Response of the Liver to Prolonged Protein Depletion

I. LIVER WEIGHT, NITROGEN, AND DEOXYRIBONUCLEIC ACID

J. N. WILLIAMS, JR.
Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

Studies in the past of the effects of a complete protein deficiency on different components of the liver have the main drawback that in general only the initial stages of a protein deprivation were studied in any detail. In those studies in which several liver components were followed as a function of time of protein deprivation, the length of the studies was usually one month, which is one-third the time required for adult rats to become moribund from the effects of a complete protein deficiency. Pair-fed controls were generally not included in the studies to observe the changes due to the deficiency of protein per se without interference from the effects of partial inanition produced by the lowered food intake occurring during complete protein deprivation.

Addis and co-workers ('36, '40) were among the first to study in some detail the relationship between dietary protein and the protein content of the liver. They found that after feeding a protein-free diet for two days the liver of the rat loses more protein than any other organ. Elman and co-workers ('43) fed rats a protein-free diet for two weeks and found no change in the liver cell size although the cells showed a loss in stainable cytoplasm and the formation of large clear spaces filled with glycogen.

Kosterlitz[1] observed that, after 7 days of receiving a protein-free ration, female rats lost both stainable cytoplasm and mitochondria from the liver cells. Using female rats fed a protein-free diet for 28 days, Kosterlitz ('47) found that the most rapid losses of protein and nucleic acid occurred by the 4th day with a gradual loss thereafter for 24 days. Nucleic acids were determined as a combination of ribo- and deoxyribonucleic acids and thus the results could not be evaluated with respect to individual nucleic acids. Upon histological examination, the liver cells showed little or no decrease in size. Using male rats, Campbell and Kosterlitz[2] observed an increase in liver deoxyribonucleic acid (DNA) concentration after feeding a protein-free ration for 7 days.

Among the many contributions of a paper by Thomson et al. ('53), these authors reported that liver DNA concentration increased by 36% in adult male rats fed a protein-free ration for 15 days. The total DNA content of the liver, however, was unchanged, and the average DNA content of the nucleus did not change significantly, thus confirming the work of Campbell and Kosterlitz ('52). Ely and Ross ('51), however, reported that feeding a protein-free diet to young animals produced an 18% increase in the average DNA content of the nucleus. The main difference in the studies of Ely and Ross compared with those of Thomson et al. and Campbell et al. is that the first workers used considerably younger animals than the last two groups of workers.

Before beginning a detailed study of the relationship of dietary protein to liver composition and metabolism the following problems were considered: (1) what liver components should be studied; (2) how long a complete protein deficiency should be continued to observe the maximum changes that would take place; and (3) what end-point should be taken as the time to begin repletion in order to discover any

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[1] Kosterlitz, H. W. 1944 Effect of dietary protein on liver cytoplasm. Biochem. J., 38: XIVPC.

[2] Campbell, R. M., and H. W. Kosterlitz 1947 Ribonucleic acid as a constituent of labile liver cytoplasm. J. Physiol., *106*: 12 p.

irreversible changes that might have taken place. It was decided to follow the variables to be studied at fairly frequent time intervals after the introduction of the protein deficiency in adult male rats until a certain proportion of the animals died from the deficiency. Then a complete ration would be fed until weight gain of the formerly deficient group reached a maximum and leveled off. Thus the entire course of complete protein deficiency as well as full repletion would be followed. Pair-fed controls were necessary throughout the study to observe the changes due to the deficiency of protein per se without concomitant effects of partial inanition. A third group of animals, fed the complete ration ad libitum throughout the investigation, was included.

The present investigation may be divided into two parts: (1) a study of liver composition with respect to nitrogen and deoxyribonucleic acid; and (2) a detailed study of an essential liver enzyme system. Liver nitrogen was chosen as an example of a liver substance that would be expected to be directly influenced by dietary protein. The extent and the manner in which it is influenced by prolonged complete protein deficiency followed by repletion is unknown, however. Since the concentration of DNA per average liver cell nucleus is considered to be constant during protein deprivation in the adult male albino rat (Campbell and Kosterlitz, '52; Thomson et al., '53), measurement of DNA should give some information concerning the effects of prolonged complete protein deficiency on the number of cells per unit weight of liver and per total liver, changes in individual cell weight, and concentration of other constituents per cell. It should be emphasized, however, that the use of DNA concentration to express the concentration of different liver constituents per cell should be considered as an approximation since according to the results of Ely and Ross ('51), an 18% increase in DNA content per liver nucleus is possible in young rats fed a protein-free ration.

The results concerning liver composition with respect to nitrogen and deoxyribonucleic acid are presented in the present paper. The enzyme studies are presented in the succeeding paper (Williams, '61).

EXPERIMENTAL

Ninety-six adult male rats of the Sprague-Dawley strain, weighing from 165 to 180 gm, were fed a complete purified ration for 10 days to accustom them to the purified diet. The complete ration (diet R1) consisted, in per cent, of casein,[3] 20; DL-methionine, 0.3; corn oil, 5; Salts N plus molybdate (Fox and Briggs, '60), 6.5; glucose monohydrate,[4] 63.5; choline chloride, 0.2; *i*-inositol, 0.02; and water-soluble vitamin mix in glucose monohydrate (Fox et al., '55), 4.5. Fat-soluble vitamins were given separately to each rat in two drops of corn oil each week so that each rat received 0.97 mg of vitamin A acetate, 0.0007 mg of vitamin D_3, 4.9 mg of DL-α-tocopheryl acetate, and 0.42 mg of 2-methyl-1,4-naphthoquinone per week.

After the preliminary feeding period, the average weight of the rats was 208 gm. The rats were then separated into three groups. Group 1 was fed diet R1 less casein, which was replaced with glucose monohydrate; group 2 was fed diet R1 daily in the amount of the ration eaten by group 1; and group 3 was fed diet R1 ad libitum. The 0.3% of methionine was retained in the ration of group 1. After various time intervals, as indicated in the figures, 6 to 8 rats from group 1, three to 6 rats from group 2, and three to 5 rats from group 3 were sacrificed, the livers removed and weighed, and homogenates prepared from portions of each liver. The remainder of each liver was stored in the deepfreeze for DNA analysis. Nitrogen was determined on aliquots of the homogenates by direct nesslerization after digestion by the author's modification of the method of Koch and McMeekin ('24): Gum ghatti was omitted, the tubes were iced before addition of Nessler's reagent, the volumes of all reagents were scaled down to one-fourth, and the upper limit of nitrogen determined was 50 µg. DNA was determined on portions of the frozen livers by Schneider's extraction procedure (Schneider, '45) followed by color development with diphenylamine (Dische, '30).

