium as chloride or carbonate: bicarbonate and potassium. The evidence indicates that in the presence of phosphorus levels exceeding recommended levels, the calcium levels (expressed as a percentage of the diet) recommended by the National Research Council for lambs and probably for beef cattle are grossly inadequate. With these rations a Ca:P ration of 1.3:1 is borderline. The data suggest that ratios of 1.5:1 or 2:1 are more desirable.

These studies were initiated to determine the cause of a metabolic disorder described as "stiffness in cattle." [6] The syndrome included swollen shoulders, stiff hind limbs and evidence of pain whenever the animals moved. The rations generally contained about 90% ground ear corn and 10% of a closed formula commercial supplement containing 0.0011% diethylstilbestrol (DES). Daily gains were about 0.5 kg/day.

When sheep were fed these rations under laboratory conditions, they exhibited symptoms similar to those observed in cattle. In addition, some lambs developed urinary calculi.

It was reasoned that, with rations borderline in calcium, certain metabolic patterns in the ruminants involved may have been altered by the relatively high level of phosphorus in the high grain ration. The minimum calcium (Ca) and phosphorus (P) requirement of ruminants and the relationship which exists in the metabolism of these two elements have been studied by several workers (1–6). However, little work, if any, has been reported on the effect of feeding high phosphorus (0.30 to 0.45%) rations in conjunction with the minimum calcium level (0.17 to 0.23%) recommended by the National Research Council (7,8).

EXPERIMENTAL PROCEDURE

The field ration supplied 0.16% calcium. The basal ration, used in trials 1 and 2, was designed to duplicate those which produced the metabolic disorder. It contained ground ear corn and a protein-mineral supplement formulated from the same ingredients at levels estimated by microscopic analysis to duplicate the commercial supplement. In the first of three consecutive feeding trials, the effects of three

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[6] This observation was reported by Dr. A. A. Case of the School of Veterinary Medicine and Dr. G. B. Garner of the Agricultural Chemistry Department, University of Missouri, Columbia, Missouri.

ration calcium levels and two ration phosphorus levels on performance of growing wether lambs, were studied. Four sources and two levels of calcium were used in trial 2 to determine if the favorable response to added calcium noted in trial 1 was due to: 1) a buffering or alkalizing effect of the $CaCo_3$ in the rumen; 2) a need for the elemental calcium for normal body function; or 3) the contribution of calcium to the total cationic load of the animal. Equal molar amounts of sodium (Na) as $NaHCO_3$:Na_2CO_3, and potassium (K) as $KHCO_3$:K_2CO_3, were added to one-half the rations to determine if these cations were as effective as the equivalent amount of calcium in the prophylaxis. Two levels of calcium and three levels of phosphorus were employed in trial 3 to study their effect upon the performance of growing-finishing lambs under feedlot conditions.

Trial 1. Thirty-two Texas crossbred wether lambs were randomly placed into four treatment groups of eight lambs each. The lambs were fed individually to appetite, for two periods of 2 hours each day, a completely mixed ration containing either 0.16% Ca and 0.35% P (ration 1); 0.19% Ca and 0.37% P (ration 2); 0.31% Ca and 0.31% P (ration 3); or 0.59% Ca and 0.36% P (ration 4), respectively, as shown in table 1. Rations 2 to 4 contained 0.53% added salt. Four samples of each ration taken at intervals during the trial were analyzed [7] and the analyses are presented in table 2.

TABLE 1

Formulation of rations [1] used (trial 1)

Ingredients [2]	Ration no.			
	1	2	3	4
Soybean meal [3]	4.01	4.33	4.52	5.00
Wheat grey shorts	4.60	3.89	3.42	2.27
Limestone	0.05	0.05	0.60	1.05
Salt, iodized	0.05	0.53	0.53	0.53
Bone meal	0.05	0.05	—	0.05
Calcium phosphate [4]	0.05	0.05	—	0.05
Rock phosphate [5]	0.20	0.20	—	0.20

[1] Kilogram of ingredients per 100 kg ration.

[2] All rations contained: (in percent) ground ear corn, 90; urea, 262, 0.17; dehydrated alfalfa, 0.2; blackstrap molasses, 0.5; sodium sulfate, 0.02; and a trace mineral mixture supplying 4.405 g $MnSO_4 \cdot H_2O$; 0.991 g $CuSO_4 \cdot 5H_2O$; 0.441 g Fe_2O_3; and 23.6 mg $CoCO_3$/100 kg ration.

[3] Processed by solvent method. Contained 44% crude protein.

[4] Supplied by International Minerals, Skokie, Ill. Eighteen percent phosphorus and 30 to 35% Ca.

[5] Supplied by International Minerals. Eighteen and one-half percent phosphorus and 20 to 24% Ca.

TABLE 2

Chemical analyses of rations [1] used (trial 1)

Ingredient	Ration no.			
	1	2	3	4
%				
Crude protein	10.9	11.0	10.9	11.3
Dry matter	89.3	89.4	89.2	89.5
Organic matter	87.1	86.8	86.6	86.2
Nitrogen-free extract	64.8	64.9	64.8	64.5
Fat	3.2	3.2	3.1	3.1
Fiber	8.2	7.7	7.8	7.3
Ash	2.2	2.6	2.6	3.3
Calcium	0.16	0.19	0.31	0.59
Phosphorus	0.35	0.37	0.31	0.36
Magnesium	0.12	0.13	0.13	0.13
Sodium	0.03	0.25	0.22	0.24
Potassium	0.50	0.49	0.52	0.52

[1] Average of samples from rations mixed on four separate dates.

The trial was conducted in two phases. Phase 1 lasted 90 days after which 50% of the animals were killed. Phase 2, which followed a metabolism trial, lasted 60 days. The lambs were weighed weekly and feed intake was recorded. The lambs had access to shelter in an earthen lot and water was available except while they were in the feeding stalls in an adjacent barn.

Trial 2. Sixty-four Northwestern crossbred wether lambs were randomly placed into eight treatment groups of eight lambs each. The lambs were fed individually the rations shown in table 3. The chemical analyses of trial 2 rations are given in table 4. The feeding trial lasted 128 days and management of the experimental animals was similar to that in trial 1.

At zero, 2, 4, 8, 16, 32, 64 and 128 days after the lambs started on test, blood samples were withdrawn from the external jugular vein 5 hours after the morning feeding. Plasma calcium, magnesium, inor-

[7] Protein, dry matter, fat, fiber and ash were determined by proximate analysis (Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D.C.), Calcium and magnesium determinations in feed, feces, urine and plasma were made with a Perkin-Elmer Model 290 atomic absorption spectrophotometer, Perkin-Elmer Corporation, Norwalk, Conn., using methods suggested by the manufacturer. The method of Fiske and Subbarow (Fiske, C. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., 66: 375) was employed for all phosphorus determinations. Sodium and potassium were determined by flame photometry.

TABLE 3

Composition of rations [1] used (trial 2)

Ingredient [2]	Ration no.							
	9	10	11	12	13	14	15	16
Wheat grey shorts	2.54	2.07	2.26	1.78	1.83	1.41	1.79	1.32
Limestone	0.05	0.05	0.05	0.05	0.71	0.71	0.47	0.47
$NaHCO_3$	0.00	0.08	0.00	0.08	0.00	0.08	0.00	0.08
$NaCO_3$	0.00	0.13	0.00	0.13	0.00	0.13	0.00	0.13
$KHCO_3$	0.00	0.13	0.00	0.15	0.00	0.15	0.00	0.15
KCO_3	0.00	0.11	0.00	0.11	0.00	0.11	0.00	0.11
Calcium gluconate	0.00	0.00	1.29	1.29	0.00	0.00	0.00	0.00
Calcium acetate	0.00	0.00	0.47	0.47	0.00	0.00	0.00	0.00
$CaCl_2$	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.33
Corn starch	1.37	1.37	0.00	0.00	1.37	1.37	1.37	1.37

[1] Kilogram of ingredients per 100 kg ration.

[2] In addition to the ingredients listed, all rations contained: (in percent) corn, 90; solvent soybean oil meal, 5.57; urea, 262, 0.17; steamed bone meal, 0.05; dicalcium phosphate (18% P, 33% Ca), 0.05; rock phosphate (18% P, 22% Ca), 0.2; iodized salt, 0.05; and a trace mineral mixture containing 4.405 g $MnSO_4 \cdot H_2O$; 0.991 g $CuSO_4 \cdot 5H_2O$; 0.441 g Fe_2O_3; and 23.6 mg $CoCO_3$/100 kg ration.

TABLE 4

Chemical analyses of rations used (trial 2)

Ingredient	Ration no.							
	9	10	11	12	13	14	15	16
%								
Crude protein	12.4	12.1	12.2	12.1	12.2	12.1	12.2	12.0
Dry matter	90.4	90.4	90.7	90.6	90.7	90.7	90.6	90.9
Organic matter	88.3	88.1	88.3	87.8	88.2	88.0	88.1	88.1
Nitrogen-free extract	64.7	64.5	64.9	64.6	64.4	64.5	64.6	64.4
Fat	4.0	3.9	4.0	3.9	3.8	3.8	4.0	3.9
Fiber	7.2	7.5	7.0	7.1	7.7	7.5	7.3	7.7
Ash	2.1	2.3	2.4	2.7	2.5	2.7	2.5	2.7
Calcium	0.16	0.15	0.37	0.40	0.40	0.40	0.41	0.42
Phosphorus	0.34	0.32	0.33	0.34	0.33	0.33	0.33	0.33
Sodium	0.06	0.11	0.06	0.11	0.05	0.12	0.06	0.12
Potassium	0.43	0.55	0.44	0.56	0.43	0.56	0.42	0.54

ganic phosphorus, sodium, potassium, hemoglobin and hematocrit were determined.[8]

Trial 3. Forty-eight wether lambs of Northwest origin were randomly divided into six treatment groups of eight lambs each. The lambs were group-fed ad libitum on the rations shown in table 5 for a period of 70 days. The chemical analyses of these rations are given in table 6. The lambs were weighed at biweekly intervals and were housed by treatment group in 3 × 4 meter stalls containing self-feeders and automatic waterers. The lambs had access to adjacent outside lots. Plasma calcium and inorganic phosphorus were determined [9] at zero, 7, 14, 21 and 70 days. The right metacarpal bone from all lambs was removed at time of killing. The length was measured and the bone divided into two equal lengths; it was dried, extracted with petroleum ether, weighed and dry-ashed in procedures similar to those described by Benzie et al. (9).

Trial 3-A. An additional eight lambs were divided into two subgroups to study the effect of ration calcium and phosphorus level on phosphorus retention and excretion. One subgroup received, in sequence, rations 1, 3 and 5; the other, rations 2, 4 and 6 (table 5). The lambs were placed in metabolism crates to facilitate urine and feces collection. For each ration tested, feed intake was determined by the maximum amount of ration each sheep would consume on each of 3 consecutive days before the collection period. This level of feed intake was maintained throughout the remainder of the trial. All animals

[8] See footnote 7.

[9] See footnote 7.

TABLE 5

Composition of rations [1] used (trial 3)

Ingredient [3]	Ration no.[2]					
	1	2	3	4	5	6
Cornstarch	9.95	9.38	9.45	8.88	8.95	8.38
Limestone	0.20	0.77	0.20	0.77	0.20	0.77
Na_2HPO	—	—	0.22	0.22	0.45	0.45
K_2HPO_4	—	—	0.28	0.28	0.57	0.57
Trace mineral [4]	+	+	+	+	+	+
Vitamins A and D [5]	+	+	+	+	+	+

[1] Kilogram of ingredients per 100 kg ration.
[2] Ration numbers shown have prefix 502–66–000–.
[3] All rations contained: (in percent) corn, 60; solvent soybean meal, 2.8; cottonseed hulls, 15; urea, 262, 1.3; corn oil, 0.3; cane molasses, 10; and iodized salt, 0.45.
[4] A mixture containing 4.405 g $MnSO_4 \cdot H_2O$; 0.991 g $CuSO_4 \cdot 5H_2O$; 0.441 g Fe_2O_3; 43.81 g sulfur; 43.81 g methionine; 1.764 g ZnO; and 23.6 $CoCO_3$/100 kg ration.
[5] Two and two-tenths grams per kilogram of mixture. Contained 400 IU vitamin D_3 and 2500 IU vitamin A/gram.

TABLE 6

Chemical analyses of rations used (trial 3)

Ingredient	Ration no.[1]					
	1	2	3	4	5	6
%						
Crude protein	12.3	12.0	12.0	12.3	12.4	11.8
Dry matter	92.7	92.4	92.0	94.1	93.2	92.7
Nitrogen-free extract	66.9	66.0	66.2	67.0	65.9	65.9
Fat	3.3	3.2	3.2	3.2	3.3	3.1
Fiber	7.3	7.6	7.3	7.8	7.6	7.7
Ash	2.9	3.2	3.3	3.8	3.8	4.2
Calcium	0.22	0.41	0.19	0.44	0.20	0.46
Phosphorus	0.22	0.21	0.33	0.33	0.44	0.42
Sodium	0.27	0.28	0.29	0.29	0.36	0.42
Potassium	0.68	0.68	0.80	0.84	0.90	1.01
Magnesium	0.13	0.13	0.13	0.13	0.13	0.13
Copper, ppm	6.10	5.85	5.67	6.02	6.60	5.50

[1] Ration numbers shown have prefix 502–66–000–.

were fed twice daily and water was available at all times. Twenty-four-hour total collections of urine and feces were made for 3 consecutive days, after which the ration was changed. Four days were allowed on the new ration before the collection sequence was repeated. The phosphorus contents of urine, feces and feed were determined as described by Hawk et al. (10).

RESULTS

Effect of ration calcium and phosphorus on growth and performance

Trial 1. The growth curves of lambs on the four treatments are shown in figure 1. The lambs which received the basal ration (ration 1, 0.16% Ca, 0.35% P) had a lower rate of gain during both phases of the test, and this difference became greater with time ($P < 0.05$). Gains of lambs fed ration 2 (0.19% Ca, 0.37% P) were greater than those of the basal group during phase 1 of the feeding trial, but not in phase 2. This response may have been due to the slightly higher calcium level in their ration, or to their increased feed intake during phase 1, or both. Stiffness and general unthriftiness were noted in the lambs in these two groups during phase 2 of the feeding trial. Weight gains and general performance improved when the sources of inorganic phosphate were removed from the basal ration and $CaCO_3$ was added to give a Ca:P ratio of 1 (ration 3, 0.31% Ca, 0.31% P). When performance for the entire 196-day period was considered, those lambs which received ration 4 (0.59% Ca and 0.36% P) gained faster than other lambs. They did not exhibit any

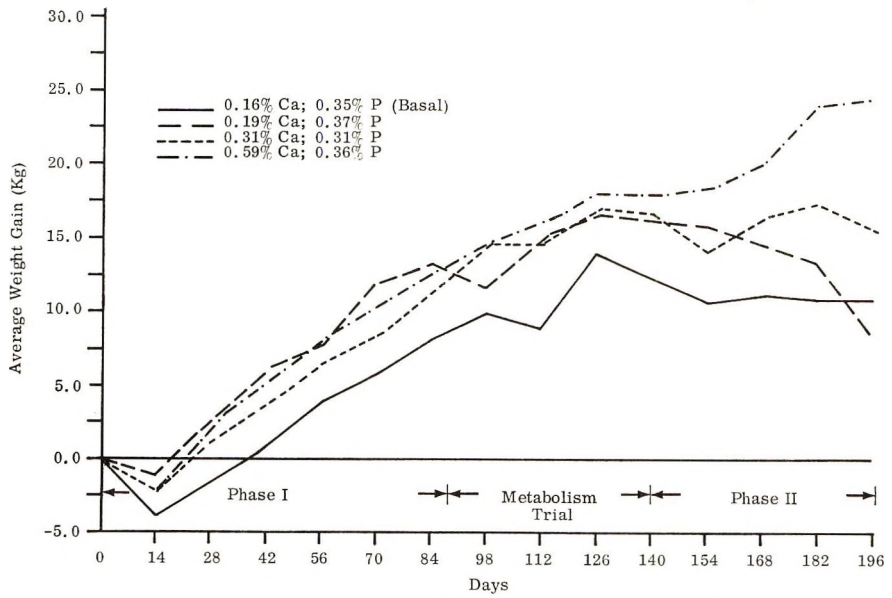

Fig. 1 Growth curves of lambs fed the basal ration and the supplemental calcium (trial 1).

TABLE 7

Performance of lambs [1] *(trial 2)*

Treatment no.	Final wt	Daily gain	Daily feed	Gain/ Feed	Mortality
	kg	*g*	*kg*	*g/kg*	%
9 (basal)	47.8	190	1.11	171	0
10 (9+sodium, potassium)	48.6	168	1.01	166	25
11 (basal+organic calcium)	52.9	242	1.08	224	12.5
12 (11+sodium, potassium)	52.8	230	1.03	223	37.5
13 (basal+lime)	53.3	232	1.15	202	0
14 (13+sodium, potassium)	53.7	222	1.16	191	12.5
15 (basal+$CaCl_2$, $CaCO_3$)	53.8	246	1.18	208	0
16 (15+sodium, potassium)	52.6	225	1.08	208	25

[1] One hundred twenty days on test, individually fed.

signs of stiffness. Average daily gain and feed efficiency were improved in phase 1 of the feeding trial by all additions to the basal ration ($P < 0.05$). Only the addition of $CaCO_3$ to give a Ca:P ratio of 1.5:1, however, resulted in an improvement in these criteria during phase 2 ($P < 0.05$). Of the lambs killed at the end of feeding phase 1, those which received the basal ration were lighter in weight ($P < 0.05$) and yielded a carcass of inferior quality ($P < 0.05$) when compared with those on the three other rations. No gross lesions or abnormalities were noted in any of the lambs killed. Three lambs died during the latter feeding phase, two from the group which received the basal ration and one from the group which received the basal ration plus NaCl.

Trial 2. The average performance of the lambs on the different treatments [10] is summarized in table 7. The lambs which received supplemental calcium consistently gained more throughout the experimental period than those fed the basal [11] ration ($P < 0.05$). Furthermore, the dif-

[10] The following calcium sources were used: 1) that coming from the basal ration only (rations 9 and 10); 2) basal plus equimolar portions of calcium gluconate and calcium acetate (rations 11 and 12); 3) basal plus $CaCO_3$ (rations 13 and 14); and 4) basal plus an equivalent amount of calcium from $CaCl_2$ and $CaCO_3$ (rations 15 and 16). An equimolar mixture of sodium and potassium carbonates and bicarbonates were added to each even-numbered ration to give a ration potassium level of 0.6%.

[11] The basal ration contains the same kind and level of ingredients as used in trial 1.

ference in weight gain between the supplemented and nonsupplemented lambs became more pronounced with time. Although there was little difference in feed consumption, the lambs which received supplemental calcium gained faster and made more efficient gains ($P < 0.05$) than those which received the basal ration.

There was a small, but consistent adverse effect on weight gain and feed efficiency when mixtures of sodium and potassium carbonates and bicarbonates were added to the rations. This effect on weight gain was significant ($P < 0.05$) when sodium and potassium were fed in conjunction with the basal ration.

Two lambs receiving $CaCl_2$, $CaCO_3$, sodium and potassium died within 30 days after the experiment was initiated. Seven other deaths occurred between 50 and 120

TABLE 8

Performance of lambs [1] *(trial 3)*

Lot	Calcium	Phosphorus	Final wt	Daily gain	Daily feed	Gain/ Feed	Plasma inorganic phosphorus		Mortality	Number with kidney damage
							Day 0	Day 70		
			kg	*g*	*kg*	*g/kg*				
1	0.22	0.22	54.3	284	1.56	182	7.3	8.6		1
2	0.41	0.22	57.9	307	1.65	186	7.9	9.3		1
3	0.19	0.33	53.0	247	1.63	151	7.1	10.4	1	1
4	0.44	0.33	54.9	270	1.60	169	7.1	10.4		1
5	0.20	0.45	54.3	258	1.68	153	8.2	10.8	1	3
6	0.46	0.45	55.2	264	1.73	153	7.7	8.9	2	3

[1] Seventy days on test, lot fed.

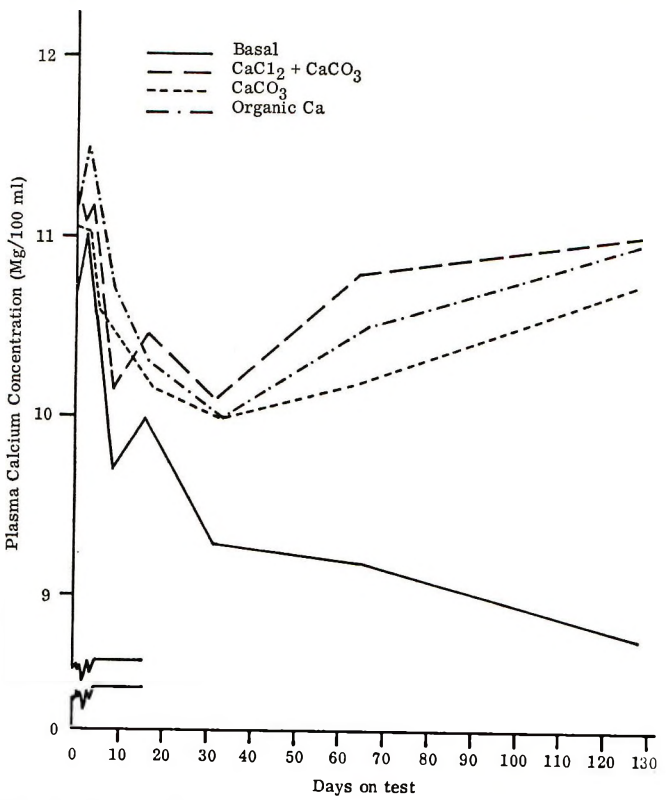

Fig. 2 Effect of ration calcium level on plasma calcium (trial 2).

days on test, with the highest loss (three) occurring in the group which received the calcium acetate and calcium gluconate mixture with supplemental sodium and potassium. Eight of the nine lambs that died during the experiment received supplemental sodium and potassium. Only one lamb that received $CaCO_3$ died. Stiffness, lack of appetite and alertness, and an arching of the back were noted in the animals that died, as well as in several animals from the basal-ration treatments which did not die or have any gross lesions at slaughter. The postmortem condition common to most lambs was a blockage of the urinary tract by uroliths or by a sludgelike plug in the urethra. Organic calcium was apparently satisfying the tissue needs for calcium but did not maintain the proper conditions in the urine to prevent sludge formation. A qualitative analysis of the sludge indicated it was composed largely of a magnesium-ammonia-phosphate complex with traces of calcium. No other gross lesions were noted.

The lambs fed rations containing sodium and potassium carbonate-bicarbonate mixtures suffered the highest mortality rate, regardless of calcium source ($P < 0.01$). This was probably because the urine pH of these animals was higher and favored the precipitation of minerals in the urinary tract.

Trial 3. The data shown in table 8 indicate that the greatest 70-day weight gain ($P < 0.05$) was obtained in lambs which received ration 2 (0.41% Ca, 0.22% P).

Four deaths occurred in trial 3; one each from the group which received rations 3 (0.19% Ca, 0.33% P) and 5 (0.20% Ca, 0.45% P), and two which received ration 6 (0.44% Ca, 0.45% P). Deaths were due to blockage of the urinary tract by calculi, or sludge, or both. Ten lambs had mineral deposits in the kidney when killed; of these, one came from each group fed

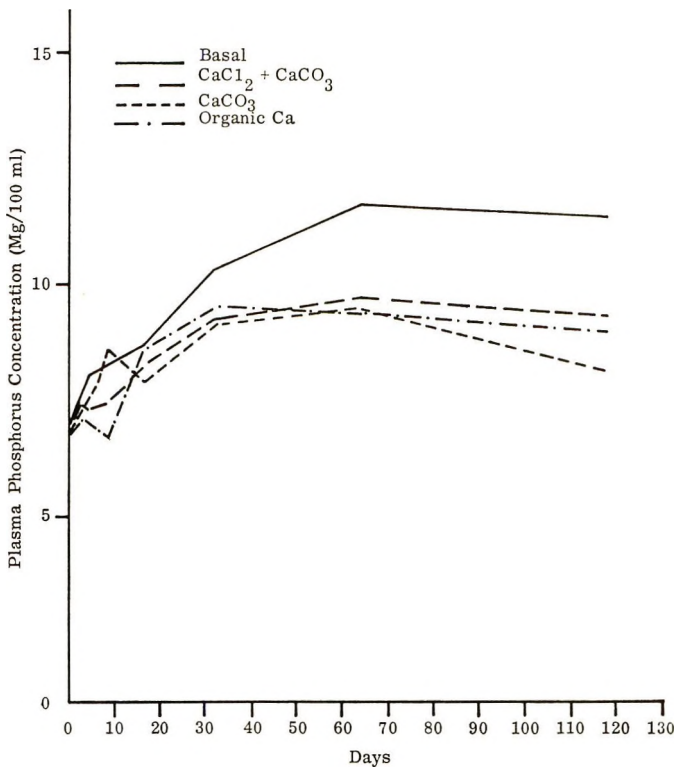

Fig. 3 Effect of ration calcium level on plasma inorganic phosphorus (trial 2).

rations 1, 2, 3 and 4 and three came from each group fed rations 5 and 6.

Effect of ration calcium and phosphorus on certain blood constituents

Trial 2. The plasma calcium level of the lambs fed the basal ration decreased throughout the experimental period whereas the plasma inorganic phosphorus and magnesium concentrations increased ($P < 0.05$) for 64 days and then plateaued. In lambs fed supplemental calcium, there was a slight increase in plasma inorganic phosphorus (fig. 3) and magnesium (fig. 4), and a decrease in plasma calcium (fig. 2) by day 8 on test. These differences continued until day 32; subsequent changes which occurred in plasma mineral content were, for the most part, in the direction of a gradual return toward initial levels. No consistent treatment differences in plasma sodium and potassium levels or in mean hemoglobin and hematocrit levels were noted.

Trial 3. Although not statistically significant, a small increase over the initial concentration was observed in plasma inorganic phosphorus at 70 days. Dietary calcium level had very little effect on plasma inorganic phosphorus concentration, except when fed in conjunction with 0.45% P. There was an indication, however, that dietary phosphorus level affected plasma phosphorus concentration. The greatest increase in plasma inorganic phosphorus over the initial values ($P < 0.05$) was noted in lambs which were fed the 0.33% P rations (3.3 mg/100 ml when 0.33% P was fed versus 1.4 mg/100 ml when 0.22% P was fed). In trial 3-A, the plasma inorganic phosphorus concentration increased from 8.18 mg/100 ml when

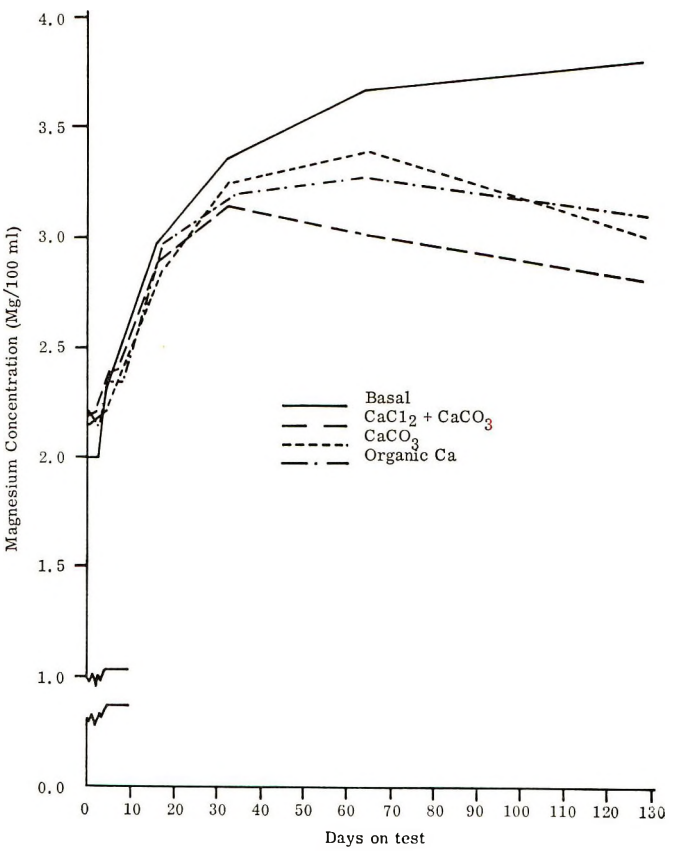

Fig. 4 Effect of ration calcium level on plasma magnesium (trial 2).

0.22% P rations were fed to 10.48 mg/100 ml when 0.33% P rations were fed, and decreased to 8.58 mg/100 ml when the rations were changed to those containing 0.45% P.

Effect of ration calcium and phosphorus level on bone

Trial 3. The metacarpal bone weight, percentage ash and mean thickness of the bone shaft were greater on 0.4% Ca than on 0.2% Ca rations ($P < 0.05$). The highest values for all three criteria were obtained when the ration containing 0.41% Ca and 0.22% P was used (ration 2). The mean values of the criteria used to measure differences in bone development, however, do not reflect the great variation noted in the metacarpal after ashing. The ashed bones from several lambs which received low calcium and high phosphorus were so fragile they could not be handled without breaking apart and were obviously more porous than those from corresponding treatments which received 0.41% Ca in the ration. Figures 5 and 6 illustrate the extremes noted in the ashed bone.

Effect of dietary calcium and phosphorus on urine phosphorus excretion

Phosphorus intake and excretion in feces and urine at each level of dietary phosphorus and calcium are shown in figure 7. Phosphorus intake was somewhat higher at each level of dietary phosphorus when 0.44% Ca was fed. The amount of phosphorus excreted in the urine, however, was less in those lambs which received the

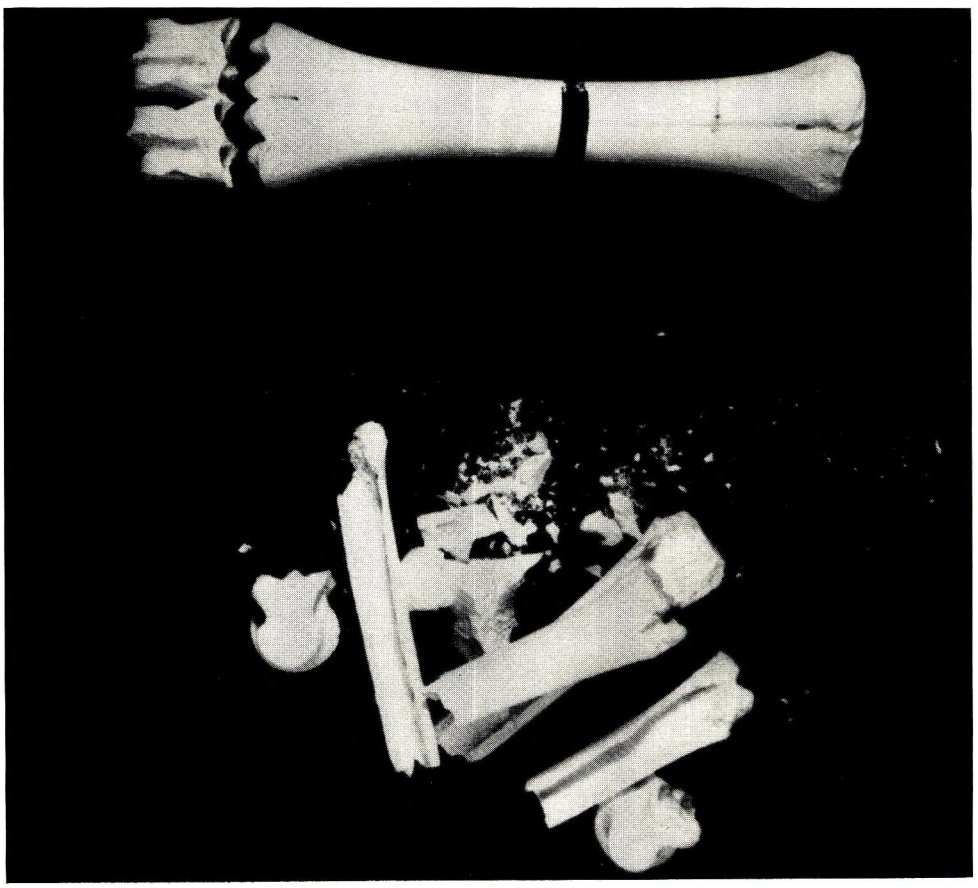

Fig. 5 Illustration of extremes noted in bone ash from lambs in trial 3 which received rations containing 0.20% Ca and 0.45% P (top), and 0.44% Ca and 0.45% P (bottom).

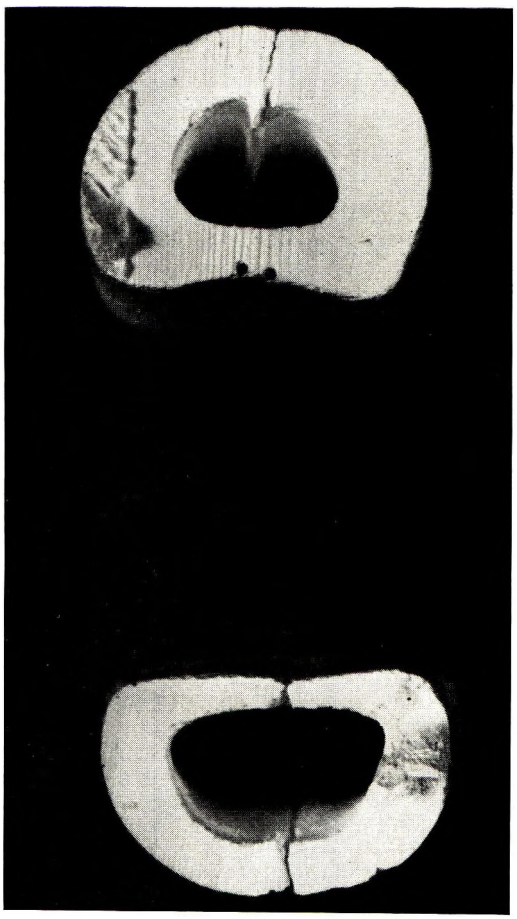

Fig. 6 Illustration of extremes noted in thickness of metacarpal shaft from lambs in trial 3 which received rations containing 0.20% Ca and 0.45% P (top), and 0.44% Ca and 0.45% P (bottom).

higher level of calcium, except when it was fed with 0.45% P ($P < 0.05$).

Phosphorus retention was highest in lambs receiving 0.33% dietary P and lowest when 0.45% P rations were fed ($P < 0.05$). Most of the decrease in phosphorus retention with the 0.45% P rations could be accounted for by an increase in urinary phosphorus excretion ($P < 0.05$).

The amount of phosphorus excreted in the urine was decreased when 0.44% Ca was fed in conjunction with the two lower dietary phosphorus levels ($P < 0.05$); however, the amount excreted in the urine increased slightly when the higher calcium level was fed in conjunction with 0.45% P. Fecal phosphorus was higher on the higher calcium and phosphorus rations.

Figure 8 shows the relationship of plasma inorganic phosphorus concentration to urinary phosphorus concentration during the 20-day experimental period. Phosphorus concentration of both plasma and urine was lower when 0.4% Ca rations were fed.

DISCUSSION

The results of these trials indicated that there was a growth response and an overall improvement in lamb performance when supplemental calcium was added to a basal ration containing 0.16% Ca and 0.33% P. This compares with the minimum requirements of 0.17% Ca and 0.15% P for fattening lambs (7) and 0.20% Ca and 0.20% P for finishing short yearlings (8). More recent estimates (11, 12) are slightly higher but appear to be grossly inadequate when viewed against the background provided by the initial field cases and the data obtained in our studies. With our present knowledge, no one calcium level can be recommended. When all observations are considered, the best performance during the three trials was obtained in the presence of 0.6% Ca, 0.36% P (trial 1); 0.4% Ca, 0.33% P (trial 2); and 0.4% Ca, 0.2% P (trial 3). In each of the above situations, however, the performance of lambs was not optimal for all criteria observed. The major feature to be emphasized is that performance was always unsatisfactory when the Ca:P ratio was 1:1 or less, and that high levels of phosphorus generally accentuated the difficulties observed. Most rations formulated from grain, oil meals and hays grown on fertilized soil will supply phosphorus in excess of NRC requirements, and high grain rations will contain at least 0.3% P. As a first step, it is suggested that sufficient calcium be added to provide an intake of at least 1.3 times the phosphorus intake, and a ratio of 2:1 may be advisable. Presumably, the levels needed will be modified by vitamin D status, length of feeding period and endocrine status of the animal.

The addition of 0.5 to 1.0% $CaCO_3$ often corrected the field conditions in cattle. Many functions of calcium that have been elucidated help establish it as a primary factor in the etiology of the field

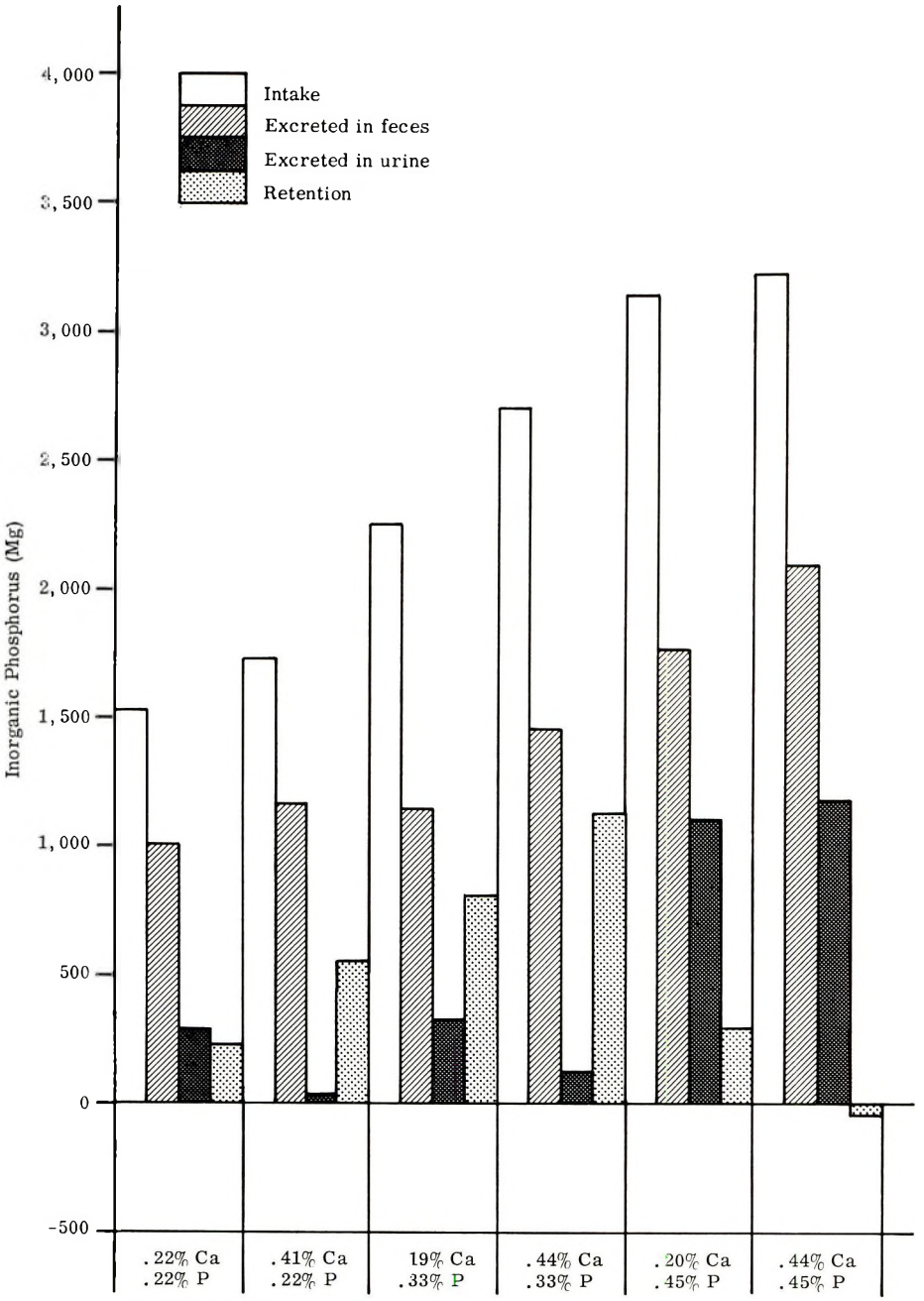

Fig. 7 Phosphorus balance as influenced by ration calcium and phosphorus levels (trial 3).

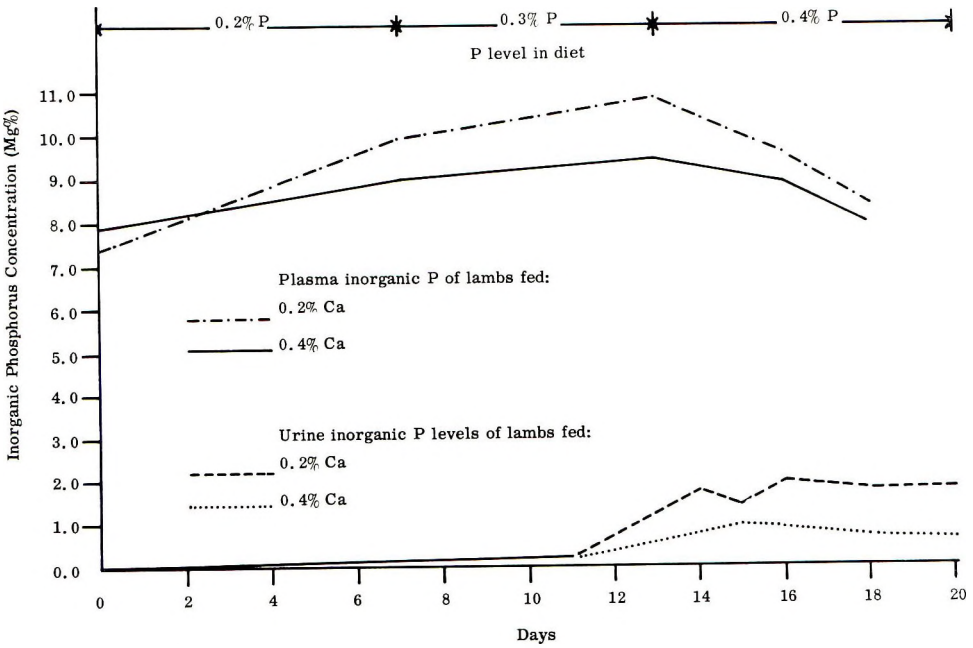

Fig. 8 Influence of calcium and phosphorus levels on plasma inorganic phosphorus and urinary phosphorus (trial 3-A).

condition and in the syndrome observed when the basal ration was fed. Calcium has been shown to have a role in the activation of certain enzymes, including lipase, succinic dehydrogenase, adenosine triphosphatase and some proteases. This could account, at least in part, for the increased weight gain noted when supplemental calcium was offered. The failure of lambs receiving 0.44% Ca and 0.45% P to retain phosphorus (trial 3-A) suggests that less phosphorus was being used by these lambs.

The stiffness noted in the lambs may have been due to soreness from bone resorption, or an effect on the neuromuscular system. Campbell (13) reported that dogs were reluctant to move about after being fed a diet low in calcium and vitamin D. Nocenti (14) summarized the basic role of Ca^{2+} concentration in determining the excitability of nerve and muscle.

An increase in plasma inorganic phosphorus and magnesium from the initial values was noted in most lambs whereas the plasma calcium decreased. Generally, the greatest increase in plasma inorganic phosphorus occurred in those sheep with the lower plasma calcium values. This increase in plasma inorganic phosphorus at the end of the experimental period was due in part to the high level of phosphorus in the rations (plasma data from trial 3-A) and to the resorption of bone (figs. 5 and 6) which was occurring to maintain plasma calcium level (15–19). There is ample experimental evidence that blood calcium is lowered in hypoparathyroidism and raised in hyperparathyroidism, but the mechanism by which this is achieved is poorly understood (20, 21). Though some researchers believe that only the plasma calcium concentration determines parathyroid activity (22), others believe an increase in plasma inorganic phosphorus is a further stimulus to the parathyroid glands to decrease tubular reabsorption of phosphorus (23, 24). The data obtained from trial 3-A suggest that the plasma phosphorus must reach some as yet undetermined threshold before there is a significant increase in urinary phosphorus and concomitant decrease in plasma inorganic phosphorus.

The ostensible inability of the parathyroid to function in the manner described above in the case of the lambs in trial 2

may have been due to: 1) hypofunction of the glands; 2) the activation of a control mechanism after a certain amount of bone has been resorbed, which prevents the total disappearance of bone, no matter how great the stimulus for resorption (25); or 3) the high phosphorus content of the plasma could have lowered the plasma calcium through some as yet undetermined mechanism. Salvesen et al. (26) reported that overloading the circulatory fluids quickly with inorganic phosphorus, given either parenterally or orally, resulted in rapid decline of the serum calcium concentration. Pfander et al. (27) reported similar results when phosphate was infused intravenously in sheep. A smaller amount in the ration fed over a long period of time may have the same effect. This effect may be mediated through calcitonin or a calcitonin-releasing factor (21, 28, 29).

The probable explanation of the increased mortality rate from sludge and calculi by the addition of the sodium-potassium carbonate-bicarbonate mixture is its effect on urine pH. The formation of calculi and sludge in these experiments occurred at much lower levels of phosphorus intake than those used by Emerick and Embry (5, 6), but the favorable effect of supplemental calcium is similar to the South Dakota results. Although the acid-base economy of the ruminant is intimately associated with events which take place in the rumen (30), the renal mechanism is an important regulator of the concentration of H^+ and the excretion of "fixed base" in the urine.

The increased intake of sodium and potassium would tend to reduce renal tubule exchange of H^+ and Na^+ ions (31), increasing the amounts of phosphate being excreted in the form of Na_2HPO_4 instead of NaH_2PO_4. Urinary phosphate compounds would be less soluble in urine with a higher pH. The increased magnesium excretion on low calcium rations would increase the probability of urolith or sludge formation, or both, from Mg^{2+} and HPO_4^{2-}. The additional urinary phosphorus resulting when high phosphate rations are fed, as indicated by the results of trial 3-A, would contribute to the adverse situation.

At least two interrelationships between calcium and phosphorus in the intestine have been reported. McCance (32) found that excess dietary phosphate reduced calcium absorption and Albright and Reifenstein (33) found that dietary calcium may limit the absorption of phosphorus from the gastrointestinal tract. Some of the effects of a Ca:P ratio less than 1:1 which were responsible for the field condition and were reproduced in detail in the laboratory recall the reports of Kintner and Holt (34) and Kintner (35) with horses.

The favorable effect of organic calcium sources and $CaCl_2$ on growth merit additional attention, especially if their contribution to increased sludge formation could be overcome.

ACKNOWLEDGMENT

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Effect of Prior High Protein Intake on Food Intake, Serine Dehydratase Activity and Plasma Amino Acids of Rats Fed Amino Acid-imbalanced Diets [1]

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ABSTRACT Relationships among food intake, growth, liver serine-threonine dehydratase activity (S-TDH) and plasma amino acid concentrations were studied in rats adapted to low or high protein diets at intervals after feeding them imbalanced diets containing 5% casein and 6, 12 or 18% of an amino acid mixture devoid of histidine (AA—His). When rats adapted to the low protein diet were fed the 6% AA—His-imbalanced diet, S-TDH was low; total plasma amino acid concentrations increased; plasma histidine, food intake and growth decreased. As time progressed S-TDH increased slowly; plasma serine plus threonine concentration fell; food intake and growth began to rise. When rats adapted to the high protein diet were fed the 6% AA—His-imbalanced diet, S-TDH decreased rapidly, but food intake remained constant until S-TDH had decreased considerably and plasma serine plus threonine had risen. When rats adapted to the high protein diet were fed the more severely imbalanced diets their diminishing ability to degrade amino acids was apparently exceeded; plasma amino acids rose; food intake and growth decreased. Both alterations in food intake and amino acid-degrading capacity appear to contribute to the ability of the rat to adjust to dietary imbalances of amino acids.

The physiological and biochemical responses of growing rats to a dietary imbalance of amino acids, commonly created by the addition of an amino acid mixture devoid of one indispensable amino acid to a low protein diet, include depressions in food intake and growth (1–5), a sharp decrease in the plasma concentration of the amino acid which is most limiting in the diet and elevated concentrations of other amino acids (1, 3, 4), and a preference for a protein-free or balanced-protein diet over the imbalanced diet when the rat is offered a choice between them (2, 6, 7). From these observations the hypothesis was proposed that an amino acid imbalance, by causing the plasma amino acid pattern to resemble that of an animal fed a much more deficient diet, may trigger appetite depression (8). If the altered plasma amino acid pattern serves directly as a signal for appetite depression, one would expect restoration of normal food intake to be associated with a change in the plasma amino acid pattern toward that of the rat fed a balanced-protein diet.

Because the food intake and growth rate of rats fed an imbalanced diet in a cold environment were not depressed (9–12) and the plasma amino acid pattern of rats fed a diet in which the imbalance was produced by only 1% of amino acids was similar to that of rats fed the control diet (10), Klain et al. (9) and Klain and Winders (10) suggested that rats in a cold environment may preferentially degrade the imbalancing portion of the amino acid mixture. There is some evidence that food intake depression is alleviated by treatments that enhance the ability of the rat to degrade amino acids. Food intake of rats consuming a low protein diet and having only a limited capacity for amino acid degradation (13–17) falls when they are fed a high protein diet, and this is associated with elevated plasma amino acid concentrations (13). Within a short time, their

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food intake returns to normal; this occurs in conjunction with a rising ability to degrade amino acids and decreasing plasma amino acid concentrations (13). Growth and food intake depressions in rats fed an imbalanced diet are smaller if the animals have previously been fed an adequate or high protein diet or if the balanced-protein content of the imbalanced diet is increased (18–21). Also, rats adapted to a high protein intake and offered a choice between a protein-free and an imbalanced diet consume 75% of their total intake as the imbalanced diet (7). If alleviations of the effects of the imbalanced diet on food intake, growth rate and food preference are related to the ability of the rat to degrade amino acids and to reduce concentrations of amino acids in the plasma, the gradual improvement with time in food intake and growth rate of rats fed an imbalanced diet might be similarly explained (22, 23). On the other hand, assuming that food intake depression is the result of a certain degree of abnormality in the plasma amino acid pattern, different amounts of food would be required to produce this degree of abnormality depending upon the capacity of the animal to metabolize amino acids.

To investigate these possibilities, associations among food intake, growth, the activity of serine-threonine dehydratase (S-TDH) and plasma amino acid concentrations were investigated in rats allowed to adjust to either low or high protein diets before being offered an imbalanced diet. Serine-threonine dehydratase was selected as being representative of enzymes that increase in activity under conditions in which amino acid catabolism is enhanced (14, 24–29). The results indicate that although growth and food intake of rats fed an imbalanced diet improved with time, the plasma amino acid pattern remained abnormal.

EXPERIMENTAL

Young male rats of the Holtzman [3] strain were used in both experiments. They were individually housed in suspended wire-bottom cages in a room with a 12-hour light-dark cycle. The rats were offered the diets, prepared as agar-gels (30), and water ad libitum throughout the experiments. The composition of the diets is presented in table 1. Body weight changes and dry matter intakes were recorded daily for each animal.

In experiment 1 rats averaging 83 g in weight were fed the 5% casein diet for 12 days. They were then separated into two groups of equal average weight (116 g). One group of animals was continued on the 5% casein diet and the other group was fed the diet containing 5% casein

[3] Holtzman Company, Madison, Wis.

TABLE 1

Composition of the diets in percent

Ingredient	Diet [1]					
	50% C	5% C	5% C + 6% AA − His	5% C + 12% AA − His	5% C + 18% AA − His	5% C + 18% AA + His
Vitamin-test casein [2]	50.0	5.0	5.0	5.0	5.0	5.0
L-Methionine	0.0	0.3	0.3	0.3	0.3	0.3
L-Threonine	0.0	0.2	0.2	0.2	0.2	0.2
Corn oil	5.0	5.0	5.0	5.0	5.0	5.0
Mineral mixture [3]	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin mixture [3]	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride [4]	0.2	0.2	0.2	0.2	0.2	0.2
Cornstarch	19.6	41.9	38.9	35.9	32.9	32.7
Glucose monohydrate	19.7	41.9	38.9	35.9	32.9	32.8
Amino acid mixture [5]	0.0	0.0	6.0	12.0	18.0	18.0
L-Histidine·HCl·H_2O	0.0	0.0	0.0	0.0	0.0	0.3

[1] C = casein; AA = amino acid mixture; His = histidine.
[2] General Biochemicals, Inc., Chagrin Falls, Ohio.
[3] Rogers, Q. R., and A. E. Harper (30).
[4] Added to the diet as a water solution.
[5] The amino acid premix contained the following: (in percent) L-methionine, 5; L-phenylalanine, 15; L-leucine, 15; L-isoleucine, 10; L-valine, 10; L-lysine·HCl, 15; L-arginine·HCl, 10; L-threonine, 7.5; L-tryptophan, 2.5; and Na acetate, 10.

plus 6% of the amino acid mixture devoid of histidine (6% AA—His; table 1). Plasma amino acid concentrations and the activity of liver serine-threonine dehydratase were determined on days zero, 2, 6, and 14 (five rats per group). In experiment 2 the rats were fed the 50% casein diet for 7 days. They were then separated into groups of equal average weight (115 g in study 1 and 135 g in study 2) and were fed the diets shown in table 1. Plasma amino acid concentrations and liver serine-threonine dehydratase activity were determined on days 2 and 6 in study 1 and on day 13 in study 2 (four or five rats per group). Ten rats in study 2 served as zero day controls.

To obtain blood and liver samples rats were lightly anesthesized with ether during the first 1.5 hours after the beginning of the daylight period at a time when most of them still had food in their stomachs; blood was obtained by heart puncture immediately thereafter and liver was excised, weighed and chilled in 0.14 M KCl.

Plasma amino acid concentrations were determined using an analyzer.[4] Equal quantities of plasma from each rat within a group were pooled and deproteinized with an amount of 15% sulfosalicylic acid to make the concentration in the resulting supernate 3% in sulfosalicylic acid. Since asparagine and glutamine could not be adequately separated from the first four amino acids on the chromatogram, each sample was heated for 3 hours in a sealed ampule with an equal quantity of 3% sulfosalicylic acid. This procedure hydrolyzed the amides and resulted in reasonably good separation of aspartic acid, threonine, serine and glutamic acid.

Serine-threonine dehydratase activity was determined by a modification (13) of the procedure of Freedland and Avery (26) using serine as the substrate.

RESULTS

Growth and food intake of rats adjusted to a low protein intake (5% casein) before they were fed the 6% AA—His-imbalanced diet were greatly depressed within 1 day (exp. 1; figs. 1 and 2). After consuming

[4] Technicon Auto-Analyzer, Technicon Corporation, Ardsley, N. Y.

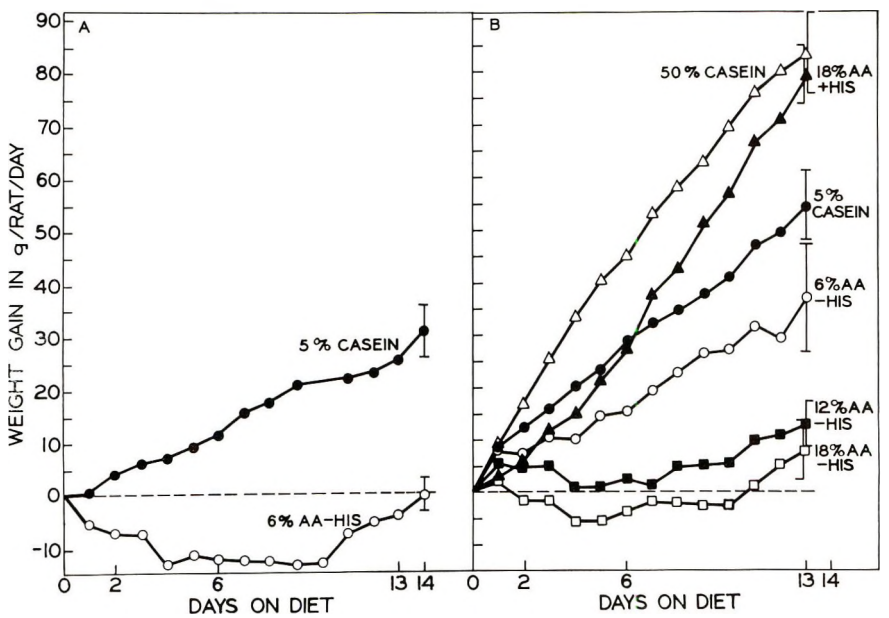

Fig. 1 Growth of rats previously fed 5% casein (A) or 50% casein (B) and then fed the experimental diets. Weight gain (grams per rat per day) is plotted versus days on the diet. Each point represents the average weight gain of five rats. ●, 5% casein; ○, 5% casein + 6% of the amino acid mixture − histidine (AA—His); ■, 5% casein + 12% AA—His; □, 5% casein + 18% AA—His; ▲, 5% casein + 18% AA+His; △, 50% casein (see table 1 for composition of the diets). I, mean ± SE.

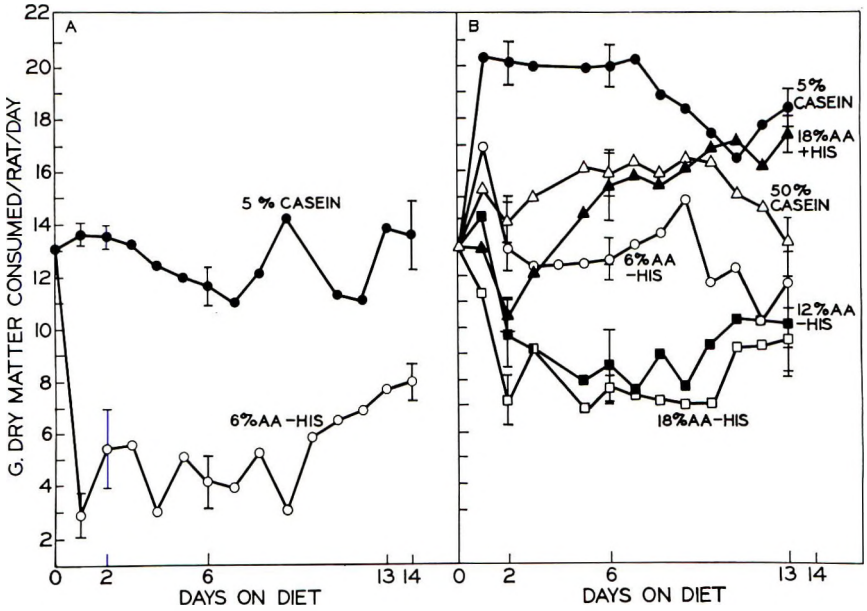

Fig. 2 Food intake of rats previously fed 5% casein (A) or 50% casein (B) and then fed the experimental diets. Dry matter consumed (grams per rat per day) is plotted versus days on the diet. Each point represents the average food intake of five rats. ●, 5% casein; ○, 5% casein + 6% of the amino acid mixture – histidine (AA–His); ■, 5% casein + 12% AA–His; □, 5% casein + 18% AA–His; ▲, 5% casein + 18% AA+His; △, 50% casein (see table 1 for composition of the diets). I, mean ± SE.

the imbalanced diet for 4 days, these rats had lost an average of 12 to 13 g of body weight, but on day 11 their food intake increased, they began to gain weight and continued to gain until the end of the experiment. In contrast, the growth of rats adjusted to a high protein intake (50% casein) before they were fed the 6% AA–His-imbalanced diet was not depressed on day 1 and only slightly on days 2, 3 and 4, after which they grew at a reasonably steady rate (exp. 2). Food intake of these rats was not depressed initially but some decrease occurred from days 9 to 13. Food intake and growth patterns of these animals were similar to those of rats fed the 5% casein diet throughout experiment 1.