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

[4] Cerelose, Corn Products Refining Company, New York.

On day 36, the first rat of group 1 died spontaneously; the second died on day 53; and three more on days 82 to 84. On day 83, 6 rats of the remaining 14 of that group became markedly edematous. Because of the frequency of spontaneous deaths and edema on days 82 to 84, the remaining rats of group 1 were shifted to diet R1 on day 84. Beginning on day 89, the rats of group 2 were pair-fed the amounts consumed by group 1 from day 84 on. The two groups were staggered this way to enable handling of enzyme measurements in the rats of group 1 within two days after repletion was begun.

Treatment of results. The analytical results are presented as nitrogen per milligram of fresh liver, DNA per milligram of fresh liver, weight of liver per 100 gm of body weight, and total calculated number of cells per liver. In order to simplify the figures as much as possible, all of the values for groups 1 and 2 have been calculated as percentage of the corresponding average for group 3. The absolute values for group 3 are presented in the lower part of each figure so that calculation of absolute values for the other groups can be made if desired.

The results have been analyzed using Student's t test to determine whether the differences between groups 1 and 2 and groups 2 and 3 for corresponding times are significant. Wherever P is less than 0.05 for group 1 versus 2 and group 2 versus 3, the point is encircled in the figure. In most cases P was less than 0.01.

In each figure a dotted line is drawn from the last point before repletion to the day repletion was begun since it is not known whether the values changed during that period or not. It is doubtful, however, that significant changes occurred during that period. An arrow denotes the day for each curve on which repletion was begun. Since only two animals could be handled satisfactorily in one day because of the time necessary for making the enzyme measurements reported in the succeeding paper, the points on the figures represent the averages for the animals sacrificed from the 5th day before to the 5th day after the indicated day.

RESULTS

Body weight. In figure 1 are presented the changes in body weight for the three groups of animals. By day 80 groups 1 and 2 had lost 45 and 20% of their initial body weight, respectively. Both groups rapidly responded to the repletion regimens and reached the weight of group 3 after about 50 days of repletion. Before repletion was begun, the rats of group 1 were thoroughly emaciated and almost moribund. The edema that developed in the animals of group 1 around day 80 was observed as a pouch mainly under the neck, as if 4 to 5 ml of fluid had been injected subcutaneously. The appearance of the edema was very similar to that shown in the photograph of an edematous rat presented by Alexander and Engel ('52) except that the subcutaneous fluid pouch was under the neck instead of the abdomen and thorax. The rats of group 2, although thin, highly active, and ravenous, were not emaciated nor edematous. By day 130 the rats of all three groups were identical in gross aspect.

Nitrogen per milligram of liver. In figure 2 are presented the results for nitrogen concentration per milligram of liver. In group 1 the concentration fell initially at a rapid rate, leveled off, and began to increase after 30 days. Within two days after pro-

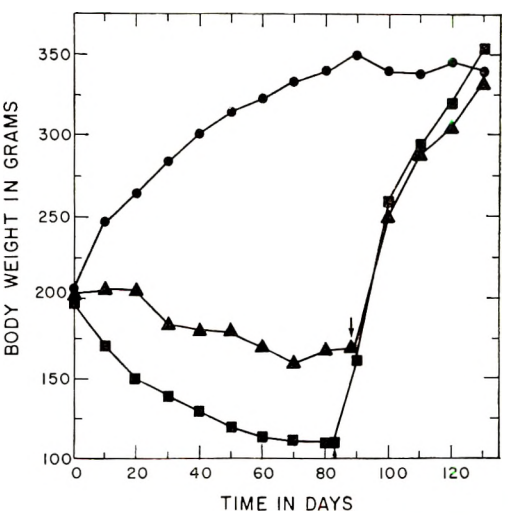

Fig. 1 Weight changes versus time of feeding for the protein-deficient rats (■), the pair-fed controls (▲), and the ad libitum-fed controls (●). Protein supplementation for the protein-deficient rats and food resupplementation for the pair-fed controls were begun at the arrows.

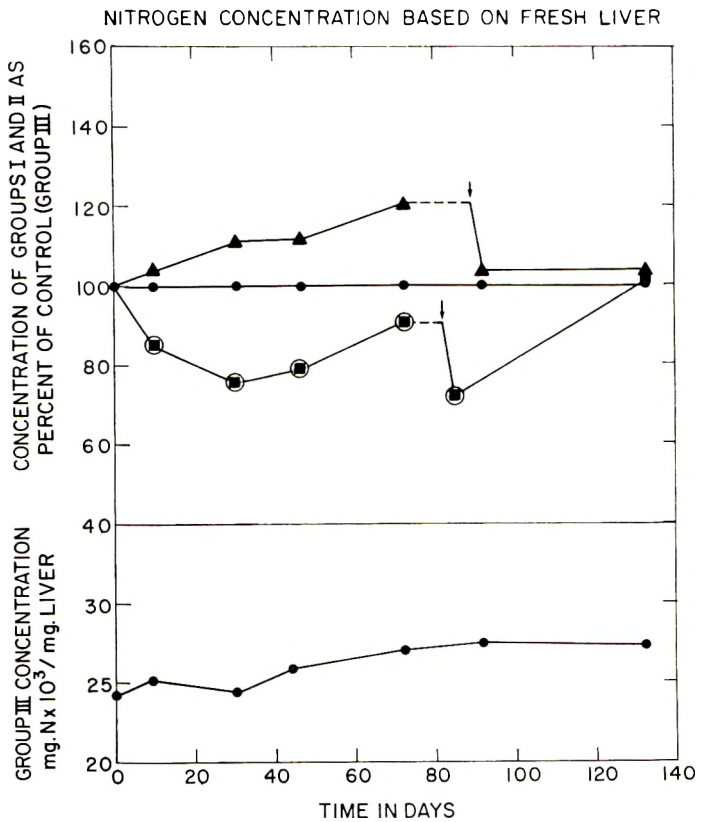

Fig. 2 Nitrogen concentration per milligram of liver versus days fed diet for the protein-deficient rats (■), the pair-fed controls (▲), and ad libitum-fed controls (●). Protein supplementation for the protein-deficient rats and food resupplementation for the pair-fed controls were begun at the arrows. The upper curves express the nitrogen concentration as percentage of the ad libitum-fed controls. The lower curve gives the actual values for liver nitrogen concentration of the ad libitum-fed controls. The points for statistically significant difference ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

tein repletion began, the concentration fell sharply to its lowest point; by 132 days it returned to normal. In the pair-fed controls (group 2), liver nitrogen concentration increased gradually throughout the first 72 days. When food was restored, the nitrogen concentration returned to normal within three days and remained there. It is quite likely that the lowered nitrogen concentration per milligram of liver up to 30 days is a result of increased deposition of glycogen and lipid. Kosterlitz ('47) found that both glycogen and lipid concentrations per unit weight of liver increased during protein deficiency. The increase in nitrogen concentration per milligram of liver between 30 and 72 days, however, is probably a reflection of the effects of inanition superimposed upon those of protein deficiency. Under those conditions it might be assumed that stored glycogen and lipid would be lost from the liver cells to be used for energy, thus increasing the relative amount of nitrogen per unit weight of liver.

DNA per milligram of liver. Thomson et al. ('53) observed that the DNA content of the nuclei of adult male rat liver cells is unaffected by sex, strain, body weight, fasting, or a nonprotein diet. Campbell and Kosterlitz ('52) also noted no change in the amount of DNA per nucleus after feeding adult male rats a protein-free ration for 28 days. Thus, estimation of DNA per milligram of liver should give an

estimate of the average number of cells per milligram of liver assuming that no marked changes in the number of nuclei per cell or of the amount of DNA per nucleus occurs. In younger male rats (130 to 160 gm), however, Ely and Ross ('51) observed an 18% increase in the amount of DNA per nucleus under conditions of complete protein deprivation. In the present studies the initial weight of the rats was about the same as that of the rats used by Thomson et al. ('53). In the discussion of the present results, however, the possibility that the amount of DNA per nucleus, and thus per cell, increased slightly during the production of the protein deficiency should be kept in mind. As will be seen later, however, an 18% increase in the DNA content of the nucleus would not be great enough to affect the interpretation of the results.

From zero time to 10 days, group 1 showed an increase in DNA at a significantly faster rate than group 2 (fig. 3). From then on, however, both groups gave the same concentration of DNA per milligram of liver. The plateau at 30 days, followed by a further rise after 46 days, is a reflection of a decrease in DNA per milligram of liver in group 3. This decrease in group 3, from 46 to 72 days, appears to be significant and may be a reflection of the leveling off of the growth curve for that group (fig. 1). When groups 1 and 2 were supplemented at the times indicated by the

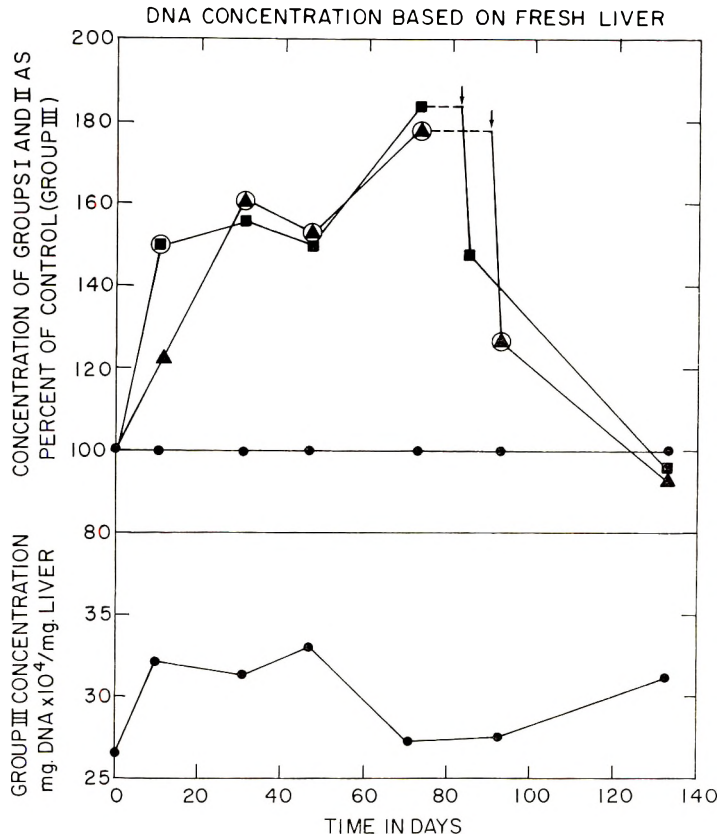

Fig. 3 DNA concentration per milligram of liver versus days fed diet for the protein-deficient rats (■), the pair-fed controls (▲), and the ad libitum-fed controls (●). Protein supplementation for the protein-deficient rats and food resupplementation for the pair-fed controls were begun at the arrows. The upper curves express the DNA concentration as percentage of the ad libitum-fed controls. The lower curve gives the actual values for DNA concentration of the ad libitum-fed controls. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

arrows, a rapid drop in DNA concentration occurred, followed by a return to normal by 132 days.

If one considers that the amount of DNA per milligram of liver is a measure of the number of cells per milligram of liver, then after 10 days of protein depletion group 1 had about 1.5 times as many cells per milligram of liver as group 3 and about 1.25 times as many cells as group 2. From 30 to 46 days the number of cells per milligram of liver for group 2 reached the same value as for group 1. Both groups then had 1.5 to 1.6 times as many cells per milligram of liver as group 3. This value increased to 1.8 at 72 days. After supplementation, the number of cells per milligram of liver for groups 1 and 2 fell to the same respective values as for 10 days. By 132 days the number of cells per milligram of liver was the same for all three groups. To allow for the increase in number of cells per unit weight of liver during the depletion period in groups 1 and 2, the cells simply must have become lighter in weight. The initial marked loss in weight of the cells of group 1 can probably be explained partially on the basis of a loss of the more dense cytoplasmic protein and substitution for it by less dense neutral lipid. Glycogen also takes the place of some of the cytoplasmic protein (Elman, '43). Then beyond 30 days, when the effects of inanition became more and more marked, glycogen and lipid were probably gradually lost until at 72 days the cells were even lighter in weight than at 30 days. Except for the values at 10 days and the first period after repletion was begun, the average liver cell weight of group 1 was the same as that of group 2, which was approximately 60% of normal.