In experiment 2 growth and food intake of rats fed the more severely imbalanced diets (12 and 18% AA–His) after they had become adjusted to a high protein intake (50% casein) were depressed within 1 or 2 days and remained depressed until day 11 when both began to increase. Growth and food intake patterns of groups fed the 6% AA–His-imbalanced diet throughout experiment 1 and the 12 and 18% AA–His-imbalanced diets throughout experiment 2 were similar, but the depression was more severe in experiment 1. The food intakes of all three groups fed the imbalanced diets throughout experiment 2 were approximately the same by day 13.

Serine-threonine dehydratase activities of all groups in both experiments are shown in figure 3. The activity of serine-threonine dehydratase in the livers of all groups of rats adjusted to the 50% casein diet and then fed the low protein experimental diets (table 1) decreased rapidly for the first 6 days of experiment 2 regardless of the quantity or quality of the amino acid mixture present in the diet. Between days 6 and 14 enzyme activity decreased more gradually. This pattern of decrease in serine-threonine dehydratase activity with time is similar to that obtained for arginase (31) under comparable conditions. On day 13 the activity of serine-threonine dehydratase in the livers of rats fed the 18% AA–His-imbalanced diet was significantly greater than that of rats fed either the 5%

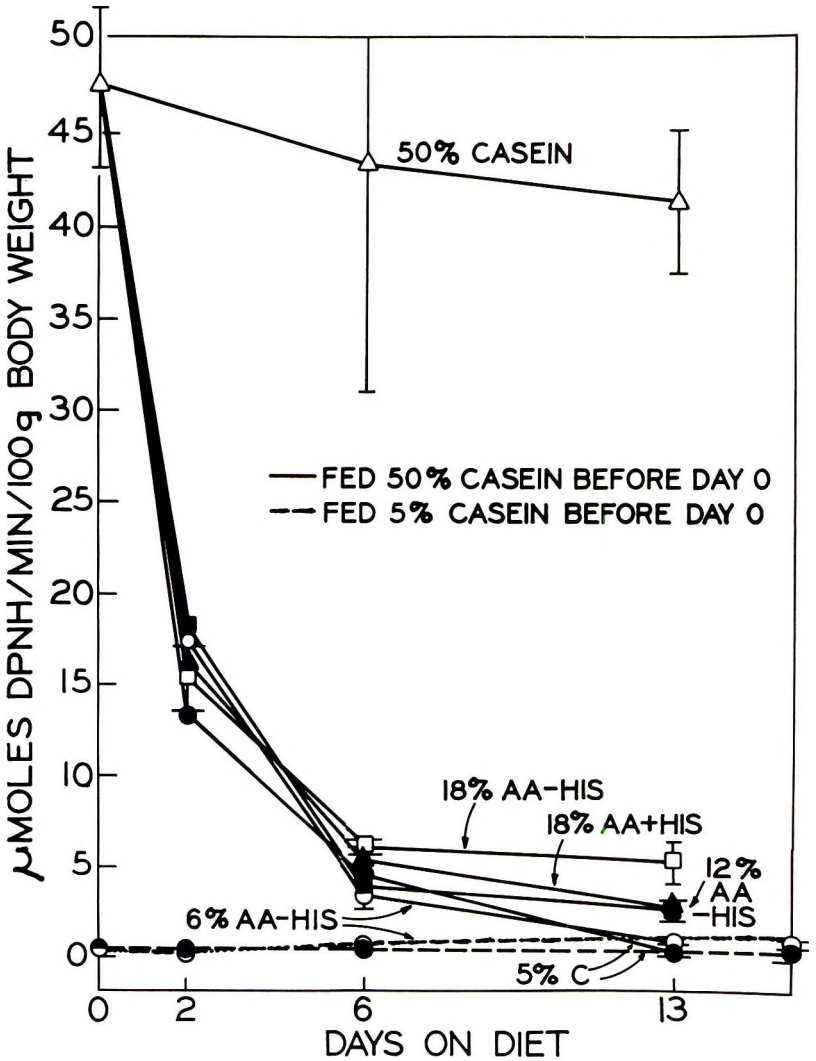

Fig. 3 Liver serine-threonine dehydratase activity of rats previously fed 5% casein(– – –) or 50% casein (——) and then fed the experimental diets. Serine dehydratase activity (μmoles DPNH per minute per 100 g body weight) is plotted versus days on the diet. Each point represents the average serine dehydratase activity of five rats. ●, 5% casein; ○, 5% casein + 6% of the amino acid mixture − histidine (AA−His); ■, 5% casein + 12% AA−His; □, 5% casein + 18% AA−His; ▲, 5% casein + 18% AA+His; △, 50% casein (see table 1 for composition of the diets). I, mean ± SE.

casein diet or the 6% AA−His-imbalanced diet ($P < 0.01$), whereas for rats fed the 18% AA + His-imbalanced diet supplemented with histidine the activity was just significantly greater than that of rats fed the 5% casein diet ($P < 0.05$).

The activity of serine-threonine dehydratase in the liver of rats fed the 5% casein diet remained constant throughout experiment 1. The activity of the enzyme rose about threefold, from 0.32 to 1.02 μmoles/minute per 100 g body weight, between day zero and day 14 ($P < 0.01$) in the livers of rats adjusted to a low protein intake and then fed the 6% AA−His-imbalanced diet, so that by the termination of the experiment the activity was fourfold greater than the value of 0.25 μmole/minute per 100 g

body weight of rats fed the 5% casein diet throughout; the activity at the end of the experiment was also similar to that of rats adjusted to a high protein intake in experiment 2 and then fed the 6% AA—His-imbalanced diet for 13 days.

Figure 4 shows that at the end of experiment 2 there was a direct logarithmic relationship between the intake of amino acids and the calculated capacity of the rat to degrade serine and threonine or threonine. Rats fed the 5% casein diet, the 6, 12 and 18% AA—His-imbalanced diets were theoretically capable of degrading 0.8, 1.8, 3.4 and 6.8 times, respectively, the amount of serine plus threonine that they consumed

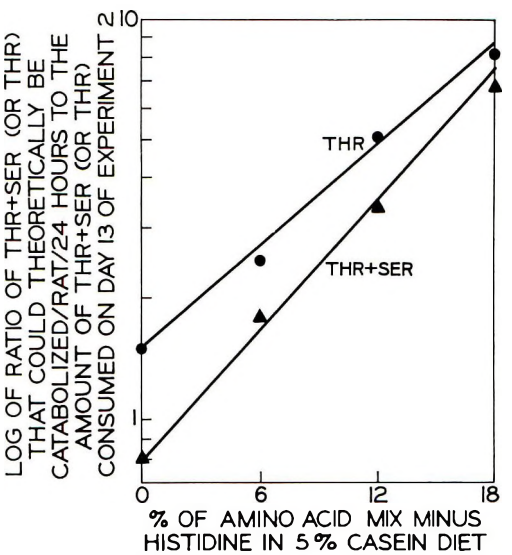

Fig. 4 Effect on serine-threonine dehydratase activity of including increasing increments of the imbalanced amino acid mixture in the 5% casein diet. The ratio of the amount of serine plus threonine or threonine(log of mmoles per rat per 24 hours) that could theoretically be catabolized to the amount of serine plus threonine or threonine consumed on day 13 of experiment 2 is plotted versus the percentage of the imbalanced amino acid mixture included in the 5% casein diet (see table 1 for composition of the diets). Theoretical ability to catabolize serine plus threonine or threonine was calculated from the actual enzyme activity determined on day 13 using the formula: activity/hour/liver × 24 hours. The serine and threonine content of the casein in the diet was estimated using the figures of Orr and Watt (32) and the threonine content of the amino acid mixture was estimated from the unknown composition (table 1, footnote 5). Each point represents the average for five rats.

on day 13. Relative liver sizes for all rats on days 13 and 14 were within the range of 3.4 to 4.1 g/100 g body weight. These differences in liver size would not influence appreciably the values calculated. The calculated values for rats adapted to low or high protein diets were essentially the same after they had consumed the 6% AA—His diet for 13 or 14 days since the value was 2.3 for the group previously fed the low protein diet and 1.8 for those previously fed the high protein diet.

Alterations in the total concentrations of amino acids in the plasma varied with the diet the rats had been fed previously, the subsequent experimental diet fed and the length of time it was fed. In experiment 1 there was a tendency for the concentrations of total amino acids in the plasma of rats that had become adjusted to a low protein intake before they were fed the 6% AA—His-imbalanced diet to rise, and for these concentrations in rats fed the 5% casein diet throughout the experiment to fall (fig. 5). In experiment 2 in which all rats had become adjusted to a high protein intake before being fed the experimental diets, the total amino acids in the plasma of all groups, except those fed the 50% casein diet throughout, decreased sharply during the first 2 days, presumably owing to both the decreased intake of amino acids and the higher degradative enzyme activity in the livers of the animals at that time than later in the experiment (fig. 3). The degree of rise in plasma amino acid concentrations after day 2 varied with the diet. The concentration of total amino acids in the plasma of rats fed the 5% casein diet remained low until day 6, after which it rose at the same time that enzyme activity was still decreasing (fig. 3). In the groups fed the three imbalanced diets the concentrations of total amino acids rose until day 6 but were higher in rats fed the 12 and 18% AA—His diets than in those fed the 6% AA—His diet; thereafter, they continued to increase in rats fed the 6% AA—His-imbalanced diet, remained the same in those fed the 12% AA—His-imbalanced diet and decreased in those fed the 18% AA—His-imbalanced diet. By day 13 when the values for enzyme activity were similar for the different groups of rats in experiment 2 (fig. 3), plasma total amino acid

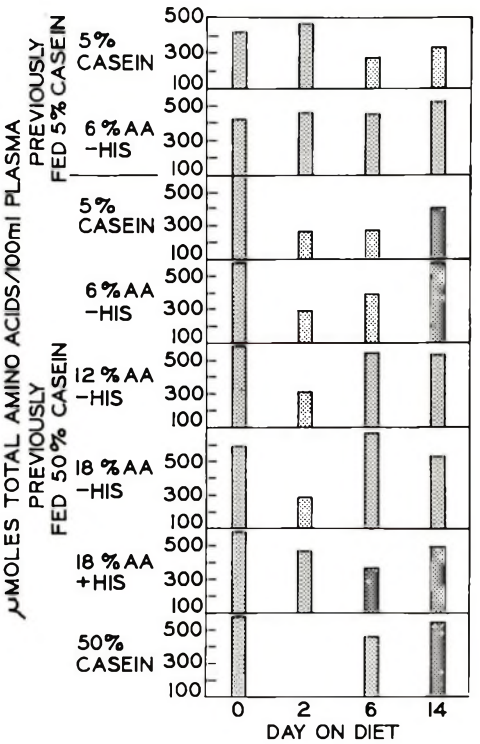

Fig. 5 Plasma concentrations of total amino acids of rats previously fed 5% casein (top two sections) or 50% casein (bottom six sections) and then fed the experimental diets. Total amino acids (μmoles per 100 ml plasma) are plotted versus days on the diet. Each bar represents a pooled plasma sample from five rats. See table 1 for composition of the diets. All diets containing the amino acid mixture also contained 5% casein.

values were also similar and approached that of the group fed the 50% casein control diet. In contrast, the total amino acid concentrations in the plasma of the rats fed the 5% casein diet in both experiments were nearly 200 μmoles/100 ml less than those for the group fed the 6% imbalanced diet regardless of the casein content of the diet fed before initiation of the experiment.

Figure 6 represents an attempt to assess changes in the ability of the different groups in the two experiments to catabolize serine plus threonine by plotting on the various days of blood sampling the ratio of the total amounts of these two amino acids circulating in the plasma to the total amounts ingested by the rat per day. If one can assume that a change in this ratio indicates a change in amino acid degradative ability, the figure appears to support the suggestion that a reduction in the ability of rats fed the imbalanced diet to clear amino acids from the plasma was associated with decreasing food intake while enhancement of this ability was associated with increasing food intake.

The plasma concentration of histidine in rats adjusted to a low protein intake prior to being fed the 6% AA—His-imbalanced diet fell rapidly and on day 6 was 31% of the day zero value (fig. 7); after day 6, at the time growth rate was increasing (fig. 1), plasma histidine concentration rose to 50% of the day zero value. In experiment 2 plasma histidine concentrations of all animals fed the imbalanced diets had fallen by day 2. This probably was a reflection of the greatly reduced histidine intake of each group after day zero. After day 2, histidine concentrations began to rise until by day 13 they were approximately the same for rats fed the three imbalanced diets. Plasma histidine concentrations of groups fed the balanced diets that supported substantial growth rates (fig. 1) were higher by the end of the experiment than those of the groups fed the imbalanced diets that depressed growth rate. Histidine concentrations in the plasma of groups fed the 6% AA—His-imbalanced diet, whether their previous diet contained 5 or 50% casein, were the same on days 6 and 14.

DISCUSSION

Plasma amino acid concentrations change with time after ingestion of a meal and differ with the quality and quantity of the protein in the diet[5] (18, 34–36); however, the values are usually at their maxima by 3 hours and remain relatively constant until about 6 hours after the meal. Comparisons at single intervals during the period of absorption cannot give as accurate a quantitative picture as comparisons made at several intervals over the time period. In most of the studies reported, however, interpretations based on comparisons of values for a single time interval during the absorptive period would not differ greatly from that based on the full curve. Differences among plasma amino

[5] Perez, L., and A. E. Harper, unpublished observations.

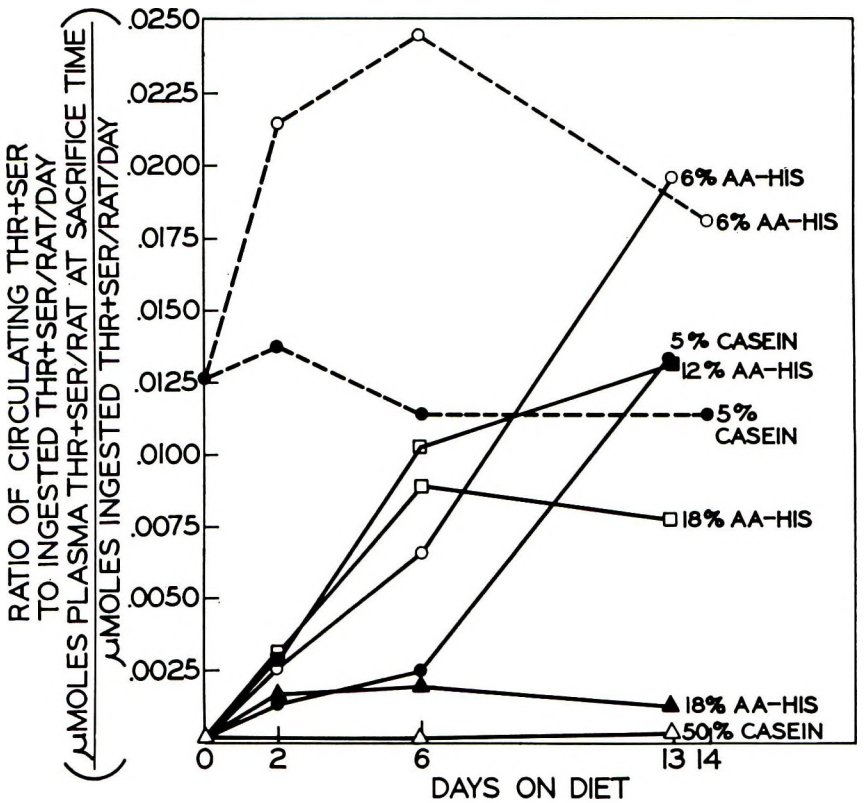

Fig. 6 Ability of the rat to degrade serine plus threonine. The ratio of serine plus threonine circulating in the plasma at time of killing to the ingested serine plus threonine

$$\frac{(\mu\text{moles plasma Thr + Ser/rat at sacrifice time})}{(\mu\text{moles ingested Thr + Ser/rat/24 hours})}$$

is plotted versus days on the diet. The values were corrected for plasma volume using the figures of Fernandez et al. (33). Each point represents the average value for five rats. ●, 5% casein; ○, 5% casein + 6% of the amino acid mixture − histidine (AA−His); ■, 5% casein + 12% AA−His; □, 5% casein + 18% AA−His; ▲, 5% casein + 18% AA+His; △, 50% casein; (– – –), rats fed 5% casein before being fed the experimental diets; (——), rats fed 50% casein before being fed the experimental diets.

acid values for the single interval during the absorptive period in these studies are therefore considered to be representative of differences among groups throughout that period.

The physiological and biochemical responses of the growing rat to a dietary imbalance of amino acids appear to depend upon the pretreatment; rats previously fed a low protein diet responded differently than those previously fed a high protein diet. This suggests that the responses to an imbalanced diet are influenced by the capacity of the animal to degrade amino acids, as affected by the diet fed initially. In animals adapted to a low protein diet both major routes for the utilization of dietary amino acids are limited: growth (13), owing to the low amount of amino acids in the diet; and degradation, owing to low activities of amino acid catabolizing enzymes (13–17). When such an animal is fed an imbalanced diet low in protein and limiting in one indispensable amino acid, the concentrations of the amino acids in excess in the diet, and which cannot be used for protein synthesis (37) or be readily catabolized, tend to rise in the plasma, and concomitantly, food intake and growth decrease (figs. 1, 2, 5). As time

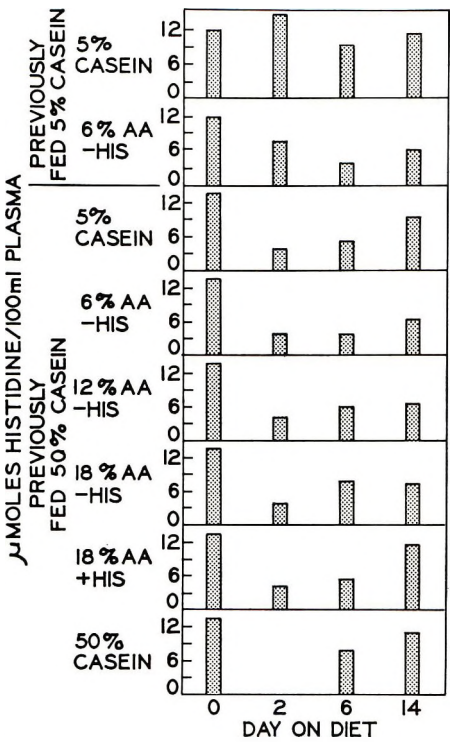

Fig. 7 Plasma concentrations of histidine of rats previously fed 5% casein (top two sections) or 50% casein (bottom six sections) and then fed the experimental diets. Histidine concentration (μmoles per 100 ml plasma) is plotted versus days on diet. Each bar represents a pooled plasma sample from five rats. See table 1 for composition of the diets. All diets containing the amino acid mixture also contained 5% casein.

progresses the rat continues to consume a small amount of the imbalanced diet, the plasma concentrations of amino acids ingested in excess of needs remain elevated, and the activity of serine-threonine dehydratase (fig. 3), taken as an indicator of the behavior of many of the enzymes of amino acid catabolism, begins to rise slowly and may constitute the initial phase of adaptation to the diet. When serine-threonine dehydratase activity has increased above the initial value, the amount of serine plus threonine present in the plasma in relation to that ingested (fig. 6) has decreased and food intake and growth begin to increase.

On the other hand, rats adapted to a high protein diet and growing rapidly (13) are synthesizing considerable amounts of protein and have the ability to catabolize large amounts of amino acids (13, 38–39). When the rat is then fed a low protein, imbalanced diet the influx of amino acids is much less than before and both the concentrations of plasma amino acids and the activity of serine-threonine dehydratase fall (figs. 3 and 5); subsequently, as serine-threonine dehydratase activity continues to decrease, the concentrations of the amino acids in surplus in the diet rise in the plasma; food intake (fig. 2) is constant for a time but also falls after enzyme activity has decreased considerably and serine plus threonine concentration in the plasma have risen. If the amino acid imbalance is severe enough, ingestion of such a diet by the rat adapted to a high protein intake apparently results in so large an influx of amino acids that must be degraded while the animal's ability to catabolize amino acids is rapidly decreasing, that, within a short time its degradative capacity is exceeded, amino acids accumulate in body fluids and coincident with this, its food intake and growth rate decrease (figs. 1, 2, 3). Serine-threonine dehydratase activity continues to decrease with time while only a small amount of the imbalanced diet is being consumed. The concentrations of plasma amino acids and enzyme activity eventually stabilize at values that depend upon the food intake and the percentage of the imbalancing amino acid mixture in the diet. Thereafter, food intake and growth, although remaining low, tend to increase. Evidence that a large quantity of dietary amino acids from either a balanced or from certain incomplete mixtures results in elevated threonine dehydratase activity was obtained by Peraino et al. (40). Thus, alterations in both food intake and in amino acid-degrading capacity contribute to the ability of the rat to cope with a dietary imbalance of amino acids.

Evidence of some adaptation of rats to the 6% AA−His diet in experiment 1 and to the 12 and 18% AA−His diets in experiment 2 was observed as the investigation progressed. Apparently, the ability of these rats to degrade amino acids (fig. 3) was great enough toward the end of the experiments to influence the concentrations of amino acids present in the plasma (figs. 5 and 6) and, associated with this, food con-

sumption was rising (fig. 2). Such increases in amino acid degradative ability with increasing amino acid intake, although small, would be a decided advantage when amino acid-imblanced diets are fed since this would enable the animal to dispose of some of the surplus of dietary amino acids while consuming a quantity of diet that provides enough balanced protein for maintenance and, perhaps, some growth.

In contrast, the apparent protective influence of adaptation to a high protein intake in the present investigation appeared to decline toward the end of experiment 2. The tendency for the total plasma amino acid concentrations (fig. 5) to increase despite the decreased food intake of the animals (fig. 2) toward the end of the 2-week period, coincides with a decreased ability to catabolize amino acids (figs. 3 and 6), and appears to be a reflection of the progressively decreasing ability of these animals to cope with the dietary influx of amino acids.

There was not a consistent correlation between changes in food intake and plasma amino acid concentrations in rats fed the various imbalanced diets in the present investigation. For example, rats adapted to a high protein diet and having high degradative ability were able to consume much more of the imbalanced diet (5% casein + 6% AA—His) throughout the 2 weeks than those adapted to a low protein diet even though the ratios of the concentrations of indispensable amino acids to histidine in the plasma (41 and 36 on day 2, 64 and 67 on day 6 and 56 and 54 on days 13 and 14 for rats previously adapted to high and low protein, respectively) were similar. Growth and food intake of rats adapted to low protein and then fed the imbalanced diet improved gradually toward the end of the study even though the plasma amino acid pattern remained quite abnormal. From a different type of study Peng et al. (18) also concluded that no direct quantitative relationship exists between the plasma concentrations of histidine or total amino acids and food intake in rats fed an amino acid-imbalanced diet.

If changes in plasma amino acid concentrations do trigger an appetite-depressing mechanism, there is no reason to assume that there should be a quantitative relationship between food intake and the concentrations of amino acids in the plasma. Changes in plasma amino acid concentrations depend upon the amount and pattern of amino acids ingested, the ability to utilize amino acids in protein synthesis and the ability to degrade any surplus. Therefore, rats previously fed a high protein diet and having a high capacity to degrade amino acids would be able to consume more of the imbalanced diet than those previously fed a low protein diet before their plasma amino acid pattern showed the same degree of abnormality. As their capacity to degrade amino acids fell, the amount of imbalanced diet to cause this degree of abnormality in the plasma amino acid pattern would be less. This is what was observed. Also, rats previously fed the low protein diet and whose degradative ability increased slowly, would gradually be able to consume more of the imbalanced diet without developing any greater degree of abnormality in their plasma amino acid pattern. This, too, is essentially what occurred. Changes in plasma amino acid patterns could, therefore, still provide the triggering mechanism for food intake depression, but as the ability of the animal to remove amino acids increased, more food would be consumed before this signal of satiety was evoked.

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Iron Restriction in the Nursing Rat: Early effects upon tissue heme proteins, hemoglobin and liver iron [1]

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ABSTRACT Rats were made iron deficient between 10 and 21 days of age by restricting their customary access to nonmaternal sources of iron. The depression of cytochrome c and myoglobin concentrations in skeletal muscle and of cytochrome c in intestinal mucosa became comparable to and, in some instances, was more profound than the concomitant 34% mean decrease in hemoglobin concentration. These conditions did not interfere with normal gain in body weight. Thus, tissue heme protein deficiency was one of the early manifestations of iron depletion during a period of rapid growth. Earlier and more stringent iron deficiency in newborns was also produced by treating the mothers with an iron-chelating agent starting at 16 days of gestation to 21 days postnatally. This treatment reduced the rate of growth of the young and resulted in a more marked diminution in the concentration of hemoglobin. There was little additional effect, however, upon cytochrome c in skeletal muscle or intestinal mucosa in comparison with the mildly iron-restricted group. A significant depression was also observed in heart muscle and in kidney. The concentration of cytochrome c in brain was least affected. The rapid rate of growth and differentiation that characterizes most organs during the newborn period is probably a predisposing factor to a more widespread and rapid tissue heme protein depletion than that observed in adult animals.

Prolonged deprivation of dietary iron reduces the concentration of iron-containing heme proteins in many tissues in humans and experimental animals (1–7). Initially, it was thought that this tissue depletion occurred only with severe or chronic iron deficiency, and that the comparatively small amount of iron in myoglobin and the cytochromes was spared until there was a profound reduction in hemoglobin concentration. Recent data indicate, however, that tissue heme proteins can also be affected rapidly even under mild conditions (8, 9). In man, cytochrome-oxidase activity in the buccal mucosa may be decreased even when there is little depression in concentration of hemoglobin (8). Studies in the growing rat indicate that cytochrome c and myoglobin deficiencies also can result from a brief exposure to a low iron regimen (9).

In the rat, as in man, iron balance is most precarious during the end of the nursing period just before the exogenous sources of iron become available (10). During this period of rapid growth, when iron stores are rapidly utilized, the diet consists almost entirely of milk, a relatively poor source of this trace element. In man, a mild deficiency of iron is common at this age and severe depletion of iron results if there is either an undue delay in the addition of supplementary foods or the iron stores of the newborn have been decreased by such factors as premature birth. Previous studies in man have shown heme protein depletion in two tissues with a rapid rate of cell turnover (11), that is, buccal mucosa (5, 8) and intestinal mucosa (12). Studies of the less approachable solid tissues in man are difficult to justify and prompt the use of an experimental animal whose iron metabolism resembles that of man in many essential respects (10).

Nursing rats begin to open their eyes and become physically more active at

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about 14 days of age. At this time, they are observed to supplement breast milk, their major source of nourishment, with the diet and drinking water provided for the mother. After about 21 days, they rely predominately upon solid food and can be weaned.

The present study is designed to investigate the manifestations of iron deficiency, particularly the response of tissue heme proteins, during the transitional last third of the nursing period when breast milk is not supplemented with other sources of iron. The results of this mild iron restriction are compared with the effects of earlier and more stringent depletion that follows treatment of pregnant and nursing mother rats with the iron-chelating agent, desferrioxamine.[4]

METHODS

Rats of the Wistar strain were placed on several regimens involving iron deprivation during gestation and the nursing period. The iron-poor diet in each case was based upon dried, partially skimmed milk (12% fat)[5] supplemented with a complete vitamin and mineral formula which contained no added iron (13). Drinking water was provided ad libitum in all experiments. Nursing rats were not restricted in their access to the diet and drinking water provided for the dams.

Experiment 1. Mothers with litters of eight male young were provided a stock-pellet diet until the pups were 10 days old. Then they were placed into one of four groups as follows: A) iron-poor diet and distilled water; B) iron-poor diet and 0.5 g ferrous sulfate added/liter of drinking water; C) stock-pellet diet (iron content 197 ppm)[6] and distilled water; and D) iron-poor diet and distilled water; mothers received one intramuscular injection of 2.5 mg of elemental iron as iron dextran[7] when the young were 10 days old. At 21 days of age the rats were killed for determination of liver iron, hemoglobin concentration, myoglobin and cytochrome c.

Experiment 2. The regimen of iron depletion during gestation was instituted as follows: four females at 15 days of gestation were provided the iron-poor diet and distilled water. The iron-chelating agent, desferrioxamine, was administered intramuscularly (50 mg once a day). The injection was omitted on the day of delivery and then resumed until the young were 21 days old. One of the litters was eliminated because it contained only three young. A control group of four females was given the stock-pellet diet and daily injections of saline. Animals from each litter were killed individually at 2, 7 and 12 days of age for determination of liver iron and hemoglobin concentration. In addition to these, cytochrome c assays were done at 21 days.

Experiment 3. A group of four females, each with eight newborn males, was subjected to a series of desferrioxamine injections, as above, starting 1 day after delivery and continuing daily until the young were 21 days old. The low iron diet was provided throughout this period. Four control mothers received saline injections and the stock-pellet diet. An additional group of four mothers was fed the iron-poor diet. All rats had received the stock-pellet diet during gestation. At 21 days of age studies were done on the young as above.

Animals were killed by decapitation and tissues were handled as before (9). Venous hemoglobin was estimated as cyanmethemoglobin (14). Cytochrome c was determined spectrophotometrically by the method of Rosenthal and Drabkin (15). Myoglobin was measured spectrophotometrically after purification by differential precipitation with ammonium sulfate and by column chromatography on carboxymethylcellulose according to the method of Åkeson et al. (16). Hydrolyzable liver iron was determined colorimetrically by the method of Kaldor (17).

RESULTS

Deprivation of exogenous iron during the nursing period (exp. 1). Nursing rats, whose access to exogenous iron was limited between 10 and 21 days of age, had a deficiency of cytochrome c and myoglobin proportional to the decrease in the concentration of hemoglobin. The limitation of iron intake in groups A and D did not re-

[4] Desferol, CIBA Corporation, Summit, N. J.
[5] Dryco, Borden Company, New York, N. Y.
[6] Purina Rat Chow, Ralston Purina Company, St. Louis, Mo.
[7] Imferon, Lakeside Laboratories, Inc., Milwaukee, Wisc.

TABLE 1

Effect of deprivation of exogenous iron during the nursing period upon 21-day-old rats

Group	No.	Weight	Hemoglobin	Total hydrolyzable liver iron	Cytochrome c		Myoglobin [3]
					Muscle [1]	Intestinal mucosa [2]	
		g	*g/100 ml*	*µg*	*µg/g*	*µg/g*	*mg/g*
A (iron-poor diet)	26	45 ± 2 [4]	6.3 ± 0.7 ***	21 ± 1	32 ± 2 **	135 ± 15 **	0.13 ± 0.01 *
B (iron-poor diet and iron-supplemented water)	24	49 ± 1	9.7 ± 0.5	103 ± 6	50 ± 2	270 ± 39	
C (stock diet)	12	43 ± 3	9.4 ± 0.6	38 ± 10	56 ± 4		0.20 ± 0.02
D (iron-poor diet and mother injected with iron)	12	45 ± 3	8.6 ± 0.3	23 ± 2	33 ± 3		

[1] Quadratus lumborum and psoas muscles pooled from three to four rats, four to six separate samples in each group; concentration is expressed on a fresh weight basis.
[2] Concentration is expressed on a dry weight basis.
[3] Muscles of the lower extremities pooled from three to six rats, four separate samples in each group; concentration is expressed on a fresh weight basis.
[4] Mean ± SE of the mean.
* Groups A and C differ ($P < 0.02$).
** Groups A and B differ ($P < 0.01$).
*** Groups A and B differ ($P < 0.001$).

sult in retardation of growth in relation to the animals in control groups B and C (table 1). The total liver iron content in group A was significantly less than in rats allowed free access to the stock-pellet diet (group C). Group B, which had access to drinking water supplemented with ferrous sulfate, had the highest liver iron content. In group A, the concentrations of the heme proteins, hemoglobin, myoglobin and cytochrome c were 30 to 50% less than in groups B or C.

In group D, the mothers had received an injection of iron dextran equivalent to the normal liver iron in an adult female (18). This was intended to compensate for a possible decrease in breast-milk iron related to the short-term exposure to a low iron regimen. Although the mean hemoglobin concentration of this group was somewhat higher than in group A, the liver iron and skeletal muscle cytochrome c concentrations were equivalent.

Severe depletion of iron during gestation and the nursing period (exps. 2 and 3). The combination of a low iron diet and injections of the iron-chelating agent, desferrioxamine, during the last 6 days of gestation (exp. 2) reduced the liver iron content

TABLE 2

Effect of desferrioxamine treatment and the low iron regimen after day 16 of gestation (days 16 to 22) upon individual newborn rats

No. in litter	Hemoglobin	Liver iron
	g/100 ml	*total µg*
	Desferrioxamine group	
7	9.1	19
	8.1	21
8	9.5	25
13	7.0	17
	7.7	19
	8.3 ± 1.1 [1] *	18 ± 1.4 [1] **
	Control group	
10	10.6	39
	10.2	34
7	9.9	31
	8.9	47
11	12.3	40
	10.2	41
13	11.6	25
	10.5 ± 1.3 [1]	37 ± 2.8 [1]

[1] Mean ± SE of mean.
* Control and experimental groups differ ($P < 0.3$).
** Control and experimental groups differ ($P < 0.001$).

TABLE 3

Severe iron deficiency in 21-day-old rats

Exp. no.	No.	Wt	Hemoglobin	Total hydrolyzable liver iron	Cytochrome c [1] in				Brain		
					Muscle	Intestinal mucosa	Heart	Kidney	Total	Cortex	Stem
		g	*g/100 ml*	*μg*	*μg/g*	*μg/g*	*μg/g*	*μg/g*			
2 (Desferrioxamine injections during pregnancy and nursing)	8	23 ± 3 [2] ***	3.2 ± 0.4 ***	13 ± 1 ***	42 ± 3 ***	132 ± 29 ***	153 ± 11 ***	68 ± 4 ***	22 ± 2 ***		
Control	9	45 ± 2	9.7 ± 0.2	80 ± 6	69 ± 8	256 ± 43	229 ± 25	119 ± 9	30 ± 1		
3 (Desferrioxamine injections during nursing period)	24	27 ± 3	4.6 ± 0.3		56 ± 7 *		271 ± 35	89 ± 6 **		30 ± 5	51 ± 7
Control	24	32 ± 2	11.0 ± 0.2		72 ± 5		305 ± 52	110 ± 5		32 ± 4	48 ± 9

[1] Concentration is expressed on a dry weight basis in intestinal mucosa and on a fresh weight basis in all other tissues.
[2] SE of the mean.
* Control and experimental groups differ ($P < 0.1$).
** Control and experimental groups differ ($P < 0.05$).
*** Control and experimental groups differ ($P < 0.01$).

in newborn animals to about one-half of the control values, as shown in table 2. The effect upon hemoglobin concentration in the newborns appeared less marked. One or two animals from each litter were used for liver iron determinations at 7 and 12 days of age to determine the rate of iron depletion with control and deficient regimens. At day 7, hydrolyzable liver iron in the control group ranged from 190 to 380 compared with 4 to 15 μg/g in the deficient group. At 12 days of age the ranges were 50 to 185 and 5 to 20 μg/g, respectively. Data obtained at day 21 are shown in the upper part of table 3. The deficient group was characterized by profound growth retardation, anemia and depletion of liver iron stores. Cytochrome c depletion was observed in skeletal muscle, intestinal mucosa, heart muscle, kidney and brain. The deficiency in the first two tissues, however, was no more severe than observed in the moderately iron-depleted group A (table 1).

Similar results were obtained when desferrioxamine injections were initiated after birth (exp. 3, table 3). The unusually low weight of this control group is unexplained and may be a response to the amount of handling required by the regimen. No depletion of brain cytochrome c was observed in this study. Offspring of four mothers exposed to the iron-poor diet and saline injections (not shown in the table) had a hemoglobin concentration of 7.2 ± 0.5 g/100 ml, or similar to that observed in group A (table 1).

DISCUSSION

The present studies with nursing rats show that a relatively brief period during which dietary intake is limited to breast milk and a cow's milk product results not only in a moderate depletion of hemoglobin but also produces deficiencies in tissue heme proteins often of comparable or greater magnitude. After iron administration, hemoglobin deficiency is repaired rapidly and completely, as hypochromic cells are replaced by newly produced normal red cells. Tissue cytochrome deficiencies in long-lived cells, however, such as skeletal muscle, are more slowly repaired (9) and may have a greater potential for resulting in permanent damage.

In experiment 1, the deficiency of heme proteins in the iron-poor young appeared to be due primarily to lack of supplemental iron from nonmaternal sources. It is doubtful that decreased breast-milk iron, resulting from acute loss of the mother's dietary iron, plays a large role. These results are consistent with the studies of Ezekial and Morgan (18) who showed that breast-milk iron in the rat can be modified only to a minor extent either by iron loading or deprivation.

In experiment 2, the rapid depletion of iron in the offspring of mothers treated with desferrioxamine appeared to be due primarily to the action of this chelating agent rather than to the low iron content of the maternal diet. This is suggested because the animals in experiment 3 exposed to the low iron diet during the entire nursing period were no more anemic than rats on the deficient regimen only between 10 and 21 days (group A, table 1). Desferrioxamine might act directly upon iron metabolism in the young after transport of the drug through the placenta or via breast milk. In addition, it could decrease the iron content of breast milk and the normal (19), rapid placental transport of iron during the last part of gestation. The ultimate influence of desferrioxamine upon hemoglobin synthesis in the young is noteworthy since no effect upon hemoglobin production has been found in the adult rat (20).

Depression of cytochrome c concentration in skeletal muscle and intestinal mucosa was maximal, even after the mild depletion of iron in experiment 1. Conservation of this respiratory enzyme in more severe iron restriction (exps. 2 and 3) appeared to occur at the expense of production of other heme proteins and ultimately at the expense of body growth (2–4, 6, 7, 9).

Heart muscle and brain have been found to be unusually resistant to cytochrome c depletion in older iron-deficient rats (7). The rapid rate of growth and differentiation that characterizes most tissues in the nursing animal undoubtedly contributes to the more widespread cytochrome deficiency found in the severely iron-depleted groups. The cytochrome c concentration in both heart muscle and brain normally increased

rapidly between 10 and 20 days of age (21). Even the most stringent restriction of iron interfered surprisingly little with this developmental rise in concentration of the respiratory enzyme, despite diminution in concentration of this and other heme proteins in most parts of the body.

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Synthesis of Phospholipids and Deoxyribonucleic Acid in Choline-supplemented and Choline-deficient Weanling Rats [1]

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ABSTRACT The incorporation of choline-1,2-^{14}C into phospholipid and thymidine-2-^{14}C into DNA of kidneys, liver and several other tissues has been measured in choline-supplemented and choline-deficient male, weanling rats. One hour after injection the kidneys had incorporated eight times as much labeled choline as heart tissue and approximately three times as much as either spleen or intestine (per unit weight). The liver was the most active tissue studied; however, the kidneys incorporated choline into phospholipid at a rate significantly faster than the other nonhepatic tissues measured. In choline-deficient rats, the specific activity (disintegration per minute per milligram phospholipid) of heart, spleen and intestine increased; liver decreased and kidney remained the same. Rats fed a choline-deficient diet incorporated more thymidine per unit weight into the DNA fraction of the kidneys than rats receiving the supplemented diet. The increased incorporation of thymidine in the choline-deficient rats is probably due to increased cellular synthesis as the kidneys become enlarged. The radioactivity in the acid-soluble nucleotide pool in the kidneys was not altered by choline deficiency.

Male weanling rats fed a diet deficient in choline for 7 days develop fatty livers and enlarged hemorrhagic kidneys. Parks and Smith (1) have presented evidence that this increased kidney size in choline-deficient rats (29 to 33 days of age) is due in part to increased renal cellular proliferation. Other physical and chemical treatments also cause enlargement of the kidney. Rats fed a 70% protein diet for 1 year had kidneys that were 20% larger than those of a control group (2). There were no pathological changes in these rats. Unilateral nephrectomy in young rats resulted in compensatory growth of the remaining kidney (3). DNA synthesis in the remaining kidney was markedly enhanced after unilateral nephrectomy (4).

Patterson et al. (5) reported that phospholipid turnover in the kidney tissue of male rats is maximum at 27 days of age. These workers (5) also reported an increased phospholipid turnover in the liver during this period. Thus the question may be raised, is the increased phospholipid turnover at this age unique to the kidneys or do the other nonhepatic tissues also exhibit this increased activity? If the kidneys do have an increased phospholipid requirement (especially choline-containing phospholipids) relative to these other tissues, it may help to explain the profound pathological changes that occur in the kidneys of choline-deficient rats at about 30 days of age.

The purpose of this study was a) to compare the rate of phospholipid synthesis in kidney, liver, heart, spleen and intestine from male rats 27 to 29 days of age and b) to compare the synthesis of phospholipids and DNA in weanling rats fed a choline-deficient or a choline-supplemented diet.

EXPERIMENTAL PROCEDURE

Male weanling rats of the Charles River CDA strain with an average initial weight of 55 g were used in these experiments. The diets have been described in a previous paper of this series (6). Essentially they were high protein, high fat (20%) diets that were limiting in methionine and vitamin B_{12}. The rats were housed in individual wire-bottom cages in an air-condi-

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tioned room and had continuous access to the experimental diets and fresh water.

Phospholipid studies. Rats 27 to 29 days of age were injected intraperitoneally with 0.3 ml of 0.15 M NaCl solution containing 15 μCi of choline-1,2-^{14}C (20 μCi/μmole). The rats were killed and the heart, liver, kidney, spleen and intestines were removed, cleaned and weighed. The time of killing and the number of rats killed at each time interval are presented under results. The tissues were homogenized in 2:1 chloroform–methanol; the crude lipid extract was washed by the method of Folch et al. (7) and the phospholipid fraction isolated as described by Parks and Smith (1). This fraction contained approximately 10% cholesterol (1). An aliquot of the phospholipid fraction dissolved in 0.2 ml of 2:1 chloroform–methanol was added to 10 ml of a scintillation mixture (8). All samples were counted in a liquid scintillation counter.[2]

The phospholipid from one rat was used to determine if the recovered radioactivity was still associated with choline. This was performed by hydrolyzing an aliquot of the phospholipid fraction in 6 N HCl for 3 hours (9) and chromatographing the hydrolysate on Dowex 50-X8 resin according to the procedure of Christianson et al. (10). Five-milliliter fractions were collected from the column and each fraction was analyzed for radioactivity and choline. Radioactivity was determined by taking 1 ml of each fraction to dryness, redissolving the residue in 10 ml of an aqueous scintillation fluid and counting in the scintillation counter. Choline was determined by the method of Wall et al. (11).

Nucleic acid studies. The rats used in these studies were approximately 23 days old when placed on the experimental diets. In one experiment (ATP experiment) 27-day-old rats were used. DNA synthesis in tissues was ascertained by injecting rats intraperitoneally with 0.3 ml of an aqueous solution containing 3 μCi of thymidine-2-^{14}C (49.7 μCi/μmole). Thirty minutes later they were killed and the tissues were homogenized in cold 5% trichloroacetic acid (TCA). After about 30 minutes the samples were centrifuged and washed once with an equal volume of cold TCA. The residue was incubated in 0.3 N KOH at 37° for 16 hours to hydrolyze the RNA. The KOH was neutralized with HCl, and DNA and protein precipitated by the addition of 20% TCA to a final concentration of 5%. After centrifugation, the pellet was suspended in 5% TCA and the DNA hydrolyzed by heating for 30 minutes at 100°. The extract was centrifuged and aliquots of the supernatant solution used to determine the amount of DNA by a diphenylamine reaction (12) and counted to determine the amount of thymidine incorporated. The TCA was removed by extraction of the solutions five times with an equal volume of ether and the aqueous solution evaporated to dryness under a stream of air. Concentrated perchloric acid (0.4 ml) was added to each tube and the tubes were heated for 1 hour at 100°. After cooling, 5 ml of water and about 200 mg of acid-washed carbon were added to each tube. After 30 minutes the solutions were filtered, washed with 2 volumes of water and the nucleotides eluted with an aqueous ethanol-ammonium hydroxide solution (13). The filtrates were evaporated to dryness, dissolved in water and chromatographed on Whatman no. 3MM filter paper in a butanol–water (86:14) solvent system. The ultraviolet-absorbing compounds that chromatographed with the same R_F as a thymine standard were eluted overnight in 5 ml of 0.1 N HCl. The concentration of thymine was estimated by its absorption at 260 mμ using a molar absorbance of 7400. An aliquot of the same sample was counted to determine the amount of radioactivity. The remaining solution was evaporated to dryness and chromatographed on Whatman no. 3MM filter paper in an isopropanol–HCl–water (170:41:39) solvent system. The paper was scanned with a radiochromatogram scanner.[3] One major spot which contained approximately 90% of the ultraviolet-absorbing material also contained all of the radioactivity and had the same R_F as a thymine standard.

Tissues were analyzed for RNA by the orcinol method (12). ATP was analyzed in the cold TCA-soluble portion (see above) of the cells by a luciferin-luciferase reac-

[2] Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.

[3] Packard Instrument Company, Inc., Downers Grove, Ill.

tion (14). TCA was removed by ether extraction before analysis.

Chemicals. The radioisotopes were purchased from a commercial source.[4]

RESULTS

The relative weights and phospholipid content of the five tissues from choline-supplemented and choline-deficient rats are compared in table 1. The organ weights and the phospholipid concentrations (milligrams per gram tissue) of the heart, liver and spleen were similar in the two groups. The kidneys in the deficient rats weighed almost twice as much as those from the control rats and their phospholipid concentration was approximately one-half of the control. The total phospholipid contents of the various tissues were similar in the supplemented and deficient rats. These results support the earlier report of Parks and Smith (1) that the concentration of phospholipid per unit weight of kidney tissue is reduced in choline-deficient weanling rats but the total renal phospholipid content is similar to that of supplemented rats.

The rats in this laboratory are normally placed on the choline-deficient diets at 23 to 26 days of age and develop hemorrhagic kidneys by 29 to 33 days of age. We have determined the relative incorporation of choline-1,2-^{14}C into the phospholipids of kidneys, liver, heart, spleen and intestine of 27- to 29-day-old choline-supplemented rats (fig. 1, table 2) and 29-day-old choline-deficient rats (table 2). Initially, separate incorporation experiments were run at 27, 28 and 29 days with the choline-supplemented rats. The results of the three experiments were similar, therefore the values have been pooled for the purpose of this report. At 29 days the rats had been on the choline-deficient diet almost 6 full days.

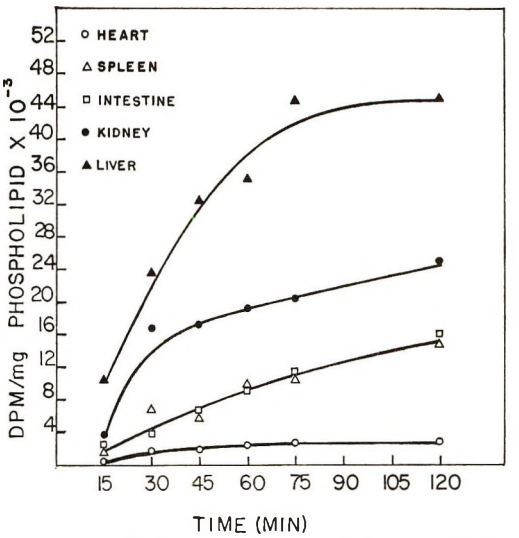

Fig. 1 The incorporation of choline-1,2-^{14}C into the phospholipids of heart, liver, kidney, spleen and intestine of male rats 27 to 29 days of age. Each rat received 15 μCi of the labeled choline.

[4] Schwarz BioResearch, Inc., Orangeburg, N. Y.

TABLE 1

Organ weight and phospholipid content from 29-day-old male choline-supplemented and choline-deficient rats

Tissue	Organ wt	Phospholipid	Total phospholipid
	g	*mg/g tissue*	*mg*
	Choline-supplemented		
Heart	0.48 ± 0.02 [1]	25.66 ± 0.44	12.21 ± 0.47
Liver	5.02 ± 0.24	25.61 ± 2.07	127.62 ± 6.38
Kidney	1.16 ± 0.02	26.33 ± 1.31	30.57 ± 1.75
Spleen	0.48 ± 0.05	18.07 ± 1.28	8.56 ± 0.77
Intestine	—	20.15 ± 0.40	—
	Choline-deficient [2]		
Heart	0.40 ± 0.02	31.90 ± 3.98	12.83 ± 1.75
Liver	5.00 ± 0.28	27.14 ± 0.98	135.37 ± 5.43
Kidney	2.01 ± 0.02	15.07 ± 1.36	30.32 ± 2.73
Spleen	0.47 ± 0.01	19.20 ± 1.31	9.01 ± 0.66
Intestine	—	19.95 ± 0.27	—

[1] Mean ± SE, three rats.
[2] These rats were removed from the choline-deficient diet on day 6 of the experiment.

TABLE 2

Relative incorporation of choline-1,2-^{14}C into the heart, liver, kidney, spleen and intestine of choline-supplemented and choline-deficient male weanling rats [1]

Tissue	No. of animals	dpm/mg tissue	dpm/mg phospholipid $\times 10^{-2}$
	Choline-supplemented		
Heart	10	65 ± 6 [2]	23 ± 2
Liver	10	984 ± 145	352 ± 49
Kidney	10	536 ± 28	192 ± 3
Spleen	10	179 ± 15	96 ± 8
Intestine	10	194 ± 12	92 ± 5
	Choline-deficient		
Heart	3	236 ± 66	78 ± 26
Liver	3	209 ± 29	78 ± 12
Kidney	3	239 ± 46	160 ± 29
Spleen	3	449 ± 140	235 ± 68
Intestine	3	288 ± 30	144 ± 14

[1] The values presented in this table were obtained 1 hour after injection of the labeled choline.
[2] Mean ± SE.

The relative incorporation of choline into the phospholipids of kidney, liver, heart, spleen and intestine, over a 2-hour period is indicated in figure 1. With the exception of 15-minute samples for spleen and intestine (based on the analyses of two rats) all points presented in figure 1 represent the tissue from at least three animals. The 60-minute values represent the tissue from 10 rats. Kidneys incorporated significantly ($P < 0.01$ at 1 hour) more choline into phospholipid (disintegration per minute per milligram phospholipid) over the duration of this experiment than either heart, spleen or intestine. The rate of incorporation by the kidneys for the first 30 minutes was approximately eight times faster than heart and three times faster than spleen or intestine. The liver incorporated more choline into phospholipid than kidney; however, the rate of incorporation did not differ greatly for kidney and liver over the first 30 minutes. The mean level of choline incorporated into the phospholipids of the five tissues at 1 hour is presented in table 2 with the standard error of the mean. At 1 hour the kidneys had incorporated eight times as much choline into phospholipid per milligram of tissue as heart ($P < 0.01$) and three times as much as spleen or intestine ($P < 0.01$). Approximately twice as much choline was incorporated into liver tissue as was incorporated into kidney tissue at 1 hour ($P < 0.01$). Wells and Remy (15) have reported similar results with liver and kidney using slightly older rats. Considerable variability was noted in the activity of hepatic tissues as evidenced by the large standard error.

The injection of labeled choline into choline-deficient rats resulted in the increased incorporation per unit weight of choline by the heart, spleen and intestine at 1 hour relative to the choline-supplemented rats. The incorporation of labeled choline per milligram of kidney tissue was decreased in the deficient rats; however, when the specific activity was related to phospholipid there was no difference in supplemented and deficient rats. There appeared to be decreased incorporation of choline in hepatic phospholipids of choline-deficient rats.

Figure 2 demonstrates that the labeled compound isolated from the phospholipid was choline rather than some metabolic product. No radioactivity could be detected in the fractions 50–60 that normally contain ethanolamine, *N*-methyl ethanolamine or *N*, *N*-dimethyl ethanolamine.[5]

The incorporation of thymidine-2-^{14}C into DNA (per 100 μg) of the tissues of choline-deficient rats showed marked differences at 7 days (table 3). The DNA of the kidneys of the choline-deficient rats contained twice as much radioactivity as that of the control group ($P < 0.01$). There was a threefold increase in the amount of thymidine incorporated into the DNA of

[5] Haggard and Strength, unpublished results.

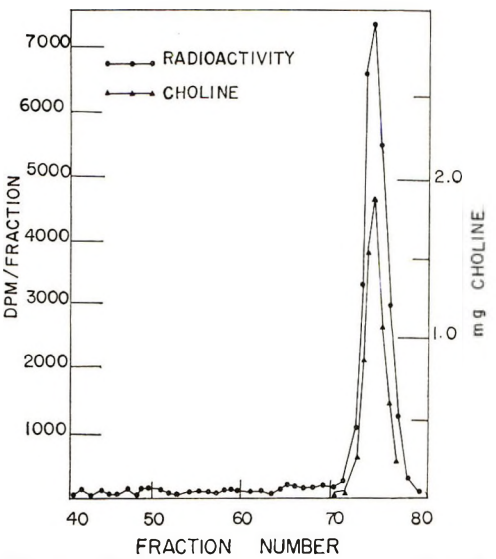

Fig. 2 Coincidence of choline and radioactivity in the renal phospholipids of rats injected with choline-1,2-^{14}C. The chromatographic separation was performed according to the method of Christianson et al. (10).

spleens of rats fed the choline-deficient diet ($P < 0.01$). Incorporation of thymidine-2-^{14}C by the spleen was two to three times that of the other tissues in the control group. The DNA in hearts of deficient rats incorporated only 30% as much thymidine as the control group ($P < 0.01$). The livers of the choline-deficient rats incorporated about the same amount of radioactivity as the control group at 7 days ($P > 0.05$). Increased incorporation of thymidine into renal DNA of choline-deficient rats is not evident on either days 5 or 6. As noted above, however, there was a twofold increase on day 7. This increase coincides with kidney enlargement observed in the choline-deficient rats on day 7. This conclusion is strengthened by the observation that one rat on day 6 had enlarged kidneys and this rat incorporated 921 dpm/100 μg DNA — some 135 dpm above the supplemented rats. In preliminary studies, we have noted increased thymidine incorporation in choline-deficient rats as early as day 5. The specific activities of thymine purified by paper chromatography showed the same patterns of incorporation. Nucleic acid and protein content of liver, heart and spleen in control and deficient rats were similar. The concentration of RNA and DNA in the kidneys was less in the choline-deficient group than in the controls.

At 5, 6 or 7 days the radioactivity (disintegration per minute per gram tissue) from thymidine-2-^{14}C in the cold TCA-soluble portion of the kidneys of control rats was greater than that of the liver, spleen or heart, which were all similar (table 4). At 5 days the radioactivity in the tissues of the choline-deficient rats was similar to that of the control group. At 7 days the TCA-soluble portion of the heart, liver and spleen of the choline-deficient rats contained two to three times as

TABLE 3

Incorporation of thymidine-2-^{14}C into DNA of organs of rats fed either a choline-supplemented or choline-deficient diet

Tissue	Days on experimental diets [1]		
	5	6	7
	dpm/100 μg of DNA		
	Choline-supplemented		
Heart	1318 ± 95 [2]	660 ± 29	1068 ± 106
Liver	1044 ± 106	1634 ± 268	1379 ± 166
Kidney	962 ± 47	786 ± 51	780 ± 33
Spleen	1535 ± 72	2144 ± 290	2178 ± 227
	Choline-deficient		
Heart	708 ± 152	541 ± 89	361 ± 85
Liver	1582 ± 229	1785 ± 224	2920 ± 882
Kidney	769 ± 88	878 ± 28	1761 ± 223
Spleen	1621 ± 71	2816 ± 611	6597 ± 446

[1] Tissues from three rats were analyzed on days 5 and 6. Tissues from six rats were analyzed on day 7.
[2] Mean ± SE.

TABLE 4

Radioactivity in the cold TCA-soluble portion of organs of rats injected with thymidine-2-^{14}C

Tissue	Days on experimental diets [1]		
	5	6	7
	dpm/g tissue $\times 10^{-2}$		
	Choline-supplemented		
Heart	237 ± 5 [2]	176 ± 15	214 ± 11
Liver	183 ± 10	318 ± 44	269 ± 17
Kidney	496 ± 1	437 ± 37	518 ± 36
Spleen	181 ± 10	203 ± 12	167 ± 15
	Choline-deficient		
Heart	288 ± 16	322 ± 14	411 ± 24
Liver	212 ± 32	317 ± 21	517 ± 97
Kidney	504 ± 73	509 ± 57	490 ± 52
Spleen	201 ± 9	230 ± 40	499 ± 37

[1] Tissues from three rats were analyzed on days 5 and 6. Tissues from six rats were analyzed on day 7.
[2] Mean ± SE.

TABLE 5

Concentration of ATP in organs of weanling rats fed either a choline-supplemented or choline-deficient diet

Tissue	Choline-supplemented	Choline-deficient
	μmoles/g tissue	*μmoles/g tissue*
Heart	1.34 ± 0.06 [1]	1.38 ± 0.09
Liver	1.40 ± 0.05	0.68 ± 0.02
Kidney	0.76 ± 0.01	0.60 ± 0.05
Spleen	1.26 ± 0.01	1.18 ± 0.04

[1] Mean ± SE, three rats.

much radioactivity as that of the controls. The kidneys of the choline-deficient rats had approximately the same activity as the control group and was comparable to the value at 5 days. The increase in the activity of the tissues did not necessarily result in increased incorporation into the DNA of these tissues (table 3).

The concentration of ATP in the livers of rats fed the choline-deficient diet for 7 days was markedly reduced in weanling rats (table 5) just as it was in older rats fed a choline-deficient diet (16). A variety of other treatments that produce fatty livers result in a lowering of the concentration of ATP (16). In this study the concentration of ATP in the kidneys of the choline-deficient group was 79% that of the control group; however, this has not been a consistent observation in this laboratory. Choline deficiency did not affect the concentration of ATP in the spleen or heart.

DISCUSSION

Several workers have reported that the kidneys of rats are not fully developed at birth but maturate during the first few weeks of life (see review by Foster (17)). Patterson et al. (5) have shown that this is also the period of most active phospholipid synthesis by the kidneys. The results reported here further demonstrate that this synthetic process (as measured by choline incorporation into phospholipids) is much more active in the kidneys than in other nonhepatic tissues. In these studies the kidneys had incorporated eight times as much labeled choline into phospholipid as heart tissue and approximately three times as much as either spleen or intestine 1 hour after injection (per unit weight). The liver was the most active tissue studied as one might expect due to the key role played by this organ in lipid metabolism. Several interesting results were obtained when rats which had been on a choline-deficient diet almost 6 full days were injected with labeled choline. Heart, spleen and intestine had an increased specific activity (disintegration per minute per milligram phospholipid) relative to rats on a supplemented diet, whereas kidneys which were beginning to exhibit pathological changes had essentially the same specific activity as those from supplemented rats. The kidneys from the deficient rats had almost doubled in size; however, there was no increase in the total phospholipid content or apparently in phospholipid synthe-

sis. The livers from the deficient rats incorporated less choline into phospholipid than livers from supplemented rats. We presently can offer no clear-cut explanation for the incorporation patterns in kidneys and liver except that they are probably related to the well-known pathological conditions (hemorrhagic kidneys and fatty liver) associated with these organs in choline deficiency. Work is continuing on this problem.