Nitrogen per milligram of DNA. Since the amount of DNA per unit weight of liver is proportional to the number of cells per unit weight of liver under the conditions of the present studies, with the limitations discussed in the preceding section, calculation of the amount of nitrogen per milligram of DNA should give a measure of the nitrogen per liver cell. These results are presented in figure 4. Thus the amount of nitrogen per cell of group 1 fell to 57% of normal after 10 days and varied between 48 and 53% of normal from 30 to 84 days. No measurable increase in nitrogen content of the cells was observed after two days of repletion. For group 2 the nitrogen per cell fell to 84% of normal after 10 days and varied between 68 and 73% of normal from 30 to 72 days. After three days of partial refeeding (89 to 92 days) the figure rose to 82%. Thus in both groups 1 and 2 the maximum loss of cellular nitrogen was reached by 30 days. By 132 days the nitrogen per cell returned to normal for both groups 1 and 2. Thus, more nitrogen was lost per cell from group 1 than from group 2 throughout the experiment, even though the average weight of the liver cells from the two groups was about the same.

Hence, the weight of the liver cell as measured by DNA per milligram of liver (fig. 3) appears to be influenced more strongly by food intake than by protein in the diet. On the other hand, while lowered food intake causes significant loss in the nitrogen content of the cell, the lack of dietary protein causes a still further loss. This effect of partial inanition on the loss of nitrogen from the cell would not have been seen if nitrogen concentration per milligram of liver only (fig. 2) had been measured.

Liver weight per 100 gm of rat. As shown in figure 5, striking differences in liver size with respect to body weight occurred in group 1 as compared with groups 2 and 3. The liver weight per 100 gm of body weight of group 1 increased almost linearly for 72 days. When protein supplementation was begun, the liver weight increased much more rapidly than body weight. By 132 days a decrease in liver weight with respect to body weight occurred in group 1, but it was still significantly higher than that of group 2. Thus, this is a change that did not return to normal even after 48 days of resupplementation with protein. In group 2 liver weight with respect to body weight was constant and slightly below that of group 3 throughout until ad libitum feeding was begun. The ratio of liver weight to body weight of group 1 then rapidly returned to normal.

Calculated number of cells per liver. Using the value of Thomson et al. ('53) for DNA per liver cell nucleus (9.56×10^{-12} gm of DNA per nucleus), the total number of cells for the livers of each group of rats may be calculated from the total liver

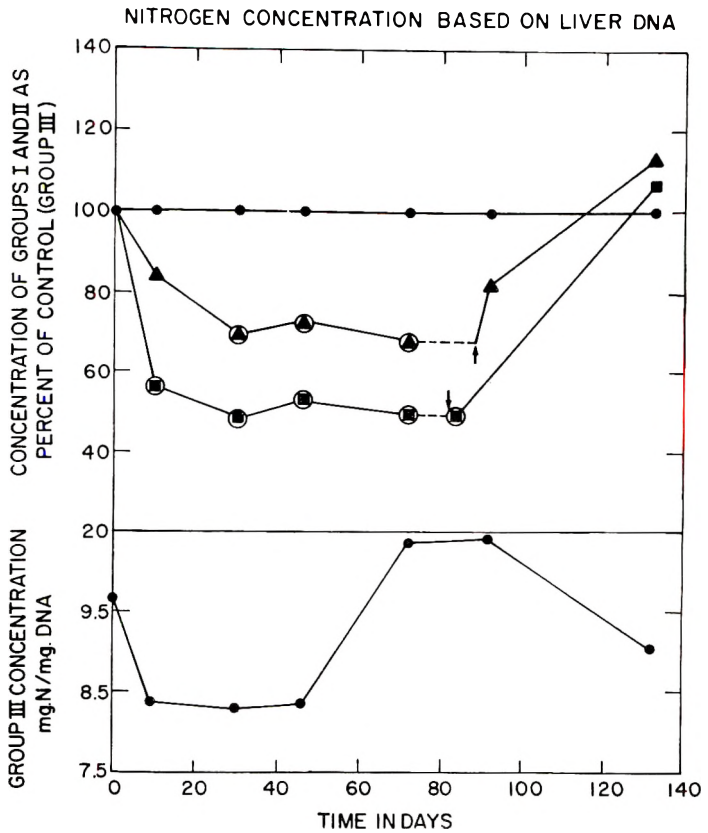

Fig. 4 Nitrogen concentration per milligram of DNA versus days fed diet for the protein-deficient rats (■), the pair-fed controls (▲), and the ad libitum-fed controls (●). Protein supplementation for the protein-deficient rats and food resupplementation for the pair-fed controls were begun at the arrows. The upper curves express the nitrogen concentration per milligram of DNA as percentage of the ad libitum-fed controls. The lower curve gives the actual values for nitrogen concentration per milligram of DNA of the ad libitum-fed controls. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

DNA. This calculation has been made and the results are presented in figure 6. It should be noted that the total calculated cells of the ad libitum-fed control group (group 3) increased over the interval from zero to 10 days and leveled off thereafter. Using the absolute values for total calculated cells per liver for group 3, it can be shown that this value for groups 1 and 2 also increased from day zero to 10, but to a less extent than for group 3. Thereafter the total calculated liver cells of both groups 1 and 2 decreased continuously through day 72. Thus the results from day 10 until day 72 indicate that an actual loss in total liver cells occurred in groups 1 and 2 during that time interval. If the DNA content of the liver nucleus increased by 18% in the present experiments, as Ely and Ross ('51) found for younger animals, this decrease in total calculated cells during the protein deficiency would have been even greater than if a constant value for DNA per nucleus is assumed. Groups 1 and 2 followed each other very closely throughout the depletion period with respect to calculated number of cells per liver. When protein was given to group 1 at 82 days, however, a sharp rise in total calculated cells per liver occurred, which was divergent from the response of group 2 to food resupplementation. After 132 days the total calculated number of liver cells of group 1 was still significantly