Much of the renal growth during the early life of the rat is related to the formation of new nephrons in the outer zone of the cortex (17). This is also the site of renal hemorrhage in choline-deficient rats 4 to 5 weeks of age (18). Older rats with mature kidneys do not normally develop renal hemorrhages on choline-deficient diets. Handler (19) has shown, however, that older, choline-deficient rats which undergo unilateral nephrectomy and the accompanying hypertrophy do exhibit hemorrhagic kidneys similar to that observed in weanling rats. Thus, the development of renal hemorrhage appears to be more closely related to the metabolic activity of the kidney (synthesis of renal tissue) than chronological age of the rat. Parks and Smith (1) have presented evidence that the kidneys from choline-deficient rats exhibit a rate of cellular proliferation which exceeds that normally observed in weanling rats. There is also a small increase in moisture concentration and probably protein concentration in hemorrhagic kidneys.

Unilateral nephrectomy in young rats also causes a compensatory type of cellular proliferation in the remaining kidney (3). This compensatory growth is marked by increased DNA synthesis (4). A similar increase in DNA synthesis was noted in the kidneys of choline-deficient rats. At 7 days rats fed a choline-deficient diet incorporated more thymidine into the DNA fraction of the kidneys than did a control group. Although the concentration of DNA in the kidneys of the choline-deficient group was less than the controls, the total kidney DNA was greater (1). The increased incorporation of thymidine in the choline-deficient rats is probably a reflection of an increase in cellular synthesis as the kidneys become enlarged. This difference in incorporation could be due to an alteration in the pool size of the thymidine nucleotides. This seems unlikely because the radioactivity in the acid-soluble pool of the kidneys was not altered by choline deficiency and the concentration of ATP, one of the major components of the pool, was altered only slightly. In the liver where there was a marked decrease in the concentration of ATP and an increase in the radioactivity of the acid-soluble pool of the choline-deficient rats, there was no significant change ($P > 0.05$) in the incorporation of thymidine into DNA at 7 days. Although neither the concentration nor total content of DNA in the spleen were altered by choline deficiency, there was a threefold increase in the incorporation of thymidine into the DNA of choline-deficient rats at 7 days. The incorporation of thymidine into the DNA of hearts of choline-deficient rats was only about 30% that of controls although the radioactivity in the acid-soluble pool was twice as high. This may be related to the cardiovascular alterations reported in weanling rats (20). After the experiments reported here were completed, Newberne et al. (21) reported that the renal epithelium of male weanling rats fed a diet low in methionine and choline had an increase in DNA synthesis after 6 weeks on the diet but not after 3 weeks.

One explanation of our results is that the developing tubular tissues in the renal cortex of young rats require large amounts of choline-containing phospholipids for membrane formation. In choline-deficient rats the limiting concentration of choline causes the synthesis of abnormal cells. This could have an effect similar to that seen in unilateral nephrectomy and trigger increased cell growth. The new cells, however, would also be abnormal. The exact cause of the renal hemorrhage remains to be determined.

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Reproductive Performance and Progeny Development in Swine as Influenced by Feed Intake During Pregnancy

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ABSTRACT First-litter gilts (nearly equal numbers of purebred Hampshires, purebred Yorkshires and crossbreds) were used to study effects of gestation-diet intake on reproductive performance and progeny development. From breeding to farrowing, gilts were fed 0.9, 1.4, 1.9, 2.4 or 3.0 kg/day of a diet which was designed to be adequate to superadequate in all nutrients when fed at 1.9 kg. All gilts received this diet ad libitum during a 3-week lactation period. Farrowing percentage (number farrowing as a percentage of those mated) was less for gilts fed 0.9 kg/day than for those fed more, and was less for purebreds than for crossbreds. Gestation-diet intake did not affect the number of pigs farrowed (total or live) or weaned, but crossbreds farrowed and weaned more pigs than purebreds. Birth weight and weaning weight of offspring increased as gestation-diet intake increased; birth weight plateauing at 1.9 kg and weaning weight plateauing at 2.4 kg. As gestation-diet intake increased, weight gain of gilts increased quadratically during gestation but decreased linearly during lactation. Birth weight per pig decreased 43 g for each additional pig in the litter.

Information pertaining to the nutritive requirements for reproduction in swine is very meager. Successful swine producers usually impose some form of feed restriction on pregnant swine, yet the minimal level of feed intake for optimal reproductive performance has not been established.

There is good evidence to suggest that underfeeding of swine during pregnancy will reduce both birth and weaning weight of the offspring (1–3).[1] Similar effects have been reported for rabbits (4) and rats (5, 6). Reduced birth and weaning weights in offspring from underfed pregnant swine appear to result specifically from energy restriction and not from protein restriction (7).

The objective of our study was to evaluate effects of levels of gestation-feed intake on reproductive performance of first-litter gilts.

EXPERIMENTAL

The experiment was conducted over 1 complete year period. Six groups of first-litter gilts farrowed at bimonthly intervals; Hampshires in October and April, Yorkshires in December and June, and crossbreds (hybrids) in February and August.

Prebreeding and breeding. For each of the six replicate groups, about 75 gilts were selected from finishing lots at an average age of 6 months. They were transferred to pasture lots where they were fed individually 2.27 kg/day of a 16%-protein-corn-soybean meal diet to an average age of 8 months. After worming (piperazine) the gilts were trucked to a confinement, slotted-floor breeding facility where environmental temperature was not allowed to fall below 10°. For 30 days prior to breeding and during the 30-day breeding period, the same diet was fed at 2.27 kg/day.

Each gilt was hand-mated to one of three boars of the same breed, and when possible remated 24 hours later. Gilts failing to show estrus were discarded. Breeding was stopped as soon as 55 gilts were mated.

Gestation. Within 24 hours after the last mating each gilt was moved to a confinement, slotted-floor gestating unit. Supplemental heat was provided as needed to keep the temperature above 10° during the winter months. As bred, and randomly within groups of five, they were immediately placed on one of the five gestation feed-intake treatments and hand-fed once per day in individual feeding stalls. Gilts

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[1] Vermedahl, L. D., R. J. Meade, H. E. Hanke and J. W. Rust 1968 Gestation and lactation feeding levels for gilts. I. Effects on reproduction and lactation performance. J. Anim. Sci., 27: 1158 (abstract).

on the same treatment were penned together, each pen containing 11 feeding stalls. The pen, therefore, became the experimental unit (five pens times three breeds times two replicates) of which there were 30 by the end of the experiment.

The experimental diet (table 1) contained 16% crude protein and 3310 kcal metabolizable energy (ME)/kg diet. This diet was fed at a daily rate of 0.9, 1.4, 1.9, 2.4 or 3.0 kg. Treatment 5 (3.0 kg) was originally intended to be ad libitum feeding, but midway through group 1 it was decided that 3.0 kg/day was close to a maximum intake when fed once per day. Thereafter, 3.0 kg/day was fed to gilts on treatment 5. The experimental diet was designed to be adequate to superadequate in all nutrients (except energy, for which there is no established requirement for gestation) when fed at a level of 1.9 kg/day. Hence, the treatments involved graded intakes of all nutrients, including energy, protein, vitamins and minerals.

Gilts remained on their designated feed-intake treatment throughout gestation. When a gilt reached day 109 of gestation she was transferred to one of three 20-sow farrowing houses where she remained on her respective feed-intake treatment until farrowing. If good evidence was obtained that a gilt was cycling, hence nongravid, she was removed from the experiment and considered to have mated but not conceived.

Gilts were weighed at mating, at day 109 of gestation, just after farrowing and when litters were weaned at 21 days of age.

Lactation. After parturition all gilts were continued on the same diet (table 1) but on a simulated ad libitum basis (8)—all they would eat in two 1-hour feedings/day. A standard creep ration was fed to baby pigs between day 7 and day 21 of lactation. Pigs were weighed at birth and at weaning.

In calculating the average number of offspring weaned per litter, all litters containing one or more live pigs at farrowing were included. For calculation of average pig weaning weight, only litters containing at least one live pig at weaning were used. Likewise, the average gilt lactation weight change and feed consumption were based on those gilts weaning at least one live pig.

Analysis of variance with orthogonal single degree-of-freedom comparisons was used to test for breed and gestation feed-intake effects and interactions.

TABLE 1

Composition of diet fed during gestation

Ingredient	%
Ground yellow corn	79.35
Solvent soybean meal	18.00
Dicalcium phosphate	1.00
Ground limestone	1.00
Trace-mineralized salt [1]	0.50
Vitamin mix [2]	0.10
Antibiotic premix [3]	0.05
	100.00

[1] Contained, as a percentage of the mix: salt, 97.0; zinc, 0.800; manganese, 0.400; iron, 0.330; copper, 0.048; iodine, 0.011; and cobalt, 0.022.

[2] Contained, per kilogram: 1.1 g riboflavin; 5.5 g calcium pantothenate; 16.5 g nicotinic acid; 110.3 g choline chloride; 18.0 mg vitamin B_{12} activity; 3.3 million IU vitamin A; and 330,000 IU vitamin D_2.

[3] Contained 33.0 g streptomycin (as sulfate) and 11.0 g procaine penicillin/kg.

RESULTS AND DISCUSSION

The breed times treatment interaction was not statistically significant for any of the parameters measured so only main effects are presented (table 2).

Farrowing percentage. Gilts fed only 0.9 kg diet/day exhibited a lower ($P<0.05$) farrowing percentage than those fed at higher levels, and purebreds were lower ($P<0.05$) than crossbreds. Because treatment was initiated after conception, it seems likely that the low farrowing percentage of gilts fed 0.9 kg/day was the result of interference with embryo implantation, which is thought to occur in swine about 18 days postconception (9). Abortions were recorded, but there was no indication that incidence was related to treatment.

Overall, 72% of the gilts mated actually farrowed, crossbreds substantially outperforming purebreds. This average value provides an estimate of breeding performance under complete confinement. Many producers have found it difficult to detect estrus in gilts confined to a concrete- or slotted-floored enclosed building. We had no difficulty in detecting estrus in our gilts. Perhaps the presence of boars in the same building aided estrus detection. A farrowing percentage of 72 is certainly acceptable for first-litter gilts for a breeding period as short as 30 days.

TABLE 2

Reproductive performance of gilts fed different quantities of diet during gestation

Criterion	Daily gestation diet intake,[1] kg					Breed [2]		
	0.9	1.4	1.9	2.4	3.0	Hampshire	Yorkshire	Crossbred
No. of gilts started [3]	62	62	62	62	62	95	105	110
Farrowing, % [4]	60.9	71.0	82.3	71.4	76.0	69.2	65.0	82.7
No. of pigs born	9.1	8.9	9.5	9.6	9.3	8.7	9.0	10.1
No. of pigs born alive	7.6	7.9	8.2	8.7	8.0	7.3	8.0	9.0
No. of pigs weaned	6.0	5.7	6.3	6.7	6.1	5.4	5.9	7.2
Birth wt, kg	1.00	1.13	1.23	1.24	1.22	1.18	1.08	1.24
21-day weaning wt, kg	4.23	4.77	5.06	5.40	5.34	4.80	4.96	5.11
Gestation wt gain, kg [5]	5.9	30.3	51.2	62.8	74.4	44.0	45.2	45.4
Lactation wt gain, kg [6]	6.1	0.9	−4.4	−7.6	−8.5	−0.1	−1.8	−6.2
Lactation-diet intake, kg	89.4	90.3	90.5	81.1	71.7	90.3	84.5	78.9

[1] Diet intake comparisons represent a pooling of all three breeds.
[2] Breed comparisons represent a pooling of results from all levels of feeding.
[3] Average weight of gilts at conception was 120 kg.
[4] Number farrowing as a percentage of those mated.
[5] Gain of gilts from conception to day 109 of gestation.
[6] Gain of gilts from immediately postfarrowing to weaning (21 days).

Number of pigs born and weaned. Levels of feed intake during pregnancy exerted no significant effect on the number of pigs born, born alive or weaned at 21 days of age. This is in agreement with other reports (1–3, 7)[2] and suggests that underfeeding during pregnancy does not influence litter size.

Crossbreds farrowed (total or live) and weaned more ($P<0.05$) pigs than purebreds. This supports the well-known effect of heterosis on criteria such as litter size (10).

Weight of pigs at birth and at weaning. Diet intake in pregnancy had a marked effect on both birth and weaning weight of offspring. Birth weight increased quadratically ($P<0.01$) and weaning weight increased linearly ($P<0.01$) as diet intake during pregnancy increased. Gilts fed 1.9, 2.4 and 3.0 kg/day farrowed heavier ($P<0.01$) pigs than those fed less, and gilts fed 2.4 and 3.0 kg/day weaned heavier ($P<0.01$) pigs than those fed less. Thus, under conditions of our study it appeared that slightly more feed was required during pregnancy to maximize weaning weight (2.4 kg/day) than to maximize birth weight (1.9 kg/day).

Crossbred gilts farrowed heavier ($P<0.01$) offspring than purebred gilts, and within purebreds, Hampshires farrowed heavier ($P<0.01$) offspring than Yorkshires. That crossbred pigs were heavier than purebreds is particularly noteworthy because of a negative regression ($P<0.01$) of birth weight on litter size. Birth weight per pig decreased 43 g for each additional pig in the litter. Thus, despite the larger ($P<0.05$) litter size in crossbreds, birth weight per pig was still greater in crossbreds than in purebreds. Weaning weight too was greater in crossbreds than in purebreds, but not significantly.

There is good evidence to support the conclusion that both gestation-feed intake (1–3)[3] and breed (10) influence weight of offspring at birth and at weaning. To date, however, there has been no good basis for selecting a minimal gestation-feed intake which will support maximal reproductive performance. Our data permit an estimate of the minimal feed intake during pregnancy for maximal birth or weaning weight, but only insofar as it applies to 1) first-litter gilts under confinement management, and 2) total feed restriction rather than restriction of individual nutrients. It is questionable, however, whether maximal birth and weaning weights contribute to most efficient swine production. Are the reduced weaning weights at lower levels of gestation-feed intake reversible during later growth or are they indicative of a permanent growth stunting as seems to be the case with rats (5); and, if permanent, is the magnitude sufficient to offset the increased cost of a greater diet intake during pregnancy? Elsley et al. (3) suggest that the greatest efficiency of overall swine pro-

[2] See footnote 1.
[3] See footnote 1.

duction may be realized at a gestation-feed intake less than that needed for maximal weaning weight. Vermedahl et al.[4] reported that growth rate subsequent to weaning was similar in offspring from gilts fed 1.36 kg/day and those fed 2.27 kg/day during gestation. However, gain/feed ratio between weaning (at 21 days) and 23-kg body weight was greater in offspring from gilts fed the lesser quantity of feed during pregnancy, but this difference did not exist for the period from 23- to 91-kg body weight. This suggests that the lower weight gain of pigs in utero and during lactation can be reversed subsequent to weaning.

An apparent species difference between swine [5] and rats (5) regarding the reversibility of the stunting effect that occurs in utero raises the question of what specific nutrient or nutrients are involved. Using individual component restriction, Hsueh et al. (6) concluded that growth retardation of young rats was the result of protein restriction and, to a lesser extent, energy restriction, and definitely not due to mineral or vitamin restriction. When the pregnancy and lactation diet intake was restricted by 50%, energy supplementation as sucrose eliminated the birth weight difference between offspring of dams fed ad libitum and dams fed a restricted diet, but only partially eliminated the reduction observed at weaning. On the other hand, it was shown that an ad libitum pregnancy and lactation diet based upon casein (high quality) produced far greater birth and weaning weights of offspring than one based upon zein (low quality). Moreover, methionine alone added to a methionine-deficient alpha-protein diet fed ad libitum during gestation and lactation markedly increased weight of offspring at birth, at 3 weeks (weaning) and even at 26 weeks.

Indeed, deleterious effects of underfeeding rats during pregnancy have been tied closely to the restriction of protein. A reduced protein intake before and during gestation has been associated with a cessation of estrus and increased embryonic mortality (11–13). Impaired protein metabolism has been observed in offspring from underfed dams (14). Moreover, that growth hormone or pituitary extract administration to offspring of dams fed restricted diets can reverse the stunting effect (5) implies that protein is involved.

In the studies with rats, it is difficult to isolate the effects of restriction during gestation from those of restriction during lactation, because in most cases the restricted regimen commenced at conception and continued uninterrupted through lactation. It is conceivable that different results might have been obtained, particularly with regard to the permanence of the stunting effect, if a liberal quantity of diet containing good quality protein in sufficient quantity had been fed during the lactation period. The rat data of Richardson et al. (15) suggest that protein intake during lactation is more critical than protein intake during gestation in terms of achieving maximal weaning weight of offspring. With swine (7) energy restriction during gestation, with high quality protein fed ad libitum during lactation, caused reduced birth and weaning weight of offspring. In the same study, rather severe protein restriction during gestation had no effect on baby pig weights. However, recent work by Mahan et al.[6] suggests that protein restriction of the dam during lactation markedly reduces weight gain of pigs from birth to weaning.

The experiments of Rippel et al. (16) indicate that pregnant gilts can perform normally on protein intakes considered very low in both quantity and quality. No differences in number or weight of pigs born or weaned resulted from feeding pregnant gilts 1.82 kg/day of a 16%-protein corn-soybean meal control diet, an 8%-protein corn diet (low in lysine and tryptophan) or 5%-protein diets (3500 kcal ME/kg) containing either sesame meal (low in lysine) or gelatin (tryptophan devoid) as the sole source of dietary protein during the last 50 days of gestation. In agreement with these results, Holden et al.[7] observed no effect on birth weight or weaning weight of pigs from feeding 1.82 kg/day of an 8%-protein corn-soybean meal

[4] Vermedahl, L. D., R. J. Meade, J. W. Rust and H. E. Hanke 1968 Gestation and lactation feeding levels for gilts. II. Performance of offspring subsequent to weaning and carcass characteristics. J. Anim. Sci., 27: 1158 (abstract).

[5] See footnote 4.

[6] Mahan, D. C., D. E. Becker and A. H. Jensen 1968 Efficacy of protein levels and opaque-2 corn for lactating sows. J. Anim. Sci., 27: 1777 (abstract).

[7] Holden, P., E. Lucas, V. C. Speer, V. W. Hays and R. C. Ewan 1967 Long term effects of protein level on swine reproduction. J. Anim. Sci., 26: 1478 (abstract).

diet to gravid swine over four successive reproductive cycles.

Strachan et al.[8] fed a protein-free diet to gilts throughout gestation. Embryo survival was not affected but birth weight was reduced as compared with a control diet. However, feeding the control diet at 1.8 kg/day up to day 24 of gestation (through the period of embryo implantation), followed by the protein-free diet at the same level of intake for the remainder of gestation, resulted in birth weights similar to those obtained when the control diet was fed throughout the 114-day gestation. This suggests that severe protein restriction (or perhaps restriction of certain amino acids) during the period of implantation may affect subsequent birth weight of offspring.

The results with rats and swine raise the question of what constitutes a proper diet intake and, more specifically, a proper nutrient intake for pregnant women. Gestation-feeding studies with swine would suggest that, although total diet or energy restriction of the dam will reduce birth weight and weaning weight of offspring, permanent stunting does not occur from underfeeding during pregnancy. Protein restriction of the dam seems to have little effect during gestation but a marked effect during lactation on performance of offspring. With rats, the consequences of protein restriction of the dam in either gestation or lactation, or both, are so severe that it seems important to isolate that which is caused by lactation restriction and that which is caused by gestation restriction. Nutritive considerations of women who breast-feed their babies could be far different from those who do not.

Weight gain of gilts. Weight gain during pregnancy (based on 109 days of a gestation period averaging 114 days) increased quadratically ($P < 0.01$) as gestation-diet intake increased from 0.9 kg/day to 3.0 kg/day (table 2, fig. 1). Conversely, subsequent weight gain during the 21-day lactation period decreased linearly ($P < 0.01$) as diet intake in pregnancy increased. These data support the conclusion of Lodge et al. (17) that the greater the weight gain of swine in gestation, the lesser the subsequent weight gain in lactation. Gilts fed gestation-diet intakes less than 1.9 kg/day lost weight during lactation.

It is remarkable that pregnant gilts fed only 0.9 kg/day throughout gestation could, in fact, gain weight. Pregnant gilts, how-

[8] Strachan, D. N., E. F. Walker, Jr., W. G. Pond, J. R. O'Connor, J. A. Dunn and R. H. Barnes 1968 Reproduction in swine fed a protein-free diet at various stages of gestation. J. Anim. Sci., 27: 1157 (abstract).

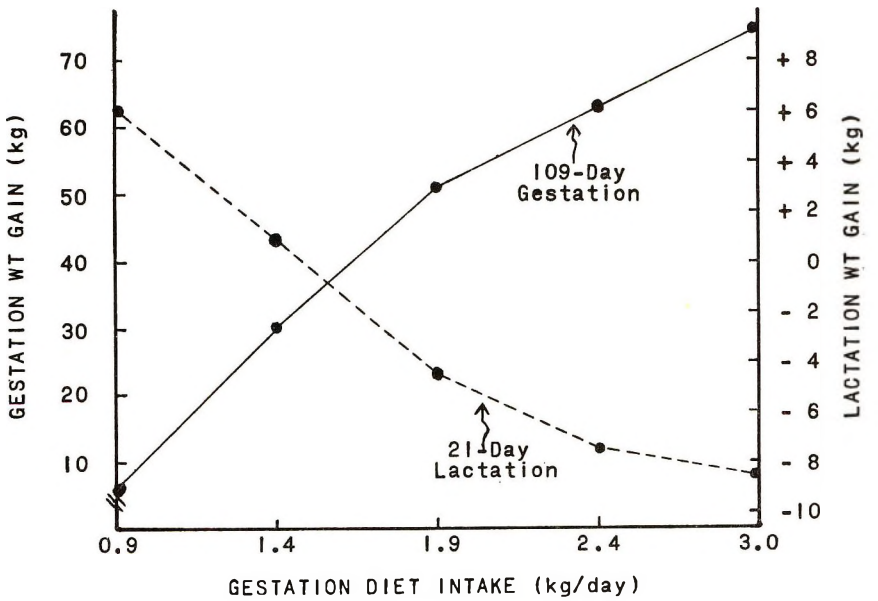

Fig. 1 Weight gain in gestation and lactation as a function of feed intake during gestation.

ever, have a remarkable propensity to gain weight, even on intakes below that which would be considered a maintenance intake for nonpregnant gilts (18). Elsley et al. (19) found that pregnant gilts gained over twice as much weight as nonpregnant gilts when fed the same quantity of diet. Part of this difference undoubtedly arises from the increased weight of the products of conception in the pregnant animal, but there is also evidence that a more efficient nitrogen metabolism accompanies the gravid state (19, 20).

An apparent change of slope in the gestation weight gain curve occurred at an intake of 1.9 kg/day (fig. 1). This suggests that feed utilization for weight gain was more efficient at intakes at or below 1.9 kg than at intakes above 1.9 kg. Perhaps it is significant that birth weight of offspring also appeared to plateau at this point. Another possible explanation for the apparent decrease in feed utilization for weight gain at the higher levels of gestation-feed intake, however, is that maternal fat synthesis may have increased relative to protein synthesis at intakes above 1.9 kg/day. Elsley et al. (3) also observed an apparent change in slope (in gain from mating to 110 days postmating) at an intake of 2.4 kg with gestation-diet intakes of 1.6, 2.4 and 3.2 kg/day fed to first-litter gilts. Litter weight plateaued at 2.4 kg also, however, so that gain from mating to immediately postfarrowing showed no change in slope.

Crossbred dams lost more ($P < 0.05$) weight during lactation than purebred dams, probably an indication that crossbred dams milked more heavily. Crossbreds nursed larger litters containing heavier pigs, and the weight advantage of crossbred pigs did not decrease during lactation.

Diet intake of gilts and creep intake of pigs during lactation. Lactating gilts that had been fed 2.4 or 3.0 kg during gestation consumed less ($P < 0.01$) feed during lactation than those that had been fed lower levels in gestation. Lactation-diet intake decreased quadratically ($P < 0.01$) as gestation-diet intake increased. Gilts on the three lower levels of gestation-feed intake voluntarily consumed virtually the same quantity of feed (approximately 4.3 kg/gilt per day). Nevertheless, weight gain during lactation decreased linearly with each increase in gestation-feed consumption. This indicates that feed intake, hence weight gain, during gestation had a greater effect on subsequent weight gain during lactation than feed consumption during lactation; and, as also noted by Lodge et al. (17), lactation weight gain can decrease with an increasing intake during gestation, even at the same rate of lactation-feed consumption.

Intake of the creep diet, which was offered to pigs at 7 days of age, was extremely low. Average creep consumption per pig was only 80 g for the entire 14 days during which it was offered. Apparently, young nursing pigs do not readily accept solid feed until sometime after 21 days of age. There was no indication that either gestation-feed intake or breed affected creep-feed consumption.

Throughout the trial, there seemed to be a rather high incidence of baby pig scours. As others conducting similar research have observed this also, we wonder if the frequent manipulation of animals in weighing and in other operations is a contributing factor. Certainly, the data collected after parturition must be interpreted in light of this condition.

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Effect of Food Restriction on Systematic Oscillations in "Control Animals" Used in Studies on Biotin-deficient Rats [1,2]

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ABSTRACT Various "control animals" used in studies on biotin-deficient rats have been evaluated. Biotin-deficient and control rats fed ad libitum, and control animals given restricted amounts of food (10 g) as one, two or four equal meals a day were used. Average food intake of control and deficient animals fed the basal diet ad libitum were 17.1 and 9.5 g, respectively. Control animals given a restricted amount of food (10 g) as one, two or four equal meals a day consumed each allotment in 6 hours, 30 and 15 minutes, respectively. The rate of food ingestion appeared to be higher in animals given multiple meals than in animals given a single meal. Systematic oscillations in body weight, liver weight, the level of hepatic glycogen and spontaneous activity, were observed in control animals given a single meal. Furthermore, these oscillations decreased proportionately in animals given multiple meals. Similar oscillations were also observed in body weights of pair-fed animals. A threefold increase in spontaneous activity was observed in animals given a restricted amount of food as a single meal compared with animals fed ad libitum. This activity increased with progress of the fast. In view of these results it is apparent that "pair-fed," "pair-weighed" and "trained-fed" control animals are different from biotin-deficient and control animals fed ad libitum.

It is generally known that biotin deficiency results in a marked reduction in food intake. In a recent study using a basal diet containing 20% spray-dried egg white, Patel and Mistry (1) observed a significant decrease in food intake of rats within 3 to 5 days from the start of the experiment. To overcome differences in energy intake between biotin-deficient and control animals the following methods were used: a) pair-feeding (2,3); b) pair-weighing (4,5); and c) trained-feeding (5). Also, "positive controls" (6) and control animals fed ad libitum (1, 4, 7) have been used. Patel and Mistry (1) observed apparent differences in body weights of control animals pair-fed a single meal or given the same amount of food in two equal portions 12 hours apart.

The consequence of food restriction (pair-feeding) on metabolic patterns in animals used as controls has not been investigated. A review of the literature reveals that diurnal variations in liver weight and tissue glycogen in rats are induced as a result of limited access to food (8–11). Gram and Okey (4) reported that the "pair-weighed" control rats consumed the daily food allotment in a short period. In view of this "meal-eating" pattern of control animals, the effect of food restriction on body weight, liver weight, the steady-state level of hepatic glycogen and the spontaneous activity of these animals has been studied. Also, the possibility of using control animals given restricted amounts of food but in multiple meals a day has been investigated.

MATERIALS AND METHODS

Animals. Nineteen-day-old weanling male rats of the Sprague-Dawley strain weighing 32 to 38 g were used. The animals were housed individually in metal cages with raised screens in a temperature-regulated room. Biotin deficiency was produced by feeding ad libitum a basal diet (12) containing 20% spray-dried egg white. The deficient animals were cured

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of biotin deficiency by injecting 100 μg biotin in physiological saline three times a week during the 2-week curative period, i.e., only during the last 2 weeks of the experiment. During the curative treatment the visible symptoms of biotin deficiency progressively disappeared. Normal animals fed the basal diet ad libitum received the same biotin treatment from the start of the experiment. Normal animals fed a commercial ration [4] ad libitum were not given additional biotin. Water was available to animals at all times.

Experiment 1. In this study 126 male rats, after 4 weeks of being fed the basal diet, were divided into five groups as shown in the experimental design in table 1. During the following 2-week curative period the control animals (groups 3 to 5) on restricted food intake received 10 g of the basal diet in one, two, or four equal allotments a day. During the same 2-week period, deficient (group 1) and control (group 2) animals were fed ad libitum and their daily food intake was recorded.

Before the animals were offered food at the zero hour (1 PM) on day 41 of the experiment, i.e., on day 13 of the treatment, body weights of animals in all groups were recorded. The daily food allotments were then offered, and food intake and body weights were recorded at various intervals over the 24-hour period.

On day 43 on experiment, i.e., day 15 of the treatment, at the zero hour (1 PM), six animals from each group were killed. The remaining animals were given their respective food allotments. At various intervals six animals from each group were killed. Portions of livers were rapidly removed and glycogen was determined as described below. The total liver weight was also recorded. Percentage of water in the liver was determined by drying approximately 1 g of sample to constant weight at 60°.

Experiment 2. Thirty-two weanling rats were divided into four groups as shown in figure 6. The animals in group 1 were fed the basal diet ad libitum. Normal animals in group 2 were pair-fed with animals in group 1. Normal animals in groups 3 and 4 were fed ad libitum the basal diet and the commercial ration, respectively. On day 16 of the experiment, the animals were offered food at the zero hour (9 AM), and the body weights were recorded at various intervals over the 24-hour period. The experiment was terminated at the end of day 16.

Experiments 3 and 4. Spontaneous activity was recorded in a revolving cage connected to a counter which registered each revolution of the cage. In experiment 3, normal animals were fed the basal diet ad libitum or the restricted amount of food (10 g/rat per day) given as a single meal for 6 weeks before they were placed in revolving cages. In experiment 4, animals were placed in revolving cages at the start

[4] Rockland Mouse/Rat Stock Diet (complete), Teklad, Inc., Monmouth, Ill.

TABLE 1

Experimental design [1]

Group	Biotin status	Feeding pattern	No. of animals
1	Deficient	Ad libitum	24
2	Cured-normal	Ad libitum	24
3	Cured-normal	Restricted food intake, 10 g once a day (10 g × 1)	30
4	Cured-normal	Restricted food intake, 5 g twice a day at 12-hour intervals (5 g × 2)	24
5	Cured-normal	Restricted food intake, 2.5 g four times a day at 6-hour intervals (2.5 g × 4)	24

[1] All animals were fed ad libitum an egg-white diet for the first 4 weeks of the experiment. The animals were then grouped and fed the same diet for another 2 weeks as indicated above. Animals in control groups were cured of biotin deficiency by injecting 100 μg biotin/animal every other day for the last 2 weeks of the experiment.

of the curative and feeding treatments, i.e., after 4 weeks of being fed the basal diet. The animals in both experiments were allowed to adjust to these cages for 1 week before data were collected. In experiment 3, the activity was measured for a 24-hour period; in experiment 4 the activity was recorded from 12 noon to 6 PM, from 6 PM to 6 AM, and from 6 AM to 12 noon for 7 days.

Determination of glycogen. Immediately after decapitation about 300 mg of the liver were removed, quickly weighed on a torsion balance,[5] transferred to a tube containing 2 ml of hot 30% KOH and digested in a boiling-water bath for 30 minutes. Glycogen was coprecipitated with Na_2SO_4 from the KOH digest using 95% ethanol (13), and the precipitate was washed once with 65% ethanol, as suggested by Fong et al. (14). The precipitate was dissolved in water and glycogen was estimated by the anthrone method (15).

RESULTS

The daily food intake of biotin-deficient (group 1) and control (group 2) animals fed ad libitum in experiment 1 during the 2-week curative period is shown in figure 1. Food intake of biotin-deficient animals (group 1) was about 10 g/day during the 2-week period. In the case of control animals (group 2), food intake increased markedly immediately after the first injection of biotin and later increased gradually. The average food intake of deficient and control animals over the 2-week period was 9.5 and 17.1 g, respectively (fig. 1).

As seen in figure 2, animals fed 10 g once (group 3), 5 g twice (group 4) and 2.5 g four times (group 5) a day consumed their individual offering in about 6 hours, 30 and 15 minutes, respectively. The rate of food consumption of animals in groups 4 and 5 appeared to be the same, whereas that of animals in group 3 was considerably lower when observed after 30 minutes from the initiation of meals (see enlarged area in fig. 2). In contrast to animals given a restricted amount of food (groups 3 to 5) animals fed ad libitum (groups 1 and

[5] Roller-Smith Precision Balances, Newark, N. J.

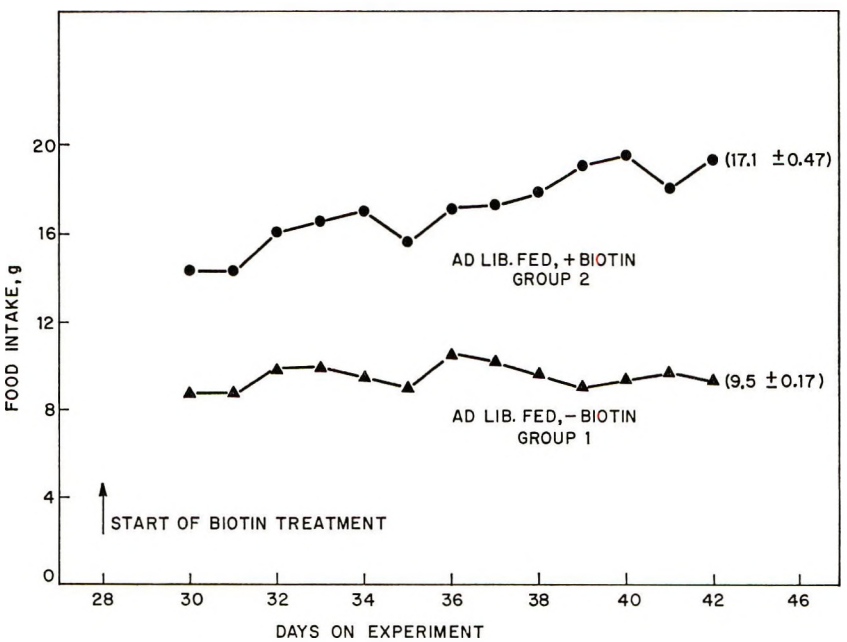

Fig. 1 Effect of biotin on food intake of rats during the curative treatment. Each result is the mean ± standard error of 24 animals. The mean ± standard error of all means in each group is shown in parentheses.

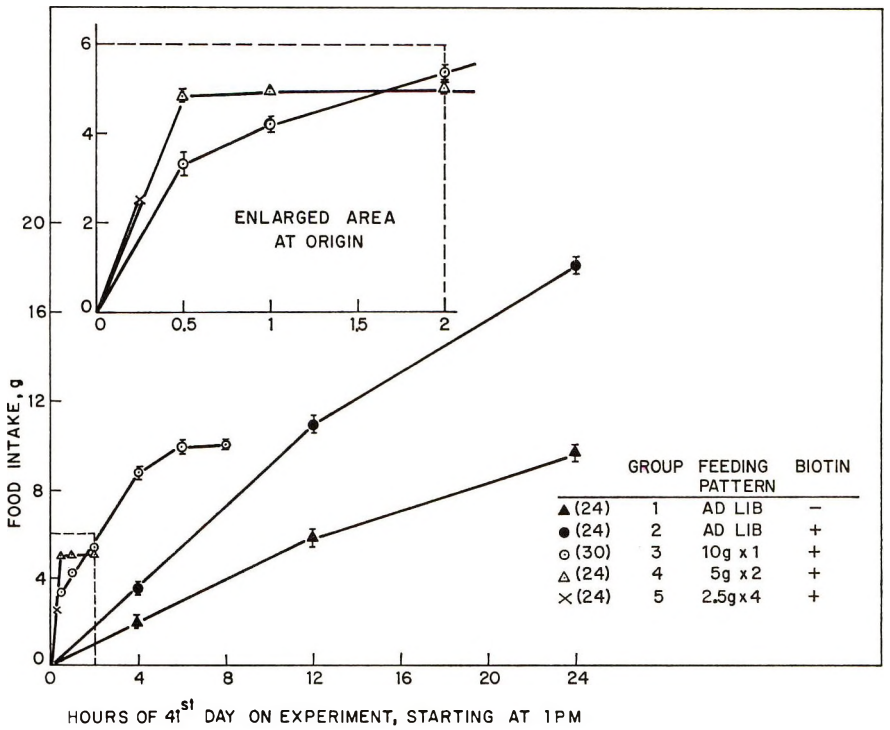

Fig. 2 Effect of various treatments on food intake of control and biotin-deficient rats. Each result is the mean ± standard error of the mean. The number of animals is shown in parentheses.

2) consumed the food steadily over the 24-hour period.

As seen in figure 3, ad libitum feeding had no oscillating effect on body weights of biotin-deficient (group 1) and control (group 2) animals over the 24-hour period, whereas the body weights of animals given 10 g once (group 3), 5 g twice (group 4) and 2.5 g four times (group 5) a day increased by about 20, 10 and 5 g, respectively. This increase was more than could be accounted for by the amount of food consumed and was due to water intake. The ratio of food to water intake was approximately 1. When the control animals (group 3) were given 10 g of food once a day, their body weights increased by 20 g about 5 hours after the commencement of the meal. After this period the body weights gradually declined and returned to the initial weight at the end of the 24-hour period. When the single meal of 10 g was given as two or four equal meals, the increase in body weight was reflected in two or four proportionately smaller peaks over the 24-hour period.

The effect of feeding patterns on liver weight is seen in figure 4. Liver weights of biotin-deficient (group 1) and control (group 2) animals did not differ at any experimental period except at the zero hour (before feeding). Control animals given 10 g of food once a day showed maximum variations in the weight of the liver, whereas the animals fed multiple meals showed proportionately smaller variations. Despite these observed changes in the total weight of the liver, the water content (69 ± 1%) of the liver per unit weight remained the same under all experimental treatments.

The effect of feeding patterns on the steady-state level of glycogen in the liver over a 24-hour period is seen in figure 5. The profiles of liver glycogen in any given group (fig. 5, compare A with B) are very similar and indicate that liver glycogen could be expressed either on the basis of

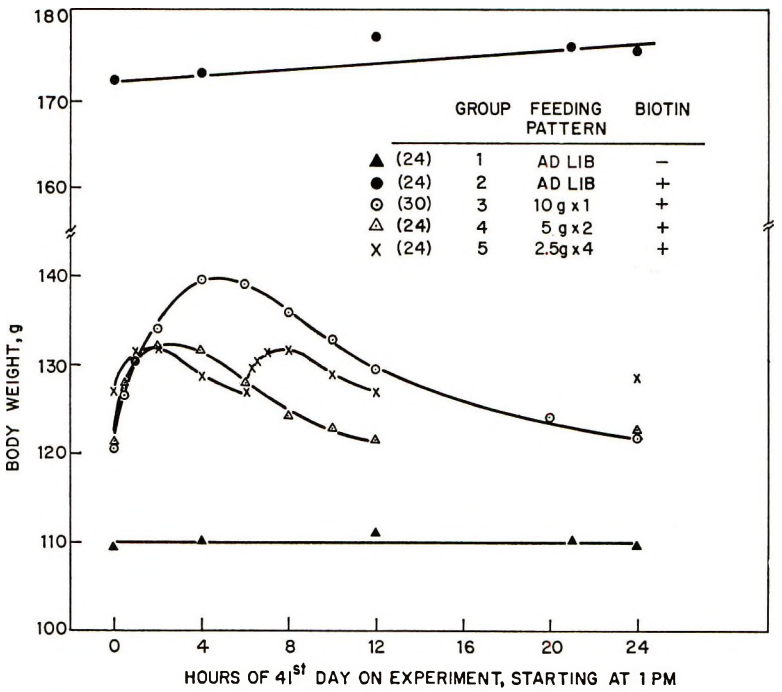

Fig. 3 Effect of feeding patterns on body weights of control and biotin-deficient rats over a 24-hour period. Each result is the mean ± standard error of the mean. The number of animals is shown in parentheses.

per gram of liver or per 100 g of body weight. The biotin-deficient (group 1) and control (group 2) animals fed ad libitum showed diurnal variations in liver glycogen; the levels did not differ during the night, but there was a significant difference during the day. Control animals fed once a day (group 3) had a very low level of glycogen in livers at the zero hour (before feeding), and was close to that observed in 24-hour-fasted normal rats. After the control animals (group 3) were offered 10 g of food the level of glycogen in the liver increased markedly in the first 8 hours and continued to increase at a slightly lower rate until it reached a peak of 95 mg/g liver, or 425 mg/100 g body weight at 12 hours, and then decreased markedly. On multiple feeding, the marked variations in liver glycogen observed in animals fed a single meal were reduced proportionately with the increase in the number of meals (compare groups 4 and 5 with group 3). The levels of liver glycogen observed in control animals fed four meals a day were in the range observed in control animals fed ad libitum (compare group 5 with group 2).

The effect of pair-feeding on body weights of control animals over the 24-hour period is shown in figure 6. As observed in the previous experiment (fig. 3, groups 1 and 2), ad libitum feeding had no oscillating effect on body weights of biotin-deficient (fig. 6, group 1) and control (groups 3 and 4) animals over the 24-hour period. Pair-fed control animals (group 2), however, showed periodic oscillations in body weights which were comparable with those observed with control animals given the restricted amount of food as a single meal (fig. 3, group 3).

The effect of restricted food intake given as a single meal on the spontaneous activity of control animals is shown in table 2. Restricted feeding resulted in approximately threefold increase in the activity of these animals as compared with similar animals fed ad libitum over the 24-hour period. Compared with animals fed ad libitum, those fed a restricted amount of food were more active even during the period

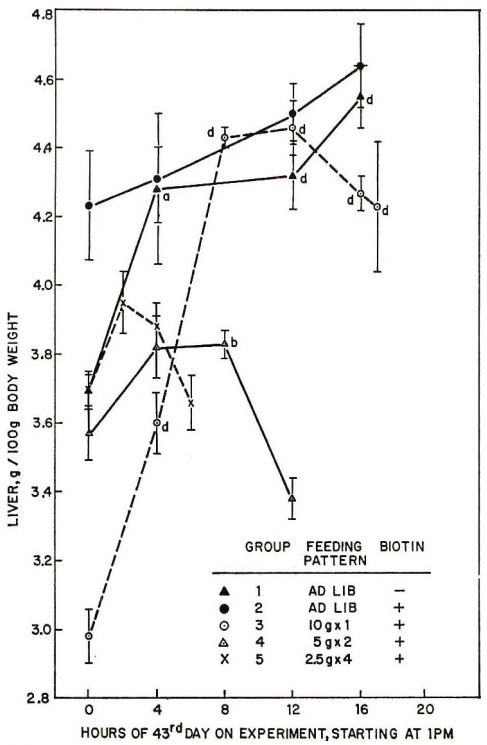

Fig. 4 Effect of feeding patterns on livers of biotin-deficient and control rats over a 24-hour period. Each result is the mean ± standard error of the mean of six animals; in group 3 at 16 and 17 hours, only three animals were used. The letter indicates the level of significance (a, < 0.05; b, < 0.02; c, < 0.01; and d, < 0.001) compared with the result at zero time of the same group.

(12 noon to 6 PM) in which they consumed their daily food allotment of 10 g. Their activity increased with the increase of the fast, whereas the activity of animals fed ad libitum increased only during the night (6 PM to 6 AM). It is of interest to note that during the period in which the food-restricted animals consumed their daily allotment there was a threefold decrease in the activity (compare 6 AM to 12 noon with 12 noon to 6 PM), whereas no change in the activity of animals fed ad libitum was observed during the same periods.

DISCUSSION

It is known that the food intake of biotin-deficient animals is markedly reduced. To restrict the energy intake of control animals, pair-feeding (2, 3), pair-weighing (4, 5) and trained-feeding (5) techniques have been used. When food intake is restricted, the animals get adapted to consume the daily food allotment in a short period after the initiation of the meal (4). As shown in the present study, the rate of food ingestion in these animals was very high as compared with animals fed ad libitum (fig. 2). Control animals given a restricted amount of food (10 g) as a single meal consumed the food in about 6 hours, but when the same amount was given as two or four equal meals, the animals consumed each allotment in 30 or 15 minutes, respectively. The time elapsed between two successive meals was greater in animals given a single meal than in animals given multiple meals; however, the rate of food ingestion in the former appeared to be slower than in the latter. No satisfactory explanation can be offered for this finding.

The rat, a nocturnal creature, consumes a greater portion of its daily food in the dark (night). This was reflected as diurnal variations in the liver weight and glycogen content of livers of animals fed ad libitum. On the other hand, in animals given restricted amounts of food, systematic oscillations in body and liver weights, and in glycogen content of livers were observed over a 24-hour period. These oscillations decreased proportionately with the increase in the number of meals offered in a day. Despite the changes observed in total weight of the liver and in hepatic glycogen of animals fed restricted amounts of food, the water content per unit weight of liver remained unaltered. On calculation, water accounted for about two-thirds of the increase in the weight of the liver, and glycogen the remaining one-third. A similar observation has been made in livers of meal-fed rats (10).

Recently several reports (8–11) have appeared demonstrating diurnal variations in liver weight and hepatic glycogen in rats adapted to a variety of experimental conditions in which access to food was limited. Leveille (9) and Leveille and Chakrabarty (10) observed diurnal variations in liver weight and tissue glycogen in rats having access to food daily for a single 2-hour period. Potter and his co-workers reported systematic oscillations in liver and in several liver enzymes of rats adapted to: a) 12 hours of feeding and 36 hours of

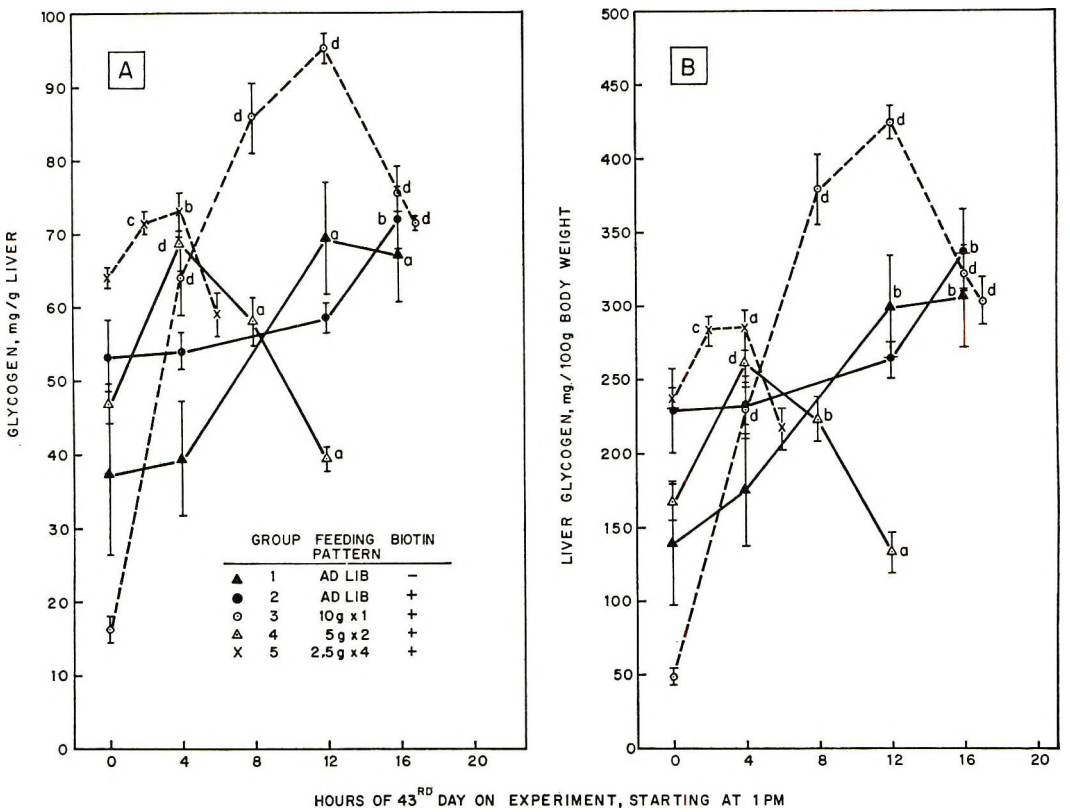

Fig. 5 Effect of feeding patterns on liver glycogen of biotin-deficient and control rats over a 24-hour period. Each result is the mean ± standard error of the mean of six animals; in group 3 at 16 and 17 hours, only three animals were used. The letter indicates the level of significance (a, < 0.05; b, < 0.02; c, < 0.01; and d, < 0.001) compared with the result at zero time of the same group.

fasting in a 48-hour period ("12 + 36" regimen) (8); b) "8 + 40" regimen (11); and c) "8 + 16" regimen (11).

Wald and Jackson (16) observed an increase in the spontaneous activity of rats deprived of food. Fabry et al. (17) and Potter et al. (8) have shown this to be true in a variety of experimental conditions in which access to food was limited. Potter et al. (8) reported that rats on a "12 + 36" regimen were more active than animals fed ad libitum. Furthermore, the voluntary activity of these animals adapted to fasting was diminished during the day (light period) as compared with the activity in the night (dark period). In the present study, the activity of animals given a restricted amount of food as a single meal was greater than that of animals fed ad libitum. Also, this activity increased with the progress of the fast. Light and dark periods had no effect on the activity of animals given restricted amounts of food, whereas a marked increase in the activity of animals fed ad libitum was observed during the night.

In the present study we have shown that the systematic oscillations in body weight, liver weight, the level of hepatic glycogen and spontaneous activity are induced in animals given a restricted amount of food as a single meal as compared with animals fed ad libitum. Thus, it is apparent that "pair-fed," "pair-weighed" and "trained-fed" control animals are different from control and biotin-deficient animals fed ad libitum. When the restricted amount of food was given in four equal meals 6 hours apart these oscillations were proportionately reduced but did not disappear com-

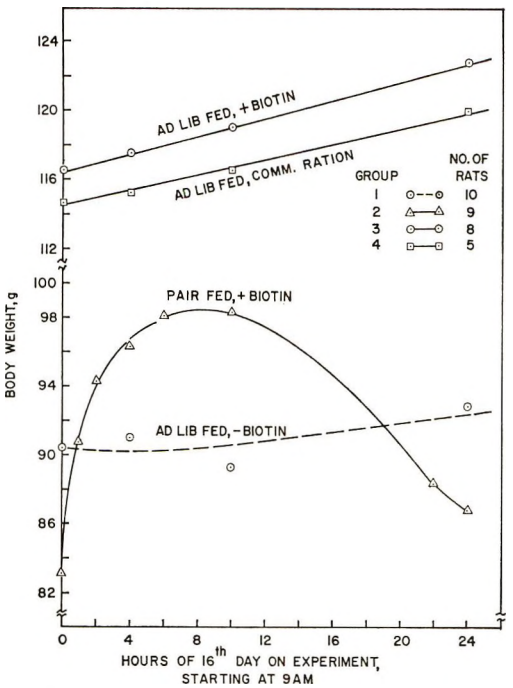

Fig. 6 Effect of pair-feeding on body weights of rats over a 24-hour period.

pletely. It is possible that these oscillations in control animals would disappear if the restricted amount of food is given in more frequent meals a day. The effect of food restriction on metabolic patterns in control animals has been investigated and will be reported in a separate paper.

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TABLE 2

Effect of feeding patterns on spontaneous activity of control animals

Exp. no.	Biotin status	Feeding pattern [1]	Time period studied: 12 Noon to 6 PM	6 PM to 6 AM	6 AM to 12 Noon	24 hr
			rev/hr	*rev/hr*	*rev/hr*	*rev/hr*
3	Normal	Ad libitum				85 ± 15(8) [2]
	Normal	10 g × 1 [3]				225 ± 16(6)
						$P < 0.001$
4	Cured-normal	Ad libitum	18 ± 4	80 ± 23	17 ± 7	46 ± 13 (4)
	Cured-normal	10 g × 1 [4]	79 ± 25	138 ± 21	273 ± 24	157 ± 18(3)
			$P < 0.05$	ns	$P < 0.001$	$P < 0.001$

[1] Animals fed once a day received their daily allotment at 12 noon.
[2] Each result is the mean ± SE of the mean of the number of animals indicated in parentheses.
[3] Fed once a day for 6 weeks from the start of the experiment.
[4] Fed once a day for the last 2 weeks of the experiment.

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Effect of Scurvy on Deiodination of ^{131}I-labeled Thyroxine in Guinea Pigs

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ABSTRACT The effect of scurvy on metabolism of thyroxine (T_4) and triiodothyronine (T_3) was investigated. Experimental scurvy was produced in guinea pigs by feeding a diet low in vitamin C for 3 or 4 weeks. Control animals received the same diet and ascorbic acid injections. Deiodination of both thyroid hormones was greatly increased in scorbutic guinea pigs. This was shown by an increasing excretion of ^{131}I in the urine and a decreasing retention of protein bound ^{131}I in the plasma, liver and kidney after a single injection of ^{131}I-labeled T_4. Similar effects were observed after ^{131}I-labeled T_3 injection. Administration of nonradioactive T_4 or cortisone to scorbutic animals failed to show any significant changes in the metabolism of ^{131}I-labeled T_4. Normal deiodinating activity was completely restored by daily injection of 15 mg of ascorbic acid for 7 days. Scurvy had no effect on the thyroid uptake of ^{131}I in vivo, but increased the amount of ^{131}I-labeled T_3 to the erythrocyte in vitro. Both plasma protein-bound iodine and thyroxine levels were significantly decreased in scorbutic guinea pigs. These low values are probably due to a decrease of plasma protein binding capacity during scurvy.

The influence of scurvy on the thyroid function has been investigated by a number of workers. Fidler et al. (1), Mosonyi and Rigo (2) and Hamne (3) observed that oxygen consumption was increased in ascorbic acid-depleted guinea pigs. Other workers were unable to show any difference in oxygen consumption and, in fact, some investigators reported decreased values (Soderstrom and Tornblom (4), Scoz et al. (5) and Baucke (6)). Studies by McCarrison (7), Bessesen (8) and Harris and Smith (9) have indicated that various types of enlargement of the thyroid gland occurred in scorbutic guinea pigs. The only work in which thyroid function was directly determined is the demonstration by Beaton et al. (10) that ascorbic acid-deprived guinea pigs exhibited a significant increase of thyroid ^{131}I uptake. Virtually nothing is known about the role of vitamin C in the degradation of thyroid hormones. Therefore, this study was undertaken to evaluate the effect of vitamin C on the rate of deiodination of injected radioactive thyroxine (T_4) and triiodothyronine (T_3). In addition, the effect of scurvy on circulating thyroxine level was assessed.

MATERIALS AND METHODS

Preparation of animals and diet. Three-to-five-week-old male guinea pigs obtained from commercial sources were used. They were housed in individual screen-bottom cages. Rabbit ration,[1] which contained a minute amount of vitamin C, was used as our basal diet for the production of experimental scurvy. Control animals received the same diet but were given intraperitoneal injections of 1 ml aqueous solution containing 15 mg of freshly prepared sodium ascorbate three times a week. Cortisone or nonradioactive thyroxine was also administered parenterally to ascorbic acid-depleted animals and controls for comparative studies.

Materials. The following radiochemicals[2] were used: sodium iodide-^{131}I (25 mCi/ug); L-thyroxine-3-^{131}I (36.7 mCi/mg); and L-triiodothyronine-3-^{131}I (23.0 mCi/mg).

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[1] Rockland rabbit chow, A. E. Staley Manufacturing Co., Decatur, Ill. Each 100 g diet contains 1.23 mg iodine and an inherent trace amount of vitamin C which was not detectable by the method of Roe and Kuether (14).

[2] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

Assessment of T_4 and T_3 deiodination. After 21 to 24 days of feeding and overnight fasting, each guinea pig received a subcutaneous injection of 2.0 ml of 1% potassium perchlorate to prevent the uptake of ^{131}I by the thyroid gland. Fifteen minutes later a single dose of ^{131}I-labeled T_4 (0.11 μg and 3 to 5 μCi in 0.25 ml) or T_3 (0.16 μg and 2 to 4 μCi in 0.25 ml) was injected intramuscularly. The animals were placed immediately into individual metabolism cages which permitted the full collection of urine. Iodide solution (25 μg KI in 100 ml of distilled water) containing 1% $KClO_4$ was offered in place of drinking water. The experiments were terminated at various time intervals; the animals were killed by cardiac puncture. Urinary excretion of ^{131}I administered as labeled thyroid hormones was measured by methods previously described (11). The results were expressed as a percentage of injected ^{131}I.

Thyroid uptake of ^{131}I. A dose of 10 μCi of sodium ^{131}I was injected intraperitoneally to each test animal. Twenty-four hours later blood was drawn from the heart through a heparinized syringe for estimation of $PB^{131}I$. The thyroid glands were removed; they were cleaned and weighed and then transferred into Pyrex counting tubes with 1 ml of 5 N NaOH. Upon completion of digestion, usually within 2 hours at room temperature, the radioactivity in the thyroid gland preparations was determined.

Measurement of labeled PBI in tissues. One gram of liver or kidney was homogenized with ice-cold water in a Potter-Elvehjem glass homogenizer. An aliquot of tissue homogenate or plasma was mixed with an equal amount of 30% trichloroacetic acid (TCA). The precipitate obtained by centrifugation was successively washed with 5% TCA three times. Both the total and protein-bound TCA ^{131}I in the plasma and tissue homogenate were measured and results were expressed as a percentage of injection radioactive T_4 or T_3.

In vitro erythrocyte uptake of ^{131}I-labeled L-triiodothyronine. The method was essentially that originally described by Hamolosky et al. (12). A measured aliquot of heparinized blood was incubated with gentle shaking for 2 hours at 37°, with a known amount of ^{131}I-labeled T_3 (0.001 to 0.012 μg/0.1 ml with 15000 to 18000 cpm/0.1 ml). The radioactivity of 1-ml aliquots of the above was measured at the end of this time. The remaining blood was then centrifuged; the plasma was removed and the erythrocytes were washed three times with isotonic saline solution at room temperature. The radioactivity of the washed erythrocytes was then measured. Results were calculated as follows:

$$\frac{\text{Net counts of washed erythrocyte (from 1 ml blood)}}{\text{Net counts of 1 ml whole blood}} \times 100$$

The percentage of erythrocyte uptake of T_3 was then corrected for an hematocrit reading of 100 and expressed as percentage uptake.

A well-type scintillation spectrometer [3] was used for the measurement of all ^{131}I activity.

Other chemical determinations. Ascorbic acid assay: Total ascorbic acid concentrations in blood and adrenal glands were assayed according to the Schaffert and Kingsley method (13), a modification of the dinitrophenylhydrazine method of Roe and Kuether (14). Blood was prepared for assay as described by the authors (14); adrenals were prepared by homogenizing with 6% TCA.

Plasma PBI and thyroxine determination. Protein-bound iodine was determined using the alkaline-ash method of Baker et al. (15). Free thyroxine (nonconjugated) was estimated by the column chromatographic technique according to the method of Pileggi et al. (16).

The results of all experiments were analyzed for statistical significance by Student's *t* test (17).

RESULTS

The effects of ascorbic acid deficiency on growth rate and certain organ weights are summarized in table 1. The animals receiving inadequate amounts of vitamin C showed a slight loss of weight, whereas vitamin C-supplemented guinea pigs grew normally. No significant effects were observed in the relative weights of the liver, kidney, heart and pituitary between the

[3] Packard Instrument Company, Inc., Downers Grove, Ill.

TABLE 1

Effect of ascorbic acid deficiency on growth and organ weights [1]

Ascorbic acid	Final body wt in 3 weeks	Organ wt						
		Liver	Kidneys	Heart	Spleen	Thyroid	Pituitary	Adrenals
	g	*g/100 g body wt*				*mg/100 g body wt*		
+	327 ± 18	4.12 ± 0.21	1.19 ± 0.18	0.22 ± 0.02	0.11 ± 0.01	10.2 ± 0.9	2.96 ± 0.14	38 ± 2.22
−	229 ± 15 [2]	4.51 ± 0.16	1.62 ± 0.03	0.25 ± 0.03	0.23 ± 0.02 [2]	16.2 ± 0.4 [3]	4.05 ± 0.28	89 ± 8.31 [2]

[1] Six ascorbic acid-supplemented guinea pigs with an initial body weight of 232 ± 7 g (mean ± SEM) and five ascorbic acid-deficient guinea pigs with an initial body weight of 252 ± 8 g were used.
[2] $P < 0.01$.
[3] $P < 0.05$.

two groups. The relative weights of the spleen, thyroids, and adrenals, however, were greater in scorbutic guinea pigs than in vitamin C-supplemented controls.

In animals kept on the scorbutigenic diet for 3 weeks, the depletion of body stores of vitamin C was evident by the findings of decreased vitamin contents in blood and in the adrenals. The mean values of six animals in each group were 0.09 ± 0.01 and 0.43 ± 0.05 (± SEM) μg/100 ml of blood, and 11 ± 1 and 125 ± 18 (± SEM) μg/100 mg of wet weight of adrenal for vitamin C-deficient and vitamin C-supplemented guinea pigs, respectively.

On day 25 of deprivation, guinea pigs showed a decrease of approximately 50% in both the PBI and thyroxine contents of the circulating plasma.

Figure 1 indicates the effect of scurvy on urinary excretion of radioiodine by guinea pigs at various times after the injection of ^{131}I-T_4. The 8-hour urinary excretion of ^{131}I obtained from the scorbutic guinea pigs was about the same as that obtained from nonscorbutic guinea pigs. By 12, 24 and 48 hours, scorbutic guinea pigs excreted significantly more than their respective controls. Because paper chromatographic analysis indicated that all the ^{131}I excreted in the urine was inorganic iodide, an increased urinary excretion of ^{131}I by

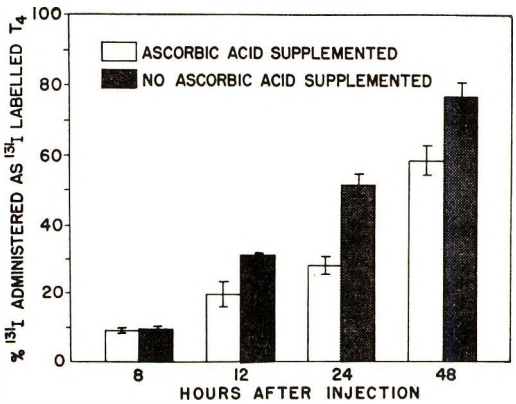

Fig. 1 Effect of ascorbic acid deficiency on urinary excretion of ^{131}I in guinea pigs following injection of ^{131}I-T_4. Each open bar is the average of five guinea pigs fed vitamin C. Each shaded bar is the average of five vitamin C-deficient guinea pigs. The vertical lines are the standard errors of the means.

vitamin C-depleted animals would suggest that scurvy increased T_4 deiodinating activity. The enhanced T_3 deiodination by scorbutic guinea pigs is illustrated in figure 2. The value at 24 hours was statistically significant.

The effects of vitamin C deficiency on tissue distribution of injected ^{131}I-T_4 and T_3 are shown in table 2. A marked reduction of PB^{131}I in the plasma, liver and kidneys was noted in scorbutic guinea pigs when these animals were killed at the end of 12, 24 and 48 hours of ^{131}I-T_4 injection. The administration of ^{131}I-T_3 for 4 hours produced a significant increase in the uptake of radioactivity in the plasma, liver and kidneys of scorbutic guinea pigs as compared with the control animals. By 24 hours these findings were reversed, the PB^{131}I values in various tissues of deficient animals were approximately one-third or less of normal values.

The effects of a single injection of ascorbic acid, thyroxine or cortisone to scorbutic animals on urinary excretion and tissue distribution of injected ^{131}I-T_4 are shown in table 3. No significant effects were observed by any of the above treatments.

In the experiment shown in table 4, guinea pigs were depleted of vitamin C for a period of 3 weeks and then received vitamin C injections for 7 days. In contrast to the scorbutic animals, urinary excretion of ^{131}I and tissue uptake of PB^{131}I in animals replenished with ascorbic acid did

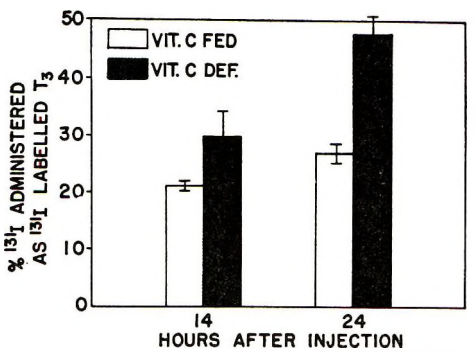

Fig. 2 Effect of ascorbic acid deficiency on urinary excretion of ^{131}I in guinea pigs following injection of ^{131}I-T_3. Each open bar is the average of five guinea pigs fed vitamin C. Each shaded bar is the average of five vitamin C-deficient guinea pigs. The vertical lines are the standard errors of the means.

TABLE 2

Effect of ascorbic acid deficiency on tissue distribution of ^{131}I in guinea pigs following injection of ^{131}I-labeled T_4 and ^{131}I-labeled T_3

Hours after injection	Compound used	Plasma		Liver		Kidney	
		+ Vitamin C	− Vitamin C	+ Vitamin C	− Vitamin C	+ Vitamin C	− Vitamin C
4	^{131}I-T_4	1.06 ± 0.05 [1] (5) [2]	1.26 ± 0.04 (6)	0.46 ± 0.09	0.49 ± 0.02	0.56 ± 0.01	0.59 ± 0.02
12	^{131}I-T_4	0.81 ± 0.09 (4)	0.29 ± 0.05 [3] (4)	0.99 ± 0.05	0.34 ± 0.06 [3]	0.40 ± 0.08	0.19 ± 0.03 [3]
24	^{131}I-T_4	0.72 ± 0.07 (6)	0.23 ± 0.06 [3] (6)	0.35 ± 0.06	0.11 ± 0.04 [3]	0.45 ± 0.06	0.16 ± 0.01 [3]
48	^{131}I-T_4	0.27 ± 0.05 (6)	0.03 ± 0.01 [3] (6)	—	—	0.18 ± 0.03	0.03 ± 0.01 [3]
4	^{131}I-T_3	0.076 ± 0.015 [1] (4) [2]	0.136 ± 0.012 [3] (5)	0.131 ± 0.052	0.401 ± 0.023 [3]	0.371 ± 0.013	0.712 ± 0.06 [3]
24	^{131}I-T_2	0.021 ± 0.003 (5)	0.007 ± 0.001 [3] (5)	0.047 ± 0.007	0.012 ± 0.003 [3]	0.102 ± 0.015	0.028 ± 0.01 [3]

[1] Mean ± SEM. The results are expressed as follows:

$$\frac{\text{Net counts of TCA-precipitable fraction of 1 g of liver or kidney or 1 ml of plasma}}{\text{Net counts of injected dose}} \times 100.$$

[2] Figures in parentheses indicate the number of guinea pigs used.

[3] $P < 0.01$.

not differ significantly from that found in the corresponding control animals.

The effect of ascorbic acid deficiency on ^{131}I uptake by the thyroid is shown in table 5. The radioactivities found in the thyroids, plasma and urinary excretion of scorbutic guinea pigs were not significantly different from the values obtained in their corresponding controls.

During the 2-hour incubation period, the amount of ^{131}I-T_3 bound to the erythrocytes in vitro was significantly increased in scorbutic guinea pigs, as compared with non-scorbutic animals (table 6). These radioac-

TABLE 3

Effect of cortisone, thyroxine or ascorbic acid on distribution of ^{131}I in scorbutic guinea pigs 24 hours following ^{131}I-T_4 injection

Group [1]	Ascorbic acid	Other treatment	Plasma	Liver	Kidney	Urinary excretion
1	+	None	1.083 ± 0.083 [2]	0.338 ± 0.002	0.433 ± 0.047	27.7 ± 1.1
2	—	None	0.272 ± 0.161 [3]	0.151 ± 0.059 [3]	—	54.5 ± 5.5 [3]
3	—	Ascorbic injection	0.371 ± 0.064 [3]	0.135 ± 0.019 [3]	0.214 ± 0.026 [3]	43.7 ± 2.6 [3]
4	—	Thyroxine	0.556 ± 0.119 [3]	0.186 ± 0.034 [3]	0.158 ± 0.020 [3]	55.5 ± 3.7 [3]
5	—	Cortisone	0.270 ± 0.039 [3]	0.172 ± 0.008 [3]	—	45.4 ± 6.1 [3]

[1] Five guinea pigs were used in each group. Groups 3 and 4 received intraperitoneally a single dose of 30 mg ascorbic acid and 40 μg L-thyroxine, respectively, 45 minutes before ^{131}I-T_4 injection. Group 5 received 4 mg cortisone daily for 4 days before ^{131}I injection.

[2] Mean ± SEM. Urinary excretion is expressed as percentage of ^{131}I administered as T_4. The other results are expressed as follows:

$$\frac{\text{Net counts of precipitable fraction of 1 g of liver or kidney or 1 ml of plasma}}{\text{Net counts of injected dose}} \times 100.$$

[3] $P < 0.01$. Values obtained from group 1 are significantly different from values obtained from the remaining groups. Values obtained from groups 2, 3, 4 and 5 are not significantly different from each other.

TABLE 4

Effect of ascorbic acid repletion on urinary excretion and tissue distribution of ^{131}I in scorbutic guinea pigs 24 hours following the injection of ^{131}I-T_4

Ascorbic acid	Plasma	Liver	Kidney	Urinary excretion
+	0.587 ± 0.116 [1](5) [2]	0.213 ± 0.053	0.351 ± 0.018	28.8 ± 2.9
—	0.231 ± 0.034 [3](4)	0.112 ± 0.003 [3]	0.182 ± 0.034 [3]	50.6 ± 3.1 [3]
— + [4]	0.579 ± 0.153 (6)	0.237 ± 0.041	0.392 ± 0.023	31.7 ± 2.3

[1] Mean ± SEM. Urinary excretion is expressed as percentage of ^{131}I administered as T_4. The other results are expressed as follows:

$$\frac{\text{Net counts of TCA-precipitable fraction of 1 g of liver or kidney or 1 ml of plasma}}{\text{Net counts of injected dose}} \times 100.$$

[2] Figures in parentheses indicate the number of animals used.

[3] $P < 0.01$.

[4] Guinea pigs were maintained on the vitamin C-deficient diet for 3 weeks and then received daily injections of 15 mg Na-ascorbate for an additional 7 days.

TABLE 5

Effect of ascorbic acid deficiency on thyroid ^{131}I uptake

Ascorbic acid	No. of animals	Final body wt in 3.5 weeks	Thyroid	Total	PBI	Urinary excretion
		g				
+	5	379 ± 18	8.79 ± 0.42 [1]	0.10 ± 0.02	0.0097 ± 0.0011	46.0 ± 3.6
—	6	211 ± 28 [2]	8.57 ± 0.42	0.14 ± 0.02	0.0158 ± 0.0011	39.8 ± 6.3

[1] Mean ± SEM. The results are expressed as follows:

$$\frac{\text{Net counts of both thyroids, 1 ml plasma or 24 hour urinary excretion}}{\text{Net counts of injected dose}} \times 100.$$

The animals were killed 24 hours after $Na^{131}I$ injection.

[2] $P < 0.01$.

TABLE 6

Effect of ascorbic acid deficiency on erythrocyte uptake of ^{131}I labeled T_3 in vitro

Trial [1]	Ascorbic acid	No. of guinea pigs	Erythrocyte uptake
			%
1	+	8	61 ± 2.7 [2]
	−	6	76 ± 2.9 [3]
2	+	5	59 ± 4.8
	−	5	76 ± 1.8 [3]

[1] ^{131}I-labeled L-triiodothyronine was diluted with isotonic saline solution to contain 0.001 to 0.012 μg/0.1 ml with 15000 to 18000 cpm/0.1 ml in trial 1. In trial 2, a phosphate buffer was used to replace isotonic saline solution for dilution, washings and counting.
[2] Mean ± SEM.
[3] $P < 0.01$.

tive T_3 values are considerably higher than those reported for human (18) and chicken red cells (19).

DISCUSSION

The data presented in this paper suggest that vitamin C is directly or indirectly involved in the degradation of thyroid hormones. This is indicated by increased urinary excretion and decreased tissue retention of radioactivity in scorbutic guinea pigs following the injection of ^{131}I-labeled thyroxine or triiodothyronine. These findings however, do not necessarily imply similar behavior of naturally occurring nonradioactive thyroid hormones.

This study also demonstrates that erythrocyte uptake of labeled L-triiodothyronine in vitro was much greater in scorbutic guinea pigs than in nonscorbutic animals. Because the amount of triiodothyronine taken up by the red cells is inversely related to the binding capacity of the serum protein carriers for thyroid hormones (20, 21), these observations may indicate a reduced binding capacity in sera of scorbutic animals which in turn resulted in an enhancement of deiodination.

It has been suggested that physiological deiodination of T_4 is mediated by a hydrogen peroxide-peroxidase system (22). When vitamin E is deficient, lipid peroxidation in several tissues is greatly increased (23). Thus, it is possible that this vitamin is required in the regulation of the rate of T_4 deiodination by its antioxidant properties. Although the sparing effect of ascorbic acid to auto-oxidation of tocopherols has been demonstrated in vitro (24) and in vivo (25), it is not clear that increased deiodination in scurvy is associated with the alterations of vitamin E contents in tissues.

The data obtained with radioiodine indicate that scurvy is not associated with an impaired uptake of ^{131}I by the thyroid. The low values of circulating PBI and thyroxine in scorbutic animals suggest that scurvy induces either hypothyroidism or enhanced deiodination, or both. These observations do not agree with the earlier report of Beaton et al. (10) that scorbutic guinea pigs showed an increased uptake of ^{131}I and therefore had an increased thyroid activity. Measurements of radioactivity in the thyroid region by these workers and in the thyroid tissues in the present study might account for the differences. The differing severities of scurvy in the animals used might also produce different effects on T_4 deiodinating activity.

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Distribution and Metabolism of Menadiol Diphosphate in the Rat [1]

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ABSTRACT Physiological doses of radioactive menadiol diphosphate, an active water-soluble form of vitamin K, were administered by intraperitoneal injection to vitamin K-deficient male rats. The compound was not concentrated by any tissue but was distributed throughout all body organs in a manner which was not influenced by the vitamin K status of the animal. Seventeen hours after an intraperitoneal injection of the radioactive vitamin about 50% of the activity had been excreted, mainly in the urine. There was a general association of the injected compound or its metabolites with the membranous fractions of the cell. The data also indicate that there was a rapid conversion of this water-soluble compound to more lipophilic forms of the vitamin.

The only observable effect of a deficiency of vitamin K in higher animals is a decrease of the blood level of prothrombin and the related clotting factors, VII, IX and X. It has been demonstrated that the production of prothrombin and its release to the plasma occurs in the hepatic parenchymal cells (1). Although details of the mechanism are not known, the current consensus is that the vitamin exerts its action at some point subsequent to the formation of a specific messenger RNA molecule, but prior to the release of the specific proteins (2–4). A better knowledge of the metabolism of vitamin K in the animal would be useful in future studies directed toward establishing the mechanism of action of the vitamin.

Previous studies utilizing radioactive-labeled compounds have indicated that phylloquinone [3] or menaquinone-4 are concentrated to some extent in the liver whereas menadione is more rapidly excreted and is distributed more evenly in various tissues (5–7). Available evidence would also indicate that the vitamin is found in highest concentration in the mitochondria (8–10). The data currently available on the metabolism and distribution of the radioactive vitamins have been obtained by using relatively massive doses, and may not be indicative of the metabolism of physiological levels of the compounds involved. This report deals with the distribution of physiological doses of radioactive menadiol diphosphate, a highly active water-soluble form of the vitamin (11), in the deficient rat and the subcellular distribution of the vitamin in rat liver.

METHODS

Animals. Male rats of the Holtzman strain (140 g) were housed in coprophagy-preventing cages (12) and fed a soy protein-low vitamin K diet (13) which in our laboratory results in a severe deficiency in 7 to 10 days. Clotting-factor activity was measured on blood obtained by heart puncture by the one-stage method of Quick using commercial thromboplastin,[4] or by the two-stage method of Ware and Seegers as modified by Shapiro and Waugh (14). Vitamin K was administered intraperitoneally as menadiol diphosphate.[5]

Tissue preparation. Deficient rats were removed from the coprophagy-preventing cages, injected intraperitoneally with radioactive menadiol diphosphate [6] and placed in metabolism cages. At the indicated times, animals were anesthetized with ether and killed by withdrawing blood from the abdominal aorta; various organs were removed for determination of radioactivity. Subcellular fractionation of the liver, ex-

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[2] Public Health Service Predoctoral fellow F-1 GM-35,372.
[3] See 1966 J. Biol. Chem., *241*: 2989 for nomenclature of the vitamin K active quinones.
[4] Simplastin, Warner-Chilcott Laboratories, Morris Plains, N. J.
[5] Synkayvite, Hoffmann-LaRoche Inc., Nutley, N. J.
[6] 2-Methyl-1,4-naphthaquinol-6,7-T diphosphate (tetra sodium salt) was obtained from Nuclear-Chicago, Des Plaines, Ill., stored at − 68° and used without further purification.

cept for nuclei and mitochondria, was carried out as described by Sarcione (15). Nuclei were isolated by the method of Blobel and Potter (16), and mitochondria from a 10% homogenate in 0.25 M sucrose by centrifuging at 7,000 × *g* for 10 minutes after an initial sedimentation for 10 minutes at 700 × *g* to remove nuclei and debris. Protein was analyzed by a modification of the colorimetric method of Lowry (17). Lipid extracts of the subcellular fractions were obtained by Bligh and Dyer (18).

Radioactivity determination. All measurements were performed in a liquid scintillation spectrometer.[7] The femoral bone, feces and red blood cells were digested and radioactivity determined by the method of Mahin and Lofberg (19). All other organs and the subcellular fractions of the liver were minced or homogenized and solubilized with an organic base [8] before addition of a toluene–PPO–POPOP solution. Radioactivity of the chloroform and H_2O extracts was measured using toluene–PPO–POPOP and Bray's (20) counting solutions, respectively. Absolute counting rates were obtained by using tritiated toluene as an internal standard.

RESULTS

The response of vitamin K-deficient rats to increasing levels of menadiol diphosphate is shown in table 1. One-stage clotting times were restored to normal at the 10-μg level but prothrombin content, as determined by the two-stage assay, did not approach normal until 25 μg of the vitamin had been administered. Based on these data all of the radioactive tracer experiments reported here utilized less than 3 μg menadiol diphosphate/rat, an amount sufficient to initiate clotting-factor synthesis but well within the physiological range.

The results of experiments designed to study gross distribution of injected menadiol diphosphate are presented in table 2. The compound or its metabolites were deposited in all tissues studied and there was no evidence that any one organ concentrated the vitamin with increasing time. With the exception of high values in the kidney, presumably because of residual urine, radioactivity per gram of the various soft tissues did not differ extensively. The amount of the compound in most tissues decreased with time and there was a concomitant increased excretion in the feces and urine. The major route of excretion was the urine, where 43% of the activity was found 17 hours after injection, as compared with 4% found in the feces. The data in table 3 illustrate the lack of an effect of the vitamin K depletion state of the body on distribution of menadiol diphosphate. Deficient and normal rats killed 6 hours after injection of the radioactive vitamin showed a marked similarity in the distribution of the compound.

The subcellular distribution of the radioactivity in the liver 3 hours after the injection of menadiol diphosphate, and that percentage of the radioactivity which was lipid extractable in each of these subcellular fractions, are shown in table 4. The relative vitamin K contents calculated in the table are values obtained by using the specific activity of the original injection

[7] Tri-Carb, model 3002, Packard Instrument Company, Inc., Downers Grove, Ill.
[8] NCS, Nuclear-Chicago, Des Plaines, Ill.

TABLE 1

Response of vitamin K-deficient rats to menadiol diphosphate

Amount of menadiol diphosphate	No. of rats [1]	Prothrombin time [2]	Prothrombin [3]
μg		*seconds*	*units/ml*
None	8	81.1(33.9–190.4) [4]	6.7± 1.9 [5]
2.5	5	20.1(13.4– 25.5)	41.1± 7.5
10	8	13.8(11.9– 15.4)	112.7±15.1
25	7	13.0(12.4– 13.4)	186.0±17.2
Normal controls	7	13.2(12.4– 14.5)	193.3±15.0

[1] Deficient male rats (140 g) were given the sodium salt of the vitamin by intraperitoneal injection, and blood drawn for prothrombin assay 20 hours later.
[2] Prothrombin times were determined by the one-stage method.
[3] Units of prothrombin were determined by the two-stage method.
[4] Mean and range.
[5] Mean ± SE.

TABLE 2

Gross distribution and excretion of radioactivity from menadiol diphosphate [1]

Organ or excretion route	Injected radioactivity		
	3 hour (7) [2]	10 hour (10)	17 hour (6)
	%	%	%
Urine	13.0 ±5.3 [3]	36.3 ±2.7 [3]	42.9 ±6.5 [3]
Feces	< 0.1	2.2 ±0.4	4.0 ±1.2
GI tract contents	27.6 ±3.6	12.4 ±0.6	13.0 ±1.9
Skeletal muscle	11.8 ±1.5	15.6 ±0.3	6.4 ±0.6
Skin and subcutaneous tissue	12.4 ±1.6	9.2 ±0.5	8.6 ±0.4
Bone	8.1 ±0.8	7.6 ±0.8	3.8 ±0.5
Liver	2.0 ±0.3	1.3 ±0.1	0.79±0.05
Kidney	4.8 ±1.3	0.99±0.08	0.54±0.04
Lung	0.51±0.04	0.39±0.08	0.25±0.02
Heart	0.20±0.01	0.20±0.01	0.13±0.01
Spleen	0.25±0.02	0.21±0.02	0.08±0.01
GI tract	6.2 ±0.5	2.7 ±0.2	2.4 ±0.2
Plasma	2.9 ±0.8	1.8 ±0.1	0.67±0.07
Erythrocytes	0.81±0.13	0.91±0.13	0.21±0.02

[1] Deficient male rats (140 g) were given an intraperitoneal dose of 160 to 210 μCi of 2-methyl-1,4-naphthaquinol-6,7-T diphosphate. Total dose was from 1.6 to 2 μg/rat in all cases. In calculating percentage of dose in tissues, skeletal muscle, skin and subcutaneous tissue, blood and bone were assumed to represent 43, 26, 7 and 17.5% of body weight. Other organs were weighed directly. Activity in adipose tissue was not determined; activity in testes was determined at the 10-hour period only, where they were found to have 0.5% of the injected dose.
[2] Number of rats per group indicated in parentheses.
[3] Mean ± SE.

TABLE 3

Effect of vitamin K status on excretion and distribution of menadiol diphosphate

Organ or excretion route	Injected radioactivity	
	Deficient rats [1] (4)	Control rats [1] (5)
	%	%
Urine	32.2 ±1.7 [2]	31.5 ±2.3 [2]
Feces	1.4 ±0.8	3.2 ±2.2
GI tract contents	13.4 ±2.2	14.8 ±1.5
Skeletal muscle	5.3 ±0.6	5.5 ±0.5
Bone	7.7 ±2.7	11.1 ±1.1
Liver	1.7 ±0.1	1.2 ±0.1
Kidney	0.97±0.02	0.56±0.06
Lung	0.45±0.03	0.40±0.04
Heart	0.14±0.02	0.11±0.02
Spleen	0.20±0.03	0.13±0.01
GI tract	4.9 ±0.6	4.6 ±0.4
Plasma	1.5 ±0.4	1.4 ±0.2
Erythrocytes	0.32±0.07	0.60±0.06

[1] Deficient or control rats (140 g) were given 2.0 or 2.76 μg, respectively, of 2-methyl-1,4-naphthaquinol-6,7-T diphosphate (105 μCi/μg) by intraperitoneal injection and killed 6 hours later. Tissue treatment as in table 2, footnote 1. Number of rats per group indicated in parentheses.
[2] Mean ± SE.

material as a conversion factor. Radioactivity not in the chloroform extract was found to be distributed between the insoluble protein residue and the H_2O extract. These data show no evidence of an extremely high concentration of the vitamin in any one fraction; however, the data do suggest a general association of the vitamin with those fractions possessing the greatest amount of membrane material. This is illustrated in the high specific activities of the mitochondrial, deoxycholate-soluble and microsomal fractions. Analyses revealed that the high specific activity in the soluble enzyme fraction was due primarily to water-extractable material. The high percentage of lipid-extractable radioactivity in all other subcellular fractions suggests that the water-soluble form of the vitamin administered is readily converted

TABLE 4

Distribution of radioactive vitamin K and vitamin K metabolites in liver [1]

Cellular fraction	Vitamin K and K metabolites	Percentage of activity chloroform soluble
	picomoles/g protein	
Total homogenate	58 ± 4	46.0 ± 2.0
Nuclei	35 ± 4	51.7 ± 3.8
Triton X-100 treated nuclei	24 ± 3	—
Mitochondria	90 ± 8	51.6 ± 3.1
Soluble protein	121 ± 8	7.3 ± 0.4
Microsomes	75 ± 5	50.3 ± 2.7
Ribosomes	31 ± 4	64.2 ± 4.4
Deoxycholate-soluble microsome fractions	90 ± 3	42.8 ± 2.4

[1] Deficient male rats (140 g) were given 2 μg of 2-methyl-1,4-naphthaquinol-6,7-T diphosphate (160 to 210 μCi) by intraperitoneal injection, and killed at 3 hours. Molar equivalents of the vitamin in the fractions were calculated from the specific activity of the compound injected. Total recovery of the radioactivity in the chloroform and aqueous phase was about 75% with the remainder in the interphase residue. Values are mean ± SE for 15 rats in the determination of the amount of vitamin in subcellular fractions, and for 5 rats in chloroform extraction.

to some lipid-soluble form. Similar results were found 3 hours after injection when livers from normal rats were used, or 10 hours after injection when livers from deficient rats were used.

DISCUSSION

Previous studies have indicated that administered doses of phylloquinone are concentrated in the liver whereas menadione is not. The studies reported here indicate that a single dose of menadiol diphosphate, in the amount needed for a physiological response, is metabolized in much the same way as has been reported for more massive doses of menadione.

Menadiol diphosphate does not have a long retention time in the body and by 17 hours about one-half the administered radioactivity has been lost. No attempt has been made to determine the chemical nature of the excretion products; however, a number of metabolites of menadione have recently been discovered (21) and presumably account for much of the activity. The data clearly indicate that at the level of administration employed there was no major shift in the tissue distribution of the vitamin in deficient and normal animals.

When the dosage differences are considered, the amount of lipid-soluble radioactivity found in the liver in these studies is in the same range as the amount of menaquinone-4 found in the liver by Taggart [9] following menadione administration. Preliminary studies indicate that much of the activity in the lipid-soluble extract is present as menaquinone-4, although some menadione and a third more polar compound appear to be present.

Chemical analyses have previously indicated a concentration of vitamin K in liver mitochondria (9) and this study also indicated that the highest concentration of lipid-soluble activity was in the mitochondrial fraction. The microsome fraction was also high in activity and the results of the deoxycholate treatment indicated that the activity was associated mainly with the membrane, rather than the ribosomal portion of the microsomes. The loss of activity from nuclei by Triton X-100 treatment would again indicate that much of the cellular vitamin K is bound to various membrane systems. A preliminary report by Bell et al.[10] also indicates that when microgram amounts of radioactive phylloquinone were administered to rats, the majority of the activity was associated with cellular membrane fractions.

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Interrelationships Among Energy Input, Body Size, Age and Body Composition of Sheep [1]

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ABSTRACT Interactions of age, body weight and energy intake on the proximate chemical composition and energy value of the body were examined in 26 Shropshire, male-castrate sheep. Body weight at a given age was manipulated by imposing on a given animal one of two levels of energy input (averaging 278 and 421 kcal of gross energy/1 kg empty-body weight $^{0.73}$ per day). The amounts of body components at a given age were greater in sheep receiving the high intake of energy than in those supplied the low level. The relationships between the body components and body weight, however, were not different for the two energy input levels. Thus, energy input, within the range studied, did not influence body composition in a manner independent of its effect on body mass. In animals containing less than 31% of fat, the amounts of body components increased linearly with increasing body weight; above this concentration of fat, the weights of water and protein increased at decreasing rates and the amounts of fat and energy increased at increasing rates, as body weight increased. The overall best fit of the relationships between the body components and body weight was provided by the model, $Y = a X^b$. For these relationships, the R^2 values for the predictands other than ash ranged from 0.939 to 0.982. The use of age in addition to body weight as the predictors increased the R^2 values by only 0.002 to 0.019 over those computed between body weight alone and the various predictands. Thus, body composition in sheep was not related to prior energy intake, only slightly associated with age, but was chiefly associated with body weight.

Attention was drawn early by the studies of Waters (1, 2) to the possibility that the tissues and parts of the animal body grow differentially relative to age. In 1908 he reported that cattle fed maintenance or slightly submaintenance rations after they had been reared on a high plane of nutrition to an age of 9 to 17 months continued to grow in height and length of skeleton for at least 1 year during which time body fat was gradually depleted. This observation indicated that certain tissues have a higher priority claim for nutrients than others at certain stages of growth.

In his theory of "differential growth," Hammond (3) set forth the viewpoints that the body proportions and conformation result from differential growth gradients between the body tissues and parts, and that these occur in a definite order. The studies of other members of his school (McMeekan, 4–6, and Pálsson and Vergés, 7, 8) amplified and extended these ideas, particularly with respect to the influence of plane of nutrition when imposed at certain stages of growth in pigs and sheep, respectively. They (4–8) concluded that the plane of nutrition imposed at certain times influences the development of the anatomical parts and of the physically separated tissues of the body differently depending upon their nutrient priorities and needs relative to the level of nutrients provided. However, significant to their conclusions is the basis on which they were derived. Both groups of workers completely dissected the total body into its component parts, organs and tissues, and they expressed the weights of these for the various treatment groups as percentages of those for a "standard" group, which was the least advanced in development.

Earlier, Child (9) had proposed that the partition of ingested nutrients among the various body tissues is determined by the metabolic rate of the tissues. Accordingly, the body tissues, organs or parts having the highest metabolic rate have the highest priority for nutrients. This viewpoint coupled with the findings of the Cambridge school (3–8) led Hammond (10) to

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the generalization that the descending order of priority for nutrients by the various body tissues is: nervous tissue, bone, muscle and fat. It is clear, however, that physiological states and the degree of maturity of the animal, and the amounts and kinds of substrates supplied can greatly influence the metabolic activity of a given tissue. In recent studies (11) with young calves and fattening older cattle, it was demonstrated that the activity of a number of adipose tissue enzymes concerned with fat synthesis is markedly greater in the mature, fat steers.

On the other hand, Wallace (12), another Cambridge worker, suggested that the apparent differential influence of nutritional plane on the relationships within tissues as reported by McMeekan (4–6) resulted chiefly from the terms in which they were expressed. He (12) proposed that the proportion of each tissue in the various carcass cuts relative to the total weight of each tissue in McMeekan's pigs was quite normal and, therefore, was not affected differentially by the plane of nutrition imposed. Elsley et al. (13), upon reanalyzing the data for pigs (McMeekan, 4–6) and for sheep (Pálsson and Vergés, 7, 8) by eliminating the effects of variation in fat content, concluded that a large part of the apparent differences in body proportions reported by those workers (4–8) is attributable to the effects of level of nutrition on fat deposition. Elsley et al. (13) noted especially that the planes of nutrition did not exert a differential effect on the weight of bone or of muscle relative to the sum of the total weights of muscle and bone together. However, as the result of applying the model, $Y = a X^b$, to the same data plus those of Hammond (3), Wallace (12, 14) and Pomeroy (15), Tulloh (16) found that 88.3 to 98.7% of the variation in the logarithms (base 10) of the weights of physically separated fat, bone and muscle is associated with the variation in the logarithm of the ingesta-free body weight.

In studies in which sheep had received a variety of diets, each imposed at two or three levels of energy input, we (17–19) observed a high degree of association between the chemical composition of the body and the body weight of animals when they had been maintained in a continuously positive energy balance. Fitting the data by means of the curvilinear model, $Y = a X^b$, in which Y = weight of chemical component or megacalories of energy and X = ingesta-free body weight, resulted in the following R^2 values for the various components: water, 0.95; protein, 0.95; fat, 0.88; and energy, 0.94. These data (19) represent a composite of 221 male castrates of six breed populations. Although most of these animals had not reached maturity, others were as old as 895 days and weighed as much as 78 kg.

In a study of 32 men ranging in age from 34 to 76 years and weighing from 50.8 to 151.8 kg, Remenchik and Bersohn (20) observed an unexpectedly high R^2 value (0.94) between estimated body fat and linear body weight. They determined body fat as the difference between total body weight and lean-body mass. Lean-body mass was assumed to contain 73% water, and body water was determined indirectly by means of tritium dilution. Also, the data of Wood and Groves (21) obtained with young pigs weighing 1 to 30 kg, represent a highly predictive relationship between body composition and linear body weight. From their data, we computed a coefficient of variation of only 2.55% between the weight of body water determined by desiccation and that predicted from linear body weight.

Although present evidence indicates that body composition and body weight are highly related and that, prior to maturity, body weight and age generally are correlated, no attempt appears to have been made to partition the relative degrees of the variance in body composition that are ascribable to the variation in age and body weight.

Accordingly, the objectives of the present study were: a) to examine the interactions among body weight, age and energy intake of sheep on the chemical composition of the body, and b) especially, to partition the variance in body composition between body weight and age.

MATERIALS AND METHODS

In this experiment body weight was varied somewhat independently of age by regulating the dietary energy input. Twenty-six purebred Shropshire wethers

were used. After slaughter and the measurement of the appropriate weights, the shorn, whole body minus ingesta (i.e., empty body) was minced; water, ether extract, protein and ash contents, and the heat of combustion were determined according to procedures outlined previously (17, 22).

The general plan of the experiment with respect to body size and age at slaughter, and to the energy levels imposed, is diagramed in figure 1. The basic experimental unit consisted of a trio of sheep, all of the same age (about 140 days) and the same full-body weight (ranging from 16.5 to 26.1 kg) at the beginning of the feeding period. One member (H) of each trio received a high allowance of energy and was killed at a body weight decided upon prior to the beginning of the experiment; this weight represented that set for high level animals at uniform intervals ranging from 26.7 to 65.0 kg. His two corresponding mates were provided a low level of energy. One (L_1) of the low level animals was killed on the same day (X_1) as the high level sheep (H); the other (L_2) was killed (X_2) when he reached the same body weight as that at which the high level sheep (H) was slaughtered. Thus, in each trio, sheep L_1 was of the same age (X_1), but of a lower body weight than sheep H, and sheep L_2 was the same body weight, but of greater age (X_2) than sheep H (the difference ranging from 61 to 240 days for a given trio).

Six such trios and one odd animal (fed the high level of energy and killed at a median full-body weight (44.2 kg)) constituted the animals on which the two dietary treatments were imposed. Seven additional

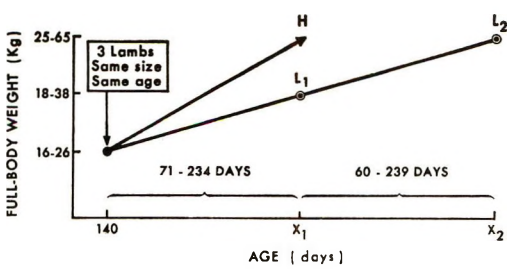

Fig. 1 Basic plan of the experiment with respect to dietary energy input and slaughter weights and ages.

sheep ranging in full-body weight from 15.3 to 29.1 kg were analyzed to determine the "base-line" body composition at the beginning of the feeding period. The beginning body composition of the animals exposed to the dietary treatments was computed by means of the equations resulting from the regression of the chemical components of the body on the body weights of the seven "base-line" animals, as described previously (18, 23).

The plan of animal allotment consisted of forming six groups, each of four animals having the same body weight and age. From each group, one animal taken at random was killed as a member of the "initial" or "base-line" composition group. These, plus one additional animal, a little heavier than the heaviest of the six grouped animals, constituted the "initial" slaughter group. Each of the remaining three animals in each of the six groups was allotted at random to one of the treatments (H, L_1 or L_2) described above. In general, the trios were assigned to slaughter-weight groups in the order of their initial body weight, that is, the smallest trio was killed at the lightest final weight and heaviest trio was slaughtered at the largest final weight.

A pelleted diet consisting of the following percentages of various ingredients was fed: alfalfa hay, 55; corn meal, 18.5; wheat middlings, 17.5; linseed meal, 2.0; molasses, 5.0; NaCl, 0.5; Ca_2PO_4, 0.5; and $CaCO_3$, 0.5. The average composition of the dry matter based on the analysis of 20 samples representing those taken continuously during the feeding period was as follows: protein, 16.13%; ether extract, 2.18%; carbohydrates, 73.81%; ash, 7.88%; and energy, 4.367 kcal/g. The daily allowance of feed was provided in three meals. Water and a trace-mineralized salt were available to the animals at all times.

As a rationing guide, full-body weight was measured at weekly intervals throughout. Accordingly, the feed allowance was readjusted on the same day each week. The following amounts of dry matter were offered daily per 1 kg of full-body weight raised to the power, 0.73: low level, 58 g; high level, 95 g. These allowances resulted in the following means and ranges of gross energy intake per day per 1 kg of empty-body weight (i.e., wool- and ingesta-free

body weight) raised to the 0.73 power (abbreviated MBS hereafter): low level, 278 kcal (range 268 to 293); high level, 421 kcal (range 384 to 442). (As a consequence of these inputs, the mean energy gains (the body-plus-wool energy) were 32.3 and 61.7 kcal/1 kg MBS per day, respectively, by the low and high level sheep.)

RESULTS AND DISCUSSION

The energy intake, body weight and age of the sheep studied, and their corresponding body composition are summarized in table 1. These data were fitted to various linear and curvilinear models and examined by correlation and regression analyses.

Relationship between level of energy input and body composition. A preliminary plotting of the data revealed that the amounts of the body components at a given age were markedly different between the low and high energy-input treatments. For example, at 380 days of age, the energy value of the body of the high level animals was of the order of 2.4 times as great as that of the low level animals. Thus, on the age basis, two distinct populations of body composition existed which are associated with the two dietary energy levels imposed.

A similar examination of the amounts of body components at the various body weights suggested that the data comprised only one population of body composition irrespective of the energy-intake level. This was then examined in a regression analysis involving all high and low energy-intake animals separately. As shown in table 2, the regression of the amounts of body water, fat, protein, ash and energy on the wool-free, ingesta-free body weight resulted in regression coefficients that are similar for both dietary treatments. None of these, within a given body component,

TABLE 1

Body composition of sheep at various body weights and ages

Animal no.	Treatment identity [1]	Energy intake [2]	EBW [3]	Age	Body components [4]				
					Water	Fat	Protein	Ash	Energy
		kcal	*kg*	*days*	*kg*	*kg*	*kg*	*kg*	*Mcal*
456	Initial		12.30	140	8.96	0.63	2.11	0.55	17.6
468	Initial		12.52	140	8.98	0.72	2.21	0.60	19.1
480	Initial		14.27	140	9.39	1.75	2.40	0.53	30.0
467	Initial		17.20	140	10.93	2.62	2.86	0.72	41.1
458	Initial		18.97	140	12.56	2.42	3.20	0.79	40.4
457	Initial		20.82	140	12.48	3.82	3.55	0.92	56.4
478	Initial		21.93	140	13.89	3.06	3.95	1.06	50.7
477	H	429	21.47	211	13.12	4.03	3.36	0.76	58.1
479	L_1	290	14.90	211	9.24	2.59	2.40	0.59	38.5
460	L_2	270	23.13	271	13.56	5.15	3.67	0.76	68.8
470	H	384	28.19	270	16.19	6.87	4.21	0.83	87.4
469	L_1	275	19.89	270	12.24	3.68	3.10	0.78	52.0
463	L_2	273	28.73	349	16.23	6.88	4.54	1.16	89.1
474	H	415	35.44	270	19.54	9.72	4.86	1.08	119.1
475	L_1	268	19.67	270	12.41	3.16	3.16	0.81	48.1
466	L_2	281	32.05	421	17.02	9.35	4.60	0.86	114.2
473	H	441	43.14	277	23.35	12.31	5.86	1.38	150.1
459	L_1	278	26.90	277	15.16	6.63	4.01	0.93	83.7
464	L_2	276	41.21	484	20.30	14.62	5.14	0.83	165.4
461	H	422	49.56	381	22.88	18.90	6.50	1.28	213.3
455	L_1	293	27.69	381	15.04	7.66	3.96	0.77	93.6
476	L_2	273	47.50	583	21.90	18.28	5.97	1.28	205.2
465	H	425	58.31	375	26.93	22.39	7.47	1.40	255.4
471	L_1	283	31.78	375	17.86	7.67	5.04	1.04	99.4
462	L_2	276	56.00	613	25.73	21.56	7.10	1.55	247.5
472	H	428	36.84	290	22.06	7.40	5.76	1.32	103.3

[1] Initial refers to animals killed just prior to beginning of feeding period; H represents animals provided high intake of energy; L_1 represents animals provided low intake of energy and killed on same day as corresponding animal H; L_2 represents animals provided low intake of energy and killed at same body weight as corresponding animal H.

[2] Energy intake is expressed as kilocalories of gross energy intake/1 kg $EBW^{0.73}$/day.

[3] EBW means empty-body weight.

[4] Body components in ingesta-free, wool-free body.

TABLE 2

Regression coefficients between body components (kg) and empty-body weight (kg) of sheep receiving two different energy levels

Energy level	Body components				
	Water	Fat	Protein	Ash	Energy
High	0.357	0.517	0.109	0.018	5.517
Low	0.376	0.498	0.106	0.019	5.344

was different at a level of probability less than $P = 0.1$. Also, the intercept values were similar for the two dietary treatments.

A more stringent test of whether the dietary energy levels exerted a differential effect on the relationships between body composition and body weight was conducted by comparing the regression data for the high level animals with those of only the low level animals killed at the same body weights. This study consisted of a test of homogeneity of the regression equations representing the two groups of data. It was revealed that dietary energy level had no differential effect on the relationship between any body component and body weight at a level of probability as low as $P = 0.1$. As a consequence, all data, irrespective of the dietary energy level imposed, were pooled to study the relationships between the body components and body size or age.

Relationship to body composition of body weight and age. The extent to which body composition is associated with body weight and age was examined by correlation and regression analyses in which body weight and age were employed singly and together as independent variables. The data were divided into 1) a population representing all sheep irrespective of their body-fat concentration and including the base-line animals, and 2) a population which included only the data for sheep containing less than 31% of fat.

Table 3 summarizes some statistics associated with the regression of the body components of all animals (irrespective of the concentration of body fat) on body weight alone and on body weight and age as independent variables. As compared with the corresponding indices obtained by the use of body weight as the sole predictor, the R^2 values were only slightly increased (e.g., by only 0.002 to 0.019), and the coefficients of variation were inappreciably reduced by the use of age in addition to body weight as the independent variables. The addition of age as a predictor did not improve significantly ($P < 0.05$) the accuracy of prediction of any of the body components derived from body weight alone.

In previous studies, we (19) observed that the increases in the weights of the individual chemical components of the body are linear functions of body weight in sheep having a fat concentration less than approximately 31%, provided that such animals are maintained in a continuously positive energy balance. Above this concentration of fat, the amounts of water and protein increase at a decreasing rate, and fat and energy increase at an increasing rate, as body weight increases. Although only 5 of the 26 animals used in the present study contained more than 31% fat, evidence was obtained to support the previous observations. Deletion of the data for the five very fat animals resulted in an appreciable reduction in the coefficient of variation between the amounts of the components determined directly and those derived by means of the linear relationships, as shown by a comparison of the data in table 4 with those in table 3. Also, in this population (that is, animals containing less than 31% fat), the use of age in addition to body weight as the predictors failed to improve ($P < 0.05$) upon the predictive efficiency of body weight alone (table 4).

Finally, the total data were fitted to the curvilinear model, $Y = a X^b$. As observed in previous studies (19), this model provided an excellent fit of the data regardless of the range in body-fat concentration (table 5). The statistical parameters shown in table 5 demonstrate that, except for ash, the variation in the $\log_{10}$ of the amounts of the gross chemical components

TABLE 3

Relationships of body composition to empty-body weight alone and to empty-body weight plus age, irrespective of degree of fatness

Body components	Prediction equations [1]	R^2	Sy. x or Sy.l..n	C.V.[2]
	Empty-body weight (X_1) as predictor			
Water	$Y = 0.394\ X_1 + 4.55$	0.968	0.978	6.08
Protein	$Y = 0.111\ X_1 + 1.02$	0.970	0.279	6.54
Fat	$Y = 0.474\ X_1 - 6.02$	0.961	1.288	16.43
Ash	$Y = 0.019\ X_1 + 0.39$	0.778	0.134	14.23
Energy	$Y = 5.124\ X_1 - 51.89$	0.975	11.05	11.28
	Empty-body weight (X_1) and age (X_2) as predictors			
Water	$Y = 0.433\ X_1 - 0.0048\ X_2 + 4.79$	0.972	0.919	5.72
Protein	$Y = 0.121\ X_1 - 0.0012\ X_2 + 1.07$	0.974	0.257	6.02
Fat	$Y = 0.422\ X_1 + 0.0064\ X_2 - 6.34$	0.966	1.220	15.56
Ash	$Y = 0.023\ X_1 - 0.0005\ X_2 + 0.42$	0.797	0.134	14.23
Energy	$Y = 4.714\ X_1 + 0.0506\ X_2 - 54.42$	0.978	10.54	10.76

[1] Y = chemical component (kg) or energy (Mcal),
X_1 = empty-body weight (kg), and
X_2 = age (days).

[2] C.V. = coefficient of variation (%) = $\frac{Sy.x}{\bar{Y}} \times 100$.

TABLE 4

Relationship of body composition to empty-body weight alone and to empty-body weight plus age in sheep containing less than 31% of fat

Body components	Prediction equations [1]	R^2	Sy. x or Sy.l..n	C.V.[2]
	Empty-body weight (X_1) as predictor			
Water	$Y = 0.478\ X_1 + 2.73$	0.978	0.605	4.23
Protein	$Y = 0.127\ X_1 + 0.69$	0.972	0.225	5.82
Fat	$Y = 0.364\ X_1 - 3.64$	0.935	0.825	16.02
Ash	$Y = 0.024\ X_1 + 0.28$	0.764	0.118	13.58
Energy	$Y = 4.145\ X_1 - 30.68$	0.966	6.693	9.62
	Empty-body weight (X_1) and age (X_2) as predictors			
Water	$Y = 0.510\ X_1 - 0.0047\ X_2 + 3.08$	0.984	0.547	3.82
Protein	$Y = 0.130\ X_1 - 0.0004\ X_2 + 0.72$	0.960	0.229	5.93
Fat	$Y = 0.324\ X_1 + 0.0058\ X_2 - 4.07$	0.947	0.766	14.87
Ash	$Y = 0.030\ X_1 - 0.0008\ X_2 + 0.33$	0.801	0.111	12.82
Energy	$Y = 3.833\ X_1 + 0.0458\ X_2 - 34.09$	0.972	6.245	8.98

[1] Y = chemical component (kg) or energy (Mcal),
X_1 = empty-body weight (kg), and
X_2 = age (days).

[2] C.V. = coefficient of variation (%) = $\frac{Sy.x}{\bar{Y}} \times 100$.

and the energy of the body is highly ascribable to the variability in the logarithm$_{10}$ of body weight, with the R^2 values for the various components ranging from 0.939 to 0.982.

Partition of variance in body composition between body weight and age. The R^2 values shown in table 6 were computed from the data representing all animals for the purpose of estimating the additional variation in the predictands (i.e., the amounts of body components and energy) which might be accounted for by including age, as an additional predictor, with empty-body weight. As indicated by the degree of reduction in the sums of squares of the predictands, the combined use of age and body weight accounted for only 0.1 to 0.6% more of the variation in body components than did empty-body weight alone. In no instance was this increase of statistical significance.

TABLE 5

Curvilinear relationships between body components and empty-body weight of sheep disregarding body-fat concentration

Body components	Prediction equations [1]	R^2	Sy. x
Water	$Y = 0.7291\ X_1 + 0.1454$	0.982	0.01998
Protein	$Y = 0.7822\ X_1 - 0.4984$	0.982	0.02069
Fat	$Y = 2.0298\ X_1 - 2.1539$	0.939	0.10348
Ash	$Y = 0.5960\ X_1 - 0.8918$	0.796	0.06061
Energy	$Y = 1.6020\ X_1 - 0.3949$	0.976	0.04971

[1] $Y = \log_{10}$ components (kg or Mcal) and
$X_1 = \log_{10}$ empty-body weight (kg).

TABLE 6

Standard partial regression coefficients and R^2 values between body components and empty-body weight or age, or both

Body component	b'_1 [1]	b'_2 [2]	Ratio $b'_1 : b'_2$	R^2 values		
				Age	EBW [3]	EBW [3] + age
Water	1.080	−0.118	9.2	0.581	0.968	0.973
Protein	1.060	−0.103	10.3	0.590	0.970	0.973
Fat	0.873	0.131	6.7	0.711	0.961	0.967
Ash	1.080	−0.241	4.5	0.407	0.778	0.797
Energy	0.909	0.097	9.4	0.702	0.975	0.978

[1] b'_1 = standard partial regression coefficients for empty-body weight.
[2] b'_2 = standard partial regression coefficients for age.
[3] EBW = empty-body weight.

As shown in table 6, when age was employed as the sole predictor, the magnitude of the R^2 values is considerable and, for most components, they are statistically significant. However, the magnitude of these values to a great degree is the reflection of the correlation between body weight and age. The correlation coefficient between these two variables was 0.82. This correlation is great enough to account for all or almost all of the R^2 values for age.

As further evidence of the importance of body weight in accounting for the variance in body composition, the standard partial regression coefficients were computed for the various body components regressed on empty-body weight (b'_1) and on age (b'_2). The coefficients and the ratios of $b'_1 : b'_2$ are summarized in table 6. The coefficients for empty-body weight are 6.7 to 10.3 times as large as those for age, except in the case of ash for which the b'_1 value is 4.5 times as large as the b'_2 value. These ratios indicate the relative importance of each predictor. Because of the relatively high correlation between the two predictors, empty-body weight and age, the emphasis put on the absolute values of the ratios must be qualified since this correlation may exaggerate the importance of the predictor which accounts for the majority of the variation. These ratios, however, substantiate the conclusions drawn from an examination of the R^2 values. Thus, the observations represented in table 6 indicate that the variation in body composition of sheep is overwhelmingly accounted for by the variability in body weight, with only a small part of it being ascribable to age.

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Effects of Chromium(III) Supplementation on Glucose and Amino Acid Metabolism in Rats Fed a Low Protein Diet [1]

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ABSTRACT The hypothesis that chromium(III) acts as a cofactor for insulin, previously derived from studies of glucose metabolism, was tested by measuring the interaction between these two agents on two parameters of amino acid metabolism. In rats fed a low protein ration (10% soy protein) and given 2 ppm chromium in the drinking water, insulin in vivo stimulated the incorporation of three amino acids into heart protein and cell transport of an amino acid analogue in heart to a greater degree than it did in chromium-deficient controls. The former dietary group also responded to insulin with a more marked hypoglycemia and glycogen formation from glucose. The results demonstrate that the insulin-chromium interaction is not restricted to glucose metabolism.

The use of a 10% soy protein, low chromium ration makes possible the production of a moderate degree of chromium deficiency, without the necessity of raising the experimental animals in a specially controlled environment (2). The slight impairment of growth observed in rats maintained on this diet offers a possibility to investigate a possible role of chromium in certain synthetic processes related to growth. Furthermore, these processes present themselves as systems to test the hypothesis that chromium acts as a cofactor for insulin. Data consistent with this hypothesis have been obtained in systems measuring various aspects of glucose metabolism in epididymal fat tissue (3), liver mitochondria (4) and crystalline lens (5). In these experiments chromium deficiency was invariably correlated with a diminished response of isolated tissue to insulin, and the response to the hormone could be significantly increased by supplements of chromium in vitro or in vivo.

It is now an accepted fact that the action of insulin is not restricted to carbohydrate metabolism. Glucose-independent effects on amino acid transport and utilization for protein synthesis have been shown by different investigators (6). The studies presented here were undertaken to test the cofactor hypothesis for chromium, by measuring the response of moderately chromium-deficient rats to insulin in vivo, and by comparing the effects of chromium supplementation on amino acid utilization with that on glucose metabolism.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain were raised from weaning in stainless steel cages and were fed a 10% soy protein-sucrose ration as described in a previous publication (2). Through careful selection and monitoring of the ingredients, the chromium content of the diet was kept below 100 ppb, as measured by atomic absorption spectroscopy. The animals had free access to food and triple-distilled, deionized water which contained 0.1 ppm vanadium and molybdenum (as vanadyl sulfate and sodium molybdate) and, for the chromium-supplemented rats, 2 ppm chromium (as $CrCl_3 \cdot 6H_2O$). The animals were fasted overnight, prior to performance of the experiments, as indicated in the tables.

Glycogen was determined by the method of Good et al. (7), and an enzymatic procedure was used to measure glucose (8). The latter also was used for determination

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[1] Preliminary reports of some of the data have been made (1); and Roginski, E. E., and W. Mertz 1967 Dietary chromium and amino acid incorporation in rats on a low-protein ration. Federation Proc., 26: 301 (abstract).

of blood glucose. The incorporation of glucose 1-^{14}C into tissue glycogen, following the intravenous injection of insulin, was studied according to the procedure of Rafaelsen et al. (9). For the determination of tissue accumulation of α-aminoisobutyric acid, fasted, functionally nephrectomized rats received per 100 g of body weight 500 μg of α-aminoisobutyric acid [2] and a sufficient amount of α-aminoisobutyric acid-1-^{14}C [3] by subcutaneous injection, with or without 50 mU of insulin. After 2 hours the animals were killed by stunning and decapitation; aliquots of blood, heart, liver and diaphragm were removed. The organs were weighed, cut into small strips and boiled in a known volume of water for 15 minutes. An aliquot of the water extract was assayed for radioactivity in a liquid scintillation detector. After correction for background and counting efficiency, the tissue concentration was expressed as disintegrations per minute (dpm) in 100 mg tissue/dpm in 0.1 ml plasma.

In the studies measuring the incorporation of amino acids into protein, 1 μCi of one of the following amino acids [4] or of the amino acid mixture was injected subcutaneously per 100 g of body weight, without added carrier substance into rats fasted overnight. Glycine-1-^{14}C, L-serine-^{14}C (uniformly labeled), L-methionine-^{14}C (uniformly labeled), L-phenylalanine-1-^{14}C, L-lysine-^{14}C (uniformly labeled), L-threonine-^{14}C (uniformly labeled) and a mixture of 14 L-amino acids-^{14}C (uniformly labeled), imitating the composition of an algal protein hydrolysate. The specific activity of the amino acids ranged from 10 to more than 200 mCi/mmole; correspondingly, the dose of amino acid injected per 100 g body weight varied from 5 to 100 nmoles. The rats receiving insulin were given 100 mU of the hormone per 100 g weight, by subcutaneous injection, together with the amino acid. The rats were killed after 2 hours and the tissue protein was isolated by precipitation with trichloroacetic acid (10). The isolated protein was washed several times with trichloroacetic acid, ethanol and ether; it was digested in 2 ml 30% KOH and a 0.1-ml aliquot of the digest was dissolved in 5 ml methanol and 10 ml scintillation fluid.[5] The samples were counted to at least 1000 counts in a liquid scintillation detector. Counting efficiency was derived from channel ratios and was used to calculate disintegrations per minute. The results are expressed as disintegrations per minute in 1 mg protein corrected for an injected dose of 2.3 million dpm/100 g body weight. Significance of differences was determined by use of Student's *t* test.[6]

RESULTS

In agreement with previous results from this laboratory (11), the moderate low chromium state did not result in fasting hyperglycemia (table 1). The reaction of blood glucose to a hypoglycemic dose of insulin, however, was significantly different between the two groups. The decline of blood glucose levels in the low chromium animals was approximately half, and not as long lasting as in the supplemented rats. Because an increased hypoglycemic response could conceivably be caused by

[2] Sigma Chemical Company, St. Louis, Mo.
[3] New England Nuclear Corporation, Boston, Mass.
[4] See footnote 3.
[5] Liquifluor, New England Nuclear Corporation, Boston, Mass.
[6] The principles of animal care as promulgated by the National Society for Medical Research were observed.

TABLE 1

Effect of insulin in vivo on blood glucose levels

	Low chromium rats (10) [1]	Chromium-supplemented rats (10) [1]
Fasting blood glucose, mg/100 ml	58	59
Change at 1 hour after insulin,[2] mg/100 ml	−14.9 ± 2.1 [3]	−26.5 ± 4.1 [3]
Change at 2 hours, mg/100 ml	−11.8 ± 3.4 [4]	−27.4 ± 5.4 [4]

[1] Number in parentheses indicates number of animals in group.
[2] Subcutaneous injection of 100 mU insulin/100 g body wt.
[3] Difference between groups significant ($P < 0.01$).
[4] $P < 0.05$.

diminished glycogen reserves, the tissue glycogen levels were determined after 18 hours of fasting in 12 rats each raised without and with chromium supplement. The results (table 2) show higher glycogen reserves in the chromium-supplemented animals, indicating increased tissue response to insulin as the cause for the greater hypoglycemic response of these rats. The same pattern evolved when the effect of non-hypoglycemic doses of insulin on glycogen formation from labeled glucose was measured (table 2). The effect of the hormone in the chromium-deficient rats was only half that in the supplemented animals, for both organs studied.