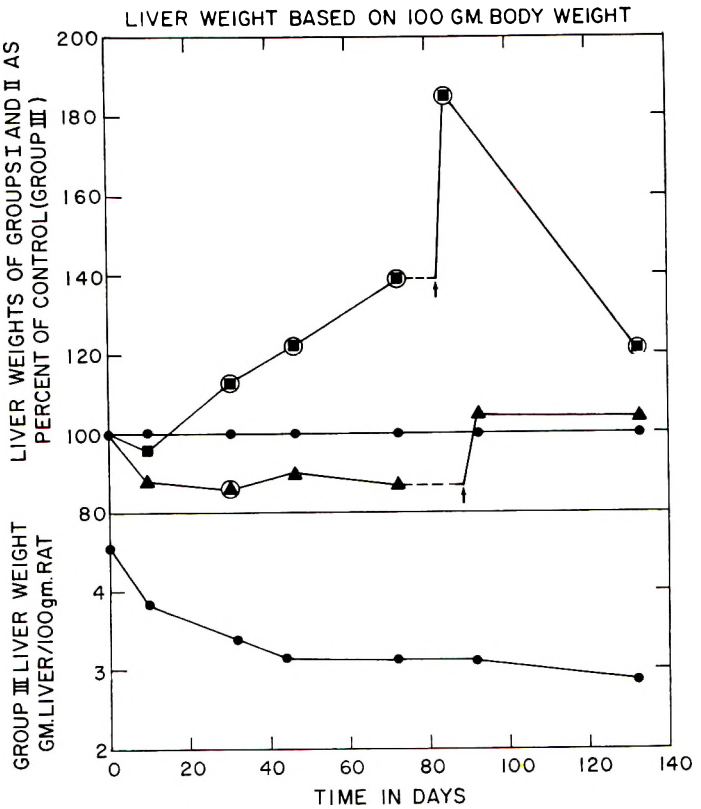

Fig. 5 Liver weight per 100 gm of body weight versus days fed diet for the protein-deficient rats (■), the pair-fed controls (▲), and the ad libitum-fed controls (●). Protein supplementation for the protein-deficient rats and food resupplementation for the pair-fed controls were begun at the arrows. The upper curves express the liver weight per 100 gm of body weight as percentage of the ad libitum-fed controls. The lower curve gives the actual values for liver weight per 100 gm of body weight of the ad libitum-fed controls. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

higher than that of the pair-fed controls. The production of DNA approximately equivalent to one billion liver cells occurred within two days when protein was fed back to the protein depleted rats. Thus, although the livers of groups 1 and 2 appear to follow the same path of degeneration during the depletion period, resupplementation of these groups brings out a marked difference with respect to their readiness for production of new liver cells.

DISCUSSION

The results presented point out sharply the absolute need for including pair-fed controls in studies concerning the effects of a complete protein deficiency upon liver components. With the possible exception of liver weight per 100 gm of body weight, the partial inanition induced by the lowered food intake during the protein deficiency produced changes in the liver that were significantly different from the ad libitum-fed controls. These changes were sometimes different from and sometimes the same as those produced by the protein deficiency per se. Thus the complete protein deficiency produced a decrease in liver nitrogen concentration, whereas partial inanition produced an increase in the same variable. With the exception of the first 10-day period, total protein deficiency and

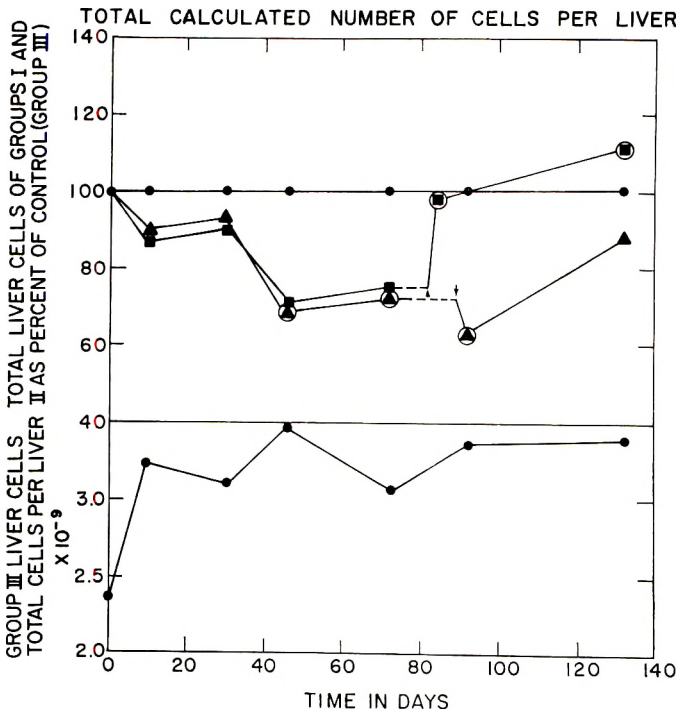

Fig. 6 Calculated number of cells per liver versus days fed diet for the protein-deficient rats (■), the pair-fed controls (▲), and the ad libitum-fed controls (●). See text for method of calculating cell number from total liver DNA. Protein supplementation for the protein-deficient rats and food resupplementation for the pair-fed controls were begun at the arrows. The upper curves express the number of cells per liver as percentage of the ad libitum-fed controls. The lower curve gives the actual values for number of cells per liver of the ad libitum-fed controls. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

partial inanition produced the same increase in DNA concentration per milligram of liver, followed by the same rate of return to normal upon supplementation. With respect to total calculated cells per liver both complete protein deficiency and partial inanition caused the same rate of loss of liver cells for 72 days; however, as pointed out earlier, regeneration of the livers of the two groups followed widely different paths upon repletion.

Changes in most of the variables studied continued to take place throughout the entire protein depletion period. Thus, liver nitrogen concentration per milligram of liver reached a minimum in the protein deficient group after 30 days. Thereafter it started to increase and continued so throughout the experiment indicating that this variable was still changing even at time of death from the protein deficiency. Liver weight per 100 gm of rat continued to rise throughout the deficiency period in group 1. Even after 50 days of protein repletion, a return to normal was not reached. The ratio of liver nitrogen to DNA, however, reached its maximum change after 30 days and did not change further.

In studies on the protein nutrition and metabolism of human beings, the slow rate at which certain variables reach their maximum changes must be carefully considered. If the rate at which changes in constituents of the body occur because of changes in the diet is a function of the life span of the animal rather than of time itself, a period of 30 days in the rat would be equivalent to slightly over three years in the human. The above observations are of

importance in planning and interpreting studies of protein metabolism of human beings.