Action on amino acid metabolism. The first insulin-responsive step of amino acid metabolism, cell transport, was investigated using the nonmetabolizable analogue, α-aminoisobutyric acid (AIB) (table 3). As had been demonstrated by others (12), the injection of insulin increased the concentration gradient between intracellular and extracellular water, as evidenced by the greater ratio AIB (tissue)/AIB (plasma) in the insulin-injected rats. The effect of the hormone was similar in the diaphragm of both dietary groups, but the hearts of chromium-supplemented animals responded to insulin with a significantly greater increase of the tissue concentration. The accumulation of AIB in liver was higher in chromium-supplemented rats in the absence of exogenous insulin, and was not further increased by additional hormone.

The second site of action of insulin in amino acid metabolism is the ribosome to which the hormone imparts a greater ability for protein synthesis (13). Protein synthesis, in response to insulin, was studied in both dietary groups, measuring the specific activity of isolated tissue protein, after injection of 6 individual and a mixture of 14 amino acids. Three of these, glycine,

TABLE 2

Effect of chromium supplementation on glycogen metabolism [1]

	Low chromium rats (12) [2]	Chromium-supplemented rats (12) [2]
	mg/g wet wt	*mg/g wet wt*
Tissue glycogen concentrations		
Muscle	2.43 ± 0.45 [3]	3.63 ± 0.33
Heart	1.88 ± 0.36	2.96 ± 0.4
Liver	2.22 ± 0.54 [4]	4.91 ± 1.05 [4]
Incorporation of glucose carbon into glycogen [5] (dpm in glycogen isolated from 100 mg tissue)		
Heart	206 ± 48 [6]	529 ± 76 [6]
Liver	333 ± 61 [4]	628 ± 122 [4]

[1] The animals were fasted for 18 hours.
[2] Number in parentheses indicates number of animals in each group.
[3] Mean and SE.
[4] Difference between groups significant ($P < 0.05$).
[5] One hour after intravenous injection of 2 μCi glucose-1-^{14}C and 1 mU insulin/100 g body weight.
[6] Difference between groups significant ($P < 0.005$).

TABLE 3

Concentration of α-aminoisobutyric acid in tissues [1]

	Low chromium rats			Chromium-supplemented rats		
	Diaphragm	Liver	Heart	Diaphragm	Liver	Heart
No insulin (12) [2]	0.8	3.9 [3]	0.9	1.0	6.8 [3]	0.8
50 mU insulin/100 g subcutaneously (17)	1.6	5.4	1.9 [4]	1.9	6.2	3.0 [4]

[1] Two hours after injection of compound into functionally nephrectomized rats. The figures given are ratios; dpm in 100 mg tissue/dpm in 0.1 ml plasma.
[2] Number in parentheses indicates number of animals in each group.
[3] Difference between groups significant ($P < 0.01$).
[4] Difference between groups significant ($P < 0.05$).

TABLE 4

Incorporation of amino acids into tissue protein 2 hours after subcutaneous injection of 1 μCi/100 g wt

Amino acid	Insulin	Low chromium rats		Chromium-supplemented rats	
		Liver	Heart	Liver	Heart
		dpm/mg protein		*dpm/mg protein*	
Glycine-1-^{14}C (12) [1]	—	116 ± 4 [2]	24.2±0.7	124 ± 6	25.2±1.1 [3]
	+	112 ± 4.7 [4]	26.2±1.3	135 ± 5.4 [4]	32.7±2.0 [3]
L-Methionine-^{14}C, UL (10)	—	197 ±13.5	72.4±4.4	203 ± 8.9 [5]	75.0±4.3
	+	218 ± 6.4 [6]	74.4±1.9 [7]	263 ±17 [5,6]	89.5±6.7 [7]
L-Serine-^{14}C, UL (12)	—	95.3± 5.3	25.2±2.5	106.3± 8.3	25.6±1.1 [8]
	+	91.8± 6.1	27.2±1.5 [9]	102.8± 5.8	33.3±2.3 [8,9]

[1] Number in parentheses indicates number of animals in each group.
[2] Mean ± SE of the mean.
[3] Values showing the same superscript are significantly different from each other.
[3],[4],[7] and [9] $P < 0.05$.
[5] and [8] $P < 0.01$.
[6] $P < 0.025$.

TABLE 5

Incorporation of amino acids into tissue protein 2 hours after subcutaneous injection of 1 μCi/100 g wt [1]

Amino acid	Insulin	Low chromium rats		Chromium-supplemented rats	
		Liver	Heart	Liver	Heart
L-Phenylalanine-1^{14}C (3) [2]	—	368±47 [3]	127±32	342±39	104±7
	+	322±16	98±0.1	312±23	95±7
L-Lysine-^{14}C, UL (3)	—	256±24	79±14	289±38	78±5
	+	295±59	70±25	300±15	81±9
L-Threonine-^{14}C, UL (4)	—	86±12	19±1	99±14	21±2
	+	99±16	21±2	105±17	21±2
L-Amino acid-^{14}C mixture (5)	—	285±23	89±5	262±15	96±13
	+	270±10	98±2	246±11	99±8

[1] None of the observed differences between groups are statistically significant.
[2] Number in parentheses indicates number of animals in each group.
[3] Mean and SE.

L-methionine and L-serine, showed a similar pattern in their incorporation into heart protein (table 4). The specific activity in the latter was significantly increased by insulin only in the chromium-supplemented rats, whereas the insulin effects were small and not significant in the low chromium groups. The liver responded to insulin with increased protein synthesis only when methionine was injected, but not with the other two amino acids. In this organ, as in heart, the response was significant only in the chromium-supplemented rats.

In contrast to these results, the incorporation of phenylalanine, lysine, threonine and a mixture of amino acids was not significantly stimulated by insulin or chromium (table 5).

DISCUSSION

The results of the studies reported in this publication demonstrate that the moderate stimulation of growth by chromium is accompanied by a greater sensitivity of the chromium-supplemented rats to insulin. The fact that the response of two synthetic processes, glycogen and protein synthesis, to insulin is enhanced by chromium does not necessarily imply that the greater effectiveness of insulin in the chromium-sufficient rats is the cause of increased growth. Growth is regulated by an interplay of many hormones and other factors, the relation of which to chromium is unknown.

The significance of the data presented here is that they support the hypothesis of

chromium acting as a cofactor for insulin. This hypothesis had heretofore been tested only in systems measuring several aspects of carbohydrate metabolism and in mitochondria. It could now be applied to two insulin-responsive steps in amino acid metabolism which are independent of the action of insulin on glucose utilization.

The pattern of response of the chromium-deficient and chromium-sufficient animals in the experiments measuring AIB transport and incorporation of glycine, methionine and serine into protein in the heart was similar to the patterns observed in carbohydrate metabolism: Chromium-supplemented animals were not different from their deficient controls in the absence of exogenous insulin, but responded to the latter with greater and more significant increases of the function under study. The relatively small increases effected by insulin even in the chromium-sufficient rats are consistent with observations by others of the small effects of insulin in protein-synthesizing systems (13). The dependence of the effect of chromium on the presence of exogenous insulin rules out one mechanism which could possibly lead to increased specific activity of the isolated protein. A reduction by chromium of the size of the amino acid pools could result in similar findings. Since no significant differences in the specific activity of the protein were found between the two dietary treatments in the absence of insulin, it is unlikely that the pool size was different.

The question as to the nature of the rather selective effects of insulin and chromium on amino acid incorporation in this system remains unanswered at the present time, particularly in view of the general stimulation of amino acid incorporation by insulin in vitro. The in vivo system used in the present studies is much more complex, and apparent effects with any one amino acid depend on the availability of others which may be limiting in protein synthesis. This dependence can be eliminated in isolated systems by adding a full complement of amino acids. Therefore, the results presented here do not speak against a general regulation of protein synthesis by insulin. One must also consider, however, the possibility that the three amino acids responsive in our system have some chemical similarities which render them more susceptible to the action of chromium (and insulin) than others. It has been shown in an independent study (14) that of six amino acids used, glycine, methionine and serine were most effective in preventing olation of trivalent chromium at neutral pH, a process leading to the formation and precipitation of large, polynucleate complexes through bridge formation between the chromium ions. It remains to be shown whether these observations are directly related to the mode of action of chromium in vivo.

The results of the experiments measuring the cell transport of α-aminoisobutyric acid show an increased response to insulin only in heart tissue of the chromium-supplemented animals. The high accumulation of the analogue in the liver of chromium-supplemented rats, even in the absence of exogenous insulin, may suggest a great sensitivity of the liver of these animals to their own, endogenous insulin, leading to maximal stimulation of cell transport. This would be consistent with the failure of additional exogenous hormone to further increase the high concentration gradient. The similar insulin response of diaphragm in both dietary groups is unexplained. However, the consistently small response of this tissue to chromium has been noted before in systems measuring glucose uptake and transport of D-galactose.[7]

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Effects of Chromium(III) Supplementation on Growth and Survival Under Stress in Rats Fed Low Protein Diets [1]

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ABSTRACT A degree of chromium deficiency resulting in impaired growth has hitherto been obtained only in rats raised in a specially controlled environment. The present study was undertaken to define methods by which growth retardation due to lack of chromium can be consistently induced under ordinary conditions. It was found that raising rats in plastic cages with a diet low in protein (10%) and chromium (<100 ppb) resulted in a moderate depression of growth which was alleviated by chromium supplementation. Subjecting the animals to controlled exercise or blood loss further aggravated the low chromium state.

For some time the impairment of various parameters of glucose utilization has been the only known symptom of low chromium states (1). Only by raising rats and mice in a strictly controlled environment has it been possible to demonstrate retarded growth and diminished longevity as a consequence of a more severe chromium deficiency (2). The difficulty with which the latter can be produced under ordinary conditions has greatly impeded studies of the action of the element in areas other than carbohydrate metabolism. This study was therefore designed to delineate conditions which would aggravate a low chromium state of experimental animals kept in a conventional laboratory. Because it is difficult at the present time to lower the chromium content of diets consistently much below 100 ppb, an attempt was made to increase the severity of symptoms of the low chromium state by applying some forms of stress. To this end the experimental animals were fed a diet of low protein content, imposing a nutritional stress. The results of these experiments and of others, with additional nutritional or physical stress, suggest that this treatment produces a more severe syndrome of the low chromium state. This makes possible the investigation of a number of biochemical symptoms of the deficiency without the precaution of using a controlled environment.

EXPERIMENTAL

It is essential to avoid as much as possible external chromium contamination. Because dust contains very high concentrations of chromium,[2] precautions are necessary to ensure a clean atmosphere. The use of plastic animal cages with plastic tops and glass grids is required where an effect of chromium on growth rates is to be demonstrated. Stainless steel housing appears acceptable for less critical experiments.

Male rats of the Sprague-Dawley strain were maintained from weaning in individual cages with free access to diet and water which were changed at least once a week. Rations containing 8, 10 and 12% soy protein were prepared once a week, stored in plastic bags under refrigeration and fed in glass cups. The composition was as follows: (in grams per kilogram) soy protein,[3] 100; distilled lard,[4] 80; Fox-Briggs salt mix (3), 40; vitamin mix (4),[5] 10; sucrose, 770; oleovitamin,[6] 5 ml, supplying 6 mg vitamin

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[1] A preliminary report of some of these data has been given: Roginski, E. E., and W. Mertz 1967 Chromium(III) in rats on low-protein diets. Proc. 7th Int. Congr. Nutr., 5: 710.

[2] Dust collected in an animal room contained 700 ppm chromium. Feldman, F. J., unpublished results.

[3] ADM Assay Protein C-1, Skidmore Enterprises, Cincinnati, Ohio.

[4] Distillation Products Industries, Rochester, N. Y.

[5] Supplying in milligrams per kilogram diet: thiamine·HCl, 10; riboflavin, 10; pyridoxine, 5; vitamin B_{12}, 1; nicotinic acid, 40; folic acid, 2; biotin, 0.3; *p*-aminobenzoic acid, 100; Ca pantothenate, 50; inositol, 300; choline chloride, 2,500; and menadione, 2.

[6] Oleovitamin A and D, NF, The Vitarine Company, Inc., New York, N. Y.

A and 0.1 mg vitamin D_2/kg of diet; and *dl*-α-tocopheryl acetate, 0.5. The amount of sucrose in the diet was adjusted for lower or higher levels of protein. For some experiments the vitamin E supplement was omitted, as indicated in the tables. The diets contained less than 100 ppb chromium, as measured by atomic absorption spectroscopy, when carefully selected ingredients of low chromium content were used. The drinking water was supplied in glass bottles with glass drinking tubes. It contained 0.1 ppm each of vanadium (as $VOSO_4$) and of molybdenum (as Na_2MoO_4) in distilled, deionized water of a resistance of approximately 18 megaohms/cm^2. The chromium-supplemented animals received, in addition, 2 ppm of chromium (as $CrCl_3 \cdot 6H_2O$ or as $Cr(CH_3COO)_3 \cdot H_2O$) in the drinking water. The solutions were prepared twice weekly. For measurement of food consumption, 20 rats received weighed amounts of diet. Every 3 or 4 days the uneaten portion of the food was carefully collected from the cups and bottoms of the cages under the grid and weighed.

Procedures. Five chromium-supplemented rats and five low chromium controls were placed in exercise cages [7] which allowed ad libitum running in an attached wheel. A counting mechanism recorded the revolutions of the wheel. By allowing or denying access to the wheel, the exercise was kept at a near-equal level for both groups. For the studies measuring the resistance of rats to acute blood loss, the animals were fasted overnight. The jugular vein was exposed under light ether anesthesia and an amount of blood equal to 3% of the body weight was withdrawn. Other groups were fasted overnight and received by intravenous injection 1.4 mg endotoxin/100 g weight (lipopolysaccharide B (*Shigella flexneri*)).[8]

Determinations. Measurements of respiration of liver slices were performed in the Warburg apparatus, as described previously (5). Serum protein was determined by a polarographic technique,[9] and serum protein pattern by electrophoresis and subsequent densitometry.[10] The protein of liver and heart homogenates was precipitated by trichloroacetic acid, washed several times with the acid and with ethanol and chloroform; it was then dried and weighed. Tissue water was measured by subtracting the weight of the dried tissue from its wet weight.[11]

RESULTS

Growth rates. No consistent stimulation of growth by chromium could be observed in animals kept in stainless steel cages during the course of 3 years. In general, the chromium-supplemented rats grew better than their controls during spring and fall, but this difference was offset by the frequent observations to the contrary during winter. This may be related to the operation of air-circulating equipment exposing the animals to excessive amounts of dust-borne chromium. In contrast, chromium-fed rats kept in plastic cages in the laboratory consistently grew better than their controls, regardless of season. In five consecutive experiments, the average weight of 46.0 and 46.0 g at the beginning of supplementation increased to 117.7 $\pm$ 2.1 g in the low chromium animals and to 126.5 $\pm$ 2.4 g in the chromium-supplemented rats ($N = 90$, $P < 0.01$).[12]

Food intake. Two groups of ten rats each were raised in plastic cages and fed the 10% soy protein ration (50 ppb chromium) with and without the chromium supplement in the drinking water. The low chromium group grew from 54.5 to 152.0 g at the end of week 12, and the supplemented animals from 54.4 to 169.5 g. The average food consumption during this period was 835 g for 97.5 $\pm$ 7.9 g weight gain in the low chromium group, and 888 g for a weight gain of 115.1 $\pm$ 5.9 g in the supplemented group. This corresponds to 11.7 and 13.0 g gain of body weight per 100 g food consumed.

[7] Wahmann Mfg. Company, Baltimore, Md.

[8] Difco Laboratories, Detroit, Mich.

[9] The authors thank Dr. G. D. Christian for performing the determinations.

[10] A microzone electrophoresis cell, cellulose acetate membranes (Beckman Instrument Co.) and a Chromoscan with Microzone scanning attachment were used (Joyce, Loebl and Co., Ltd., Gateshead, England). The procedures are described in Beckman Manual RM-IM-2 (1963) and Spinco Technical Bulletin RB-TB-004 (1963).

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

[12] Since growth rates of the control animals were different in different experiments, the values were obtained by averaging the weights of the control groups closest to 117 g and those of the corresponding chromium-supplemented groups kept at the same time and at the same location. This weight was reached between 5 and 9 weeks.

Superimposed stress of vitamin E-factor 3 deficiency. In each of three groups of rats fed vitamin E-deficient diets with 8, 10 or 12% soy protein, chromium supplementation increased growth rates only during the first few weeks, before the effects of vitamin E and factor 3 deficiency became apparent. In rats receiving the 8% protein level the average weight at 6 weeks for the low chromium and the chromium-supplemented groups was 62.4 ± 2.7 and 81.8 ± 4.1 g, respectively. At 9 weeks, rats from both groups began dying and at 12 weeks, the end of the experiment, 5 of 10 low chromium rats and 3 of 10 supplemented animals survived. In the experiment using the 10% protein ration, average weights at 8 weeks were 104.5 and 118.3 g for controls and supplemented rats, respectively. At this time, the difference between the groups began to diminish, symptoms of vitamin E deficiency appeared, and at 14 weeks, the low chromium rats weighed more than the supplemented group. After 2 of the 10 low chromium controls and 4 of 9 supplemented animals had died, the survivors were given approximately 100 mg *dl*-α-tocopheryl acetate by stomach tube, and growth resumed. The groups gained an average of 12.9 g (low chromium) and 16.6 g (chromium supplemented) in the 2 weeks following the vitamin supplement, before the experiment was terminated. In the group fed the 12% soybean protein diet, chromium did not stimulate growth (127.2 versus 123 g). Rats fed low chromium diets began dying at 13 weeks and all were dead at 17 weeks (N = 10) despite administration of approximately 100 mg *dl*-α-tocopheryl acetate by stomach tube at the end of week 15. Of the 10 chromium-supplemented rats, only 5 died, and the survivors began growing again after the vitamin was given.

To further study a possible relation between chromium and dietary necrotic liver degeneration, respiratory metabolism of liver slices was studied in rats raised on two levels of protein. Chromium supplementation did not influence oxygen consumption and was without effect on respiratory decline, one of the symptoms preceding the terminal phase of dietary necrotic liver degeneration. There appeared to be a diminished severity of the liver lesion in the chromium-supplemented rats, however, as judged by two observers independently (table 1). This observation which is in agreement with previously published results (6) suggests that chromium can delay the appearance of dietary necrotic liver degeneration, but only when the dietary protein (or factor 3 active selenite) is not too severely limiting. Chromium does not substitute for tocopherol, as is borne out by its ineffectiveness on respiratory decline of liver slices which vitamin E completely prevents (5).

Effect of added physical stress on growth rates and survival. When rats were allowed to exercise in a wheel attached to their cages, the difference in growth rates between the groups became greater (fig. 1). The rats ran an average of 11.5 km/week.

TABLE 1

Liver necrosis, oxygen consumption and respiratory decline of liver slices from vitamin E-deficient rats

	8% protein ration		10% protein ration	
	Low chromium	Chromium supplemented	Low chromium	Chromium supplemented
No. of rats	15	14	29	26
Severity of liver necrosis [1]	1.27 ± 0.34 [2]	0.29 ± 0.16	1.27 ± 0.24	0.46 ± 0.15
No. of livers assayed	13	13	21	19
Oxygen uptake of liver slices [3]	9.2 ± 0.8	9.3 ± 0.6	9.7 ± 0.3	10.8 ± 0.4
Respiratory decline [4]	67 ± 4	67 ± 5	67 ± 3	74 ± 1

[1] Lesions were graded by two observers from zero to 4.
[2] Mean and SE of the mean.
[3] Oxygen (microatoms per 100 mg tissue) consumed during first 30 minutes of incubation. Necrotic tissue was not used for these studies. When not enough normal-appearing slices could be prepared, liver was discarded.
[4] Respiratory decline = (A − B)/A × 100, where A is the oxygen consumption from zero to 30 minutes and B that from 90 to 120 minutes.

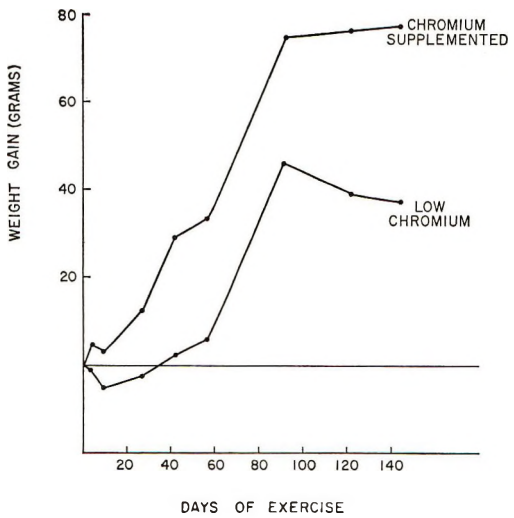

Fig. 1 Cumulative weight gain of rats during exercise.

Access to the wheel was regulated so that the performance was similar in both groups. The chromium-supplemented rats gained weight from the beginning of the exercise period, whereas the low chromium animals lost weight for a period of 12 days. Weight differences were still apparent at the end of the experiment, even though the metal cages must have contributed a certain degree of chromium contamination.

The protective action of chromium against death from acute hemorrhage is demonstrated in table 2, A. In these experiments, carried out in May 1964, dietary chromium supplementation increased the survival of rats after hemorrhage from 67 to 92%. It did not affect the initial weight loss, but increased the subsequent weight gain by more than threefold. Repetition of these experiments in September 1966 yielded similar results. Mortality was generally higher: only 27% of the low chromium animals survived, as compared with 60% of the chromium-supplemented rats. Correspondingly, the weight loss was more severe in these experiments, and the chromium-supplemented group recovered its weight at a faster rate (table 2, B). Chromium supplementation did not affect weight changes and survival of rats subjected to sublethal irradiation (600 r) or to the injection of 1.4 mg endotoxin.

Nature of weight gain. The increased body weight of the chromium-supplemented rats is not due to an increased water content of their organs. No differences were found in average water content, plasma protein concentration, hematocrit or albumin/globulin ratio (table 3). Gravimetric analyses of the protein concentration of heart and liver revealed a slightly higher protein content in the organs of the chromium-supplemented animals. Thus, the observed difference in body weight of both dietary groups is real and is accompanied by at least a proportional increase of organ protein.

DISCUSSION

The slight, but significant depression of growth resulting from a low chromium state is not impressive when compared with the cessation of growth which can be easily observed in deficiencies of other trace elements. The nutritional significance of the data presented here lies in the fact that a mild chromium deficiency state, in which

TABLE 2

Survival and recovery after experimental blood loss

	A (1964)		B (1966)	
Dietary chromium	−	+	−	+
No. of rats	27	25	30	30
No. of deaths	9 [1]	2 [1]	22 [2]	12 [2]
Survival, %	67	92	27	60
Initial wt change,[3] g	−6.7 ± 1.4	−6.2 ± 1.3	−9.5 ± 1.4	−8.8 ± 0.7
Wt change during 5-day recovery,[4] g	+2.3 ± 1.5 [1]	+7.3 ± 1.3 [1]	+10.0 ± 2.7	+17.3 ± 2.5 [5]

[1] Difference between groups significant ($P < 0.05$).
[2] Difference significant ($P < 0.01$).
[3] Rats of experiment A lost weight until day 4; those of experiment B only until day 1.
[4] In group A, recovery evaluated from days 5 to 10; in group B, from days 2 to 6.
[5] If one rat which developed pneumonia and kept losing weight is eliminated, the weight gain of this group is 19.4 ± 1.5 g; $P < 0.01$.

TABLE 3

Tissue composition

	No. in each group	Low chromium rats	Chromium supplemented rats
%			
Water, fat tissue	4	17.2	16.5
Water, muscle	4	70.8	69.6
Water, liver	4	72.5	72.8
Water, kidney	4	74.5	75.7
Protein, liver	108	21.2	21.5
Protein, heart	117	18.7 [1] ± 0.1	19.1 [1] ± 0.1
Protein, plasma	5	5.79	5.72
Ratio: albumin/globulin in plasma	14	1.08	1.09

[1] Difference between groups significant ($P < 0.05$).

only biochemical lesions are observable can be aggravated by applying some forms of stress to such a degree that growth becomes affected. Application of this principle, which has been used to great advantage in vitamin deficiencies, may provide a useful tool until diets can be developed which contain only a few parts per billion chromium.

With increasing severity of the applied stress, for example, after hemorrhage, the differences between chromium-deficient and chromium-supplemented groups widen, not only with regard to growth, but particularly with regard to survival. It can be stated then, that under the extreme conditions of experimental blood loss, chromium becomes an element essential for life. The mechanism by which the element protects against this type of injury, but not against endotoxin shock or radiation, is unknown.

Chromium supplementation does not protect against death from vitamin E-factor 3 deficiency. These results are in agreement with previous findings of the ineffectiveness of the chromium-containing glucose tolerance factor in dietary necrotic liver degeneration (7). They are at variance with more recent observations of a very substantial protection resulting from chromium supplementation in rats fed a vitamin E and factor 3-deficient Torula yeast ration (6). These latter experiments, however, had been performed under conditions which resulted in a more severe low chromium state and with a diet of higher protein content than the ration used in the present studies. The observations that the severity of liver lesions was less in the chromium-supplemented rats, confirm previous results (6). The action of chromium, however, is not related to that of vitamin E, because the former does not affect at all the respiratory failure of liver slices, a symptom preceding liver necrosis (5). It is more likely that the greater glycogen reserves of chromium-supplemented animals (8) afford a certain protection, particularly since it has been shown that glucose by itself delays death from dietary necrotic liver degeneration (9).

The fact that growth stimulation by chromium was consistently observed only in rats fed the 10% soy protein ration, but not the Torula yeast diet, raises the question which properties of the diets are responsible for the observed differences. Torula and soybean rations are not much different in total chromium content, with both well below 100 ppb. It is possible that Drackett protein contains chromium in a less available form, but this would not explain the fact that growth stimulation by chromium was observed only when the diets with the 8 and 10% protein levels were fed. The additional amounts of chromium introduced by a 2% increment of protein are minimal. The different composition of soy protein may be a factor; for example, by supplying a critical amount of one or more amino acids whose metabolism is affected by chromium. It appears more likely, however, that the nutritional stress of a suboptimal protein supply increased the chromium requirement of the animals, resulting in a slight growth retardation, the appearance of corneal opacity (4) and the various biochemical lesions to be described separately (8).

If the results of the present study with rats can be applied to problems of human nutrition, it appears that protein malnutrition may be a promising field for further study of the role of chromium. Animal protein is among the best sources of dietary chromium (10); thus, a low intake of animal protein might often result in a low intake of chromium as well. If protein deficiency aggravates the low chromium state, as our data suggest, it is possible that certain population groups might suffer from a substantial chromium deficiency and might profit from supplementation with the element in diet or water. This possibility is borne out by the studies of Hopkins et al. in malnourished children (11).

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Nutritional Value of Opaque-2 Corn for Young Chicks and Pigs [1]

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ABSTRACT One-day-old chicks and 25-day-old pigs were used to study the effect of replacing normal hybrid corn with opaque-2 corn in diets containing varying levels of supplemental soybean meal. At suboptimal protein levels, both chicks and pigs gained significantly ($P < 0.01$) more with lower feed/gain ratios when opaque-2 corn replaced an equal amount of normal corn in the diet. There was a significant ($P < 0.01$) quadratic increase in body weight gain and decrease in feed required per unit of gain by chicks and pigs fed increasing levels of soybean meal. Because of the higher protein and lysine content of opaque-2 corn as compared with normal hybrid corn approximately 5% less soybean meal was required in diets containing opaque-2 corn to result in maximum performance of chicks or pigs.

Mertz et al. (1) reported that endosperm of corn with the mutant opaque-2 gene contained 69% more lysine than endosperm of normal corn. Mertz and coworkers (2) also reported that this mutant gene affected the nutritional value of corn as evidenced by weanling rats fed diets containing 90% opaque-2 corn growing at a rate 3.6 times as fast as rats fed a similar diet containing normal hybrid corn.

Cromwell et al. (3) observed that, with the addition of methionine to the diet, chicks fed opaque-2 corn gained weight significantly faster on less feed than chicks fed similar diets containing normal corn. These authors presented data which suggest that the improved nutritional value of opaque-2 corn is largely due to its higher lysine content.

Pigs fed opaque-2 corn have been shown to grow significantly faster and more efficiently than pigs fed normal hybrid corn. Pigs fed opaque-2 corn performed as well as pigs fed an isonitrogenous corn-soybean meal diet (4).

This study was designed to further evaluate the nutritional value of opaque-2 corn for young chicks and pigs, and to determine the optimum level of supplemental soybean meal in diets containing opaque-2 and regular corn.

EXPERIMENTAL

Four hundred, one-day-old, male broiler chicks averaging 40 g body weight were allotted at random to four replications of a 2×5 factorial arangement of 10 diet treatments. The chicks were housed in wire-floor batteries with ad libitum access to feed and water during a 4-week experimental period. Battery hover temperature was maintained initially at 35° and reduced 4° each week, and room temperature was maintained at 20°. Weight gain and feed consumption data were collected weekly.

In trial 1, 80 pigs averaging 25 days of age and 5.13 kg body weight were allotted at random from outcome groups of initial weight to two replications of a 2×5 factorial arrangement of 10 diet treatments. The pigs were housed in concrete-floored pens with ad libitum access to feed and water during the 4-week experimental period. Pig gain and feed consumption data were collected biweekly.

In trial 2, 120 pigs averaging 25 days of age and 5.18 kg body weight were allotted as in trial 1 to three replications of a 2×5 factorial arrangement of diet treatments. Pig gain and feed consumption data were collected after 14, 28 and 35 days on test.

The data were analyzed statistically by analysis of variance according to methods described by Steel and Torrie (5).

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TABLE 1

Composition of experimental diets

Ingredient	Chick	Pig
	%	%
Ground corn	63.6	59.5
Soybean meal [1]	28.8	28.7
Lard	3.0	2.0
Sucrose	—	5.0
Calcium carbonate [2]	1.4	0.7
Dicalcium phosphate [3]	1.7	1.4
Iodized salt	0.5	0.5
Vitamin-additive premix	1.0 [4]	2.0 [5]
Trace mineral premix [6]	—	0.2

[1] Levels of soybean meal substituted for corn as shown in tables 3, 4 and 5.
[2] Varied to provide 1.0% Ca in chick diets and 0.7% Ca in pig diets.
[3] Varied to provide 0.65% P in chick diets and 0.6% P in pig diets.
[4] Contributed per kilogram diet: (in milligrams) vitamin K, 1.0; riboflavin, 5.0; pantothenic acid, 10; niacin, 25; penicillin, 6.6; streptomycin, 33; methionine, 750; ethoxyquin, 125; Mn, 79; Zn, 44; and (in IU) vitamin A, 7000; vitamin D3, 1000; vitamin E, 10; and (in μg) vitamin B_{12}, 10; choline added to provide 2.68 g.
[5] Contributed per kilogram diet: (in milligrams) riboflavin, 8.8; pantothenic acid, 17.6; niacin, 39.6; choline, 44.0; Chlortetracycline, 220; sulfamethazine, 220; penicillin, 110; and (in IU) vitamin A, 4400; vitamin D_2, 1100; and (in μg) vitamin B_{12}, 44.
[6] Contributed per kilogram diet: (in milligrams) Mn, 100; Zn, 100; Fe, 100; Cu, 10; Co, 1.0; and I, 6.0.

Composition of experimental diets is shown in table 1. The 10 dietary treatments consisted of five levels of soybean meal replacing an equal amount of either opaque-2 or normal corn (hybrid corn not containing the opaque-2 gene). Levels of soybean meal fed are presented in headings of tables 3, 4 and 5. Diet samples were analyzed for protein content (N × 6.25) by the method of Ferrari (6). Hydrolysates of soybean meal and normal and opaque-2 corn were prepared by refluxing 1-g samples in 50 ml of 6 N HCl for 72 hours. The hydrolysates were filtered, diluted to known volume and aliquots were analyzed for amino acid content by ion-exchange chromatography on an amino analyzer.[3] Tryptophan was measured by the method of Spies and Chambers (7).

RESULTS AND DISCUSSION

Crude protein and amino acid levels in soybean meal and normal and opaque-2 corn are shown in table 2. All essential amino acids except leucine, methionine and tryptophan were higher in opaque-2 than in normal corn. Lysine content was 34% higher in opaque-2 corn than in the regular hybrid corn. When expressed as

TABLE 2

Protein and amino acid composition of soybean meal, normal and opaque-2 corn

Nutrient	Soybean meal	Normal	Opaque-2
	% of air-dry feed		
Protein [1] (N × 6.25)	49.0	8.9	10.4
Arginine [2]	4.41	0.52	0.73
Histidine	1.80	0.34	0.46
Isoleucine	2.40	0.34	0.36
Leucine	4.14	1.02	0.94
Lysine	4.29	0.38	0.51
Methionine	0.62	0.14	0.13
Phenylalanine	2.64	0.41	0.42
Threonine	1.68	0.29	0.33
Tryptophan	0.57	0.10	0.10
Valine	2.88	0.47	0.56
Glycine	2.25	0.37	0.50
Glutamic acid	11.04	1.74	1.79
Cystine	0.12	0.14	0.20
Tyrosine	1.40	0.27	0.29
Alanine	2.28	0.62	0.61
Aspartic acid	5.90	0.60	0.91
Proline	2.46	0.74	0.74
Serine	1.98	0.30	0.31

[1] Average of six samples.
[2] Amino acid values are the averages of duplicate samples.

percentage of the protein, opaque-2 corn was higher in arginine, histidine, lysine, valine, glycine and aspartic acid, and normal corn was higher in isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, glutamic acid, tyrosine, alanine, proline and serine. The tryptophan level of opaque-2 corn was not higher, as has been reported by Mertz et al. (1) and Cromwell et al. (3,4). Zucker et al. (8), however, analyzed samples of the same corns used in these studies and found higher levels of tryptophan in opaque-2 than in normal corn.

Total gain and feed efficiency were significantly ($P < 0.01$) improved when chicks were fed opaque-2 corn as compared with normal corn (table 3). With increasing levels of soybean meal, significant ($P < 0.01$) quadratic responses in both gain and feed efficiency were observed.

Cromwell et al. (3) observed no nutritional advantage of opaque-2 corn over normal corn unless methionine was added to chick diets to provide a total of 0.8% sulfur amino acids. In this study, 0.075% methionine was added to all diets. As soybean meal was increased in the diets, the

[3] Technicon Amino Acid Analyzer, Technicon Instruments Corporation, Chauncy, N. Y.

TABLE 3

Performance of chicks fed normal or opaque-2 corn

Soybean meal level, %	8.8	13.8	18.8	23.8	28.8
Calculated crude protein, %					
Normal	11.7	13.7	15.7	17.7	19.7
Opaque-2	13.0	14.9	16.8	18.8	20.7
Analyzed crude protein, % [1]					
Normal	12.5	14.8	16.4	18.4	20.3
Opaque-2	14.4	16.7	18.3	20.3	21.6
Total 4-week gain, g [2,3]					
Normal	183.0	312.5	438.3	514.8	514.8
Opaque-2	294.0	439.5	496.3	534.0	529.5
Feed/gain [3,4]					
Normal	3.39	2.42	2.09	2.00	1.85
Opaque-2	2.48	2.04	1.84	1.90	1.80

[1] Average of duplicate samples.
[2] Average of four pens of 10 chicks each per treatment.
[3] Difference between kinds of corn and quadratic response to soybean meal levels significant ($P < 0.01$).
[4] Coefficients of variability equal 8.25 and 8.83% for gain and feed/gain, respectively.

TABLE 4

Performance of pigs fed normal or opaque-2 corn (trial 1)

Soybean meal level, %	18.7	23.7	28.7	33.7	38.7
Calculated crude protein, %					
Normal	15.5	17.5	19.5	21.5	23.5
Opaque-2	16.5	18.5	20.5	22.5	24.5
Total 4-week gain, kg [1,2]					
Normal	9.07	9.74	10.38	10.18	11.24
Opaque-2	10.76	9.67	11.28	8.99	7.06
Feed/gain [3,4]					
Normal	2.03	1.90	1.77	1.74	1.66
Opaque-2	1.87	1.66	1.68	1.62	1.80

[1] Average of two pens of four pigs each per treatment.
[2] Interaction of kind of corn and level of soybean meal significant ($P < 0.05$).
[3] Linear effect of level of soybean meal significant ($P < 0.05$).
[4] Coefficients of variability equal 9.15 and 6.29% for gain and feed/gain, respectively.

total sulfur amino acid content ranged from a low of 0.3 to a high of 0.6% of the diet. Chicks fed opaque-2 corn gained more and were more efficient than chicks fed normal corn at all levels of soybean meal.

These data demonstrate that the protein in opaque-2 corn is superior in nutritive value to the protein found in normal corn for supporting weight gain and feed efficiency of young chicks fed equal amounts of corn in the diet.

Averaged over all protein levels, pigs in trial 1 (table 4) fed normal corn gained more than pigs fed opaque-2 corn, although the difference was not statistically significant. On diets with up to 28.7% soybean meal, pigs fed opaque-2 corn averaged greater gain and required less feed per unit of gain than pigs fed normal corn; at levels of soybean meal above 28.7%, however, the reverse was true.

The interaction of kind of corn and level of soybean meal on gain was significant ($P < 0.05$). At the two higher levels of soybean meal pigs fed opaque-2 corn gained less than pigs fed the lower levels of soybean meal and less than pigs fed normal corn at the same levels of soybean meal. The apparent depression of gain in

TABLE 5

Performance of pigs fed normal or opaque-2 corn (trial 2)

Soybean meal level, %	8.7	13.7	18.7	23.7	28.7
Calculated crude protein, %					
Normal	11.5	13.5	15.5	17.5	19.5
Opaque-2	12.5	14.5	16.5	18.5	20.5
Analyzed crude protein, % [1]					
Normal	12.3	14.8	17.8	18.9	19.6
Opaque-2	15.2	17.3	18.7	20.7	21.0
Total 5-week gain, kg [2,3]					
Normal	4.89	8.71	12.20	12.72	13.59
Opaque-2	6.72	10.53	13.45	13.44	14.73
Feed/gain [4,5]					
Normal	3.10	2.44	1.94	1.83	1.72
Opaque-2	2.29	2.04	1.85	1.70	1.61

[1] Average of duplicate samples.
[2] Average of three pens of four pigs each per treatment.
[3] Difference between kind of corn and quadratic response to soybean meal levels significant ($P < 0.01$).
[4] Interaction of source of corn and level of soybean mean significant ($P < 0.01$).
[5] Coefficients of variability equal 8.89 and 6.78% for gain and feed/gain, respectively.

pigs fed opaque-2 does not appear to be a result of excessive protein or an imbalance of amino acids, but additional data will be needed to verify such a conclusion. Feed/gain ratios averaged over both sources of corn decreased linearly ($P < 0.05$) with increasing levels of soybean meal.

In trial 2, two lower protein levels were used in the diets in an attempt to assure less than optimal levels or balance of amino acids in the diet. If opaque-2 corn does contain an improved balance of amino acids for the pig, the performance of the pigs fed opaque-2 corn should be improved over the performance of pigs fed normal corn. As shown in table 5, when pigs were fed opaque-2 both gain and feed efficiency, averaged over all levels of soybean meal, were significantly ($P < 0.01$) improved over pigs fed normal hybrid corn. There was a significant ($P < 0.01$) quadratic response of both total gain and feed/gain ratio to levels of soybean meal. The response of pigs fed increasing levels of soybean meal tended to plateau at levels above 18.7%. That the performance of pigs fed corn-base diets is improved as the protein content of the diet is increased is well documented (9–11).

Cromwell et al. (4) observed that the addition of lysine alone would not improve the growth rate of pigs fed normal corn to that of pigs fed opaque-2 corn diets. The addition of both lysine and tryptophan to normal corn diets did improve the performance of pigs to levels comparable with those of pigs fed opaque-2 corn diets.

In these experiments the substitution of opaque-2 corn for normal corn resulted in a plateau of gains and feed conversion in pigs and chicks, with approximately 5% less soybean meal in the opaque-2 corn diet than in the diets containing normal corn.

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Influence of Microorganisms on Oxygen Consumption, Carbon Dioxide Production and Colonic Temperature of Rats [1,2]

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ABSTRACT The O_2 consumption and CO_2 production by germfree rats and their colonic temperature are significantly lower than those of conventionalized rats; the respiratory quotient (RQ) is not different. When germfree rats are monocontaminated with *Clostridium welchii* or Bacteroides sp. there were no changes in O_2 consumption or CO_2 production. When *Escherichia coli* (a facultative anaerobe) was the monocontaminant, colonic temperature did not change but there were prompt and parallel rises in O_2 consumption and CO_2 production to levels close to those of conventionalized rats. The changes in metabolic rate lagged behind the increase in viable bacteria in the feces. The feeding of large quantities of heat-killed *E. coli* to germfree rats did not change their O_2 consumption, CO_2 production or colonic temperature. Serum protein-bound iodine and serum thyroxine iodine levels were similar in the germfree, conventionalized, *E. coli* and Bacteroides sp. rats. When neomycin was given to rats harboring Bacteroides sp., *E. coli* and Proteus sp., the numbers of these bacteria were reduced and oxygen consumption and carbon dioxide production fell; colonic temperature did not change. It appears that the bacteria and mechanisms involved for the metabolic and colonic temperature changes noted may be different.

Our studies of the influence of microorganisms on oxygen consumption, carbon dioxide production and colonic temperature of rats began several years ago as an outgrowth of our observations of striking differences in behavior of "germfree," [6] conventionalized,[7] and open-animal-room rats subjected to various metabolic and nutritional stresses, e.g., acute and chronic choline deficiency (1, 2) and starvation (3). We have found that certain intestinal bacteria (e.g., *Escherichia coli*) have a profound influence on oxygen consumption and carbon dioxide production by rats independent of any effect on colonic temperature, whereas other intestinal bacteria (e.g., Bacteroides sp.) have no effect. We also found that the colonic temperature of germfree rats is significantly lower than that of conventionalized rats.

METHODS

Fischer rats [8] were used. They were housed in plastic cages in flexible film plastic isolators of the type described by Trexler (4). "Conventionalization" of rats was carried out as follows: germfree rats were divided by litter and sex into two comparable groups. One group was kept germfree; the other group was moved into another sterile isolator. Fresh cecal contents of an open-animal-room rat of the same strain were mixed with 25 ml tap water and entered into the second isolator. Some of this suspension was swabbed on the fur and mouth of each rat, and the rest added to their drinking water. When rats were purposefully contaminated with one or more specific species of bacteria, actively

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[2] Preliminary report of these data was presented at the 50th Annual Meeting of the Federation of American Societies for Experimental Biology, 1966. Federation Proc., 25: 482 (abstract).

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[6] "Germfree" rats are rats free of viable bacteria, parasites and fungi as judged by methods published elsewhere (8).

[7] "Conventionalized" rats were littermates of the germfree rats contaminated with cecal contents of open-animal-room rats on the day after weaning.

[8] Purchased from the Charles River Breeding Laboratories, Wilmington, Mass.

growing cultures of the bacteria sealed in glass ampoules were entered into the isolator, spread on the bodies and mouths of the rats, and added to their drinking water. The conventionalized or monocontaminated, dicontaminated, etc., rats were maintained in isolators under the same general conditions (except microbial) as the germfree, i.e., their food, supplies, air, and the like, were sterilized, and the caging, bedding, temperature, humidity and light were the same in the germfree and conventionalized isolators. Materials were entered and removed through locks sterilized by peracetic acid. Food (Diet 5010C) [9] and water were offered ad libitum to the rats until 16 hours before the metabolic and temperature measurements were made, when the solid food was withdrawn. This was generally 5 to 6 PM; the metabolic measurements were made at 9 to 10 AM the next day.

The rats were moved from their respective holding plastic isolators into small sterile plastic isolators containing glass metabolic chambers for measuring oxygen consumption and carbon dioxide production. The size of each metabolic chamber was approximately 850 ml; it had a place for insertion of a thermometer for measuring the chamber temperature and openings for incoming and outgoing air. Using a small vacuum pump, approximately 50 ml air/minute per 100 g rat body weight were drawn through the metabolic chamber. The CO_2 in the incoming air was absorbed in 2.5 N NaOH before passing into the metabolic chamber. The outgoing air from the chamber was drawn into a gas washing bottle containing 250 ml of 2.5 N NaOH (which was freshly made from a 50% NaOH solution with boiled distilled H_2O) to trap the CO_2 from the expired air. The air in the gas washing bottle had been evacuated before connecting it to the metabolic chamber. The CO_2-free expired air was then drawn into a 4-liter bottle filled with oxygen-saturated mineral oil (specific gravity, 0.880; viscosity, 340 to 355). The oil displaced by the gas was collected in an empty 4-liter bottle of known weight.

The colonic temperature of the rat was measured by a glass thermometer or a thermistor probe at the start and at the end of the metabolic run. The body weight of the rat was measured just before placing the rat into the metabolic chamber. The rat was allowed to come to a restful state after being placed in the metabolic chamber; this usually took about 30 minutes. An additional 30 minutes was taken to allow the rat to grow accustomed to its new environment. Two one-half-hour collections were then made. The weight of the mineral oil displaced by the trapped outgoing air was measured and the volume of outgoing air calculated. Correction of gas volumes to standard conditions (273.18° K and 760 mm Hg pressure) was made. Oxygen in the CO_2-free expired air was measured with an oxygen analyzer.[10] For CO_2 analysis, 3 ml of the NaOH, Na_2CO_3 solution was heated to boiling in a 50-ml Erlenmeyer flask, topped with a CO_2 absorbing tube. One milliliter of 0.2 M $BaCl_2$ was added rapidly to precipitate $BaCO_3$. The flasks were immersed in a boiling water bath for 20 minutes. After cooling, the precipitates were filtered through Whatman no. 42, 2.4-cm circle filter paper. The precipitate was washed twice with boiled distilled water and once with absolute ethyl alcohol and then dried under an infrared lamp. The dried precipitate was cooled in a desiccator before weighing. In later experiments, the outgoing air was led directly into the mineral oil collecting bottle and the CO_2 concentration measured with an infrared spectrophotometer.[11]

RESULTS

Experiment 1 (table 1). A group of germfree rats was divided at weaning into two groups of littermates. One group was kept germfree and the other conventionalized. Three and a half months later the first metabolic measurements were made. We found that the oxygen consumption and carbon dioxide production were higher (each about 20%) in the conventionalized rats. When the values for oxygen consumption and carbon dioxide production were expressed per kilogram body weight minus gut content weight, these values were still significantly higher for the conventionalized rats. These calculations were made because of the significantly larger

[9] Ralston Purina Company, St. Louis, Mo.
[10] Model E-2, Beckman Instruments, Inc., Fullerton, Calif.
[11] Perkin-Elmer Corporation, Norwalk, Conn.

TABLE 1

Influence of microorganisms on oxygen consumption, carbon dioxide production, colonic temperature, body weight and gastrointestinal tract weight of rats [1]

Exp. no.	No. of rats	Microbial status	Age	Body wt	GI tract empty	GI tract contents	Colonic temperature	Before correction for GI contents		After correction for GI contents		RQ
								O_2 consumption	CO_2 production	O_2 consumption	CO_2 production	
			months	*g*	*g*	*g*	*degrees*	*liters/kg body wt per day*		*liters/kg body wt per day*		
	6	CONV [2]	4.5	190 ± 14 [3]	7.5 ± 0.5	7.7 ± 0.8	37.6 ± 0.1	25.5 ± 0.3	22.8 ± 0.4	26.6 ± 0.4	23.7 ± 0.7	0.89 ± 0.0
1	6	GF [2]	4.5	225 ± 26	10.9 ± 0.7	21.9 ± 1.2	36.5 ± 0.2	20.9 ± 0.4	18.0 ± 0.6	23.1 ± 0.4	19.9 ± 0.4	0.86 ± 0.0
		P		ns	0.01	0.001	0.02	0.001	0.005	0.005	0.02	ns
	4	CONV	3	149 ± 17	5.3 ± 0.2	3.9 ± 0.7	37.0 ± 0.1	25.3 ± 0.4	21.6 ± 0.2	25.9 ± 0.4	22.2 ± 0.2	0.8 ± 0.0
2A	4	GF	3	168 ± 13	5.8 ± 0.2	22.9 ± 2.2	36.4 ± 0.1	20.4 ± 0.5	17.3 ± 0.2	23.6 ± 0.4	20.1 ± 0.2	0.8 ± 0.0
		P		ns	ns	0.001	0.02	0.005	0.005	0.05	0.01	ns
	4	*Cl. welchii*	2.5	127 ± 9.9	5.5 ± 0.3	12.0 ± 1.1	37.3 ± 0.1	25.8 ± 0.3	22.9 ± 0.5	28.2 ± 0.5	25.3 ± 0.7	0.89 ± 0.0
2B	4	GF	2.5	139 ± 5.0	5.9 ± 0.1	17.8 ± 1.4	36.7 ± 0.3	25.2 ± 0.1	22.1 ± 0.6	28.9 ± 0.4	25.4 ± 0.2	0.88 ± 0.0
		P		ns	ns	0.02	ns	ns	ns	ns	ns	ns
	6	*E. coli*	3	175 ± 9.0	7.7 ± 0.4	12.3 ± 1.1	37.1 ± 0.1	25.4 ± 0.3	22.2 ± 0.7	27.4 ± 0.5	23.9 ± 0.7	0.87 ± 0.0
2C	6	GF	3	191 ± 14.2	7.0 ± 0.4	14.2 ± 1.1	36.8 ± 0.1	22.8 ± 0.2	19.9 ± 0.3	24.6 ± 0.3	21.4 ± 0.2	0.87 ± 0.0
		P		ns	ns	ns	ns	0.01	0.05	0.01	0.02	ns

[1] Experiments 1, 2A, 2B: each group consisted of equal numbers of male and female Fischer rats. In experiment 2C, all rats were females.
[2] CONV = conventionalized; GF = germfree.
[3] Mean ± SE.

weight of the gut contents of the germfree rats. The respiratory quotients were similar in both groups of rats. The colonic temperatures of the germfree rats were significantly lower (about 1°) than those of the conventionalized rats.

Experiment 2 (table 1). A group of germfree Fischer weanling rats was split into six groups, two of which were purposefully monocontaminated with either *Cl. welchii* or *E. coli;* group 3 was conventionalized. Each of these groups had its own control group of littermates which were kept germfree. These rats were studied 6 weeks later. Those rats harboring only the *Cl. welchii* did not differ from their germfree littermates in respect to oxygen consumption and carbon dioxide production, whereas these parameters were significantly higher in those rats harboring *E. coli* and in the conventionalized rats than in their respective germfree controls. These differences were evident whether values were expressed per kilogram body weight or per kilogram body weight minus gut contents. (The size of the gastrointestinal tract and its contents is only slightly smaller in the *E. coli* and the *Cl. welchii* than in the germfree rats.) The *E. coli* monocontaminants showed levels of oxygen consumption and carbon dioxide production similar to those of the conventionalized rats. The respiratory quotients were similar in all groups. The colonic temperatures of the germfree and *Cl. welchii* and *E. coli* rats were similar, whereas conventionalized rats had temperatures higher than their germfree controls. All these findings were independent of sex.

Experiments 3, 4 and 5 (fig. 1, tables 2 and 3). We carried out these experiments to determine how soon the effects of *E. coli* would be evident on the metabolic rate of the rats. The increase in oxygen consumption and carbon dioxide production were very prompt indeed (as early as 12 hours), but lagged somewhat behind the number of viable *E. coli* found in the feces (exps. 3 and 4, table 2 and fig. 1). The mod-

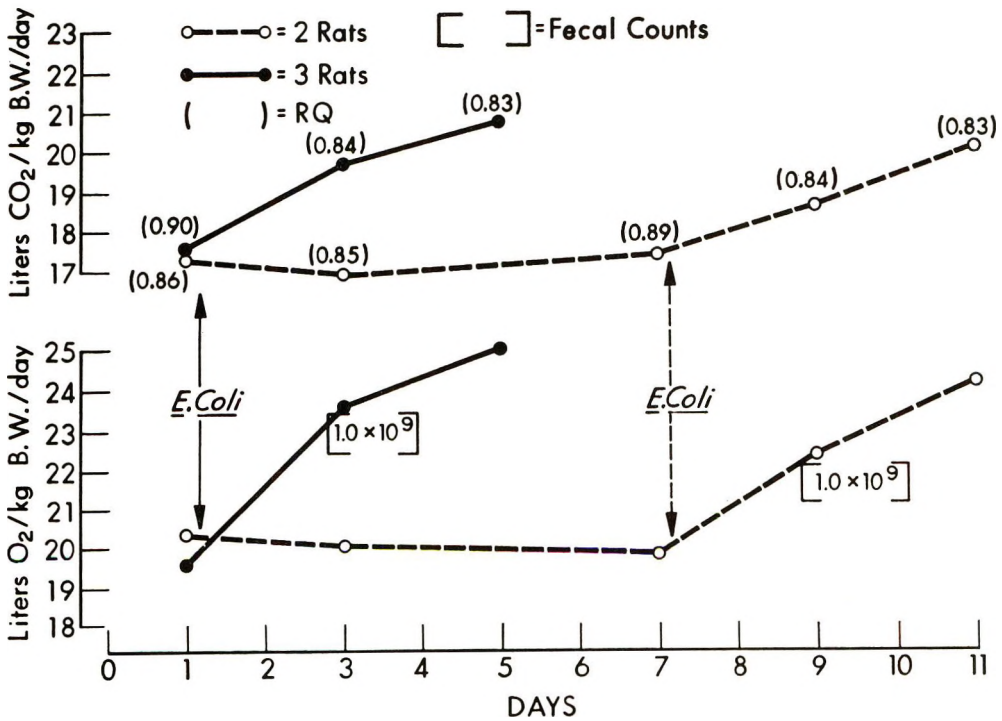

Fig. 1 O_2 consumption and CO_2 production by germfree rats and following their monocontamination with *E. coli* (exp. 4). Fecal counts expressed as number of viable bacteria per gram feces.

TABLE 2

O_2 consumption and CO_2 production by germfree and E. coli monocontaminated rats [1]

Hours after contamination	Time of day	Group	Microbial status	Rat no.	Colonic temperature	O_2 consumption	CO_2 production	RQ	Fecal count
					degrees	*liters/kg body wt per day*	*liters/kg body wt per day*		*per g*
0	0700	A	GF [2]	1	36.7	22.5	18.2	0.81	0
				2	36.7	22.8	18.9	0.83	0
		B	GF	3	36.9	22.4	18.8	0.84	0
				4	37.0	23.3	19.6	0.84	0
6	1300	A	GF	1	36.8	23.3	18.9	0.81	0
				2	37.0	23.5	19.2	0.82	0
		B	*E. coli*	3	36.8	23.7	19.9	0.84	10^3
				4	37.0	24.8	20.1	0.81	10^3
12	1900	A	GF	1	36.9	23.8	19.4	0.81	0
				2	36.9	24.0	19.6	0.82	0
		B	*E. coli*	3	36.8	25.8	21.2	0.82	10^{10}
				4	36.8	26.0	21.0	0.81	10^9
34	1700	A	GF	1	36.8	22.8	17.8	0.78	0
				2	37.0	22.7	17.3	0.76	0
		B	*E. coli*	3	36.5	27.4	22.4	0.82	10^{10}
				4	36.5	27.6	22.6	0.82	10^9
44	0300	A	GF	1	36.2	21.3	16.9	0.79	0
				2	36.8	21.1	16.6	0.79	0
		B	*E. coli*	3	36.8	27.1	22.1	0.81	10^9
				4	36.4	27.2	22.2	0.82	10^9
72	0700	A	GF	1	36.3	21.1	16.9	0.80	0
				2	36.2	21.1	17.0	0.81	0
		B	*E. coli*	3	36.3	25.4	20.4	0.80	10^9
				4	36.4	25.2	20.5	0.81	10^9
96	0700	A	GF	—	—	—	—	—	—
		B	*E. coli*	3	36.5	26.0	21.1	0.81	10^9
				4	36.5	25.2	20.3	0.81	10^9

[1] Eighty-day-old male rats of the Fischer strain, two in each group. Rats in Group B purposefully monocontaminated with *E. coli* immediately after the zero hour measurements. Solid food withdrawn 16 hours before the zero hour measurements and given back immediately after the fourth set of measurements, 34 hours after contamination, for 5 hours. Rats were given solid food again after the sixth set of measurements, 72 hours after contamination, for 5 hours. Drinking water was available ad libitum throughout the experiment, except during the measurements of oxygen consumption and carbon dioxide production (exp. 3).

[2] GF = germfree.

TABLE 3

O_2 consumption and CO_2 production by germfree, E. coli and conventionalized rats [1]

Group	Days	Colonic temperature	O_2 consumption	CO_2 production	RQ	Fecal counts
		degrees	*liters/kg body wt per day*	*liters/kg body wt per day*		*per g*
GF	1	36.8 ± 0.03 [2]	22.0 ± 0.7	17.6 ± 0.6	0.79 ± 0	0
	5	36.9 ± 0.19	22.7 ± 0.7	18.4 ± 0.4	0.81 ± 0.01	0
	7	36.7 ± 0.23	21.2 ± 0.5	17.2 ± 0.4	0.81 ± 0.01	0
E. coli mono-contaminants	9 [3]	36.5 ± 0.03	23.9 ± 0.4	19.2 ± 0.4	0.81 ± 0	10^{10}
	12	36.6 ± 0.33	27.7 ± 0.8	22.7 ± 0.2	0.82 ± 0.02	10^{9}
	14	36.7 ± 0.12	27.1 ± 0.4	22.1 ± 0.4	0.82 ± 0	10^{9}
	16	36.6 ± 0.25	27.4 ± 0.5	21.8 ± 0.1	0.80 ± 0.02	—
CONV	21 [4]	37.1 ± 0.15	29.2 ± 0.2	24.4 ± 0.3	0.83 ± 0.01	—
	23	37.0 ± 0.09	28.9 ± 0.4	23.5 ± 0.7	0.81 ± 0.01	—
	28	37.0 ± 0.09	29.2 ± 0.7	24.5 ± 0.4	0.84 ± 0.01	—

[1] The same three male rats of the Fischer strain were used for all measurements; 12 weeks old at start of exp. 5.
[2] Mean ± SE.
[3] The rats were purposefully contaminated with *E. coli* on day 7 immediately after the last measurements while the rats were germfree.
[4] The rats were purposefully contaminated with cecal contents of an open-animal-room rat on day 17.

est fluctuations in oxygen consumption and carbon dioxide production seen in the germfree rats in experiment 3 (table 2) were due, we believe, to the effects of fasting and feeding and time of day that these several measurements were made. The peak effect on oxygen consumption and carbon dioxide production occurred in 3 to 5 days (exp. 5, table 3). The respiratory quotient did not change nor did colonic temperature. The littermates of the *E. coli* rats in experiment 4 (fig. 1) which were kept germfree did not show changes in oxygen consumption and carbon dioxide production, but very soon after they were contaminated with *E. coli*, the same rises in these parameters occurred. There was a modest further rise in oxygen consumption and carbon dioxide production when the *E. coli* rats were conventionalized (exp. 5, table 3).