In group 1, the ratio of liver weight to body weight increased markedly, whereas in group 2 liver weight was lost at about the same rate as body weight (fig. 5). When protein was refed to group 1 for two days, liver weight at first increased much more rapidly than body weight. This increase appeared to be due to a rapid synthesis of approximately one billion new cells per liver (fig. 6) assuming that no significant change in DNA per cell occurred. When the amount of nitrogen per cell is considered, these new cells contained the same amount of nitrogen as before repletion began (fig. 4), although the weight of the cells increased to that seen after 30 days of protein depletion (fig. 3). Thus, this increase in cell weight was probably due to redeposition of glycogen and lipid. This interpretation is supported by the fact that nitrogen concentration per unit weight of liver (fig. 2) decreased initially after protein resupplementation was begun, indicating that a dilution of nitrogen per unit weight of liver occurred. Hence, immediately after repletion is begun, the protein-depleted rat appears to produce a great many new nitrogen-poor liver cells at an extremely rapid rate rather than refilling the ones it has with protein.

SUMMARY

A study of changes in nitrogen and deoxyribonucleic acid (DNA) content of rat liver cells associated with prolonged dietary protein deprivation followed by repletion has been presented. Pair-fed and ad libitum-fed controls were included throughout. The following conclusions were reached. (1) The number of cells per unit weight of liver as measured by DNA concentration increased to 1.5 to 1.8 times normal during protein deficiency. The pair-fed controls showed the same increase. (2) The amount of nitrogen per cell of the protein deficient group dropped to 68 to 74% of that of the *pair-fed controls*. The nitrogen per cell of the pair-fed controls fell to 68 to 84% of the *normal ad libitum controls*. (3) These changes began to show up by 10 days although the maxima were not reached until at least 30 days and in some cases until the animals became almost moribund at 72 days. (4) The ratio of liver weight to body weight increased markedly in the protein deficient rats, whereas in the pair-fed controls it remained about the same as in the ad libitum-fed controls. (5) Although protein repletion returned the total calculated number of liver cells to the same level as that of the ad libitum-fed controls within two days, the state of the liver cells with respect to nitrogen content per cell and number of cells per unit weight of liver was the same as after 30 days of protein deficiency. Body weight did not return to normal until 48 days after repletion was begun. (6) After 48 days of repletion, nitrogen per milligram of liver, nitrogen per cell, and number of cells per milligram of liver of the protein-deficient group and the pair-fed controls returned to normal. However, liver weight per 100 gm of rat and total calculated number of cells per liver were significantly higher than those of the pair-fed controls after 48 days of repletion.

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Response of the Liver to Prolonged Protein Depletion

II. THE SUCCINIC OXIDASE SYSTEM AND ITS COMPONENT ENZYMES

J. N. WILLIAMS, JR.
Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

Cell mitochondria contain those enzyme systems which are necessary for the oxidation of foodstuffs and for storage as high energy phosphate of the energy released by the oxidations. Each mitochondrial oxidative system is composed of an initial dehydrogenase linked to a series of associated electron-transporting enzymes. Since all enzymes are proteins, the deprivation of dietary protein should influence profoundly the ability of animals to synthesize these enzymes. During protein deficiency, if these oxidative enzymes cannot be made, utilization of food with respect to storage of energy as high energy phosphate may not be carried out normally. Thus, the energy necessary for various processes which require high energy phosphate would not be available. Even if the animal attempted to conserve amino acids in vital organs or was able to transport amino acids from outlying organs to more vital ones, they could not be resynthesized into proteins and nucleic acids, for example, unless high energy phosphate were available. Other processes requiring high energy phosphate, such as muscular activity, transport of ions across membranes and nerve cell activity, would be expected to be strongly affected by improper maintenance of high energy phosphate.

Succinic oxidase is an example of a mitochondrial system whose component enzymes have been fairly well characterized. This system is composed of the initial enzyme succinic dehydrogenase, followed by cytochrome *b*, cytochrome c_1, cytochrome c, and cytochrome oxidase. It is not known to what extent the succinic oxidase system is compartmentalized in the mitochondrion or to what extent the cytochrome chain is shared by succinic dehydrogenase and other dehydrogenases. It is likely, however, that any changes in cytochrome components as measured in the whole homogenate would reflect a change in the activities of those components throughout the mitochondrion, whether succinic oxidase is localized as a unit in the mitochondrion or not.

A study of the complete succinic oxidase system and its individual components, as a model mitochondrial system, in a protein deficiency should give information on the activity of the over-all system as it is influenced by dietary protein. The study of the individual components should show which component, if any, controls the activity of the system and whether all components are influenced in the same way by protein deficiency. The latter observations would be particularly important in relating those components most closely allied to oxidative phosphorylation (succinic dehydrogenase and cytochrome oxidase) to the maintenance of high energy phosphate during protein deficiency.

EXPERIMENTAL

The plan of the experiment, care of the animals, and preparation of the diets were presented in detail in the preceding paper (Williams, '61). The present studies were performed in the same animals used in the preceding study of liver weight, nitrogen, and deoxyribonucleic acid (DNA) in protein deprivation. Briefly, adult male rats were separated into three groups. Group 1 was fed diet R1 less casein; group 2, diet R1, pair-fed with group 1; and group 3, diet

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R1 ad libitum. At various time intervals rats from each group were sacrificed and the enzyme determinations carried out.

Enzyme assays. On the day the animals were decapitated, the livers were removed and weighed. A part of the liver was used in preparing a 20% homogenate in 0.25 M sucrose. From this homogenate 5, 1, and 0.1% homogenates were prepared by appropriate dilutions with 0.25 M sucrose. All homogenates were kept on ice until used in the enzyme assays. The remainder of each liver was stored in a deepfreeze for direct analysis for cytochrome c. All enzyme assays were run individually for each animal. Because of time limitations only two rats could be handled in one day.