Experiment 6 (fig. 2, table 4). Germfree rats fed enormous numbers of heat-killed *E. coli* in their drinking water showed no increase in oxygen consumption or carbon dioxide production. Similarly, when these animals were contaminated with two strains of Bacteroides sp. there were no changes in these facets of the rats' metabolism. When these same rats were fed viable *E. coli*, however, there was a prompt increase in the number of viable *E. coli* in the gut (10^9/g feces 24 hours after inoculation) and oxygen consumption and carbon dioxide production. The respiratory quotient did not change. A culture of Proteus sp. was then fed to the *E. coli* rats in an attempt to increase the number of facultative anaerobes in the gut to see how this would modify oxygen consumption and carbon dioxide production. The rats were tested 5 and 7 days later. We did not achieve our objective since there were no demonstrable changes in the total number of viable bacteria in the feces; the *E. coli* and Proteus sp. were each present in 10^8 viable bacteria/g feces. Oxygen consumption and carbon dioxide production did not change. Neomycin was then given to these rats in amounts of 0.7 mg/ml drinking water daily. Five and 7 days later, the number of viable organisms per gram of feces had dropped, *E. coli*, 10^4–10^5, Proteus sp., 10^6. There was a concomitant drop in oxygen consumption and carbon dioxide production. Neither dead *E. coli*, viable Bacteroides sp., *E. coli*, Proteus sp., nor neomycin changed the colonic temperature of the rats.

Influence of intestinal bacteria on the E_h of cecal contents (table 5). The E_h of the cecal contents was measured in several experiments. It averaged about + 30 mv in the germfree, about − 70 mv in the Bacterioides sp. rats, about − 110 mv in the *E.*

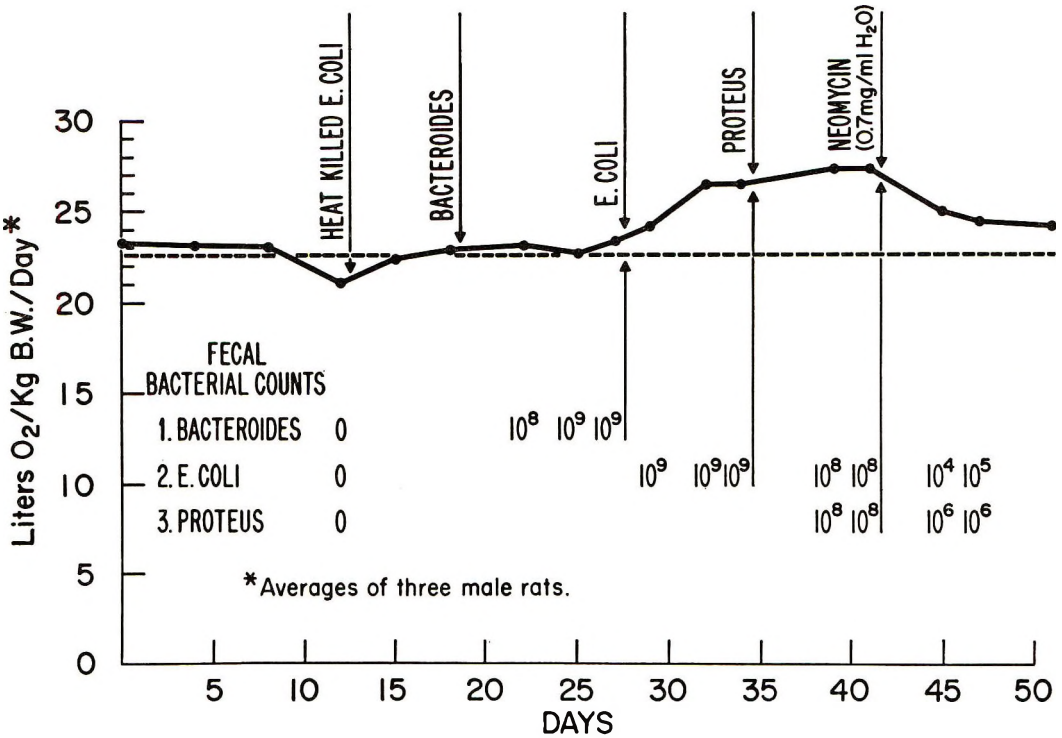

Fig. 2 Oxygen consumption of germfree rats and following the sequential feeding of heat-killed *E. coli*, viable Bacteroides sp., *E. coli*, Proteus sp. and neomycin (exp. 6). Bacterial counts expressed as number of viable bacteria per gram feces.

TABLE 4

Effect of E. coli (heat killed and viable), Bacteroides, Proteus and neomycin on O_2 consumption and CO_2 production by rats [1]

Microbial status	Days	Colonic temperature	O_2 consumption	CO_2 production	RQ	Fecal counts
		degrees	*liters/kg body wt per day*	*liters/kg body wt per day*		*per g*
GF	1	36.3 [2] ± 0.08	23.2 ± 0.30	19.0 ± 0.20	0.82 ± 0.01	0
	4	36.4 ± 0.09	23.1 ± 0.10	18.6 ± 0.29	0.80 ± 0.01	0
	8	36.5 ± 0.09	23.0 ± 0.20	18.6 ± 0.38	0.81 ± 0.01	0
	12	36.3 ± 0.09	21.1 ± 0.26	17.4 ± 0.44	0.82 ± 0.01	0
Fed heat-killed *E. coli*	15	36.2 ± 0.07	22.3 ± 0.34	18.2 ± 0.28	0.81 ± 0	0
	18	36.1 ± 0.03	22.8 ± 0.17	18.8 ± 0.17	0.83 ± 0.01	0
Bacteroides	22	35.8 ± 0.03	23.0 ± 0.18	18.8 ± 0.09	0.82 ± 0.01	10^8
	25	35.9 ± 0.09	22.6 ± 0.63	18.6 ± 0.74	0.82 ± 0.01	10^9
	27	36.0 ± 0.07	23.3 ± 0.35	19.4 ± 0.62	0.83 ± 0.01	10^9
E. coli, Bacteroides	29	36.1 ± 0.07	24.8 ± 0.51	20.1 ± 0.60	0.81 ± 0.01	10^9
	32	36.0 ± 0.12	26.7 ± 0.69	21.5 ± 0.62	0.81 ± 0.01	10^9
	34	36.0 ± 0.12	26.7 ± 0.23	21.2 ± 0.23	0.79 ± 0	10^9
Proteus, *E. coli*, Bacteroides	39	35.9 ± 0.06	27.2 ± 0.70	21.5 ± 0.47	0.79 ± 0	10^8
	41	35.8 ± 0.09	27.2 ± 0.55	21.3 ± 0.49	0.78 ± 0	10^8
Neomycin, Proteus, *E. coli*, Bacteroides	46	35.9 ± 0.12	24.9 ± 0.38	20.0 ± 0.23	0.81 ± 0.01	10^4
	48	35.9 ± 0.06	25.0 ± 0.52	20.4 ± 0.45	0.81 ± 0.02	10^5

[1] Three male rats of the Fischer strain were used; 13 weeks old at start of exp. 6.
[2] Mean ± SE.

coli rats and − 200 mv in the conventionalized.

Influence of intestinal bacteria on serum PBI and serum thyroxine iodine concentrations (table 5). Serum PBI and serum thyroxine iodine concentrations were similar in germfree, *E. coli* monocontaminants, Bacteroides sp. monocontaminants and conventionalized rats. These observations were made 2 weeks after the purposeful contaminations.

Colonic temperatures in germfree and conventionalized rats. Table 6 lists the results of a series of observations of colonic temperatures of germfree rats and their conventionalized littermates in a number of studies carried out during several years. In all but one experiment (exp. 5, table 3) the colonic temperature of the germfree rats was significantly lower than that of the conventionalized. In the one exception, the measurements were made after the rats had been conventionalized for 11 days; in the others, the rats had been conventionalized for a minimum of 6 weeks.

DISCUSSION

A number of investigations have been carried out for many years to delineate the specific nature and significance of the complex influences of microorganisms on mammalian metabolic and nutritional processes. An idea of the variability of these influences is evident when one considers the effects of the ordinary microflora present in healthy laboratory animals on their responses to various nutritional deficiencies. The reported effects range from accentuation of the deficiency (e.g., vitamin C (5), acute choline deficiency (1)) to amelioration of the deficiency (e.g., vitamin K (6), and, in some cases, there are no major effects (e.g., vitamin A (7)). As mentioned in the introduction to this paper, we began investigating the effects of

TABLE 5

Influence of intestinal bacteria on cecal E_h, serum PBI, and thyroxine iodine of rats [1]

Microbial status	No. of rats	Cecal wt	Cecal E_h	PBI	Thyroxine iodine [2]
		% body wt	*mv*	*μg/100 ml serum*	*μg/100 ml serum*
Germfree	14	15.3 ± 0.4 [3]	+ 35 ± 5.7	2.7 ± 0.49	0.5
Conventionalized [4]	15	2.1 ± 0.1	−201 ± 6.6	3.2 ± 0.59	0.5
E. coli [4]	15	12.9 ± 0.5	− 85 ± 4.7	3.0 ± 0.32	0.7
Bacteroides [4]	15	12.3 ± 0.7	− 72 ± 4.0	3.1 ± 0.54	—

[1] Male rats of the Fischer strain, 3 months old; five in each group.
[2] Pooled samples.
[3] Mean ± SE.
[4] Rats monocontaminated or conventionalized for 2 weeks.

TABLE 6

Colonic temperatures in germfree and conventionalized rats [1]

Exp. no.	Sex	Age	Colonic temperature [2]		P values
			Germfree	Conventionalized	
		months	*degrees*	*degrees*	
1	Male	3	36.4 ± 0.1 [3] (4) [4]	37.0 ± 0.1 (4)	0.02
2	Male	4.5	36.5 ± 0.5 (6)	37.6 ± 0.1 (4)	0.02
3	Male	6	36.6 ± 0.2 (9)	37.0 ± 0.1 (5)	0.05
4	Both	5	36.3 ± 0.1 (8)	37.1 ± 0.1 (8)	0.001
5	Both	4	36.0 ± 0.1 (6)	36.4 ± 0.1 (5)	0.05
6	Both	6.5	36.1 ± 0.1 (24)	36.6 ± 0 (7)	0.001
7	Both	4.5	37.3 ± 0.1 (25)	37.7 ± 0 (20)	0.001
8	Male	8	37.3 ± 0.2 (16)	38.0 ± 0.2 (16)	0.001
9	Both	1.5	35.0 ± 1.0 (12)	37.9 ± 0.2 (12)	0.001

[1] Rats of the Fischer strain were used; littermates in each pair of groups. The rats in experiments 1 through 8 ate a rat ration (diet 5010C, Ralston Purina Company, St. Louis, Mo.); rats in experiment 9 ate a chemically defined liquid diet (#116, Winitz and Greenstein).
[2] Temperatures taken at about 9 AM, after 16 hours without food except water.
[3] Mean ± SE.
[4] Numbers in parentheses indicate number of rats.

intestinal bacteria on the oxygen consumption, carbon dioxide production and body temperature of the rat for these reasons, and because of the striking differences in body composition (8), fecal nitrogen excretion (8) and response to food deprivation (3) we had noted in germfree, conventionalized and open-animal-room rats.

The influence of age, sex, nutritional status, certain hormones, activity, fever, injury and disease on basal metabolism is well recognized, but the dramatic effect of the infectious state (but not disease) which characterizes normal mammalian life on basal metabolism has not been realized until recently. We have reference in particular to certain of the ordinary bacteria present in the gastrointestinal tracts of healthy animals and humans. This is demonstrated by the observations on oxygen consumption by open-animal-room and germfree rats of Desplaces et al. (9), the observations of Windmueller et al. (10) of carbon dioxide production by open-animal-room and germfree rats and our observations of oxygen consumption and carbon dioxide production by conventionalized littermates of germfree rats. Wostmann and co-workers (11) have also just reported that germfree rats have lower oxygen consumption rates than open-animal-room or conventionalized rats. Our experiments indicate that it is the facultative anaerobes, such as *E. coli,* and not the strict anaerobes which increase the O_2 consumption and CO_2 production by rats, directly or indirectly. We found that these changes were independent of sex and any change of colonic temperature in the *E. coli* experiments. No mention of body temperature was made by Sacquet and her colleagues (9) or by Windmueller and his associates (10). Wostmann et al. (11) found higher temperatures in the germfree than in the open-animal-room rats (including those housed for a period of time in isolators). This is diametrically opposed to our findings. This will be discussed later.

The fact that we found the changes in O_2 consumption and CO_2 production are prompt, beginning within hours after purposeful contamination with *E. coli* and that they do not follow contamination with Bacteroides sp. makes us think that at least part of the effect is direct utilization of O_2 and production of CO_2 by facultative anaerobic bacteria. That a direct effect of bacteria may be involved has also been suggested by Windmueller and his associates (10). There is some information available about the rate of O_2 consumption by *E. coli* growing in culture with an excess of O_2 present, but not under conditions similar to those present in the gut. Under the former condition, the rate of O_2 consumption is very high.

Another effect may be the formation of certain amines, e.g., tryptamine, by gut bacteria, which are then absorbed. This thought occurred to us because of the demonstration that such amines may increase O_2 consumption of chicks (12). In this same vein, but conversely, Wostmann et al. (11) have recently postulated that the lower O_2 consumption and CO_2 output of germfree rats may result from the absorption of one or more bioactive substances from their enlarged ceca which might, by some unknown mechanism, exert a depressant action on O_2 consumption and cardiac output. In this regard, they cite that Gordon (13) has described toxic and musculoactive principles present in greater amounts in the cecal contents of germfree animals. Wostmann et al. base their view on their observation that germfree rats 3 to 4 months old whose ceca had been excised at the age of 6 weeks had cardiac outputs and O_2 consumption rates significantly higher than germfree rats whose ceca were intact. Similar operations did not influence these parameters in their conventional rats. The increase in cardiac output of the cecectomized germfree rats was to the same level as seen in their conventional rats (with and without previous cecectomy). Though the increase in O_2 consumption by the cecectomized germfree rats was similar to that of their conventionalized rats, it was significantly lower than that of the conventional rats. Data as to the gastrointestinal contents of these various groups of rats used in these experiments are not given, so the possible effects of this factor on the calculations cannot be made.

Metabolic rate has been considered to be more closely related to lean body mass than to body weight. We pointed out in the experiments reported in this paper that

although the differences in O_2 consumption and CO_2 production by germfree and conventionalized rats was somewhat less when expressed in terms of body weight minus gut content weight rather than body weight alone, these differences were still highly significant. This type of calculation did not affect the relative values for the *E. coli, Cl. welchii,* Bacteroides sp. and Proteus sp. rats since these bacteria had little, if any, influence on the amount of gut contents.

In other experiments,[12] we have found that the body composition of germfree and conventionalized rats differ not only in regard to gut contents, but also in several other important respects. For example, the concentrations and contents of carcass and body fat and calories are greater in conventionalized rats. There were no significant differences, however, in total body, carcass or liver protein. Therefore, the differences in O_2 consumption and CO_2 production by germfree and conventionalized rats cannot be explained by differences in lean body mass.

We lean toward the point of view that a major reason for the higher O_2 consumption and CO_2 production rates of *E. coli,* conventionalized and open-animal-room rats is the metabolic activity of the facultative anaerobes in their gastrointestinal tracts. The fact that we have found a lag, at times of a few days, between the peak rise in O_2 consumption and CO_2 production and the number of viable bacteria in the feces of rats purposefully contaminated with *E. coli* suggests to us that indirect effects on the host are involved also. These would include possible alteration of thyroid function as suggested by Desplaces et al. (10). Their data are not convincing to us, however. The apparent differences in radioactive iodine uptake by the thyroids of open-animal-room and germfree rats found by them do not seem large enough to account for the marked differences in O_2 consumption observed. Our own data show no differences in serum PBI and serum thyroxine iodine concentrations of germfree and conventionalized rats.

Alterations in the function of other endocrine glands are among some of the possible changes induced by certain of the intestinal bacteria which may influence O_2 consumption and CO_2 production. For example, an increase in the secretion of norepinephrine may be associated with an increase in O_2 consumption. The information regarding the effects of specific microbes on mammalian catecholamine metabolism and the metabolism of other hormonal agents is meager indeed.

Possible differences in the rates of turnover of intestinal mucosal cells of germfree, *E. coli,* Bacteroides sp. and conventionalized rats may also underlie the differences in metabolic rates of these groups of rats. In regard to this possibility, Abrams and associates (14) found that the renewal rate of ileal mucosal epithelial cells was about twice as fast in conventional mice as in germfree mice. In studies to be reported elsewhere, however, we found relatively little difference among the renewal rates of ileal mucosal epithelial cells of germfree, conventionalized, *E. coli* monocontaminants, and Bacteroides sp. monocontaminants.[13]

The lower metabolic rate of germfree animals may explain in part their greater resistance to immunosuppressant drugs such as azathioprine (15), and to whole-body X-irradiation (16), particularly in the supralethal ranges where factors other than (or in addition to) secondary bacterial infection and toxemia seem to play a role.

The lower metabolic rate of the germfree rats may modify in important ways their responses to various nutritional deficiencies. We have pointed out elsewhere (2) that this factor may explain in part the greater resistance of weanling germfree rats to acute choline deficiency as compared with conventionalized or open-animal-room rats.

Although the rise in basal metabolism of the *E. coli* rats is independent of any rise in colonic temperature, it should be noted that we found a higher colonic temperature (about 1°) in conventionalized rats compared with their germfree littermates provided the conventionalization had gone on for at least 6 weeks. In contrast to our findings, Wostmann et al. (11) found little difference in the rectal temperatures of 10 germfree and 9 conven-

[12] Levenson, S. M., B. Tennant, L. Crowley, A. Nagler and D. Kan, unpublished data.

[13] Levenson, S. M., H. Ragins, D. Kan, F. Wincze and C. A. Gruber, unpublished data.

tional rats, the latter housed in an isolator for 3 days prior to the measurements. In fact, the mean rectal temperature for the germfree rats was somewhat higher, though not significantly so statistically. It should be noted that in our experiments, the conventionalized rats were littermates of the germfree and were housed in isolators throughout their lives, just as were the germfree. Whether these factors account for the differences in our findings and those of Wostmann et al., we do not know. As mentioned previously, we found higher colonic temperatures in conventionalized rats in a number of experiments carried out at various times during several years.

The reason for the higher colonic temperature of conventionalized rats as compared with germfree, and the possible effects of this colonic temperature differential on the responses of the rats to a variety of challenges are under study in our laboratory. It would seem, though, that the factors underlying the differences in the colonic temperatures of germfree and conventionalized rats are not the same as those underlying the differences in oxygen consumption and carbon dioxide production, although the former may include the latter.

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Influence of Sulfur on Incidence of White Muscle Disease in Lambs [1,2]

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ABSTRACT Two trials were conducted with sheep to investigate the effect of sulfur on the incidence of white muscle disease (WMD). Neither methionine nor sulfate increased the incidence of WMD in either trial, but sulfate significantly increased the number of lambs with degenerative lesions of the heart. There was a fivefold increase of plasma glutamic-oxaloacetic transaminase (GOT), lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) of the lambs at 2 weeks of age with the nonsupplemented ewes as compared with those from ewes receiving selenium. The GOT, LDH and MDH levels of the lambs from ewes fed sulfate or methionine were twofold greater than comparable values of the control lot. At 6 weeks of age, however, the levels of these enzymes of the sulfate and methionine lots had approached the levels of the nonsupplemented group. White muscle disease had no effect on blood glucose, lactate or cholesterol. Based on the enzyme levels, the sulfur sources appeared to delay the onset of WMD. A significant "carryover" of selenium was noted.

Due to the chemical similarity of selenium and sulfur, it would be expected that a biological interaction between these two elements exists. This is supported by work with several species of animals which indicates that sulfur does alter the metabolism of selenium (1–5). Muth et al. (5) and Hintz and Hogue (3) reported the addition of sulfur as Na_2SO_4 to the diet decreased the effectiveness of dietary selenium as Na_2SeO_3 in preventing white muscle disease (WMD). Furthermore, Schubert et al. (6) observed an increase in the incidence of WMD in sheep grazed on alfalfa after the field was treated with gypsum. Analysis showed an increase in the total sulfur of the plants, largely as sulfate. Sulfate, however, had a slight effect on the metabolic fate of ^{75}Se-selenate in the gestating [3] or lactating ewe (7).

Serum enzyme levels have been used to follow the incidence of WMD. Serum glutamic-oxaloacetic transaminase (GOT) (8–15), lactic dehydrogenase (LDH) (10, 11, 16) and alanine transaminase and glutamate dehydrogenase (10) have been reported to be elevated in lambs with white muscle disease (WMD). In addition, elevated serum malic dehydrogenase (MDH) has been reported in humans with muscular dystrophy (17). Therefore, to get a better understanding of the sulfur-selenium interrelationship, some of these enzymes were assayed in lambs nursing ewes fed various forms of sulfur with high and low selenium rations. In addition to the enzyme assays, blood glucose, lactate and cholesterol were measured to determine whether these components were influenced by the sulfur sources or by WMD. A preliminary report of some of this work has been presented.[4]

METHODS

The sheep used in these studies were similar to and maintained like those in previously reported experiments (6, 18). The ewes of trial 1 were divided into four lots with 12 ewes/lot in lots 1 and 2, and 24 ewes/lot in lots 3 and 4. Those in lots 1 and 2 were fed 1816 g high selenium (0.23 ppm) alfalfa hay plus 100 g ground oats (0.01 ppm selenium) daily per ewe.

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[2] Supported in part by Public Health Service Research Grant no. NB 07413-01 from the National Institute of Neurological Diseases and Blindness.
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Those in lots 3 and 4 were fed 1816 g low selenium (0.01 ppm) alfalfa hay plus 100 g ground oats (0.01 ppm selenium) daily per ewe. Potassium sulfate was mixed with the ground oats given to those ewes of lots 2 and 4 at a level calculated to be 1.0% of the total ration.

The ewes of trial 2 were divided into five lots (15 ewes/lot), and all of them were fed 1816 g low selenium alfalfa hay (0.01 ppm) and 100 g oats daily per ewe. Lot 1 was injected with 5 mg selenium as selenite 1 month before calculated parturition. Lot 2 did not receive any supplement, but lots 3 and 4 were fed ground oats containing 1.0% K_2SO_4 or 0.5% DL-methionine calculated on the basis of the total ration. Some ewes from lots 1 and 2 of trial 1 were selected for lot 5, and were fed the same ration as those of lot 2. These ewes, which had been fed the high selenium hay the previous year, were used to determine whether the previous selenium status of an animal would influence the incidence of WMD in the offspring, and this was referred to as the "carryover" lot. The supplementation was started 100 days before estimated parturition and continued for about 80 days after.

Blood was taken from the ewes before the morning feeding about 4 weeks before and after parturition for enzyme assays and selenium-75 uptake by red blood cells. Blood was taken from the lambs for chemical analysis and enzyme assays at 2 weeks and when they were killed for necropsy at 6 weeks of age. All lambs were weighed at birth, at 2 weeks, and terminally at 6 weeks of age. Blocks of tissue were routinely selected, processed, and examined histologically as previously described by Muth et al. (18).

Red blood cell selenium-75 uptake as described by Weswig et al. (19) and plasma GOT [5] were determined on blood taken in both trials. In addition, in trial 2, plasma LDH and MDH, and blood glucose, lactate and cholesterol were determined on the blood from both ewes and lambs. LDH and MDH [6] were determined by the methods of Wroblewski and LaDue (20) and Siegel and Bing (21), respectively. Blood glucose (22) and lactate (23) were determined on a protein-free filtrate. Plasma cholesterol was determined by the method of Pearson et al. (24). Selenium concentration was determined on all feeds and sulfur sources (25). The sulfur sources contained negligible amounts of selenium. The alfalfa hays used in both trials were shown by analysis to contain approximately 0.39% total sulfur (6).

Data were subjected to analysis of variance and correlation coefficient calculations (26).

RESULTS

Sulfate did not result in elevated levels of plasma GOT (table 1). There were no differences between the levels of plasma

[5] Colorimetric determination of glutamic-oxalacetic and glutamic-pyruvic transaminases at 505 mμ. Sigma Chemical Company, St. Louis, Tech. Bull. no. 505, 1964.

[6] Although this assay is not completely specific for MDH, it will be designated as such for convenience.

TABLE 1

Plasma GOT levels of 2- and 6-week-old lambs nursing ewes fed high or low selenium alfalfa hay, with and without added sulfate (trial 1)

Diet	Plasma				Lambs with over 200 U/ml plasma	
	Average		Range			
	2 weeks	6 weeks	2 weeks	6 weeks	2 weeks	6 weeks
	units [1] per ml		*units [1] per ml*		%	%
High selenium alfalfa	104	71	79–152	18–124	0	0
High selenium alfalfa plus sulfate	99	94	67–138	20–193	0	0
Low selenium alfalfa	121	385 [2]	69–345	36–2060	4.0	35.0
Low selenium alfalfa plus sulfate	127	406 [2]	69–375	66–1970	6.0	25.0

[1] Sigma unit as defined (20).

[2] Significantly higher ($P < 0.05$) than high selenium alfalfa lots.

GOT between the lots at 2 weeks of age; at 6 weeks of age, however, there was a significant difference ($P < 0.05$) between the lots fed high selenium alfalfa and those fed low selenium alfalfa. With the data from all the lots combined, analysis of variance revealed that those lambs with WMD had significantly higher ($P < 0.05$) plasma GOT levels than those without WMD. Although not shown in this table, there were no differences between plasma GOT of ewes fed the various diets. Elevated GOT levels of WMD lambs are in agreement with other reports (8–10, 12–15).

The uptake of selenium-75 by red blood cells from low selenium ewes was significantly greater ($P < 0.01$) than that of ewes fed high selenium hays (table 2). With both high and low selenium hays, sulfate appeared to slightly suppress selenium uptake by maternal red blood cells. There was a significant difference ($P < 0.05$) in the uptake of selenium by red blood cells from 2-week-old lambs fed high and low selenium. This difference disappeared at 6 weeks of age. The uptake of selenium-75 by red blood cells from 2-week-old lambs with WMD was significantly greater ($P < 0.05$) than from 2-week-old lambs without WMD. This difference only approached significance, however, when the lambs were 6 weeks old. Thus, it appears selenium uptake by red blood cells may be more indicative of a potential WMD lamb at 2 weeks of age than when the lamb becomes 6 weeks of age. These results are in agreement with other reports, indicating there is a relationship between the selenium status of the animal and uptake of selenium by red blood cells (19, 27, 28).

Sulfate did not increase the incidence of WMD (table 3), and there appeared to be little difference in the number of lambs which died before 6 weeks of age between the treatment groups. Sulfate in the low selenium ration appeared to reduce the incidence of WMD slightly. These data are inconsistent with previous reports (3, 5, 6) and prompted the second trial discussed next.

TABLE 2

Percentage ^{75}Se-selenite uptake by red blood cells (RBC) from 2- and 6-week-old lambs, and from ewes about 1 month before parturition (trial 1)

Diet	RBC uptake		
	Lamb		Ewe
	2 weeks	6 weeks	
High selenium alfalfa	1.7 ± 0.4 [a]	3.0 ± 0.4	1.5 ± 0.5 [c]
High selenium alfalfa plus sulfate	2.5 ± 0.3 [a]	2.3 ± 0.3	1.3 ± 0.45 [c]
Low selenium alfalfa	4.2 ± 0.2 [b]	4.0 ± 0.7	8.1 ± 1.3 [d]
Low selenium alfalfa plus sulfate	3.9 ± 0.8 [b]	4.5 ± 0.7	6.6 ± 3.0 [d]

[a] and [b] are significantly different at 5% level.
[c] and [d] are significantly different at 1% level.

TABLE 3

Incidence of white muscle disease of lambs nursing ewes fed high and low selenium alfalfa, with and without sulfate (trial 1)

Diet	Lambs dying			Incidence of WMD [1]
	At birth	Before 2 weeks	Before 6 weeks	
	%	%	%	%
High selenium alfalfa (16) [2]	0	18	25	0
High selenium alfalfa plus sulfate (17)	0	23	23	0
Low selenium alfalfa (33)	0	7	21	45
Low selenium alfalfa plus sulfate (26)	11	27	38	31

[1] Histological examination of semitendinosus muscle.
[2] Number in parentheses indicates number of lambs used.

In trial 2, there were no significant differences between lots in plasma LDH, MDH or GOT, or blood glucose or lactate levels of ewe blood taken either 1 month before or after parturition. Glucose and lactate have previously been shown to be elevated in sulfur-deficient sheep (29). Selenium-deficient sheep appear to differ from sulfur-deficient sheep in these respects. There were no significant differences between the lots in uptake of selenium-75 by red blood cells from either the ewes or lambs. Furthermore, there was no significant correlation of selenium-75 uptake by red blood cells and WMD of lambs in this trial. At first, this appeared to be in contradiction with trial 1. The ewes of trial 1, however, were fed 0.23 and 0.01 ppm selenium hays, whereas those of trial 2 were fed 0.01 ppm selenium hays with the control group getting an injection of 5 mg selenite 1 month before lambing. The allotted daily amount of 1816 g hay/day for the ewes of trial 1 provided a selenium intake of about 0.4 mg. Over a period of about 180 days, the approximate length of our trials, this would be about 15 times as much selenium as the control ewes received in trial 2. Therefore, a difference in red blood cell uptake of selenium is apparent only when ewes differ widely in their selenium status. Consequently, enzyme levels and selenium-75 uptake by red blood cells indicate there is still no reliable method to determine whether a ewe will produce a WMD lamb. The uptake of selenium-75 by red blood cells, however, may be used to determine the selenium status of an animal; this status could indicate the probability of ewes producing WMD lambs.

There were no differences in serum cholesterol of 2- or 6-week-old lambs from the various treatment groups; all groups averaged about 140 mg/100 ml blood. Poukka (30) reported an increase of free cholesterol in degenerated areas of skeletal muscle from calves with white muscle disease. Assuming the same is true for lambs with WMD, it is apparently not reflected by an increase of serum cholesterol.

There was a significant increase ($P < 0.05$) in plasma GOT of the nonsupplemented 2-week-lambs over those of the other groups (table 4). At 6 weeks of age, however, the GOT levels did not differ significantly from those of the lambs from the sulfate and methionine lots, but was significantly higher ($P < 0.05$) than that of lambs from the injected lot. The plasma GOT levels of the carryover lot were about half those of the nonsupplemented, sulfate or methionine lots. In trial 1, there was no difference between the GOT levels of lambs from high and low selenium treatments at 2 weeks, which is inconsistent with the results of trial 2. This inconsistency may be due to the ewes being more completely depleted of their selenium reserves in this latter trial.

The MDH and LDH levels followed the same trend as the GOT levels (table 5). Only in the nonsupplemented lot was there a significant increase of MDH and LDH levels over those of the injected lot at 2 weeks of age. However, at 6 weeks of age

TABLE 4

Plasma glutamic-oxalacetic transaminase levels of 2- and 6-week-old lambs nursing ewes fed low selenium alfalfa hay plus selenium injection, sulfate or methionine administration (trial 2)

Ewe treatment	Plasma				Lambs with over 200 U/ml plasma	
	Average		Range			
	2 weeks	6 weeks	2 weeks	6 weeks	2 weeks	6 weeks
	units [1] per ml		*units [1] per ml*		%	%
Injected selenium	88 [c]	104 [d]	73–116	86–139	0	0
No supplement	616 [b]	903 [e]	66–4400	82–4000	30	60
Sulfate supplement	193 [c]	1287 [e]	88–5760 [f]	169–2820	33	80
Methionine supplement	141 [c]	1303 [e]	71–1010	97–6520	25	78
No supplement (carryover)	141 [c]	595	90–410	95–1560	7	53

[1] Sigma unit as defined (20).
[b] and [c] are significantly different ($P < 0.05$).
[d] and [e] are significantly different ($P < 0.05$).
[f] One lamb had a value of 5760 and the next highest was 1640.

TABLE 5

Plasma lactic and malic dehydrogenases of 2- and 6-week-old lambs nursing ewes fed low selenium alfalfa plus selenium injection, sulfate or methionine administration (trial 2)

Ewe treatment	Enzyme	Plasma				Lambs with over 2000 U/ml plasma	
		Average		Range			
		2 weeks	6 weeks	2 weeks	6 weeks	2 weeks	6 weeks
		units [1] per ml		*units [1] per ml*		%	%
Injected selenium	LDH	889 [b]	1093 [f]	690–1190	750–1500	0	0
	MDH	333 [e]	503 [h]	250–380	310–630	0	0
No supplement	LDH	4450 [c]	9274 [g]	690–16850	250–34500	27	74
	MDH	2204 [d]	1683 [i]	250–11250	400–5950	20	27
Sulfate supplement	LDH	1841 [b]	4904 [g]	1000–6120	1130–10000	18	83
	MDH	810 [e]	2477 [i]	250–3150	810–5650	6	41
Methionine supplement	LDH	1441 [b]	5673 [g]	810–5650	940–20000	8	79
	MDH	698 [e]	3474 [i]	250–3880	440–8150	4	37
No supplement (carryover)	LDH	1313 [b]	3875	750–2310	750–12100	7	64
	MDH	511 [e]	1465	310–1130	530–3150	0	29

[1] One unit is defined as that amount of enzyme which results in an OD change of 0.001/minute (21, 22).
[b] and [c] are significantly different ($P < 0.05$).
[d] and [e] are significantly different ($P < 0.05$).
[f] and [g] are significantly different ($P < 0.05$).
[h] and [i] are significantly different ($P < 0.05$).

the levels of these enzymes in all lots were significantly greater ($P < 0.05$) than the injected lot. The levels of these plasma enzymes were considerably lower in the carryover lot than the nonsupplemented, sulfate or methionine lots.

There was no difference in the blood glucose or lactate levels of the various treatment lots (table 6). As expected, the 6-week glucose levels were significantly lower than the 2-week levels. The lactate levels did not follow the same trend; the levels of these two components are generally correlated in ruminants.

There was a 70 to 81% incidence of WMD in the nonsupplemented, sulfate or methionine lots (table 7), and approximately half this in the carryover lot. The incidence of WMD in this carryover lot was very similar to the level observed in trial 1, which might suggest that the ewes of trial 1 were not as completely depleted of their selenium storage as those of trial 2. None of the lambs from the carryover lot had gross lesions in the heart, whereas 12 and 16%, respectively, of the lambs in the nonsupplemented or methionine-supplemented lots had lesions. Over half the lambs from the sulfate group had lesions in the heart. The reason for this high incidence is unknown and is being further investigated. More recent studies, however, indicate that selenium will prevent this condition, but the mechanism of this interaction is likewise unknown.

Plasma GOT levels from 2-week-old lambs were significantly correlated with the 2-week levels of LDH and MDH in all lots. At 6 weeks of age plasma GOT levels were correlated with plasma LDH in all except the nonsupplemented lot, and with MDH in all except the methionine-supplemented lot. When data from all lots were analyzed together, the 6-week values for plasma GOT were significantly correlated ($r = 0.57$) with MDH, but not with LDH. Only in the sulfate-supplemented group was there a correlation ($r = 0.54$) between muscle damage determined histologically and plasma GOT. There was a correlation, however, between plasma GOT levels and heart lesions in the sulfate ($r = 0.77$) and methionine ($r = 0.55$) lots.

Only in the sulfate groups was there a correlation between plasma LDH levels and the degree of heart damage ($r = 0.54$). The levels of LDH were correlated ($r = 0.72$) with muscle damage only in the me-

TABLE 6

Blood glucose and lactate from 2- and 6-week-old lambs nursing ewes fed low selenium alfalfa hay plus selenium injection, sulfate or methionine administration (trial 2)

Ewe treatment	Glucose [1]		Lactate [1]	
	2 weeks	6 weeks	2 weeks	6 weeks
Injected selenium	117±7 [2]	78±6 [3]	18.5±3.5	22.4±1.8
No supplement	109±9	68±3	25.0±1.7	24.8±3.1
Sulfate supplement	100±4	82±3	23.3±1.7	21.4±2.9
Methionine supplement	101±8	73±3	24.2±2.5	27.2±3.5
No supplement (carryover)	107±6	74±3	23.9±2.5	30.6±2.8

[1] Milligrams per 100 ml blood.
[2] SE.
[3] Six-week glucose values are significantly lower than 2-week values ($P < 0.05$).

TABLE 7

Incidence of white muscle disease of lambs nursing ewes fed low selenium alfalfa hay plus selenium injection, sulfate or methionine administration (trial 2)

Ewe treatment	Lambs dying			Histological lesions	
	At birth	Before 2 weeks	Before 6 weeks	Heart	Muscle (semi-tendinosus)
	%	%	%	%	%
Injected selenium (20) [1]	0	10	10	0	0
No supplement (22)	0	9	9	12	81
Sulfate (22)	0	21	30	65	70
Methionine (27)	0	11	11	16	74
No supplement (carryover) (20)	15	25	25	0	35

[1] Number in parentheses indicates number of lambs.

thionine-supplemented lot. Plasma MDH was correlated with muscle damage in the methionine ($r = 0.53$), carryover ($r = 0.75$) and nonsupplemented ($r = 0.50$) lots, but was not correlated with this myopathy in lambs fed the sulfate supplement. When the data from all lots were combined, there was a correlation of MDH and muscle damage. Thus, it is evident that dietary treatments have an effect on these plasma enzymes, and further that precautionary measures might usefully be taken to evaluate potential WMD on the basis of plasma GOT, LDH and MDH levels.

Analysis of variance was calculated with the data combined from all lots comparing GOT, MDH and LDH levels in lambs with heart and muscle lesions against those without these lesions. The GOT and MDH levels of lambs with heart lesions were significantly ($P < 0.01$) greater than those without heart lesions. This difference with LDH was significant only at the 5% level. Lambs with muscle damage had a significantly higher plasma MDH ($P < 0.01$) and GOT ($P < 0.05$) than normal lambs, but LDH was not significantly different between lambs with muscle damage and those without this myopathy. This may suggest that other tissues are damaged in WMD in addition to the semitendinosus muscle. For example, the kidney has recently been implicated in connection with WMD (15). Plasma MDH levels appear to be the most sensitive indicators of muscle damage, followed by GOT levels, with LDH levels being the least indicative of muscular degeneration among the criteria studied.

There were no significant differences between the rate of gain in lambs from any of the treatment groups or from those with degenerative lesions as compared with those without lesions. Birth weight and subsequent weight gains for single lambs were significantly greater ($P < 0.01$) than for twin lambs, and the plasma GOT levels in 2-week-old and the MDH levels in 6-week-old twins were significantly higher ($P < 0.05$) than in the single lambs. The type of birth (single or twin) did not affect the incidence of WMD, but if one twin became affected with WMD, the other

twin had a greater chance ($P < 0.05$) of also being affected than an unrelated lamb within the same treatment group. The last observation has been reported previously (3).

DISCUSSION

The addition of various sulfur compounds did not alter the incidence of WMD in these trials. This is in agreement with Boyazoglu et al. (31) who found no evidence that dietary sulfate in rations fed to ewes during late pregnancy contributed to WMD in lambs. Based on the enzyme data (GOT, LDH and MDH; tables 4 and 5), the sulfur sources appeared only to delay the onset of WMD. Paradoxically, sulfur did not prevent the beneficial effects of selenium in forages nor did it increase the incidence of WMD. Moreover, Allaway and Hodgson (32) were unable to demonstrate a consistent effect of increased sulfur content of forages in contributing to selenium deficiency in livestock, and suggested additional studies were necessary to demonstrate the effect of indigenous sulfur on the biological efficiency of selenium in forages. Other work by Mathias et al. (33) indicated that selenium from selenite, low sulfur alfalfa, and high sulfur alfalfa tended to be of similar value for prevention of liver necrosis in rats and in promoting growth of rats and chicks. The selenium in the high sulfur alfalfa, however, was about 25% less effective than selenium from the other sources for prevention of exudative diathesis in chicks.

Muth et al. (5) and Hintz and Hogue (3) indicated that Na_2SO_4 given with Na_2SeO_3 decreased the effectiveness of selenium. Sodium sulfate appeared to increase the clinical incidence of WMD, but cystine and methionine administration had no significant effects (3). Thus, it appears that inorganic sulfur may alter the metabolism of inorganic selenium more than organic sulfur. This concept is supported by work from our laboratory [7] which indicated that the sulfur analogues are more inhibitory to selenium uptake by rumen microorganisms than other forms of sulfur. The addition of Na_2SO_4 to diets has been shown to reduce the toxicity of high levels of selenate for rats more than if the element was supplied as selenite or as wheat-containing selenium (2). The work of Ganther and Baumann (1) with rats indicated that sulfate was much less effective in altering the distribution or excretion of selenite than selenate. Their data appear to substantiate the idea of a greater alteration of selenium metabolism by sulfur analogues than other forms of sulfur. This suggests that when one considers interference of sulfur with selenium metabolism, he must designate the forms of selenium and sulfur used to correctly interpret the results.

The selenium in alfalfa hay in this study was probably organically bound. Reasoning along these lines, sulfate added to the ration would not be expected to increase the incidence of WMD because it was not present as the selenium analogue. Similarly, methionine was possibly not antagonistic because the selenium in alfalfa was bound in some other form than selenomethionine. Alternatively, methionine may be ineffective in altering the metabolism of selenomethionine bound in protein. This appears to be the most logical interpretation of these results.

The incidence of WMD was reported from our Station to be more prevalent in lambs whose dams were fed hay grown on fields fertilized with gypsum than those lambs whose dams were fed hay from fields without gypsum application (6). The conclusion was that sulfur interfered with the action of selenium and increased the incidence of WMD. From such an experiment, it would be difficult to determine whether the antagonism of sulfur and selenium was in the forages or the animals.

The results from lot 5 (trial 2) indicated that there can be a significant carryover effect of selenium. Although the incidence of WMD and the plasma enzyme levels were higher than in the injected group, these variables were approximately 50% lower than in the deficient lots. The selenium status of an animal has been shown to significantly influence the metabolism of administered selenium-75 (34). The low incidence of WMD in trial 1 could be due to animals which were not depleted of their selenium reserves. The lack of response in animals to selenium adminis-

[7] Whanger, P. D., O. H. Muth, J. E. Oldfield and P. H. Weswig, unpublished observations.

tration reported in the literature is most probably due to experiments with animals not depleted of this element. Our work with rats suggests that trace amounts of selenium are retained tenaciously, and that it takes two generations to deplete the animal's selenium storage.[8] Therefore, it is of paramount importance to know the selenium status of animals before experiments are performed and conclusions drawn.

It is debatable whether selenium is an essential element. Certainly under the conditions of our experiments, one would consider this element to be essential. It must be admitted, however, that a deficiency of selenium per se may not be the sole factor responsible for WMD (15). Although we have not observed it consistently, Hartley and Grant (35) reported that selenium administration results in faster weight gains of lambs. Other work suggesting selenium to be essential is that by Thompson and Scott (36) with chicks [9] and quail, and by Scott et al. (37) with young poults. Recently found selenium responses not related to vitamin E in second generation-depleted rats from our laboratory further suggest an essential role for selenium.[10]

With all this evidence for selenium being essential, the molecular role for this element is still obscure. Selenium compounds have been advocated by Tappel and Caldwell (38) to function as biological antioxidants. Schwarz (39) and Bull and Oldfield (40), on the other hand, found evidence for a direct involvement of selenium in the oxidative processes of the tricarboxylic acid cycle. Other work by Pendell et al.[11] and by McCoy and Weswig [12] suggests the involvement of selenium in long-chain fatty acid metabolism. Only further experimentation will elucidate the exact metabolic role(s) for selenium.

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AGING, NUTRITION AND HEPATIC ENZYME ACTIVITY PATTERNS IN THE RAT

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Aging, Nutrition and Hepatic Enzyme Activity Patterns in the Rat [1]

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ABSTRACT A correlation between life expectancy of the rat and the levels of hepatic enzyme activity has been established. When the age-associated enzyme activity patterns were modified by dietary means, there were corresponding modifications in life span. This study of lifetime duration entailed approximately 1000 male rats divided into five dietary groups; the dietary variables were limited to the intake of protein, carbohydrate and calories. The hepatic enzymes investigated were ATPase, alkaline phosphatase, histidase and catalase, and the activities were determined at seven age periods ranging from 21 to 1000 days of age. The activities were expressed on the following bases: per unit weight (fresh, dry and fat-free), per unit nitrogen, per organ and per cell. From the cellular and enzymological data it was found possible to determine how much of the change in total enzyme activity, from one age to another, was directly attributable to change in number of hepatocytes and in volume of the hepatocytes. For each of the enzymes studied, the activity levels were found to vary with the age of the rat and with the diet fed. Similarly, the volume of the individual hepatocyte and the total number of hepatocytes also varied throughout the life of the rat and both were significantly influenced by the dietary regimen imposed. When parameters other than age were used as a basis of comparison, these discrete multiple patterns disappeared. The levels of activity of each of the enzymes, irrespective of age or diet, formed a single, continuous slope when related to nitrogen content, number of hepatocytes, hepatocytic volume, body weight and caloric intake. The progressive changes with age in the activity level of the organ or of the cell, represent an adaptation to the change in requirements of a larger animal or a larger cell. Rapid growth rates, structural or biochemical, are not commensurate with prolonged life span. The dietary regimen which evoked the greatest rate of change with age was most detrimental and such rats had the shortest life expectancy. With long-term caloric restriction, the levels of any of the biochemical constituents as well as of cell and animal size, were more like that of the young rat, and the longest life spans were obtained.

During aging, the activity levels of a number of hepatic enzymes in the rat had been found to change; some increased, others decreased (1). These enzymes were not only sensitive to modification in the proportion and intake of the various ingredients in the diet at any age period, but the degree of response to a specific diet was predictable (2–5). Thus, it was possible, within limits, to evoke arbitrarily in the young or mature rat the unique pattern of enzyme activities characteristic of any other chronologically aged rat, younger or older, or to reduce the rate of change in activity with age. These relationships suggested the hypothesis that biological aging was a manifestation of naturally occurring changes in the enzyme activities, and that dietary regimens which reduced these rates of change would reduce the rate of aging. The increase in life span and the reduction in degenerative disease incidence resulting from long-term caloric restriction (6–8) would appear to support the nutritional aspects of this concept.

A further study of lifetime duration was undertaken to determine within the same population of animals the relationship between the nutritionally modifiable enzyme activity rate pattern and the aging processes. Mortality and age-associated organic disease risk patterns were considered to afford suitable criteria for assessing the rate of aging of a population. They were both found to be greatly influenced by the caloric intake of the rat and by the ratio

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and intake of the protein and carbohydrate components of the diet (9–12). In support of the hypothesis, the levels and rates of change in the activities of the several hepatic enzymes which have been studied and reported upon only in part (8, 11) correlated with the life expectancy of the different dietary groups of rats. Similarly, Fanestil and Barrows (13), employing the rotifer as a test organism, reported that a change in life span through dietary restriction was associated with a temporal displacement of the pattern of dehydrogenase activity of the total animal.

In addition, the concomitant cellular studies made on the livers of the same rats used for the biochemical studies, revealed that the number of parenchymal cells in a stated volume of tissue also varied with the age of the rat and was influenced by the amount and type of diet consumed. This, then, raised the question: were the progressive changes with age in the enzyme activity levels, in reality, attributable to the changes in the number, size or type of cells comprising the mass of tissue?

In this paper, the data for four enzymes, adenosine triphosphatase, alkaline phosphatase, histidase and catalase, are expressed on a number of bases including that of the cell. Such information allows a more comprehensive evaluation of the age-related changes in enzyme activities, their modification by dietary means and the pertinence of their relationships to the age-specific diseases and mortality patterns for the same population of rats. The biochemical entities have been correlated also with body and organ weight and with size and number of cells during growth, maturation and senescent phases of life.

MATERIALS AND METHODS

The rat. The environmental conditions and care of the rats, approximately 1000 of the Charles River SD strain, have been described earlier (9). Only male rats were used and they were 21 days old when the investigations were begun. At this time they were weighed and divided at random into five dietary groups. Each rat was housed individually and all were kept in one large room throughout their lifetime. The number of rats beginning the study in each of the groups, termed control, A, B, C and D, were 210, 210, 120, 210 and 195, respectively. During the more than 1600 days that they remained under observation no other rats were permitted into these quarters.

TABLE 1

Composition of purified diets

	Diet A	Diet B	Diet C	Diet D
	%	%	%	%
Casein [1]	30.0	50.85	8.0	21.62
Sucrose	61.0	33.90	83.0	54.05
Corn oil [2]	5.0	8.47	5.0	13.52
Salt mixture (USP 12)	4.0	6.78	4.0	10.81
Vitamins and trace elements [3]				

[1] Vitamin-free Test Casein, 89% protein nitrogen.
[2] Mazola Oil, Corn Products Company, New York.
[3] Vitamin and trace element content of diets adjusted to afford identical allotments each day to each rat (see ref. 9). Note: Choline allotments were increased at 640 days of age from 10 mg/rat per day to 25 mg.

Dietary regimen. Rats of four of the five groups were maintained on restricted intakes of the purified diets. The rats of the remaining group were maintained on a commercial ration [2] ad libitum, and their consumption of food was determined daily throughout life.

The daily amounts of the purified diets [3] allotted differed according to the diet fed and to the age of the rat. The composition of the diet (table 1) and regimen of allotment were designed to afford differences only in intake of the carbohydrate component (sucrose), the protein component (casein) and of total calories. The four regimens with regard to intake of protein and of carbohydrate can be characterized as follows: group A, intakes high in protein and high in carbohydrate; group B, intakes high in protein and low in carbohydrate; group C, intakes low in protein and high in carbohydrate; and group D, intakes low in protein and low in carbohydrate.

[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.

[3] The author acknowledges with gratitude the generosity of the following organizations for their donations of ingredients used in the diets: sugar from the Sugar Research Foundation, New York, N. Y.; Mazola Oil from the Corn Products Company, New York, N. Y.; vitamins from Hoffmann-LaRoche Inc., Nutley, N. J.; Lederle Laboratories (Division, American Cyanamid Company), Princeton, N. J.; Merck Institute for Therapeutic Research, West Point, Pa.; and Mead Johnson and Company, Evansville, Ind.

The protein intakes by the rats in groups A and B were similar but the intakes of carbohydrate differed. The intakes of protein by rats in groups C and D were also identical to each other though smaller in amount than by rats in the former two groups; the carbohydrate intake of these two groups also differed. The carbohydrate intakes of groups B and D were similar; the caloric intakes of groups A and C were identical. Rats of group D had the lowest caloric intake.

The amount of the purified diet offered to each rat was increased in increments of 0.1 g from 4.9, 2.9, 4.9 and 1.8 g on day 21 in dietary groups A, B, C and D, respectively, to a maximum at 685 days of age of 14.3, 8.5, 14.3 and 5.3 g (8). The maximum levels of allotment were then maintained until death. Except when a rat became moribund, the daily allotment of the purified diet was completely consumed. The casein and caloric intakes at the different age periods, when members of each dietary group were killed, are presented in table 2.

Assays. The activities of hepatic ATPase, alkaline phosphatase, histidase and catalase, and the concentration of hepatic nitrogen and total lipids as well as moisture content, were determined at seven age periods: 21, 121, 221, 385, 621, 795 and 995 days. Assay values for the 21-day-old rats fed the commercial diet provided common base values for each of the dietary groups. Enzyme activity levels are expressed on the basis of fresh weight, dry weight, fat-free, per unit nitrogen, per organ and per hepatocyte.

At each period a minimum of 10 rats was chosen at random from each of the groups and killed for assays of the above and for cytological and histological studies. Except for the 21-day-old rats, not all assays for all rats could be performed in 1 day. The assays, therefore, which were made in duplicate, were carried out each day for 10 days (starting 5 days prior to the age chosen) on at least one rat from each of the dietary groups. The sequence in which rats of different groups were killed was rotated each day to assure equivalent conditions. Due to the small amount of liver available from the 21-day-old rats and from 121-day-old rats fed diet D, twice the number had to be killed at each of these times to supply sufficient material for the various procedures. Because there were too few survivors in that group of rats fed the commercial diet, as they approached 1000 days of age, to warrant their killing, the rats of this group were kept to provide nonbiochemical data; i.e., age-specific disease and mortality rates.

The rats were weighed and killed by decapitation. The entire liver was quickly removed, blotted and weighed. A specimen at least 7 to 8 mm in one dimension from the left lateral lobe was fixed in Bouin's solution for subsequent histological processing. Another, of known weight, approximately 1 g, adjacent to the former, was homogenized without delay in cold, distilled water for 30 seconds in a blender.[4] One-milliliter aliquot samples, after dilution with phosphate buffer 1:20, were immediately employed for catalase activity

[4] Waring Blendor, Waring Products, Inc., Winsted, Conn.

TABLE 2

Casein and caloric allotments of purified diets at time of killing

Age	Dietary group							
	A		B		C		D	
	Casein	Calories	Casein	Calories	Casein	Calories	Casein	Calories
days	*g*		*g*		*g*		*g*	
21	1.47	20.0	1.47	12.0	0.39	20.0	0.39	7.6
121	3.06	41.7	3.06	24.9	0.82	41.7	0.82	16.1
221	3.30	45.0	3.30	27.0	0.88	45.0	0.88	17.4
385	4.05	55.2	4.05	33.2	1.08	55.2	1.08	21.2
621	4.20	57.3	4.20	34.4	1.12	57.3	1.12	22.0
795	4.29	58.5	4.29	34.9	1.14	58.5	1.14	22.5
995	4.29	58.5	4.29	34.9	1.14	58.5	1.14	22.5

determinations by an iodimetric method (14); *K*, or activity constant values were computed from the changes in the rate of activity with time (zero, 3, 6, 9 and 12 minutes) of incubation at 0°

Portions of the undiluted homogenates were also used in the determination of ATPase activity at pH 8.4 (15), for alkaline phosphatase activity with β-glycerolphosphate as the substrate at pH 9.1 (16, 17) and for nitrogen content (18). The ATPase and alkaline phosphatase activity determinations were made 1.5 and 2 hours, respectively, after the animals were killed; in the interim the homogenates were stored at 4°.

Another specimen of liver of known weight, approximately 2 g, which had been homogenized in 100 ml 1/45 M phosphate buffer, pH 8.0, supplied the material for the determination of histidase activity (19) and of lipid content. The lipids were extracted from 30-ml samples of the homogenates which had been precipitated with hot, absolute, ethyl alcohol. After centrifugation and three additional extractions with hot alcohol, the supernates were taken to dryness. The remaining precipitate was then extracted four times with hot ether; these supernates were added to the residue from the alcohol extractions and also taken to dryness. The residue was then reextracted with hot petroleum ether. The extracts were filtered through cotton into tared containers and dried. The amount of extracted lipids was determined by gravimetric methods.

Moisture content was determined on another portion of liver; the samples of known weight, approximately 1 g, were dried at 60° to constant weight.

Histometric determination of number of hepatocytes and its rationale. It was learned that the number and dimensional characteristics of mononucleated and binucleated hepatocytes and their nuclei were influenced by the diet fed and the age of the rat. Moreover, when segments of liver lobules were examined in depth by photo-reconstruction techniques of serial sections, binucleated cells were found to account for a considerable proportion (up to 42%) of the liver parenchyma. These variables precluded quantitative determination of the numbers and types of hepatocytes in a unit volume of liver, either from counts of isolated nuclei in suspension or from the assay values for any biochemical constituent. For the same reason, direct counts of the nuclei in sectioned tissues alone would be misleading unless excessively thick sections are employed. Data obtainable from thin sections of liver, however, can be used to derive accurate and verifiable information as to the number and type of hepatocytes in a given volume or weight of tissue.

The essential feature of the method to be described is the determination of the probability values that a cell in thin section will or will not contain nuclear material. These values can be computed since the disposition of the hepatocytes to a sectioned surface in a liver lobule is random and, thus, any section will contain a random aggregation of cell faces. Inasmuch as an infinite number of slices of a cell are possible, a cut made tangent to a cell has the same probability as that made through any other plane of the cell. That a mononucleated hepatocyte in section does or does not contain nuclear material depends upon the size and shape of the cell, the size and shape of its nucleus and the thickness of the section. For the binucleated hepatocyte, the probability value that the cell in section contains portions of one or two nuclei or none at all will, in addition, be influenced by the distribution of the two nuclei in space, one to the other. The methods for computing the five probability values have been developed for us by W. F. G. Swann and L. de Branges.

Similar approaches to the solution of this problem have been made (20–25). When applied to liver these earlier methods, however, had to be modified because the wide variation had been overlooked in 1) both the size of the hepatic nuclei and the hepatocytes, and 2) the changing proportion of the mononucleated to the binucleated cells that are found to occur during aging. Furthermore, corrections were not introduced for tissue shrinkage during histological processing or for the amount of nonparenchymal mass present.

Probability expressions: (A) The "mononucleated" hepatocyte. The hepatocyte has been considered to be a highly irregular 14-sided solid with variations between 10

to 18 sides (26–28), but for purposes of probability determinations, the shape of the mononucleated cell may be assumed to have a spherical, topological configuration in space. Furthermore, since the nucleus approaches the shape of a sphere it, too, is assumed for these calculations to have a spherical shape. Because the relationship between the mononucleated cell and its nucleus is the same in two dimensions as it is in three, whether the nucleus and cell are concentric or not, the probability of observing the nucleus in thin section (Pm_1) is:

$$Pm_1 = \frac{2r + T}{2R + T}$$

Where

r = Mean radius of mononucleated nucleus
R = mean radius of mononucleated cell
T = thickness of tissue

(B) The binucleated hepatocyte. The binucleated cell appeared, on the basis of the observations made of the "photo-reconstruction" of serial sections, to be almost invariably elongated although hepatocytes approaching the shape of "spheres" and oblate "spheroids" were encountered. The idealized form was assumed to be best characterized as a prolate spheroid with the long axis passing through the centers of both nuclei; cells were also observed in which the short axis passed through the center of the nuclei. By assigning various geometric forms in which the length was longer than the two other dimensions, further refinement of the assumed shape of the cell beyond an ellipsoid contributed only negligibly to the expression for the probability of intersecting the nuclei. To compute the volume of the binucleated cell, however, it was practical to define the shape of the individual cell according to the disposition of its nuclei, as a prolate spheroid, an oblate spheroid or a sphere.

The expression of the probabilities relating to a three-dimensional system was obtained by considering equal areas on the surface of a sphere in which the system is located. If two of the three dimensions are identical as in a solid of revolution, the sphere may be reduced to a circle and necessary weighting to include all three dimensions accomplished by appropriate segmentation of the circle. For each segment, the length of the cell through which a nucleus or nuclei are included in a section, is related to the total length through which the cell is intersected to give ratios representing the probability of including in a slice one nucleus or two. Integrating the expression for the length of these segments yielded the following general probability statements:

The probability of observing one nucleus only in a sectioned binucleated hepatocyte (Pb_1):

$$Pb_1 = \frac{D}{\sqrt[3]{\frac{3V}{4\pi}} + \frac{1}{2}T}$$

Where

D = ½ mean distance between centers of nuclei in microns

$\sqrt[3]{\frac{3V}{4\pi}}$ = mean computed radius, in microns, of a sphere having a volume equal to the empirically determined volume (V) of the cell

$$V = \frac{1}{6}\pi C^3 L W^2$$

$$V = \frac{1}{6}\pi C^3 L^3 \text{ (when } W = L)$$

Where

L = axis of rotation; length in microns of the individual binucleated cell measured through the centers of the two nuclei
W = length in microns of individual binucleated cell perpendicular to L
C = magnification factor

The probability of observing two nuclei of a binucleated hepatocyte (Pb_2):

$$Pb_2 = \frac{2r_2 - D + T}{2\sqrt[3]{\frac{3V}{4\pi}} + T}$$

Where

r_2 is the mean nuclear radius of the binucleated cell.

Verification of the expressions given here for the probability that a nucleus of a mononucleated cell is seen and for the probability that in the binucleated cell, two nuclei, one, or none at all are seen, was obtained by empirically identifying in depth through serial reconstructions the number and type of hepatocytes in 4-μ thick sections. At least 30 sections were employed for each of the reconstructions. The computed probability values were in close agreement with those obtained empirically; the deviation was in the order of 1%.

In addition to the probability values, the data required to estimate the number of hepatocytes and their type consist of the following: direct microscopic counts, in situ, of hepatocytic nuclei; the proportion of hepatocytes actually observed as binucleated cells; the measurable characteristics of the binucleated hepatocytes and of the presumed mononucleated hepatocytes and their respective nuclei; the weight and volume of the liver; the proportion of the liver not occupied by hepatocytes and the changes in the liver due to histological processing.

The number and type of hepatocytes were computed as follows:

$$N = \frac{(S)(H)(K)}{(B)(V) + (1-B)(Y)}$$

Where

N = total number of hepatocytes in liver
S = 1.00 − (fraction of liver volume not occupied by hepatocytes)
H = liver weight in grams

$$K = \frac{1}{(\text{sp gr in g}/\mu^3)(\text{shrinkage factor})}$$

Where

sp gr in $g/\mu^3 = 1.10 \times 10^{-12}$
Shrinkage factor (fresh to fixed) = 1.22

$$B = \frac{\frac{G}{(Pb_2)} \times 100}{\frac{A}{(Pm_1)} + G\left[\left(\frac{1}{Pb_2}\right) - \left(\frac{2}{Pm_1}\right) - \frac{(Pb_1)}{(Pb_2)(Pm_1)}\right]}$$

Where

B = actual percent binucleated hepatocytes
G = mean binucleated cell count (observed)
A = mean nuclear count (observed)
V = mean volume of binucleated cell
Y (mean volume of mononucleated cell)

$$= \frac{4}{3}\,\pi\left(\frac{fc_1}{\pi}\right)^{3/2}$$

Where

fc_1 = cross sectional area (μ^2)

And, therefore,

$$\text{number of hepatocytes/g liver} = \frac{N}{H}$$

Histological technique. The specimens of liver collected each day were handled throughout the fixation and dehydration process as a unit. A composite paraffin block was prepared containing a portion of liver from a rat in each of the dietary groups; a piece from a 21-day-old rat was included to be used as a standard of "age" reference.

All sections were cut at 4 μ; a single stroke of the microtome knife assured that all of the specimens in a section were of equal thickness and, thus, of uniform volume per unit area examined. The sections to be used for nuclear and cell counts were stained with hematoxylin-aniline yellow and aniline blue; other sections which were to be used for the determination of the dimensional characteristics of the hepatocytes were stained with a tetrachrome stain (29) which adequately delineated "cell membranes."

Cell and nuclear counts. Every hepatocyte which contained a recognizable nucleus was counted and classified according to type; i.e., presumed mononucleated hepatocytes and binucleated hepatocytes. The counts were made under high-dry magnification; field size, 150 by 150 μ, was defined by an ocular reticle. Nonhepatocytic cells exclusive of blood cells were also counted. Of the nonhepatocytic cells, the fibroblasts only were noted as to type.

The first field of each section was chosen at random regardless of its orientation within the lobule; successively adjacent fields were then counted provided they included less than approximately 15% of nonhepatocytic material. The counts made on any one section thus represented a sampling of cells within a number of lobules and of random planes within the lobules. Initially, the counts were made on 50 fields/specimen. After statistical treatment of these data indicated that significant values could be obtained from as few as 15 fields, all remaining counts were made on 25 fields (representing in excess of 1000 hepatocytic nuclei).

Cell and nuclear dimensions. (A) The "mononucleated" hepatocyte. All measurements were made indirectly from projections of photographic negatives. At least 30 randomly chosen fields of each tetrachrome-stained section were photographed on 35 mm high-contrast film through a Wratten G filter. A high-dry plano (40 ×) objective was used. The films were devel-

oped in Dektol [5] under conditions which would further increase the contrast of the cellular and nuclear outlines.

An enclosed, first surface, silvered mirror projection system permitted distortionless enlargement of the entire negative onto the back of a thin, frosted-glass plate. The overall magnification was 3000 ×. A minimum of 250 cells which appeared to have been cut through the center of their nuclei was used. The cross-sectional outlines of the images of each of these cells and of their nuclei were traced on paper, cut out and weighed individually. Changes in paper weight of a weight area standard resulting from changes in relative humidity were also determined at the time of the weighing of the "cut-outs."

(B) The binucleated hepatocyte. All measurements of the binucleated cell were obtained directly from the same tetrachrome-stained sections used above. The high-dry plano objective was used in conjunction with a Filar micrometer eye piece. Only those cells were measured which appeared to have been cut through the polar axis. The length of the long and short axes, diameters of both nuclei and the intranuclear distances were measured for each of 50 cells.

Volume changes of tissues during histological processing. The volume of small samples of fresh liver of known weight, approximately 0.2 g, was determined by employing a custom-made, wide-mouth pycnometer with a capacity of 1.3 ml. From these data the specific gravity of the tissues was also determined. To avoid loss of fragments of liver, each specimen was then encased in a hollow, thin-walled, plastic-foam chamber for fixation in Bouin's fluid and for histological processing. After paraffin impregnation, deparaffination and hydration, the sample was removed from the foam chamber and its volume and weight determined again. The percentage changes in volume and specific gravity were then computed.

Confirmation of these values was obtained by a planimetric method: photographs were made of the cut face of the tissue prior to fixation and of the same face after the tissues had been processed, sectioned and stained. Planimetric determinations of the areas from enlargements of the photographs were then made and the change in area computed.

Nonparenchymal volume. The principle of random hits was applied in estimating for each specimen the volume of tissue not occupied by hepatocytes. The photographic negatives prepared for measuring the dimensional characteristics of the mononucleated hepatocytes were also used in these determinations. Fifty points were placed at random on the ground glass plate of the projection apparatus, and only those points falling outside the hepatocyte were counted. The average percentage hits from at least 30 frames/rat was taken as a measure of nonparenchymal volume. These values were identical to those obtained when the total projected areas occupied by every hepatocyte in a frame were determined and related to the total area of the field of projection.

Thickness of section. Thickness of the sections cut at a microtome setting of 4 μ was determined by the method of Marengo (30). Calibration of the microtome was also made at different positions of the microtome wedge; the variation was found to be within 2%.

Disease and mortality rates. Members of the various dietary groups permitted to live out their lives provided information of disease and mortality patterns, and the like. At time of killing or natural death, a thorough necropsy was performed on every rat and tissues routinely secured for histopathological examination. In deriving age-specific disease incidence and life expectancy patterns, adjustments were made for the changes in size of the population at risk (9, 10, 12). These data which had been presented in 100-day periods were recomputed so that the age periods for these actuarial values coincided with the time when the rats were killed for the biochemical assays.

Computations. The large number of mathematical conversions of raw mensuration data of the individual cell and of their use in subsequent computations, the collation and derivations for each parameter for the individual rat, regressions analysis, coefficients of correlation and other statistical treatments (31) of the data classed

[5] Eastman Kodak Company, (Distillation Products Industries), Rochester, N. Y.

according to groups of rats, were expedited by the use of a computer. In estimating the level of enzyme activity on a cellular basis, it was necessary to consider that all the activity was in the hepatocytes.

RESULTS

1. *Influence of age and diet on body weight, liver weight and number of hepatocytes*

The average body and liver weights at time of killing for each of the five dietary groups are shown in table 3. The rates of increase in body weight in each of the groups were significantly different. The body weight attained, however, was proportional to the caloric intake (fig. 1). The rate of increase with increase in caloric intake for four of the five groups was nearly identical but that of group C, while also linear, was higher.

The average liver weight at any age, and regardless of dietary group, related directly and linearly to body weight; several values for group C rats, however, were displaced from the single pattern indicative of relatively large livers for the weight of the rat.

The influence of age and diet on number of hepatocytes in the liver and their average volume are presented in table 4.

TABLE 3

Influence of age and diet on liver and body weight

Dietary group	Age	No. of rats	Body wt	Liver wt	
	days		*g*	*g*	*%*
Commercial	21	20	51 ± 1.4 [1]	1.99 ± 0.07	3.9 ± 0.08
	121	10	413 ± 9.9	14.13 ± 0.51	3.4 ± 0.07
	221	10	527 ± 15.9	15.31 ± 0.59	2.9 ± 0.06
	385	10	549 ± 18.4	15.87 ± 0.75	2.9 ± 0.07
	621	10	606 ± 15.2	19.50 ± 1.13	3.2 ± 0.16
	795	10	610 ± 39.4	17.61 ± 1.28	2.9 ± 0.12
A	21	[2]	[2]	[2]	[2]
	121	10	226 ± 4.7	6.99 ± 0.21	3.1 ± 0.09
	221	10	314 ± 4.3	8.87 ± 0.20	2.8 ± 0.06
	385	10	377 ± 6.6	11.17 ± 0.36	3.0 ± 0.08
	621	10	420 ± 7.3	11.77 ± 0.38	2.8 ± 0.08
	795	10	399 ± 20.7	10.42 ± 0.53	2.6 ± 0.08
	995	10	383 ± 15.6	10.19 ± 0.26	2.7 ± 0.10
B	21	[2]	[2]	[2]	[2]
	121	10	138 ± 1.8	4.02 ± 0.11	2.9 ± 0.07
	221	10	172 ± 6.5	4.62 ± 0.19	2.7 ± 0.12
	385	10	228 ± 3.6	5.81 ± 0.18	2.6 ± 0.09
	621	10	287 ± 4.3	7.09 ± 0.21	2.5 ± 0.09
	795	10	254 ± 12.9	6.22 ± 0.21	2.5 ± 0.11
	995	10	258 ± 11.2	6.51 ± 0.23	2.6 ± 0.09
C	21	[2]	[2]	[2]	[2]
	121	10	132 ± 7.5	5.21 ± 0.40	4.0 ± 0.21
	221	10	203 ± 12.3	7.32 ± 0.51	3.6 ± 0.20
	385	10	300 ± 11.9	7.79 ± 0.45	2.6 ± 0.14
	621	10	326 ± 17.7	10.88 ± 0.74	3.4 ± 0.21
	795	10	390 ± 17.1	10.48 ± 0.42	2.7 ± 0.09
	995	10	358 ± 15.7	9.72 ± 0.44	2.7 ± 0.09
D	21	[2]	[2]	[2]	[2]
	121	20	77 ± 1.3	2.50 ± 0.06	3.3 ± 0.07
	221	10	114 ± 1.6	3.16 ± 0.06	2.8 ± 0.05
	385	10	148 ± 2.5	3.90 ± 0.07	2.6 ± 0.05
	621	10	162 ± 2.3	4.03 ± 0.09	2.5 ± 0.05
	795	10	151 ± 3.2	4.07 ± 0.11	2.7 ± 0.06
	995	10	150 ± 3.9	4.24 ± 0.12	2.8 ± 0.10

[1] Mean ± SE of the mean.

[2] Values for 21-day-old rats fed the commercial diet provide common base values for each of the dietary groups.

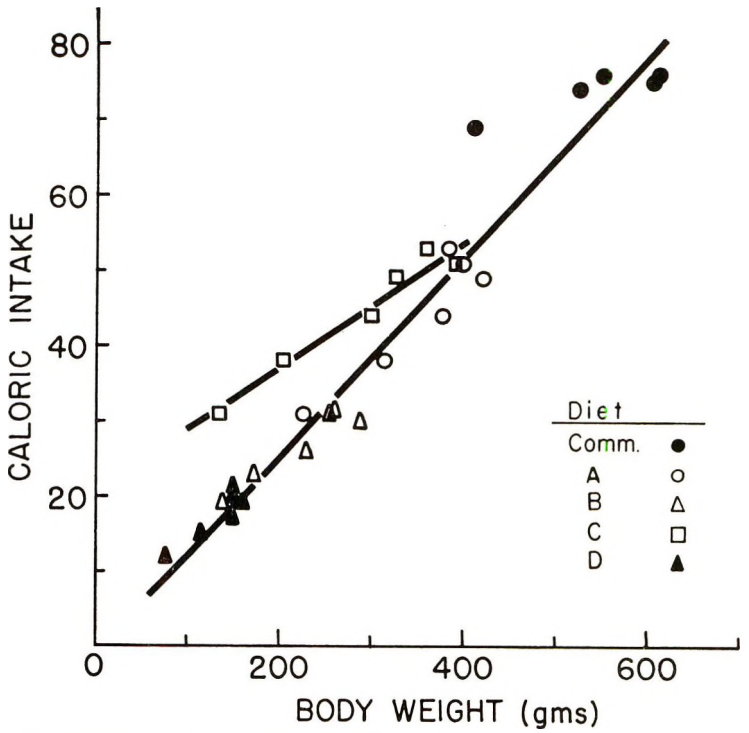

Fig. 1 Correlations between average body weight at time of sacrifice and average daily caloric intake. Key to dietary groups: ●, commercial laboratory ration; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

The total number of hepatocytes increased exponentially with increasing age; the rates of increase varied according to the diet fed and were, in decreasing order, commercial, A, C, B and D. Each curve, in general, appeared to parallel the age-body weight pattern of that dietary group until such time when "senescent body weight loss" occurred. When the total number of hepatocytes in the liver was plotted against body weight, a single, direct and remarkably uniform relationship was obtained regardless of diet or age. A single pattern was also found between cell volume and body weight.

A series of U-shaped curves was found when the number of hepatocytes per gram of liver in each dietary group was related to age (table 4). The number of cells at any one time and the time of reversal in trend differed according to the diet fed. Here, too, the distinct differences between dietary groups were lost in that a single curvilinear, inverse pattern was obtained when the number of hepatocytes per gram of liver was related to body weight.

No significant differences with age or between dietary groups in relative number of fibroblasts were observed.

2. *Influence of age and diet upon hepatic water, fat and nitrogen content*

Water. The water content of the liver and the estimated water content of the hepatocyte differed with the age of the rat and with the diet fed (table 5). At any age or dietary regimen the water content of the hepatocyte was found to be a direct function of the volume of the hepatocyte. In addition, the level of hepatocytic water correlated directly with the total water content of the liver and with the body weight of the rat.

Fat. The proportion of fat in the liver, on a fresh weight basis in four of five dietary groups (commercial, A, B and D) remained relatively constant within each dietary series (table 6); on a dry weight

TABLE 4

Influence of age and diet on number and volume of hepatocytes

Dietary group	Age	Hepatocytes per liver	Hepatocytes per gram liver	Avg hepatocytic volume
	days	*no.* $\times 10^{-6}$	*no.* $\times 10^{-6}$	μ^3
Commercial	21	585 ± 49 [1]	295 ± 24	2915 ± 235
	121	1772 ± 81	127 ± 7	5819 ± 290
	221	2252 ± 67	150 ± 8	5263 ± 234
	385	1988 ± 120	125 ± 4	5831 ± 195
	621	2563 ± 149	132 ± 3	5664 ± 140
	795	2891 ± 175	167 ± 7	4558 ± 217
A	21	[2]	[2]	[2]
	121	1377 ± 87	197 ± 10	4196 ± 188
	221	1736 ± 70	196 ± 9	4222 ± 173
	385	1939 ± 66	175 ± 6	4754 ± 206
	621	2130 ± 52	182 ± 6	4468 ± 154
	795	2097 ± 135	201 ± 7	4075 ± 142
	995	2134 ± 132	209 ± 11	3925 ± 214
B	21	[2]	[2]	[2]
	121	907 ± 35	226 ± 8	3529 ± 123
	221	977 ± 35	214 ± 9	3889 ± 158
	385	1028 ± 64	176 ± 9	4768 ± 260
	621	1237 ± 51	175 ± 6	4703 ± 163
	795	1206 ± 51	194 ± 6	4124 ± 130
	995	1407 ± 68	218 ± 11	3802 ± 167
C	21	[2]	[2]	[2]
	121	998 ± 70	196 ± 12	4198 ± 298
	221	1192 ± 72	168 ± 11	5083 ± 281
	385	1384 ± 61	183 ± 12	4701 ± 366
	621	1632 ± 114	151 ± 5	5558 ± 200
	795	1874 ± 73	182 ± 12	4677 ± 257
	995	1901 ± 111	196 ± 9	4233 ± 223
D	21	[2]	[2]	[2]
	121	623 ± 36	249 ± 11	3230 ± 167
	221	746 ± 23	237 ± 7	3486 ± 112
	385	835 ± 19	214 ± 5	3816 ± 102
	621	979 ± 28	245 ± 10	3399 ± 141
	795	940 ± 35	232 ± 10	3578 ± 144
	995	1111 ± 39	263 ± 10	3161 ± 146

[1] Average of at least 10 rats ± SE of the mean.
[2] Values for 21-day-old rats fed the commercial diet provide common base values for each of the dietary groups.

basis there was, however, an increase after 1 year of age. Although the differences between dietary groups were small, there was a direct and linear relationship between the proportion of fat in the liver and liver weight or body weight. The high values at several age periods for rats consuming diet C, were in agreement with the histological appearance of the tissues.

The total fat content of the liver generally increased with age; the values for each dietary group, however, formed a separate pattern. Except for several values of group C rats, the total amount of fat in the liver related directly to body weight.

The hepatocytic fat content was consistently the lowest in the cells of rats fed diet D and, with the exception of several values from rats fed diet C, the highest content was found in the cells of rats fed the commercial diet. Except for the several erratic values for group C rats a single, direct and linear correlation was found between the fat content of the hepatocyte and liver or body weight (fig. 2). Furthermore, the average fat content of the cell and its concentration related directly and as a continuum to the average volume of the hepatocyte; the hepatocytes of the rats fed diet C, however, contained more fat

TABLE 5

Influence of age and diet on hepatic water content

Dietary group	Age	Water content		
		Per unit weight of liver	Of total liver	Per hepatocyte [1]
	days	%	*g*	$g \times 10^{11}$
Commercial	21	71.32 ± 0.08 [2]	1.42 ± 0.05	229 ± 9.2
	121	68.47 ± 0.11	9.67 ± 0.35	439 ± 21.6
	221	68.37 ± 0.14	10.47 ± 0.41	396 ± 17.6
	385	68.37 ± 0.21	10.84 ± 0.51	439 ± 15.7
	621	68.75 ± 0.17	13.42 ± 0.79	428 ± 10.5
	795	69.04 ± 0.32	12.17 ± 0.90	346 ± 16.3
A	21	[3]	[3]	[3]
	121	68.71 ± 0.27	4.80 ± 0.14	317 ± 13.8
	221	68.54 ± 0.21	6.08 ± 0.13	318 ± 12.3
	385	67.92 ± 0.35	7.58 ± 0.23	355 ± 14.0
	621	68.41 ± 0.23	8.05 ± 0.25	336 ± 11.2
	795	68.80 ± 0.33	7.16 ± 0.35	308 ± 10.7
	995	68.60 ± 0.11	6.97 ± 0.13	297 ± 17.8
B	21	[3]	[3]	[3]
	121	68.84 ± 0.21	2.77 ± 0.08	267 ± 9.8
	221	68.90 ± 0.16	3.18 ± 0.14	295 ± 12.1
	385	68.95 ± 0.20	4.01 ± 0.13	361 ± 19.2
	621	68.77 ± 0.18	4.87 ± 0.15	356 ± 12.5
	795	69.61 ± 0.29	4.33 ± 0.14	316 ± 10.7
	995	69.92 ± 0.19	4.55 ± 0.15	292 ± 12.8
C	21	[3]	[3]	[3]
	121	68.89 ± 0.62	3.57 ± 0.25	317 ± 21.9
	221	66.56 ± 0.82	4.84 ± 0.29	371 ± 18.9
	385	69.52 ± 0.34	5.41 ± 0.30	359 ± 26.9
	621	64.13 ± 1.76	6.85 ± 0.30	391 ± 14.0
	795	68.06 ± 0.59	7.12 ± 0.26	350 ± 19.6
	995	69.45 ± 0.75	6.72 ± 0.23	323 ± 16.5
D	21	[3]	[3]	[3]
	121	68.70 ± 0.15	1.72 ± 0.04	244 ± 12.7
	221	68.90 ± 0.09	2.18 ± 0.04	265 ± 8.6
	385	69.19 ± 0.15	2.70 ± 0.05	290 ± 7.7
	621	69.20 ± 0.23	2.79 ± 0.06	259 ± 10.8
	795	69.24 ± 0.27	2.82 ± 0.08	273 ± 11.3
	995	69.76 ± 0.16	2.96 ± 0.08	242 ± 10.8

[1] Estimate of gram of H_2O per average hepatocyte =

$$\frac{\text{Fresh liver wt} - \text{wt of dry liver} \times (1 - \text{fraction of liver volume not occupied by hepatocytes})}{\text{Total number of hepatocytes}}$$

[2,3] See footnotes 1 and 2 of table 4.

than was found in cells with similar volume in rats fed the other diets. The deviation for values of group C rats was still evident even after the volume of the cell was adjusted for that volume occupied by fat.

The amount of fat in the liver was directly related to the caloric intake of the rat. Similarly, the amount of fat in the hepatocyte also related directly to the caloric intake of the rat and except for three values from group C rats this relationship persisted after adjustment for differences in cell volume.

As the fat content in the hepatocytes or in the liver increased, there was a corresponding increase in the amount of water in the cell and in the liver. For group C rats only, an increase in the proportion of fat in the liver was accompanied by a decrease in the proportion of water.

Nitrogen. The nitrogen content of the liver on a fresh weight, dry weight or fat-free basis, was found to vary with age and with the diet fed (table 7). On a percentage basis, at any one age, the livers of rats fed diet B had the highest levels, followed

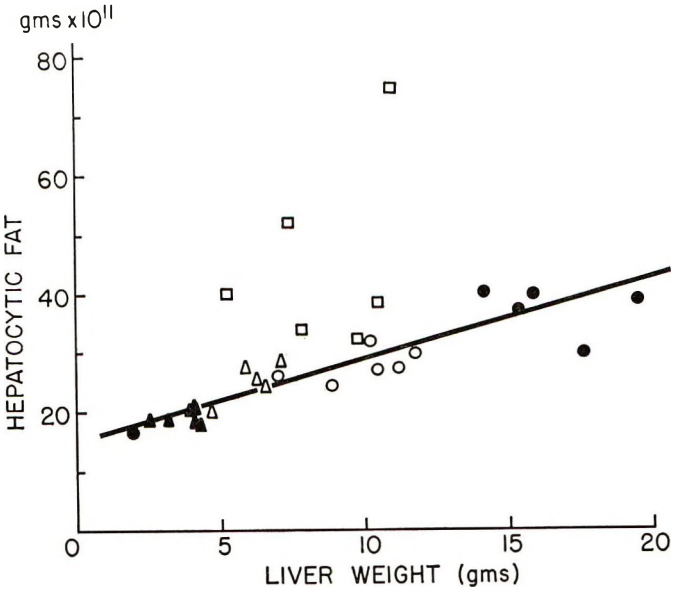

Fig. 2 Relationship between the amount of fat in the hepatocyte and the weight of the liver. Assays performed at seven age periods (21 to 995 days). Values for group C rats not included in computations for least square line. See legend of figure 1 for key to dietary groups.

in descending order, by D, A, commercial and C. Although, in each of the former four groups, the rates of change with age after 121 days were small, they were in general linear and nearly identical. For group C rats, there was initially a decrease in concentration and the subsequent pattern showed considerable variation. Unlike that found for proportion of water or of fat in the liver, no correlation was obtained between the percentage of hepatic nitrogen and liver weight or body weight. Instead, the percentage hepatic nitrogen on a fresh weight, dry weight, fat-free, or dry weight–fat-free basis correlated directly at each age with the proportion of protein in the diet consumed.

Total nitrogen content of the liver differed also in accordance with the diet fed (table 7). For each dietary group, the increase up to 621 days of age proved to be a function of the logarithm of the age; in later life, however, a decrease in nitrogen content occurred in all groups except that of group D. When the total nitrogen content of the liver for all dietary groups was plotted against body weight, a single, direct and linear correlation was obtained, indicating that the pattern of change in total nitrogen with age was similar to the pattern of change in body weight with age. Unlike that found for percentage nitrogen, the total nitrogen content related directly to the caloric intake of the rats rather than to the proportion of protein in the diet.

The nitrogen content of the hepatocyte also varied with the age of the rat and with the diet consumed (table 7). In each dietary group the hepatocytic nitrogen increased initially and then at later ages decreased to form a series of inverted U-shaped curves. The nitrogen content of the hepatocytes, irrespective of dietary group, gave rise to single, direct and linear relationships when plotted against the total nitrogen content of the liver, liver weight or body weight.

The differences in hepatocytic volume among the different groups of rats resulted in the expected inverse relationship between nitrogen content of the hepatocyte and the number of cells in a unit volume or weight of liver (fig. 3). In contrast to that found for either percentage nitrogen or for total nitrogen, nitrogen content of the hepatocyte was found to relate directly to the level of intake of the protein component in the diet.

TABLE 6

Influence of age and diet on hepatic fat content

Dietary group	Age	Fat content		
		Per unit weight of liver	Of total liver	Per hepatocyte [1]
	days	%	$g \times 10^2$	$g \times 10^{11}$
Commercial	21	4.53 ± 0.05 [2]	9.0 ± 0.15	16.48 ± 0.69
	121	4.95 ± 0.06	70.0 ± 2.88	40.28 ± 2.30
	221	5.45 ± 0.18	83.7 ± 4.75	37.38 ± 2.20
	385	5.01 ± 0.21	80.2 ± 5.75	40.12 ± 1.24
	621	5.14 ± 0.20	98.8 ± 4.60	39.06 ± 1.57
	795	5.74 ± 0.17	101.2 ± 8.00	34.98 ± 1.78
A	21	[3]	[3]	[3]
	121	5.09 ± 0.27	35.6 ± 2.10	26.29 ± 1.56
	221	4.74 ± 0.14	42.1 ± 1.51	24.60 ± 1.25
	385	4.75 ± 0.09	52.9 ± 1.26	27.51 ± 0.94
	621	5.36 ± 0.32	63.0 ± 4.33	29.93 ± 2.37
	795	5.42 ± 0.46	57.9 ± 7.02	27.32 ± 2.41
	995	6.65 ± 1.13	69.8 ± 14.31	32.14 ± 4.93
B	21	[3]	[3]	[3]
	121	4.72 ± 0.08	19.0 ± 0.67	21.13 ± 0.81
	221	4.22 ± 0.13	19.6 ± 1.08	20.01 ± 0.86
	385	4.74 ± 0.16	27.6 ± 1.57	27.37 ± 1.43
	621	4.96 ± 0.15	35.2 ± 1.60	28.71 ± 1.31
	795	4.90 ± 0.15	30.5 ± 1.43	25.35 ± 0.73
	995	5.15 ± 0.32	33.5 ± 2.22	24.19 ± 1.74
C	21	[3]	[3]	[3]
	121	7.49 ± 0.82	41.4 ± 7.85	40.14 ± 5.43
	221	8.13 ± 1.16	64.3 ± 12.57	52.05 ± 8.96
	385	5.41 ± 0.35	42.6 ± 4.32	31.49 ± 3.67
	621	11.05 ± 1.42	128.7 ± 23.51	75.04 ± 11.30
	795	6.80 ± 0.74	72.4 ± 8.87	38.49 ± 4.03
	995	6.16 ± 1.06	63.4 ± 15.06	32.42 ± 5.67
D	21	[3]	[3]	[3]
	121	4.50 ± 0.17	11.2 ± 0.25	18.47 ± 0.86
	221	4.34 ± 0.17	13.7 ± 0.28	18.54 ± 0.62
	385	4.32 ± 0.13	16.8 ± 0.44	20.17 ± 0.48
	621	4.42 ± 0.24	17.7 ± 0.95	18.19 ± 0.95
	795	4.81 ± 0.12	19.6 ± 0.72	21.01 ± 0.80
	995	4.64 ± 0.18	19.7 ± 1.01	17.91 ± 1.06

[1] Estimate of grams of fat per average hepatocyte = $\frac{\text{Grams of fat per liver}}{\text{Total number of hepatocytes}}$.

[2,3] See footnotes 1 and 2 of table 4.

Although the amount of nitrogen in the hepatocyte was found to be a direct function of the volume of the hepatocyte (fig. 3) a single, linear relationship was obtained for the values of four of the five groups (commercial, A, B and D). A separate but still linear pattern was formed by the values for group C rats. The displacement of this pattern from that of the other dietary groups indicated that the cells of the rats in group C contained less nitrogen than was present in the other hepatocytes with similar volume. Similar deviations were also found for the values of group C rats from the direct and linear relationships between the content of hepatocytic nitrogen and hepatocytic water, and between hepatocytic nitrogen and hepatocytic fat. Since the relatively high fat content of the group C hepatocytes might be responsible for these displacements, the average cell volume was adjusted for the volume occupied by fat; the extent of the deviations in each of these correlations was only slightly reduced.

TABLE 7

Influence of age and diet on hepatic nitrogen content

Dietary group	Age	Nitrogen content				
		Per unit wt, fresh	Per unit wt, fat free	Per unit wt, dry	Total liver	Per hepatocyte
	days	*mg/100*	*mg/100*	*mg/100*	*mg*	*mg × 10⁸*
Commercial	21	29.6 ± 0.09 [1]	31.0 ± 0.16	103.2 ± 0.4	58.8 ± 0.9	10.8 ± 0.54
	121	32.2 ± 0.15	33.8 ± 0.15	102.0 ± 0.5	454.5 ± 16.6	26.1 ± 1.26
	221	34.4 ± 0.41	36.3 ± 0.45	108.6 ± 1.3	525.0 ± 18.6	23.5 ± 1.11
	385	33.5 ± 0.34	35.3 ± 0.34	105.9 ± 1.3	531.8 ± 26.6	27.1 ± 1.01
	621	32.9 ± 0.55	34.7 ± 0.60	105.3 ± 1.9	638.1 ± 32.9	25.0 ± 0.40
	795	33.7 ± 0.40	35.7 ± 0.44	108.9 ± 1.7	591.0 ± 40.6	20.6 ± 1.04
A	21	[2]	[2]	[2]	[2]	[2]
	121	34.9 ± 0.57	36.7 ± 0.64	111.4 ± 1.6	243.1 ± 6.4	18.1 ± 0.82
	221	34.6 ± 0.61	36.3 ± 0.63	110.1 ± 2.3	307.2 ± 9.4	17.9 ± 0.75
	385	35.5 ± 0.60	37.3 ± 0.65	110.9 ± 2.6	396.2 ± 12.6	20.6 ± 0.70
	621	35.3 ± 0.69	37.3 ± 0.67	112.0 ± 2.6	414.4 ± 10.7	19.5 ± 0.42
	795	35.0 ± 0.59	37.1 ± 0.64	112.4 ± 2.3	365.1 ± 18.4	17.6 ± 0.49
	995	36.8 ± 0.57	39.4 ± 0.52	118.5 ± 4.3	374.3 ± 10.1	18.1 ± 0.95
B	21	[2]	[2]	[2]	[2]	[2]
	121	37.0 ± 0.33	38.9 ± 0.33	118.9 ± 1.1	148.7 ± 3.2	16.6 ± 0.57
	221	37.4 ± 0.58	39.1 ± 0.60	120.4 ± 1.6	172.4 ± 6.5	17.8 ± 0.74
	385	38.3 ± 0.23	40.2 ± 0.24	123.4 ± 1.0	222.1 ± 5.7	22.2 ± 1.12
	621	38.1 ± 0.35	40.0 ± 0.35	121.9 ± 1.2	269.4 ± 7.3	22.0 ± 0.67
	795	38.4 ± 0.53	40.3 ± 0.56	126.2 ± 1.4	238.6 ± 8.5	19.9 ± 0.62
	995	38.5 ± 0.48	40.6 ± 0.41	128.1 ± 1.2	250.5 ± 8.6	18.1 ± 0.84
C	21	[2]	[2]	[2]	[2]	[2]
	121	28.5 ± 0.86	30.8 ± 0.87	92.2 ± 4.0	146.3 ± 9.5	14.8 ± 0.63
	221	28.6 ± 1.33	31.1 ± 1.39	86.1 ± 5.0	207.0 ± 15.6	17.5 ± 1.12
	385	32.2 ± 0.96	34.1 ± 0.97	106.1 ± 4.1	247.8 ± 9.5	18.2 ± 0.97
	621	28.8 ± 0.64	32.4 ± 0.67	82.5 ± 4.6	311.6 ± 19.2	19.3 ± 0.77
	795	31.3 ± 0.84	33.6 ± 0.77	98.7 ± 4.0	326.6 ± 10.9	17.8 ± 0.98
	995	29.2 ± 0.86	31.2 ± 0.98	96.2 ± 3.5	283.1 ± 12.5	15.1 ± 0.60
D	21	[2]	[2]	[2]	[2]	[2]
	121	35.1 ± 0.50	36.8 ± 0.52	112.3 ± 1.7	87.5 ± 1.4	14.4 ± 0.73
	221	34.1 ± 1.23	35.7 ± 1.29	109.7 ± 3.9	107.8 ± 4.5	14.6 ± 0.74
	385	36.6 ± 0.47	38.2 ± 0.51	118.7 ± 1.3	142.4 ± 2.8	17.1 ± 0.34
	621	36.2 ± 0.74	37.8 ± 0.82	117.4 ± 2.0	145.4 ± 3.3	15.0 ± 0.60
	795	36.7 ± 0.69	38.6 ± 0.73	119.5 ± 2.2	149.5 ± 4.6	16.1 ± 0.70
	995	37.7 ± 0.55	39.5 ± 0.55	124.7 ± 2.4	159.4 ± 3.6	14.5 ± 0.46

[1,2] See footnotes of table 4.

3. *Influence of age and diet upon hepatic enzyme activity levels*

For each of the enzymes studied, adenosine triphosphatase, histidase, catalase and alkaline phosphatase, the activity levels were found to vary consistently and significantly with the age of the rat and with the diet fed.

Adenosine triphosphatase. The activity of ATPase in each of the dietary groups increased exponentially during the early phases of life and decreased in later life. This obtained regardless of method of expression: per unit weight of fresh, dry or fat-free liver, or per unit nitrogen, per total organ or per cell (table 8). The rate of change and the time of change in direction differed with the diet fed and, to some degree, according to the method of expressing the enzyme activity levels. For dietary groups B and D, the transition time occurred at approximately 800 days; for groups fed the commercial diet and diet A, the change in direction occurred at approximately 200 and 600 days, respectively. At any one age, both the level of activity of the total organ and the rate of change in activity were the lowest in rats fed diet D (fig. 4). Progressively higher levels and rates of change were found for rats fed diets B, C, A and the commercial diet. In later life the rate of decrease in total ac-

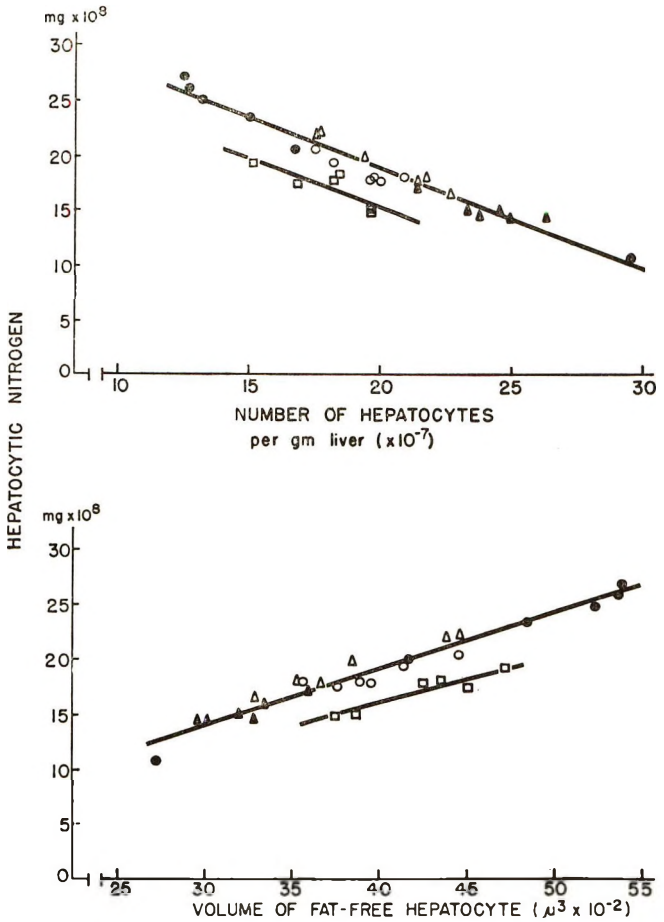

Fig. 3 Relationships between number of hepatocytes per gram liver (upper graph), the volume of fat-free hepatocytes (lower graph) and the amount of nitrogen in the hepatocyte. (Least square lines for group C values computed separately.) Assays performed at seven age periods (21 to 995 days). See legend of figure 1 for key to dietary groups.

tivity for the latter four groups simulated the pattern of increasing activity observed in the earlier stages of life.

When the total activity level was related to body weight the differences in activity-age patterns between dietary groups disappeared and a single pattern was obtained. The linear and direct relationship is shown in figure 5.

The computed hepatocytic ATPase activity level was found to be a function of the volume of the hepatocyte; the larger the cell, the greater the activity level, particularly when the volume of the hepatocyte was expressed on a fat-free, dry volume basis (fig. 6). Since the average volume of the hepatocyte is body weight associated, it was to be expected that the activity per cell related also to body weight; in this relationship, however, the activity increased asymptotically with increase in body weight.

At higher body weights and correspondingly larger liver masses, there was a greater number of hepatocytes as well as a greater level of activity for the organ; regardless of age or the diet fed, the increase in level of activity of the organ was linearly related to the increasing number of hepatocytes. The reduction in number of hepatocytes per unit weight of liver with increase in volume of the hepatocytes

TABLE 8

Influence of age and diet on hepatic adenosine triphosphatase activity

Dietary group	Age	Level of activity: Per milligram liver, fresh wt	Per milligram liver, dry wt	Per milligram N	Per liver	Per hepatocyte
	days	*units*	*units*	*units*	*units* × 10^{-2}	*units* × 10^{6}
Commercial	21	5.36 ± 0.43 [1]	18.7 ± 0.94	181.1 ± 6.9	106.4 ± 4.1	19.5 ± 1.26
	121	9.27 ± 0.36	29.4 ± 1.16	288.0 ± 11.0	1310.8 ± 71.1	75.0 ± 4.35
	221	13.32 ± 0.27	42.1 ± 0.86	387.6 ± 6.8	2032.7 ± 73.9	90.7 ± 3.44
	385	12.94 ± 0.22	40.9 ± 0.86	386.8 ± 8.2	2045.4 ± 89.0	105.1 ± 4.93
	621	11.51 ± 0.40	36.8 ± 1.18	350.2 ± 11.4	2205.9 ± 73.4	87.7 ± 3.50
	795	11.77 ± 0.37	38.0 ± 0.99	349.5 ± 10.6	2049.9 ± 129.3	71.8 ± 3.80
A	21	[2]	[2]	[2]	[2]	[2]
	121	9.16 ± 0.34	29.3 ± 1.04	262.4 ± 7.5	639.9 ± 29.6	47.3 ± 2.12
	221	11.28 ± 0.24	35.9 ± 0.73	326.4 ± 6.3	1003.4 ± 38.6	58.5 ± 2.97
	385	12.55 ± 0.37	39.2 ± 1.35	353.6 ± 10.4	1399.2 ± 55.9	73.0 ± 3.71
	621	12.85 ± 0.34	40.7 ± 1.28	363.5 ± 6.3	1506.4 ± 47.8	71.0 ± 2.33
	795	12.27 ± 0.42	39.4 ± 1.41	350.2 ± 10.7	1268.9 ± 58.6	62.0 ± 3.17
	995	11.58 ± 0.37	37.3 ± 1.63	314.6 ± 6.8	1179.8 ± 48.9	57.2 ± 3.79
B	21	[2]	[2]	[2]	[2]	[2]
	121	8.65 ± 0.37	27.8 ± 1.23	233.7 ± 10.1	347.2 ± 15.8	38.9 ± 2.37
	221	12.68 ± 0.47	40.7 ± 1.34	339.1 ± 12.2	587.2 ± 35.3	60.5 ± 3.59
	385	12.06 ± 0.39	38.8 ± 1.21	314.8 ± 9.8	698.6 ± 26.6	69.9 ± 4.03
	621	13.09 ± 0.24	41.9 ± 0.80	344.2 ± 7.2	929.6 ± 38.5	75.8 ± 2.96
	795	13.77 ± 0.27	45.3 ± 0.86	359.5 ± 7.5	858.1 ± 36.4	71.4 ± 2.19
	995	12.00 ± 0.17	39.9 ± 0.48	311.5 ± 4.2	781.3 ± 31.3	56.3 ± 2.53
C	21	[2]	[2]	[2]	[2]	[2]
	121	5.09 ± 0.65	16.5 ± 2.17	174.7 ± 20.0	262.1 ± 39.2	25.3 ± 2.62
	221	9.57 ± 0.73	29.1 ± 2.71	331.4 ± 13.6	678.7 ± 49.0	57.4 ± 3.60
	385	11.68 ± 0.48	38.5 ± 1.80	362.3 ± 10.2	897.0 ± 44.0	65.8 ± 4.22
	621	12.64 ± 0.40	36.3 ± 2.34	439.3 ± 12.4	1362.2 ± 80.2	84.6 ± 3.86
	795	12.75 ± 0.51	40.2 ± 2.12	407.0 ± 12.4	1326.9 ± 54.1	72.2 ± 4.49
	995	10.42 ± 0.77	34.3 ± 2.56	353.5 ± 20.0	1009.0 ± 80.5	53.7 ± 3.97
D	21	[2]	[2]	[2]	[2]	[2]
	121	7.53 ± 0.37	24.1 ± 1.21	213.9 ± 9.0	187.6 ± 8.9	31.1 ± 2.29
	221	11.79 ± 0.72	37.9 ± 2.31	347.6 ± 23.1	371.9 ± 23.5	50.5 ± 3.71
	385	12.37 ± 0.36	40.1 ± 1.09	338.6 ± 9.6	481.4 ± 14.8	58.0 ± 2.23
	621	12.97 ± 0.31	42.1 ± 0.95	359.3 ± 7.7	522.5 ± 16.3	54.2 ± 3.12
	795	14.03 ± 0.43	45.7 ± 1.59	382.5 ± 11.2	572.9 ± 28.5	61.9 ± 3.98
	995	12.81 ± 0.29	42.4 ± 1.05	340.3 ± 7.1	541.8 ± 14.7	49.2 ± 1.79

[1,2] See footnotes of table 4.

affords the explanation for the single but inverse relationship found between the activity level per cell and the number of cells per unit weight of liver.

Although the concentration of nitrogen in the liver (milligrams N per gram liver) does not appear to correlate with the activity level of ATPase expressed on a unit weight basis (activity per milligram liver) the activity of ATPase per hepatocyte increased exponentially with a logarithmic increase in the nitrogen content of the cell, and the activity of the organ increased directly and linearly with increase in the total nitrogen content of the organ.

Because of the relationships that exist between body weight or hepatocytic volume and caloric intake, correlations should also be found between caloric intake and activity content of the organ and of the hepatic cell. For rats consuming the commercial diet and diet A, the activity per cell or per organ increased with increase in caloric intake to a maximum at 385 days of age and then decreased; but for those rats consuming diets B, C and D the maximum activity level was reached at 600 days of age. A single, linear and direct relationship was found between activity level of the organ and caloric intake when

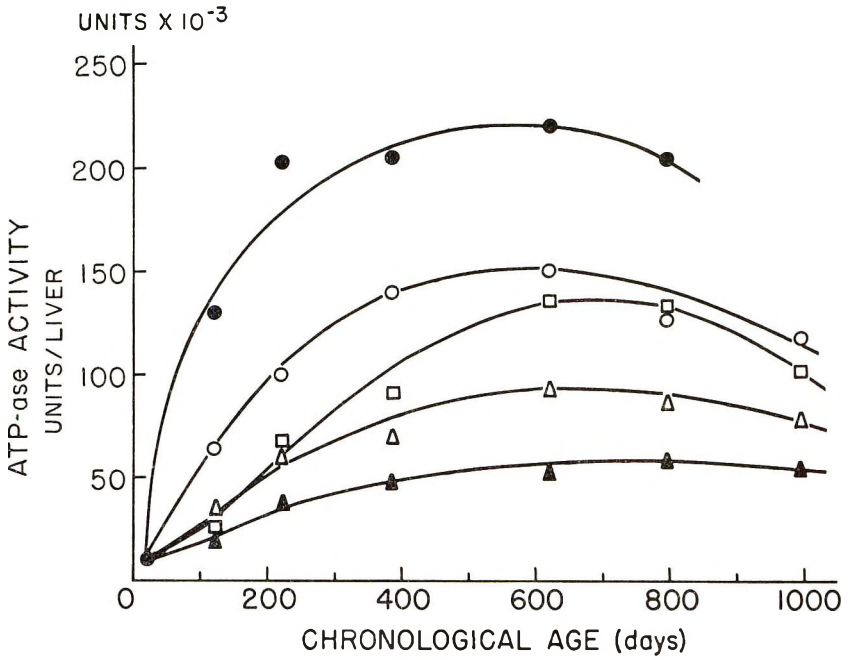

Fig. 4 Influence of age and diet on hepatic ATPase activity. See legend of figure 1 for key to dietary groups.

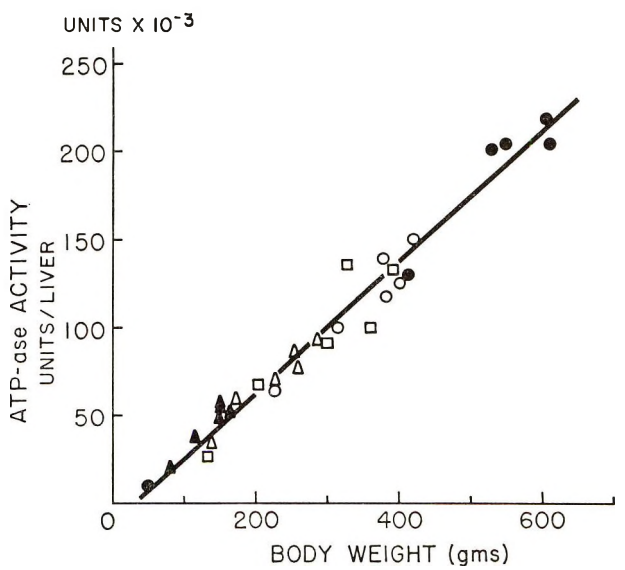

Fig. 5 Relationship between level of ATPase activity of the liver and body weight of the rat. Assays performed at seven age periods (21 to 995 days). See legend of figure 1 for key to dietary groups.

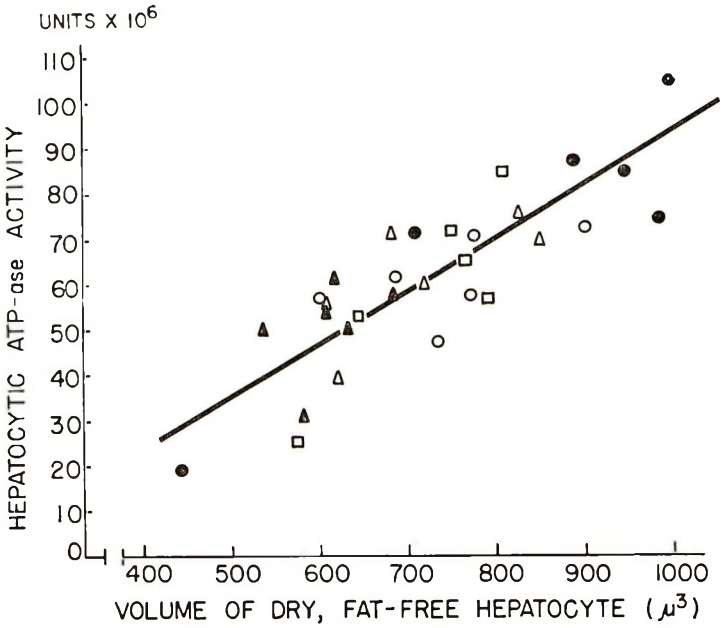

Fig. 6 Relationship between level of ATPase activity of the hepatocyte and volume of the dry, fat-free hepatocyte. Assays performed at seven age periods (21 to 995 days). See legend of figure 1 for key to dietary groups.

the activity values of the five dietary groups were limited to only those periods of life when the levels of activity were increasing.

Histidase. Initially, histidase activity per unit weight of liver, fresh, dry or fat-free, or per unit N increased exponentially with age in each dietary group (table 9). In later life it decreased; the downward trends, however, for several of the dietary groups were erratic. The highest levels and the greatest rate of change with age were found among rats fed the diet containing the greatest proportion of casein, 51%; the lowest levels and the smallest rate of change were found among rats fed the diet containing the lowest proportion of casein, 8%. The relationships between proportion of casein in the diet and the level of activity computed as an average for all ages are shown in figure 7.

The level of activity of the entire liver in each dietary group also increased exponentially with age. The activity values of rats in group D appeared to level off at approximately 1 year of age, whereas in the other four dietary groups, trends of decreasing amounts of activity were observed in the late phases of life. At any age, rats fed the commercial diet had the highest levels; the activities in decreasing order were groups A, B, C and D, respectively (table 9). Exclusive of group C values, a high coefficient of correlation was obtained in the exponential relationship between the level of activity and caloric intake (fig. 8).

The differences between dietary groups and those due to age, disappeared when the total activity of the liver was related to body weight or to the number of hepatocytes in the liver; with increase in weight of the rat or the number of cells, the total activity increased proportionately. When total activity was adjusted for differences in body weight and plotted as a function of age, the resulting activity patterns for each dietary group and the relationship between groups were similar to those observed on a unit weight basis of liver.

On a cellular basis, the activity in each dietary group also increased during the first portion of life, but in later life the trends were reversed and the level of activity decreased (table 9). The values, however, appeared to be clustered according to the amount of protein consumed rather than

TABLE 9

Influence of age and diet on hepatic histidase activity

Dietary group	Age	Level of activity				
		Per gram liver, fresh wt	Per gram liver, dry wt	Per milligram N	Per liver	Per hepatocyte
	days	*units*	*units*	*units* × 10^2	*units*	*units* × 10^9
Commercial	21	1.14 ± 0.06 [1]	4.0 ± 0.20	3.9 ± 0.16	2.3 ± 0.05	4.1 ± 0.25
	121	2.85 ± 0.20	9.1 ± 0.65	8.9 ± 0.62	40.4 ± 3.22	22.7 ± 1.25
	221	3.20 ± 0.17	10.1 ± 0.57	9.3 ± 0.46	48.8 ± 2.73	21.8 ± 1.34
	385	3.27 ± 0.13	10.3 ± 0.38	9.7 ± 0.35	52.0 ± 3.38	26.4 ± 1.09
	621	2.67 ± 0.14	8.5 ± 0.43	8.2 ± 0.46	51.7 ± 3.73	20.4 ± 1.28
	795	2.83 ± 0.26	9.1 ± 0.82	8.4 ± 0.79	50.7 ± 6.24	17.5 ± 1.97
A	21	[2]	[2]	[2]	[2]	[2]
	121	2.74 ± 0.12	8.8 ± 0.40	7.9 ± 0.33	19.2 ± 1.04	14.1 ± 0.68
	221	3.35 ± 0.19	10.7 ± 0.62	9.7 ± 0.66	29.6 ± 1.63	17.3 ± 1.12
	385	3.30 ± 0.16	10.3 ± 0.50	9.3 ± 0.50	37.0 ± 2.44	19.3 ± 1.48
	621	3.37 ± 0.23	10.7 ± 0.74	9.5 ± 0.53	39.4 ± 2.57	18.4 ± 0.98
	795	3.34 ± 0.14	10.7 ± 0.48	9.5 ± 0.41	34.7 ± 2.22	16.8 ± 0.72
	995	4.04 ± 0.18	13.0 ± 0.62	11.0 ± 0.49	41.2 ± 2.15	19.9 ± 1.29
B	21	[2]	[2]	[2]	[2]	[2]
	121	3.46 ± 0.27	11.2 ± 0.91	9.4 ± 0.73	13.9 ± 1.02	15.6 ± 1.42
	221	3.69 ± 0.18	11.9 ± 0.56	9.8 ± 0.42	16.9 ± 0.95	17.4 ± 0.91
	385	4.14 ± 0.26	13.3 ± 0.80	10.8 ± 0.65	23.8 ± 1.32	24.0 ± 1.85
	621	4.51 ± 0.17	14.4 ± 0.57	11.9 ± 0.46	31.8 ± 1.12	26.2 ± 1.45
	795	4.02 ± 0.25	13.2 ± 0.73	10.4 ± 0.58	24.9 ± 1.72	20.7 ± 1.09
	995	4.58 ± 0.15	15.2 ± 0.47	11.9 ± 0.30	29.7 ± 1.16	21.3 ± 0.78
C	21	[2]	[2]	[2]	[2]	[2]
	121	1.78 ± 0.25	5.7 ± 0.80	6.4 ± 1.05	9.0 ± 1.28	9.6 ± 1.78
	221	1.88 ± 0.19	5.7 ± 0.66	6.5 ± 0.50	13.1 ± 0.95	11.1 ± 0.69
	385	2.60 ± 0.12	8.5 ± 0.39	8.2 ± 0.55	20.6 ± 2.03	15.2 ± 1.70
	621	2.08 ± 0.18	5.9 ± 0.58	7.2 ± 0.55	23.2 ± 2.65	14.0 ± 0.41
	795	2.62 ± 0.10	8.2 ± 0.39	8.4 ± 0.32	27.4 ± 1.25	14.7 ± 0.73
	995	1.89 ± 0.12	6.2 ± 0.41	6.4 ± 0.32	18.3 ± 1.34	9.8 ± 0.69
D	21	[2]	[2]	[2]	[2]	[2]
	121	2.22 ± 0.14	7.1 ± 0.36	6.3 ± 0.28	5.5 ± 0.12	9.1 ± 0.64
	221	2.86 ± 0.23	9.2 ± 0.46	8.5 ± 0.36	9.0 ± 0.19	12.2 ± 0.73
	385	3.27 ± 0.15	10.6 ± 0.46	8.9 ± 0.39	12.6 ± 0.43	15.3 ± 1.07
	621	2.83 ± 0.15	9.2 ± 0.51	7.8 ± 0.43	11.3 ± 0.44	11.7 ± 0.70
	795	3.02 ± 0.14	9.8 ± 0.45	8.2 ± 0.35	12.2 ± 0.54	13.3 ± 0.93
	995	2.70 ± 0.11	8.9 ± 0.38	7.2 ± 0.31	11.4 ± 0.48	10.3 ± 0.61

[1,2] See footnotes of table 4.

to the proportion of protein in the diet, with the lowest levels occurring in groups C and D. Within each dietary group, hepatocytic histidase activity levels increased with increase in the average volume of the hepatic cell, but a continuum was formed for only those rats fed the commercial diet, diet A and diet D. Separate activity-cell size patterns were found for rats fed diets B and C, with more activity being present in the cells of rats fed diet B for the size of their cells than found in rats fed the commercial diet, diet A or diet D, but less activity, for the size of the cell, was found in the cells of rats fed diet C.

A single and direct proportionality existed between histidase activity and the nitrogen concentration or content regardless of the method of expressing enzyme activity levels; i.e., unit weight of liver, per liver or per hepatocyte (fig. 9).

Hepatic catalase. The activity of hepatic catalase per milligram of liver, per unit N, per organ and per hepatocyte increased with age initially, but at later ages the upward trend reversed and the activity levels progressively decreased (table 10). The time when the reversal in the activity patterns took place was dependent upon the diet fed and the method of expressing

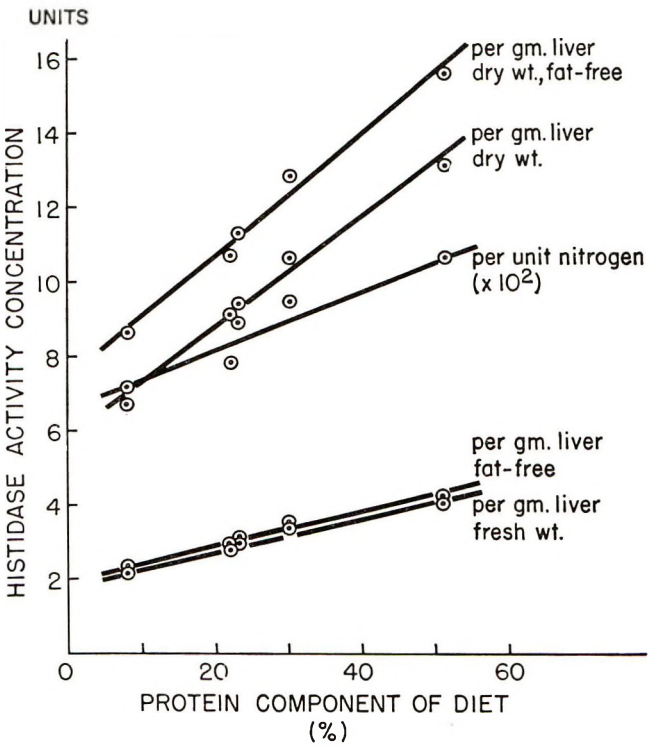

Fig. 7 Relationship between histidase activity concentration as expressed on several bases and percentage protein component in diet. Assays performed at seven age periods and activities are presented as an average for all ages.