Succinic oxidase was assayed by the method of Potter (Umbreit et al., '59). Succinic dehydrogenase was determined by a modification of a system reported by Singer and Kearney (Glick, '56). Since whole liver homogenates were used in the present experiment, their system had to be modified and checked for linearity of activity versus liver concentration. Also it had to be shown that activity of the dehydrogenase was equal to or greater than activity of succinic oxidase in an equivalent system, as suggested by Giudetta and Singer ('59). The following system met these requirements: to the main compartment of duplicate Warburg flasks were added 1.1 ml of 0.25 M sucrose, 0.5 ml of 0.3 M sodium phosphate (pH 7.6), 0.3 ml of 0.01 M KCN (adjusted to pH 7.6 just before use), and 0.4 ml of 20% liver homogenate in 0.25 M sucrose (added last); to the side arm of each flask were added 0.2 ml of 1% phenazine methosulfate [1] and 0.3 ml of water or 0.2 M sodium succinate (pH 7.6); water was added to each side arm to make the final side arm volume 0.7 ml. After adding the homogenate, the flasks were placed on the bath at 37°C, the stopcocks were closed to prevent escape of cyanide, and the flasks shaken for 7 minutes. The stopcocks were opened, the manometers quickly adjusted to 150, and the stopcocks reclosed. Readings were taken at two, 4, and 6 minutes. The oxygen uptake over the second two-minute interval was taken as succinic dehydrogenase activity. Beyond 5 minutes the activity rapidly leveled off.

Since no direct assay for cytochromes *b* and c_1 in whole liver homogenates exists, the flow of electrons through these components was estimated by titrating out succinate-cytochrome c reductase activity with antimycin A. Present information indicates that the latter inhibits the respiratory chain between cytochromes *b* and c_1. This assay was performed as follows. To a series of 14 test tubes were added 0.5 ml of 0.1 M sodium phosphate (pH 7.4), 0.6 ml of 0.25 M sucrose, and 0.2 ml of 0.06 M KCN (adjusted to pH 7.4 just before use). To alternate tubes beginning with tube 2 was added 0.2 ml of 0.3 M sodium succinate (pH 7.4); to the other tubes was added 0.2 ml of water. Antimycin A in ethanol (0.08 μg/ml ethanol) was added as follows: tubes 1 and 2, none; 3 and 4, 0.02 ml; 5 and 6, 0.04 ml; 7 and 8, 0.05 ml; 9 and 10, 0.06 ml; 11 and 12, 0.08 ml; 13 and 14, 0.1 ml. Ethanol was added to the tubes to make the final volume of ethanol 0.1 ml. The contents of each pair of tubes were transferred to cuvettes, 0.5 ml of 0.1% homogenate was added, and the mixtures were incubated for one minute. Then 1 ml 5×10^{-4} M cytochrome *c* (horse heart, type III)[2] was added and readings were taken of the second cuvette (+succinate) versus the first (−succinate) at 550 mμ at 30-second intervals for two mintues. The interval from 30 to 90 seconds was taken as succinate-cytochrome *c* reductase activity. The activity for each level of antimycin A was plotted against inhibitor concentration and a smooth curve drawn through the points. The antimycin A concentration corresponding to one-half the maximal inhibition was taken as the antimycin A-sensitive activity of the homogenate.

The results for both succinic dehydrogenase and antimycin A-sensitive activity were quite reproducible. The former assay was reproducible within ± 5% and the latter by ± 4% using the same liver homogenate. These values represent the average deviation from the mean encountered by the author.

Cytochrome *c* was estimated by the method of Rosenthal and Drabkin ('43).

[1] Sigma Chemical Company, St. Louis, Missouri.

[2] Sigma Chemical Company.

Cytochrome oxidase was determined by the method of Potter (Umbreit et al., '59).

RESULTS

The results are expressed as activity or concentration per milligram of fresh liver, per milligram of nitrogen, and per milligram of DNA. Calculation of the results as per milligram of fresh liver indicates how the enzymes vary with other liver components, such as nitrogen, water, and lipids. Calculation as per milligram of nitrogen indicates how the enzymes vary with the total protein in the cell. Calculation as per milligram of DNA indicates how the enyzmes vary per cell, regardless of the size of the cell, since the DNA content of the average liver cell is constant (Thomson et al., '53). Each point in the figures represents an average of 5 to 8 animals for group 1, three to 6 for group 2, and three to 5 for group 3. Since only two rats could be handled in one day, each point in the figure represents an average of values for that group spread over a 10-day interval. For example, a point at 30 days is the average of values spread from 25 to 35 days. The differences between the points for groups 1 and 2 and for 2 and 3 have been analyzed using Student's t test. If the difference is significant ($P < 0.05$), the point is encircled in the figures. To

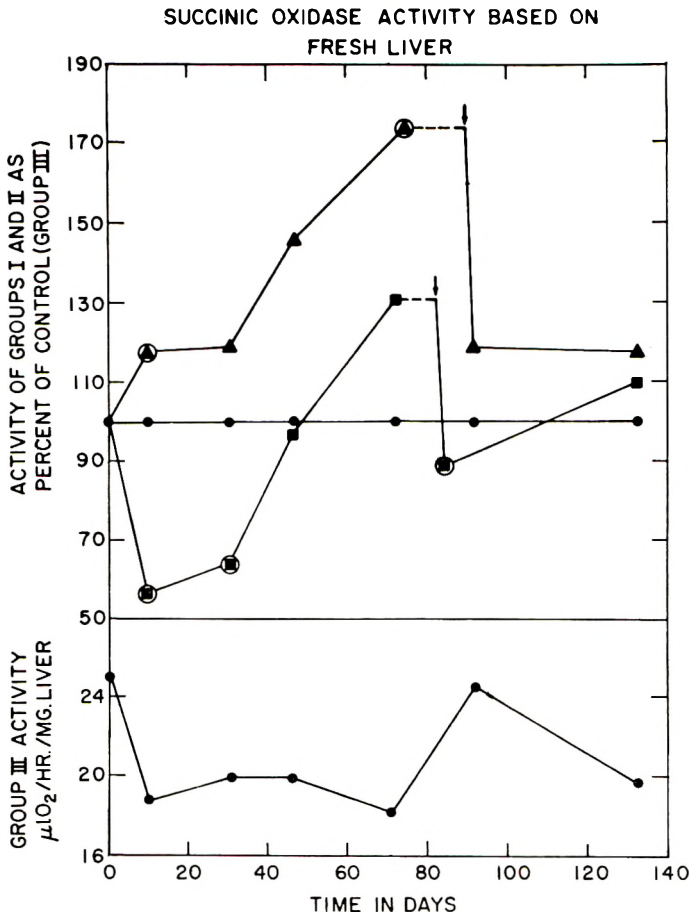

Fig. 1 Succinic oxidase activity per milligram of fresh liver versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

simplify the figures, the points for groups 1 and 2 are expressed as percentage of the ad libitum-fed controls. The absolute enzyme levels for group 3 are presented in the lower part of each figure.