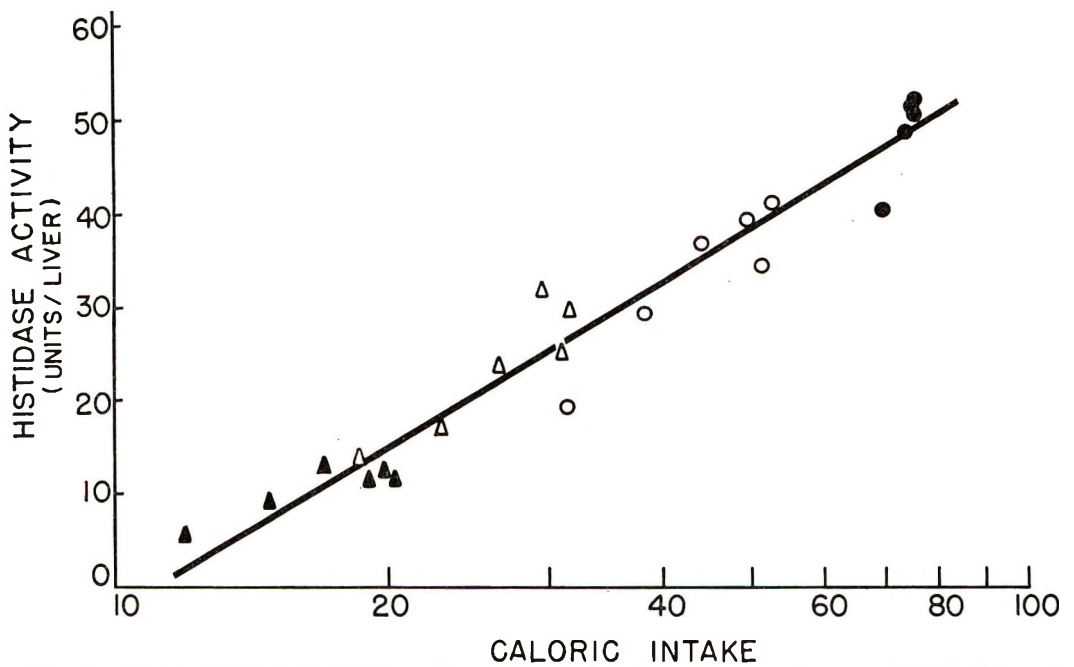

Fig. 8 Relationship between level of histidase activity of the liver and average daily caloric intake. (Least square line computed without the values for group C.) See legend of figure 1 for key to dietary groups.

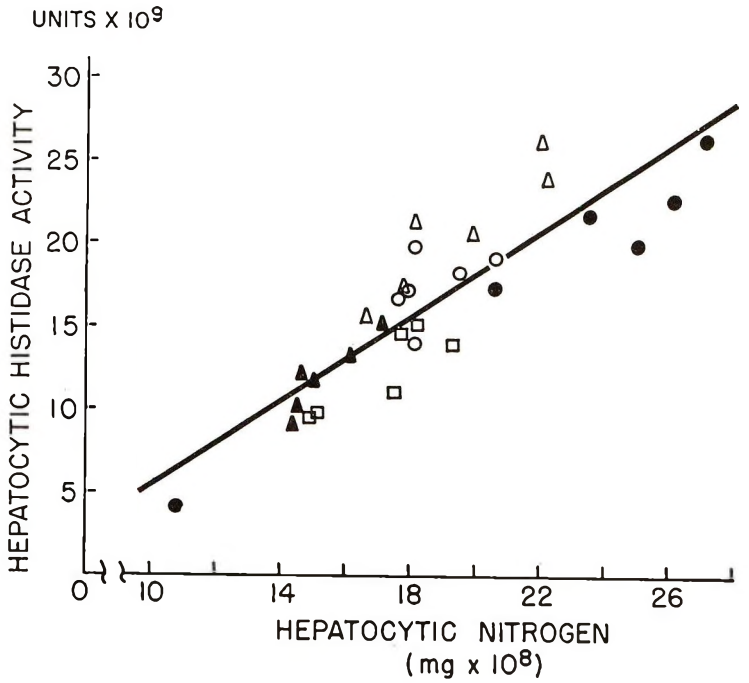

Fig. 9 Relationship between level of histidase activity of the hepatocyte and nitrogen content of the hepatocyte. Assays performed at seven age periods (21 to 995 days). See legend of figure 1 for key to dietary groups.

the activity levels. Consistently, rats fed the commercial diet reached the highest level, had the earliest reversal and the greatest rate of increase during early life, as well as the greatest rate of decrease in later life. In contrast, the lowest activity values, on the basis of a unit weight of liver, were found among the two dietary groups of rats (C and D) whose intakes of protein were low.

On the total liver basis, the activity values for rats fed diet D were throughout life more like those of young rats than was found for any of the other four dietary groups. For groups A and B the change in trend took place at approximately 600 days, but was delayed until approximately 800 days for the rats in group C. The patterns of change with age in catalase activity of the liver for each dietary group appeared to simulate the patterns of change with age in both body weight and in total number of hepatocytes. When activity values at different ages were plotted against body weight or number of cells of the respective groups, single and direct relationships were obtained. Since the body weight of the rats proved to be a direct function of caloric intake, a high correlation was also found between caloric intake and total hepatic catalase activity; there was, however, a displacement for group C values from the single pattern. When the total liver activity levels were adjusted for body weight (total activity per 100 g body weight) and plotted against age, the differences due to age and diet persisted in that a series of inverse and nearly parallel lines were obtained.

The activity level of the hepatocyte increased as the average volume of the hepatocyte increased. The exponential decrease that was noted between activity per hepatocyte and the number of hepatocytes per gram of liver was, therefore, expected (fig. 10). On the other hand, except for erratic values of group C, with increase in the volume of the hepatocyte, there was a linear increase in the level of activity per unit weight or volume of tissue. The discrepancy for group C values persisted even after the cell volume was adjusted for lipid

TABLE 10

Influence of age and diet on hepatic catalase activity

Dietary group	Age	Level of activity				
		Per milligram liver, fresh wt	Per milligram liver, dry wt	Per milligram N	Per liver	Per hepatocyte
	days	*units* × 10^2	*units* × 10^2	*units* × 10^1	*units*	*units* × 10^8
Commercial	21	4.8 ± 0.26 [1]	16.8 ± 0.80	16.3 ± 0.65	96 ± 4.6	17.6 ± 1.1
	121	7.6 ± 0.20	24.2 ± 0.61	23.7 ± 0.62	1083 ± 61.1	62.3 ± 4.2
	221	8.5 ± 0.18	26.9 ± 0.50	24.8 ± 0.52	1298 ± 49.7	58.2 ± 2.9
	385	7.9 ± 0.25	25.1 ± 0.75	23.7 ± 0.73	1267 ± 79.9	64.0 ± 2.2
	621	7.3 ± 0.46	23.5 ± 1.44	22.3 ± 1.25	1400 ± 82.1	55.6 ± 3.0
	795	6.9 ± 0.70	22.4 ± 2.24	20.5 ± 1.89	1175 ± 85.3	42.6 ± 4.9
A	21	[2]	[2]	[2]	[2]	[2]
	121	6.0 ± 0.17	19.3 ± 0.47	17.4 ± 0.51	425 ± 22.2	31.3 ± 1.5
	221	6.4 ± 0.33	20.2 ± 0.98	18.4 ± 0.99	570 ± 41.1	33.4 ± 2.8
	385	6.3 ± 0.26	19.8 ± 0.82	17.9 ± 0.75	704 ± 25.4	36.5 ± 1.1
	621	6.4 ± 0.20	20.4 ± 0.53	18.3 ± 0.79	762 ± 41.4	35.8 ± 1.9
	795	5.0 ± 0.36	16.0 ± 1.14	14.3 ± 1.01	521 ± 43.8	25.5 ± 2.5
	995	4.4 ± 0.37	14.1 ± 1.29	11.7 ± 0.90	446 ± 41.8	21.4 ± 2.3
B	21	[2]	[2]	[2]	[2]	[2]
	121	5.9 ± 0.17	18.9 ± 0.49	16.0 ± 0.53	238 ± 12.1	26.4 ± 1.1
	221	5.8 ± 0.20	18.8 ± 0.70	15.6 ± 0.60	270 ± 15.8	27.7 ± 1.4
	385	6.2 ± 0.20	19.9 ± 0.59	16.2 ± 0.55	359 ± 14.9	36.4 ± 2.9
	621	6.7 ± 0.25	21.4 ± 0.78	17.6 ± 0.65	474 ± 22.1	38.9 ± 2.4
	795	6.1 ± 0.35	20.0 ± 1.05	15.9 ± 0.91	380 ± 26.6	31.7 ± 2.2
	995	5.1 ± 0.29	16.8 ± 0.92	13.2 ± 0.77	335 ± 29.3	24.1 ± 2.2
C	21	[2]	[2]	[2]	[2]	[2]
	121	4.3 ± 0.19	13.8 ± 0.76	14.9 ± 0.36	219 ± 15.6	22.0 ± 0.8
	221	4.8 ± 0.38	14.6 ± 1.36	16.7 ± 0.78	340 ± 24.2	28.7 ± 1.6
	385	5.3 ± 0.29	17.5 ± 0.96	16.7 ± 0.99	418 ± 34.4	30.8 ± 3.0
	621	4.6 ± 0.28	13.2 ± 0.96	16.1 ± 0.82	507 ± 45.1	31.3 ± 2.5
	795	5.2 ± 0.38	16.5 ± 1.38	16.5 ± 0.95	544 ± 41.6	30.1 ± 3.1
	995	3.7 ± 0.36	12.2 ± 1.18	12.7 ± 1.28	356 ± 36.2	19.6 ± 2.5
D	21	[2]	[2]	[2]	[2]	[2]
	121	5.7 ± 0.30	18.4 ± 0.98	16.3 ± 0.69	143 ± 6.9	23.8 ± 2.0
	221	5.4 ± 0.12	17.4 ± 0.38	16.1 ± 0.55	171 ± 4.0	23.1 ± 0.8
	385	5.8 ± 0.25	18.7 ± 0.77	15.7 ± 0.61	225 ± 10.5	26.9 ± 1.2
	621	6.0 ± 0.31	19.6 ± 0.95	16.7 ± 0.81	243 ± 12.7	25.0 ± 1.5
	795	6.1 ± 0.27	19.9 ± 0.96	16.7 ± 0.67	250 ± 15.3	27.0 ± 2.0
	995	5.2 ± 0.25	17.2 ± 0.81	13.8 ± 0.72	220 ± 11.1	20.1 ± 1.4

[1,2] See footnotes of table 4.

material. Because of the reciprocal relationship between number of cells in a unit volume of tissue to the volume of the cell there was, except for group C, an inverse and exponential relationship between activity per unit weight of liver and the number of hepatocytes in an equivalent mass of liver.

Although catalase activity per unit weight of liver did not correlate with the concentration of nitrogen in the liver, a direct proportionality was found between catalase activity and nitrogen content on both an organ and a cellular basis (fig. 11).

Alkaline phosphatase. The pattern of change in hepatic alkaline phosphatase activity with age differed from those of the former enzymes in that the activity values per milligram of liver initially decreased with age and then later in life increased (table 11). The rate of change, increase or decrease, was significantly affected by the diet consumed; among rats fed the purified diets the values for group D rats on fresh weight, dry weight or fat-free basis, were more like that of the young rat than those of any of the other groups. Rats fed diet A, on the other hand, had the

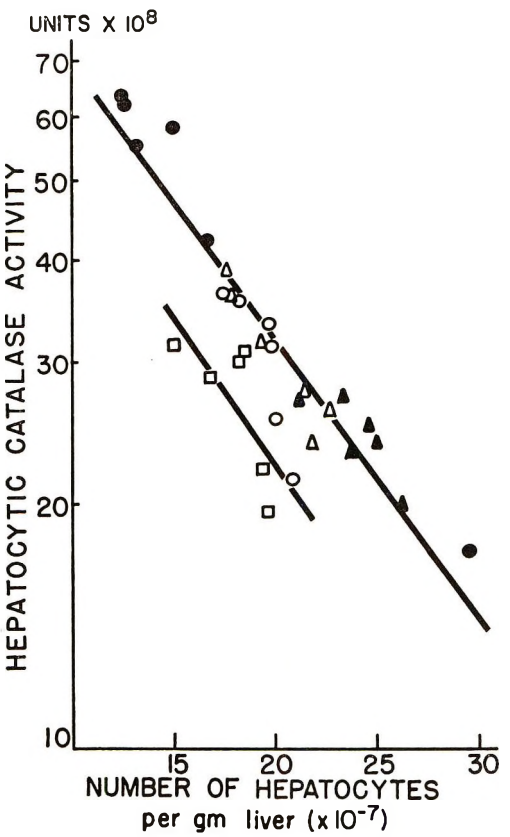

Fig. 10 Relationship between level of catalase activity of the hepatocyte and number of hepatocytes per gram fresh weight liver. Assays performed at seven age periods (21 to 995 days). (Least square line for group C values computed separately.) See legend of figure 1 for key to dietary groups.

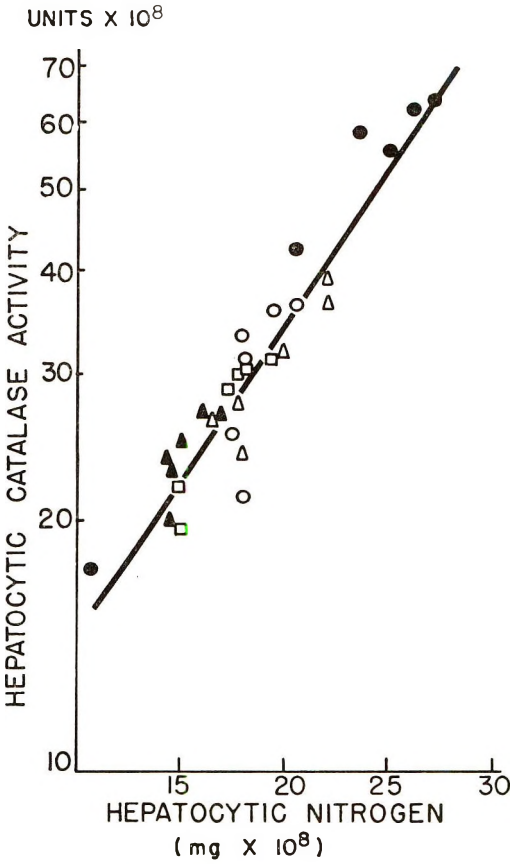

Fig. 11 Relationship between level of catalase activity of the hepatocyte and nitrogen content of the hepatocyte. Assays performed at seven age periods (21 to 995 days). See legend of figure 1 for key to dietary groups.

greatest rate of decrease, but after 221 days of age there was little change in the activity level.

Unlike that of the other enzymes, the level of alkaline phosphatase activity per milligram of liver appeared to be related as an inverse function of the body weight or liver weight among those groups of rats fed the purified diet. Although still inverse and linear, completely separate activity-body weight or activity-liver weight patterns were noted for the rats fed the commercial diet (fig. 12).

Despite the fact that the activity per unit weight of liver decreased in the first stages of life, on a total liver basis the activity values for the rats fed the purified diets generally increased with age throughout life. The differences in rate of change in activity level between dietary groups remained. Rats fed diet D had the lowest values. Conversely, rats fed the commercial diet had activity levels that were nearly five times greater at any age than found for the 21-day-old rat, and nearly two times greater than any of the values for rats fed the purified diets. That the dietary regimens influenced the activity level of the liver became particularly evident in the single and exponential pattern obtained when the total hepatic activity was related to caloric intake (fig. 13). On the other hand, the activity per milligram of liver relative to body weight correlated as an

TABLE 11

Influence of age and diet on hepatic alkaline phosphatase activity

Dietary group	Age	Level of activity				
		Per milligram liver, fresh wt	Per milligram liver, dry wt	Per milligram N	Per liver	Per hepatocyte
	days	*units* × 10^2	*units* × 10^2	*units*	*units* × 10^{-2}	*units* × 10^7
Commercial	21	119.0 ± 14.60 [1]	415 ± 24.9	40.2 ± 2.21	23.6 ± 1.40	43.3 ± 2.64
	121	90.7 ± 7.58	287 ± 24.0	28.1 ± 2.27	127.6 ± 10.19	71.8 ± 4.59
	221	74.7 ± 5.14	237 ± 17.0	21.8 ± 1.48	115.1 ± 9.75	51.1 ± 3.90
	385	71.7 ± 4.29	227 ± 14.1	21.4 ± 1.25	113.2 ± 7.58	58.2 ± 5.10
	621	76.7 ± 5.39	246 ± 17.6	23.3 ± 1.64	149.8 ± 13.82	58.2 ± 3.92
	795	65.1 ± 6.94	211 ± 23.3	19.3 ± 1.95	112.4 ± 11.90	40.1 ± 4.88
A	21	[2]	[2]	[2]	[2]	[2]
	121	57.8 ± 4.07	185 ± 12.9	16.5 ± 0.98	39.9 ± 2.17	29.8 ± 2.20
	221	38.1 ± 1.83	121 ± 6.0	11.0 ± 0.43	33.8 ± 1.93	19.6 ± 1.18
	385	40.5 ± 2.36	126 ± 7.3	11.5 ± 0.72	44.8 ± 2.21	23.6 ± 1.78
	621	39.4 ± 2.07	125 ± 7.0	11.1 ± 0.45	46.3 ± 2.61	21.8 ± 1.20
	795	37.2 ± 3.24	120 ± 10.9	10.7 ± 0.97	37.5 ± 2.23	18.8 ± 1.82
	995	45.1 ± 4.67	148 ± 17.7	12.3 ± 1.31	45.2 ± 3.98	22.7 ± 3.06
B	21	[2]	[2]	[2]	[2]	[2]
	121	74.0 ± 4.29	237 ± 13.4	20.0 ± 1.17	29.7 ± 1.90	32.9 ± 1.72
	221	59.9 ± 3.62	193 ± 12.1	16.1 ± 1.08	27.9 ± 2.41	28.7 ± 2.48
	385	56.8 ± 3.81	183 ± 12.1	14.8 ± 1.02	33.1 ± 2.68	33.0 ± 2.72
	621	60.6 ± 2.57	194 ± 8.2	16.0 ± 0.74	43.1 ± 2.53	35.4 ± 2.29
	795	58.0 ± 3.73	191 ± 12.7	15.2 ± 1.04	36.2 ± 2.82	30.3 ± 2.43
	995	78.6 ± 4.64	261 ± 15.4	20.4 ± 1.24	50.7 ± 2.78	37.7 ± 3.74
C	21	[2]	[2]	[2]	[2]	[2]
	121	101.5 ± 7.98	329 ± 28.6	35.7 ± 2.56	53.1 ± 6.01	53.0 ± 4.39
	221	86.5 ± 8.14	257 ± 22.3	31.9 ± 4.16	65.1 ± 8.87	55.3 ± 7.86
	385	64.3 ± 4.38	211 ± 14.9	20.0 ± 1.28	49.9 ± 3.87	36.8 ± 3.48
	621	64.4 ± 2.76	184 ± 12.2	22.5 ± 1.19	69.7 ± 4.82	43.1 ± 2.28
	795	44.4 ± 4.73	141 ± 16.2	14.0 ± 1.30	46.3 ± 4.64	25.9 ± 3.29
	995	58.0 ± 4.53	192 ± 16.9	20.3 ± 1.92	56.8 ± 5.32	30.3 ± 2.62
D	21	[2]	[2]	[2]	[2]	[2]
	121	91.1 ± 5.09	292 ± 17.3	26.1 ± 1.61	22.9 ± 1.51	37.6 ± 2.97
	221	62.5 ± 3.23	201 ± 10.5	18.5 ± 1.11	19.9 ± 1.26	26.7 ± 1.61
	385	73.9 ± 4.12	240 ± 13.3	20.3 ± 1.19	28.7 ± 1.54	34.7 ± 2.02
	621	81.6 ± 5.00	264 ± 15.6	22.7 ± 1.56	33.1 ± 2.44	34.5 ± 3.15
	795	91.1 ± 4.25	296 ± 14.3	25.0 ± 1.54	37.2 ± 2.18	40.1 ± 2.70
	995	97.3 ± 8.05	323 ± 27.1	25.9 ± 2.24	41.5 ± 3.93	37.3 ± 3.22

[1,2] See footnotes of table 4.

inverse and exponential function of the logarithm of the caloric intake.

A single, linear and positive correlation was obtained when the level of alkaline phosphatase activity for the liver was plotted against body weight; there was, however, a greater variance in this correlation than was observed when the activity levels of the former three enzymes were related to body weight.

On a cellular basis, whereas the patterns of change in activity with age were erratic, the computed hepatocytic activity levels of rats fed diet A consistently had the lowest level of activity. The activity per milligram of liver did not relate to the number or average volume of hepatocytes in a comparable weight of liver; the level of activity relative to body weight, however, increased exponentially as the number of hepatocytes in a unit weight of liver increased, and decreased exponentially as the average volume of the hepatocyte increased logarithmically.

A direct proportionality was found between the total activity level of the liver and the nitrogen content of the liver, but no correlation was evident between the level

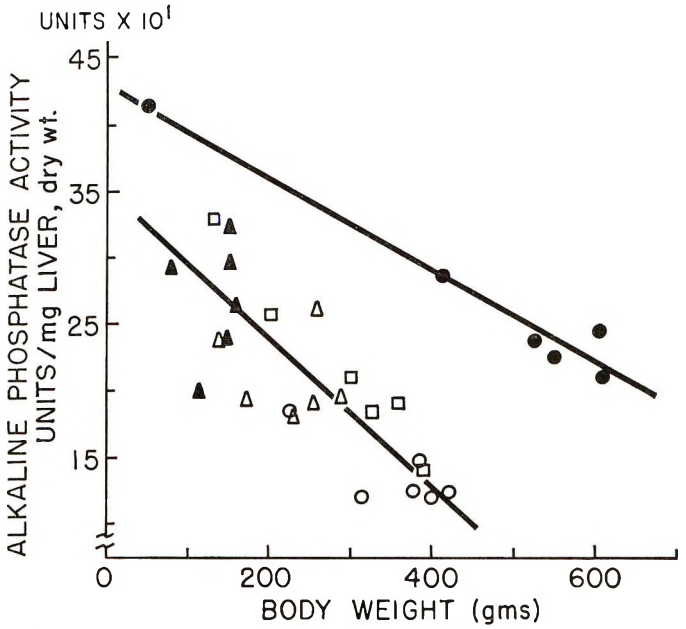

Fig. 12 Relationship between level of alkaline phosphatase activity per milligram liver, dry weight and body weight. Assays performed at seven age periods (21 to 995 days). (Least square line for values of rats fed the commercial diet computed separately.) See legend of figure 1 for key to dietary groups.

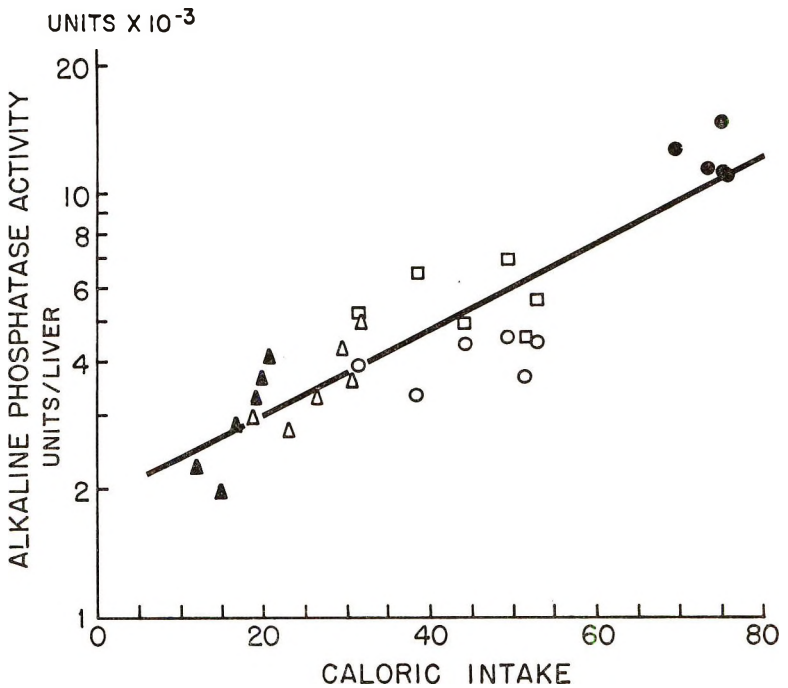

Fig. 13 Relationship between level of alkaline phosphatase activity of the liver and average daily caloric intake. See legend of figure 1 for key to dietary groups.

of activity per milligram of liver or per hepatocyte and the corresponding nitrogen concentration or content.

Enzyme-enzyme activity correlations. The activity values of each of the enzymes were plotted one against the other as a means of determining whether an incremental change in level of activity of one enzyme was accompanied by a proportional change in the activity level of another enzyme. On a total liver basis, linear and direct relationships were found between the levels of activity for each of the following: histidase and ATPase (fig. 14), alkaline phosphatase and catalase, and between alkaline phosphatase and histidase (table 12). Thus, as the level of activity of one enzyme in each pair increased, there was a corresponding increase of uniform proportion in the activity level of the other

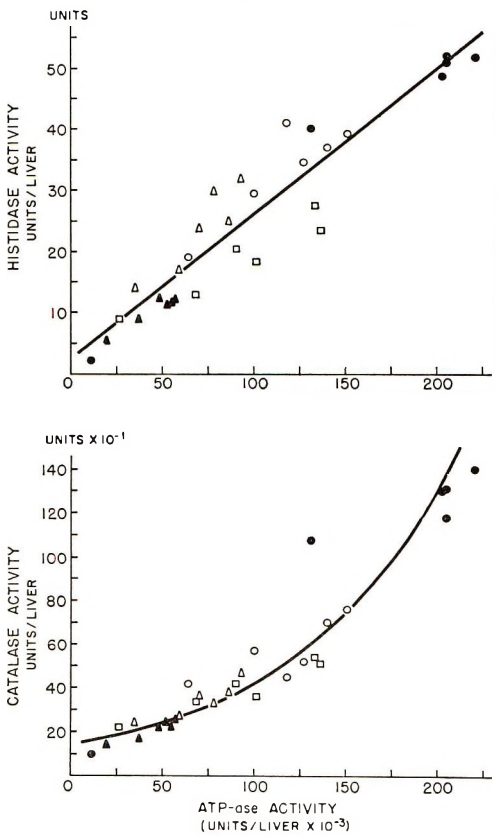

Fig. 14 Representative enzyme-enzyme activity relationships. Assays performed at seven age periods (21 to 995 days). See legend of figure 1 for key to dietary groups.

TABLE 12

Enzyme-enzyme activity relationships

Coordinates		Dietary groups included	Regression equation	Correlation coefficient	Level of significance
X	Y			r	
Activity per liver					
ATPase	Alkaline phosphatase	All [1]	$\log Y = 3.3571 + (3.2386 \times 10^{-6})X$	0.825	< 0.0005
ATPase	Catalase	All	$\log Y = 2.1456 + (4.8201 \times 10^{-6})X$	0.951	< 0.0005
ATPase	Histidase	All	$Y = 2.34 + (2.37 \times 10^{-4})X$	0.942	< 0.0005
Alkaline phosphatase	Catalase	All	$Y = -20.83 + 0.098\ X$	0.906	< 0.0005
Alkaline phosphatase	Histidase	A,B,D	$Y = -16.76 + 0.011\ X$	0.770	< 0.0005
		Commercial	$Y = -3.16 + (4.13 \times 10^{-3})X$	0.918	0.010
Catalase	Histidase	All	$Y = -97.35 + 46.95 \log X$	0.945	< 0.0005
Activity per hepatocyte					
ATPase	Catalase	All	$\log Y = \overline{7}.0885 + (6.4455 \times 10^{3})X$	0.819	< 0.0005
ATPase	Histidase	All	$Y = (2.73 \times 10^{-9}) + (2.20 \times 10^{-4})X$	0.768	< 0.0005
Catalase	Histidase	A,B,C,D	$Y = (-2.19 \times 10^{-9}) + 0.063\ X$	0.713	< 0.0005
		Commercial	$Y = (-2.94 \times 10^{-9}) + 0.043\ X$	0.984	< 0.0005

[1] Includes all dietary groups and at all ages.

enzyme. On the other hand, this unique balance was not observed in the patterns formed between the values for ATPase and catalase (fig. 14), ATPase and alkaline phosphatase, and for catalase and histidase; in these comparisons exponential relationships were obtained (table 12).

On the basis of the hepatocyte, an increase in the level of histidase was associated with a proportional increase in the level of ATPase activity. Except for values for rats fed the commercial diet, a similar relationship was found between catalase and histidase. Between ATPase and catalase, however, an exponential pattern was obtained (fig. 15) indicating that for each unit change in the level of activity in one of the enzymes there were progressively greater changes in the other (table 12).

4. Activity changes attributable to change in number and volume of hepatocytes

From the cellular and enzymological data, it was found possible to estimate how much of the change in total enzyme activity, from one age to another, could be directly attributed to the amount of activity associated with change in 1) number of hepatocytes and volume of the hepatocytes, and 2) how much could be attributed to change in "activity concentration."

To determine the effect of changes in the number and volume of the hepatocytes, the level of activity per unit volume of the hepatocyte was considered to remain constant from time period 1 to time period 2. The expected incremental change in activ-

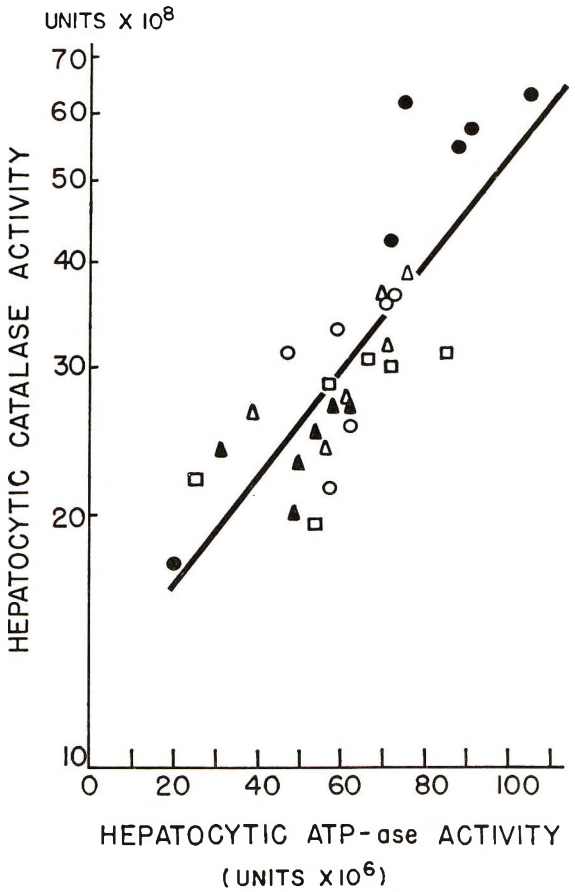

Fig. 15 Representative hepatocytic enzyme-enzyme activity relationships. Assays performed at seven age periods (21 to 995 days). See legend of figure 1 for key to dietary groups.

TABLE 13

Enzyme activity of liver attributable to change in number and volume of hepatocytes [1]

Diet	Age	ATPase	Catalase	Histidase	Alkaline phosphatase
	days	*units* × 10^{-2}	*units*	*units*	*units* × 10^{-2}
Commercial	121	573	517	12.2	127
	221	681	614	14.5	151
	385	665	600	14.2	148
	621	863	778	18.4	192
	795	773	697	16.5	172
A	121	271	245	5.8	60
	221	378	341	8.0	84
	385	506	456	10.8	112
	621	529	477	11.2	117
	795	458	413	9.7	102
	995	438	395	9.3	97
B	121	106	96	2.3	24
	221	146	132	3.1	33
	385	213	192	4.5	47
	621	279	252	5.9	62
	795	225	203	4.8	50
	995	246	222	5.2	55
C	121	171	154	3.6	38
	221	297	268	6.3	66
	385	323	291	6.9	72
	621	500	451	10.6	111
	795	471	425	10.0	105
	995	422	381	9.0	94
D	121	25	22	0.5	6
	221	66	60	1.4	15
	385	106	96	2.3	24
	621	114	103	2.4	25
	795	116	105	2.5	26
	995	126	114	2.7	28

[1] Expected activity due to change in parenchymal volume (includes both number and volume of the hepatocytes) = $[(V_x - V_1)\,B]$

To determine expected activity due to change in volume of the hepatocytes only,

$$[(V_x - V_1)B] - [(N_x - N_1)A]$$

To determine activity due to change in "activity concentration" only,

$$(T_x - T_1) - [(V_x - V_1)B]$$

Where: V_1 = parenchymal volume in μ^3, 21 days

$V_{x\,1,2,3\ldots}$ = parenchymal volume in μ^3, consecutive ages

B = activity/μ^3 of hepatocyte, 21 days

A = activity/cell, 21 days

N_1 = total number hepatocytes in liver, 21 days

$N_{x\,1,2,3\ldots}$ = total number hepatocytes in liver, consecutive ages

T_1 = total enzyme activity level of liver, 21 days

$T_{x\,1,2,3\ldots}$ = total enzyme activity of liver, consecutive ages.

ity was obtained from the product of the hepatocytic activity per μ^3, for the 21-day-old rat, and of the incremental change in total volume of the hepatocytes of a later age (see footnote to table 13). To calculate the change in amount of activity due to change in number of hepatocytes only, the activity per hepatocyte of the 21-day-old rat was assumed to remain constant; the expected change in activity was then determined as the product of the activity per cell of the prior period and the incremental change in number of cells. Any deviation in values between the expected activity level due to numbers and volume, and that of the observed activity level, denotes the amount of activity that can only be ascribed directly to change in the activity concentration.

The level of activity attributable to changes in number and volume of the hepatocyte varied with the age of the rat and with the diet fed (table 13). For each of the enzymes the disposition of these

newly derived age-activity patterns among the five dietary groups was, in general, similar to those presented earlier for total activity of the liver. Also similar to that found for total activity, single and linear correlations were obtained for each of the enzymes when the expected activity due to change in number and in volume of the hepatocyte was related to body weight (fig. 16). For ATPase, histidase and catalase, the amount of activity due to change in activity concentration was also a direct function of body weight. In the case of ATPase and histidase, as the total level of activity increased with increase in body weight, a greater proportion was consistently contributed by that amount ascribable to activity concentration than by that due to increase in number and volume of the hepatocyte (fig. 16). Furthermore, with increase in body weight, the patterns diverged one from another so that the amount afforded by the activity concentration became progessively greater. For catalase, however, the greater contribution to the whole was the result of the increase in total mass of hepatocytic material rather than of the changes in activity concentration (fig. 16).

For phosphatase, the computed amount of activity attributable to change in number and volume of hepatocytes exceeded the level of activity actually observed. This indicated, therefore, that there was a compensatory reduction in the activity concentration; this conclusion was supported

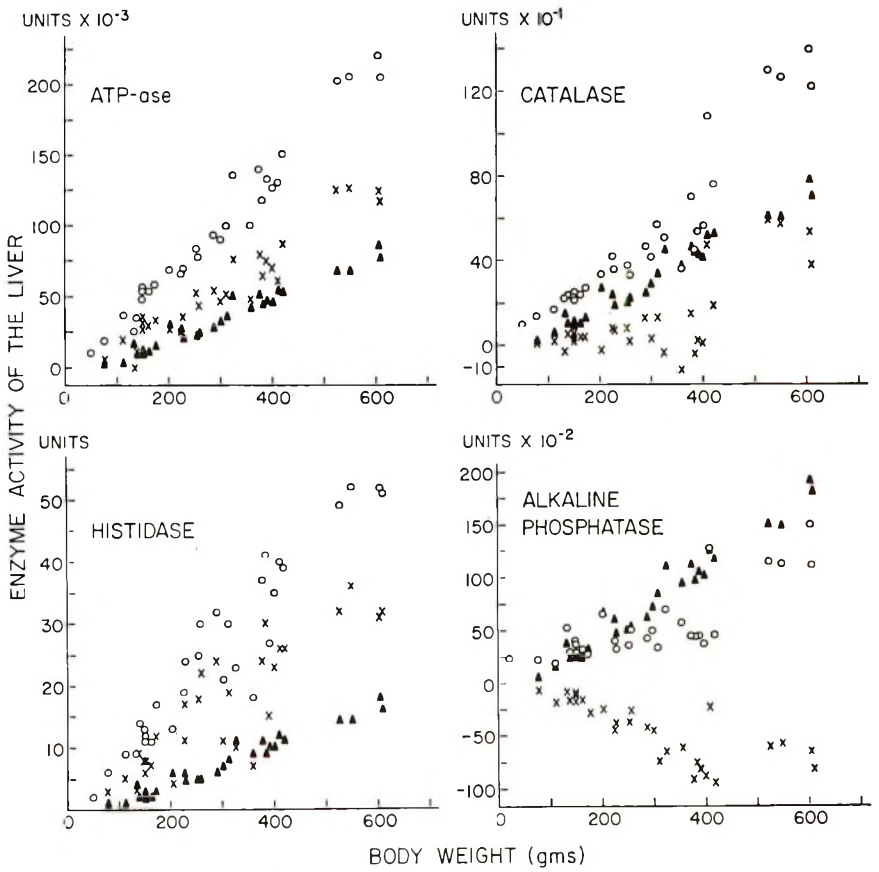

Fig. 16 Comparison of enzyme activity levels of the liver, actual (○), with computed amounts of activity attributable to change in both number and volume of hepatocytes (parenchymal volume) (▲), and with change in activity concentration (×). Values for each of the enzymes represent five dietary groups; assays performed at seven age periods.

by the fact that the observed activity per unit weight of liver or per unit volume of cell, decreased with increase in body weight. With increase in body weight, the pattern of change for activity concentration and that for change in number and volume of cells progressively diverged (fig. 16). The change in enzyme activity attributable to change in number and volume of the hepatocyte was directly related to body weight but the change due to activity concentration was inversely related; the rate of dilution in concentration was smaller than the rate of increase in expected activity due to the increase in number and volume of hepatocytes.

A similar type of analysis was also applied to the changes in level of activity when the activity was expressed on a unit weight basis of liver. With change in volume of the hepatocyte there was, for a given volume of liver, a compensatory adjustment in the number of cells. Thus, when the changes in both number and volume of the hepatocytes were considered together as a unit the differences in level of activity per unit weight of tissue could only be attributed to changes in activity concentration. When, however, the effects of change in the number of hepatocytes were considered separately from the change in volume of the hepatocytes, differences in "activity content" of the hepatocyte were found. This was due in part, to the dependence of the activity level of the hepatocyte on cell volume, and in part, to the differences in activity concentration of the hepatocyte.

5. *Relationships between enzyme activity and life expectancy*

Of the several ways of expressing mortality risk of a population, life expectancy values at different age periods proved to be most valuable in that they were found to correlate inversely not only with caloric intake (fig. 17) and body weight but also with enzyme activity levels. At each age period and for each of the enzymes, the activity level of the liver was found to relate inversely to the life expectancy of the nonsacrificed members of the various dietary groups (fig. 18). Uniformly, activity values which were more like those of the young rat had the longest life expectancy (rats fed diet D) and conversely, those with the highest activity values (rats fed the commercial diet) had the shortest ex-

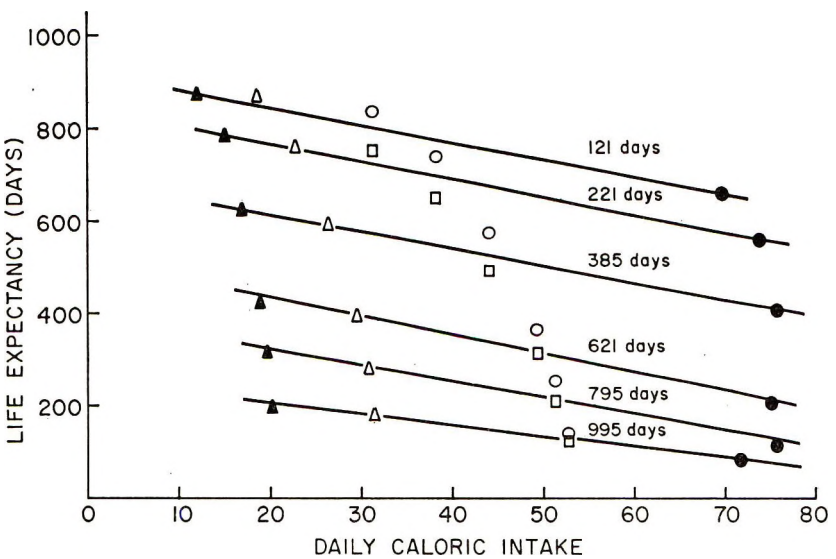

Fig. 17 Relationship between life expectancy of nonsacrificed members of the five dietary populations of rats and daily caloric intake at six age periods. Life expectation values computed to coincide with the age when members of the same population were killed for biochemical assays. See legend of figure 1 for key to dietary groups.

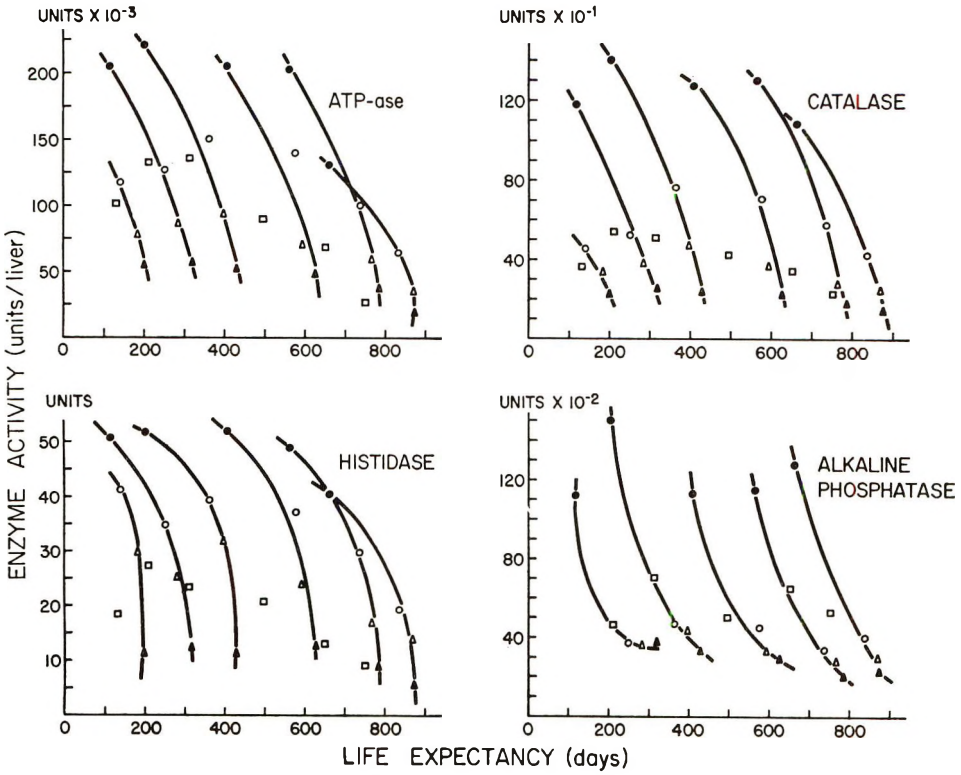

Fig. 18 Life expectancy-hepatic enzyme activity patterns. For each of the four enzymes, the series of curves represents the correlation between the activity level of the liver and the corresponding life expectancy of the nonsacrificed members of the same dietary populations of rats at different age periods. Reading from *right to left*, these ages are: 121, 221, 385, 621, 795 and 995 days, respectively. The ATPase, catalase and histidase activity values for group C rats are not included in the best fit line for the earlier age periods. See legend of figure 1 for key to dietary groups.

pectancy. In the early stages of life there were for ATPase, catalase and histidase some deviations in the values for group C rats; these deviations became negligible at later ages.

When the activity values adjusted for differences in body weight were related to life expectancy, the patterns obtained were similar to those formed when the activity per unit weight of liver was related to life expectancy. Relationships, however, were limited to catalase and phosphatase only. Catalase activity per milligram of liver (fresh, dry or fat-free) or per unit nitrogen, related inversely and linearly to life expectancy through the 621-day period; subsequent to this time there was a reversal in trend and the values for the four dietary groups fed the purified diets related directly to life expectancy. The direct and linear correlation between alkaline phosphatase activity per milligram of liver (fresh, dry or fat-free) or per unit nitrogen, and life expectancy was limited to those groups fed diets A, B and D until 621 days of age; after this time the deviations in group C values became minimal.

On a cellular basis the level of activity with certain limitations also correlated with life expectancy. For both ATPase and catalase (fig. 19), the patterns for each age period, excluding the terminal values, were roughly curvilinear and inverse. The deviation in the values for group C rats was present at all ages for catalase, but for ATPase it became negligible after the rats were 1 year old. The relationship between hepatocytic histidase and expectancy,

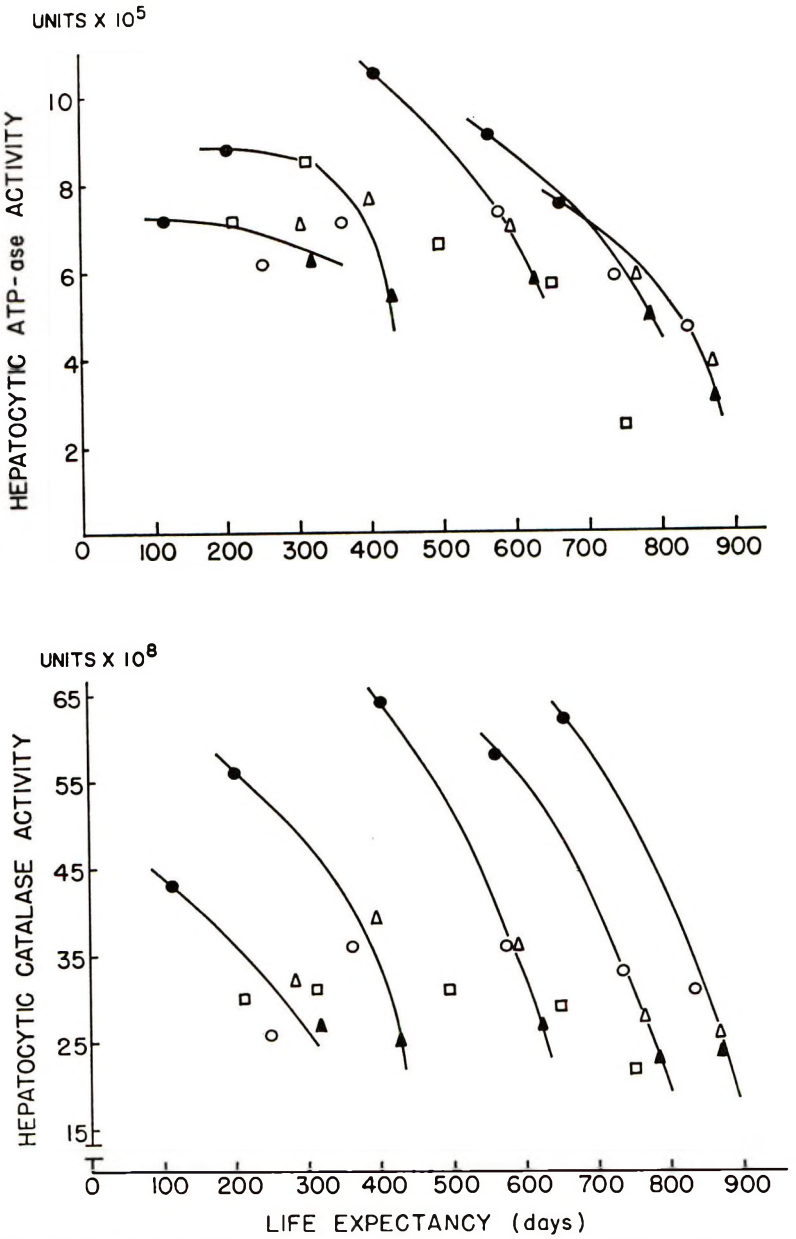

Fig. 19 Representative life expectancy-hepatocytic enzyme activity patterns. Each series of curves represents the correlation between the activity level of the hepatocyte and the corresponding life expectancy of the nonsacrificed members of the same dietary populations of rats at different age periods. For age sequence, see legend of figure 18. See legend of figure 1 for key to dietary groups.

again exclusive of group C, was also curvilinear and inverse, but correlations were evident for only year 1 of life. Hepatocytic alkaline phosphatase differed from the other enzymes in that a direct relationship was found but, like that noted when the activity level was expressed on a unit weight basis of liver, the correlation was limited to the values from dietary groups A, B and D.

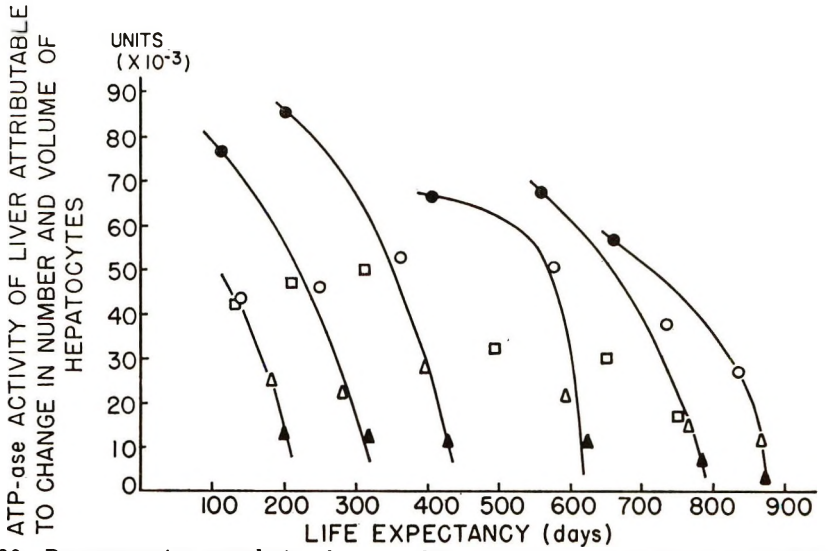

Fig. 20 Representative correlation between life expectancy at various age periods and the expected enzyme activity level of the liver attributable to changes only in number and volume of the hepatocytes (parenchymal volume). These enzyme values do not include that amount of activity contributed by the changes in activity concentration. For age sequence, see legend of figure 18. See legend of figure 1 for key to dietary groups.

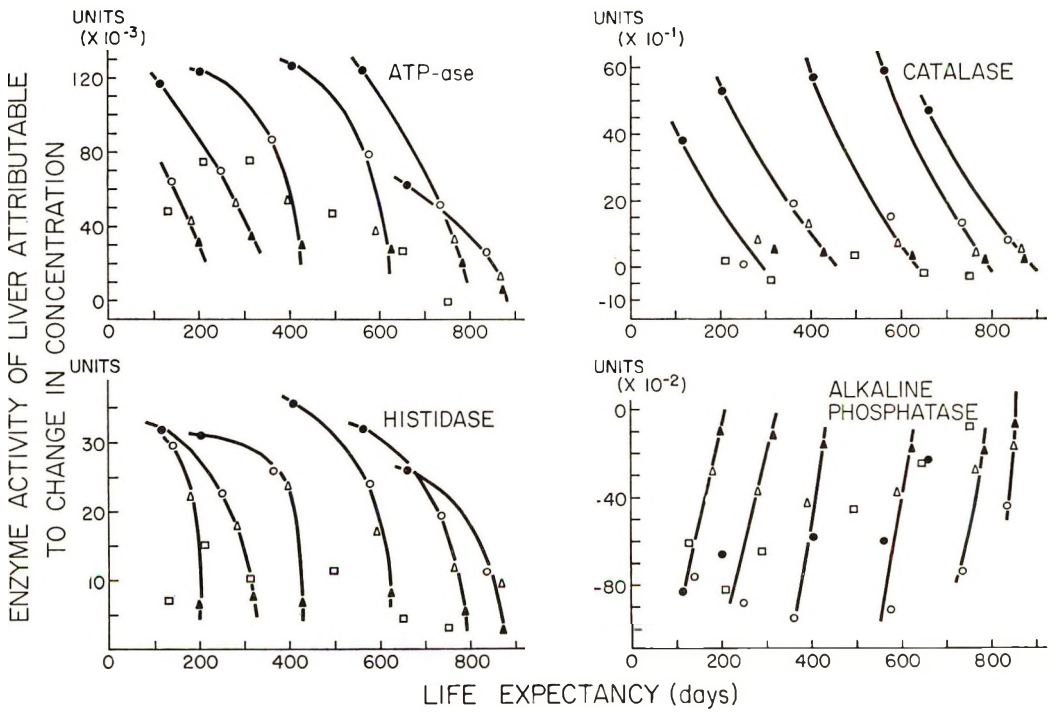

Fig. 21 Correlation between life expectancy at various age periods and the expected enzyme activity level of the liver attributable to change in activity concentration apart from that amount of activity due to change in parenchymal volume. For age sequence, see legend of figure 18. See legend of figure 1 for key to dietary groups.

Adjustment for differences in cell volume resulted in patterns which were nearly identical to those found when the activity per organ was adjusted for differences in body weight.

Because it was possible to estimate the change in amount of activity of the liver attributable to change in number and volume of hepatocytes and to change in activity concentration, the correlations between these factors and life expectancy were also assessed. Consistently for each of the enzymes, the expected change in activity due to change in number and volume of hepatocytes, separately or together, was inversely related to life expectancy (fig. 20). Since the change in number and volume of the hepatocytes is a common feature, the patterns are identical for all enzymes. During year 1 of life the patterns were curvilinear, with group C values deviating; at later ages the curves included group C values and there was, in addition, an increasing tendency toward a linear pattern.

The expected change in activity attributable to change in activity concentration for each of the enzymes also correlated to life expectancy; for three of the enzymes the relationship was inverse, for alkaline phosphatase, it was direct (fig. 21). In all cases, regardless of the direction of change, the smaller the change the greater the life expectancy.

DISCUSSION AND CONCLUSIONS

An association between life expectancy of the rat and the levels of hepatic enzyme activity has been established for each of the four enzymes investigated. When the age-associated enzyme activity patterns were modified by dietary means there were, within certain limitations, corresponding modifications in life span. The manner, however, in which each enzyme related to life expectancy was dependent upon the basis of expressing its level of activity.

Uniformly, correlations were obtained for all four enzymes, particularly at later age periods, when the levels of total liver activity in the different dietary groups were related to life expectancy. Apart from the effects of change in cell type or in distribution of an enzyme, the differences in enzyme activity level among dietary groups or at different ages may be considered as the result of concomitant changes in volume of the parenchymal cells, in number of cells or in "activity concentration." In a unit mass of liver a change in the volume of the hepatocyte was found to be compensated by a change in the number of hepatocytes; the parenchymal volume for that given mass must, therefore, be taken as remaining relatively constant. Despite the fact that there may be differences in activity on a cellular basis, a change in the level of activity per unit weight of liver (fresh weight, dry weight or fat-free) represents, in actuality, a change in activity concentration. The pattern of change with age in catalase activity concentration is diametrically opposite to that obtained for alkaline phosphatase; yet, with minor deviations, the activity levels of both enzymes, through the greater part of the rat's life span, related to life expectancy. In the course of an earlier study (11) in which rats were fed semipurified diets on an ad libitum basis, a similar correlation was found between life span and alkaline phosphatase activity per milligram of liver.

On a cellular basis, also, the activity level of each of the enzymes consistently correlated with life expectancy. Thus, an interrelationship can be said to exist among life span, diet and hepatic enzyme "activity content" at two levels of structural organization, the liver and the cell. The validity of this conclusion is further strengthened by the finding that the expected change in enzyme activity attributable directly to increase in number of hepatocytes per se, or to increase in hepatocytic volume, is also related to life expectancy.

The apparent absence of a correlation between activity concentration and life expectancy for two enzymes may be, in part, a masking effect of the changes in activity due to change in cell volume and in cell number. When the activity concentration was considered separately from that amount of activity attributable to change in parenchymal volume, the activity concentration of all four enzymes related to life expectancy.

The association among caloric restriction, life span and enzyme activity levels has been questioned by Barrows et al. (32–34). The lack of agreement with our con-

clusions may be due to the limited basis of expressing activity levels as well as the absence of mortality data for the population of rats in their study for such enzyme correlations. Positive correlations, however, were reported by Barrows' group (13) when the life span of the rotifer was related to enzyme activity levels for the total organism.

It is only when calender age was used as the basis of comparison that each dietary group gave rise to separate enzyme activity patterns. When parameters other than age were used as a basis of comparison, these discrete, multiple patterns disappeared. The level of activity of each of the enzymes formed a single and continuous slope when related to nitrogen content, number of hepatocytes, hepatocytic volume and body weight. Each of the latter, in turn, interrelated. These orderly progressions suggest that among the different dietary groups there may also be, for each of the enzymes, a singular pattern in the rate of synthesis and loss of enzymes. The differences between dietary groups at any age and the reversal in direction in change during the senescent phases of life appear to be an expression of differences in rate and level of biochemical and structural development attained.

Discrete, temporal displacements in the time of onset and in the incidence rate of certain age-associated diseases have also been found among these dietary groups (10, 12). Here, too, a single relationship becomes manifest between increase in risk to disease, particularly to spontaneous tumors, and increase in body weight, irrespective of age or of the diet imposed. These data reaffirm our earlier conclusion (12) proposed for the disease relationship that among populations of varying life expectancy, there is a remarkable uniformity in the sequence and in the proportion of time required for biochemical, structural and pathological events to occur.

The higher fat content of the liver and of the hepatocyte of rats whose intake was low in protein but high in calories does not completely explain the deviation in some of the enzyme values for this dietary group. An increase in the level of choline in the diet did have a beneficial effect and it is possible that higher levels of choline should have been provided earlier. At these later ages when the deviation in fat content was less evident, there were also fewer deviations in the values for this group in the enzyme activity-life expectancy arrays. Among the other four dietary groups whose protein-to-calorie ratio was not so severely imbalanced, the concentration of fat in the hepatocyte also changed but in a progressive and uniform manner; the increase in volume of the hepatocyte associated with increase in caloric intake was accompanied by significantly higher levels of concentration of fat. This increase in concentration, per se, may adversely affect the functional capacity of the hepatocyte, or it may be one of the consequences of altered functional activity resulting from the relative decrease in surface area of the cell as the cell volume increases.

The lability of hepatic enzymes, particularly to modification by dietary means, and its hormonal regulation has been impressively documented (35–43). The similarity in response to diet in the activity patterns of certain liver enzymes and in the growth rate of the rat has led to the suggestion that the levels of activity per unit weight of liver may be used as an index of the nutritive value of a diet or of specific dietary components (37, 38, 40, 41, 44). Fabry and Braun (44) have indicated that the changes in enzymatic level to changes in diet are not limited to the liver and are indicative of the preferential metabolism of the predominant metabolite. In the present study, however, it would appear that the progressive change with age in the activity level of the organ or the cell, for any of the dietary regimens, represents an adaptation to the change in requirements of a larger animal or a larger cell. This same phenomenon of enzyme adaptation is evident even in the late phases of life when there is loss in both body weight and cell volume. Rapid growth rates, structural or biochemical, are not commensurate with prolonged life span and reduced risk to age-associated diseases. Indeed, the dietary regimen which evoked the greatest rate of change with age was most detrimental and such animals had the shortest life expectancy and the greatest incidence and severity of disease. Conversely, through long-term caloric restriction, the

levels of any of the biochemical constituents as well as of cell and animal size, were more like that of the young rat, and the longest life span and the lowest incidence of disease were obtained.

Though only four, arbitrarily chosen, enzymes were studied in depth, they have similar patterns of change with age and show similar response to diet as that observed for a considerably larger number of systems investigated earlier (1–5). For relatively short periods of time, the small order of change in activity in any one enzyme may have little immediate, functional significance. Ultimately, over a longer time period, however, the accretion of such changes and those resulting from the progressive disproportion in the relative activities among the enzymes may have an additive effect with other intrinsic conditions that eventuate in the gross aberrations attendant with aging.

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