An arrow in the figures indicates the day on which repletion was begun; this is supplementation of group 1 with diet R1 ad libitum and of group 2 with an amount of food equal to that eaten by group 1 after repletion was begun. Supplementation of group 2 follows that of group 1 by 7 days to allow the analyses for group 1 to be run as rapidly as possible after repletion was begun.

Succinic oxidase activity (SO). The results for the complete enzyme system are presented in figures 1 to 3. Per milligram of liver activity of group 1 dropped initially to about one-half normal. After 30 days an

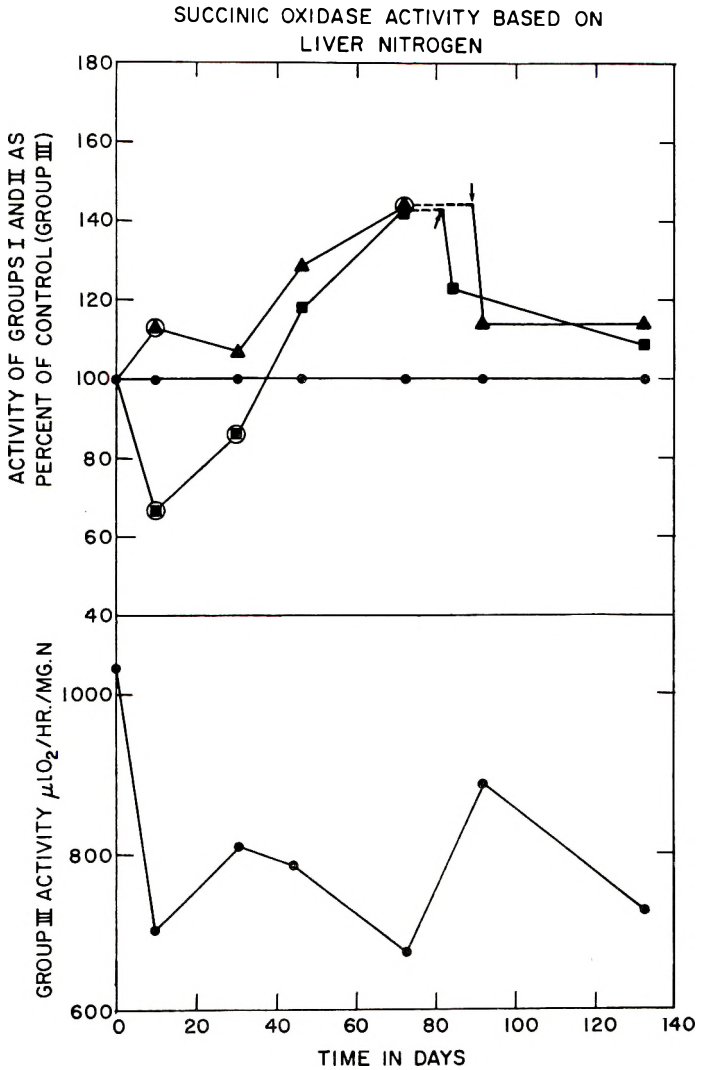

Fig. 2 Succinic oxidase activity per milligram of liver nitrogen versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

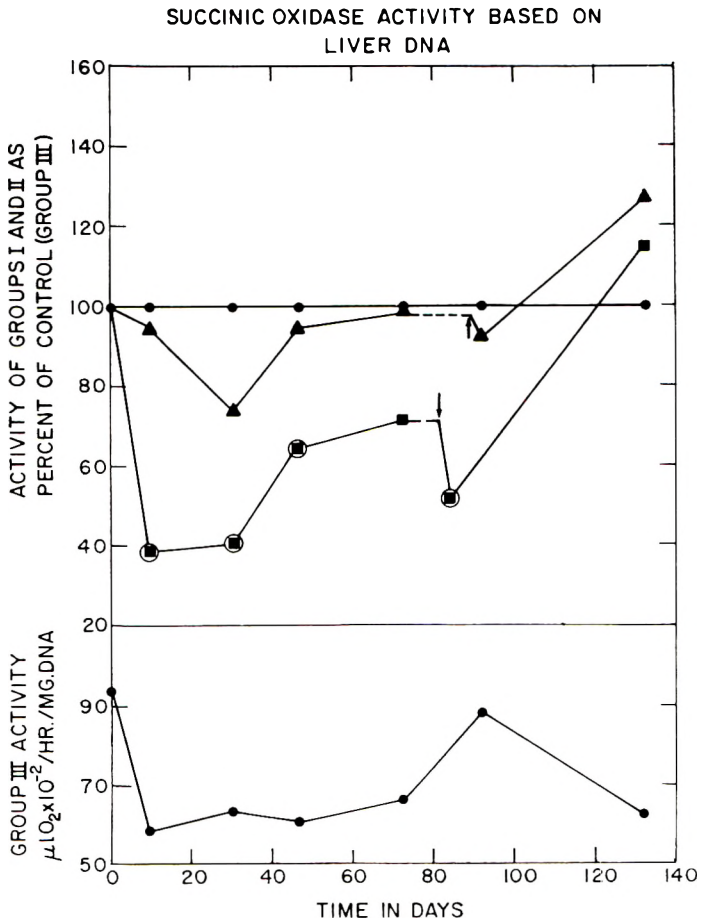

Fig. 3 Succinic oxidase activity per milligram of liver DNA versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

increase in activity began which by the 72nd day exceeded that of the ad libitum-fed controls. The deficiency was at its terminal stages at 72 days so that it could not be continued further. Within two days after repletion, SO per milligram of liver returned to normal and continued there. The pair-fed controls showed an increase in activity per milligram of liver throughout the initial periods. When supplementation was begun, an immediate drop to normal activity occurred. The increase of SO per milligram of liver in group 2 during the depletion period can probably be accounted for by a concentrating effect within the unit weight of liver because of the markedly lowered food intake. This means that other substances were lost from the liver whereas SO was retained. This explanation is upheld by the results for DNA per milligram of liver in the preceding paper (Williams, '61). Those results indicated that the number of cells per milligram of liver increased to 180% of normal by 72 days in the pair-fed controls. This is the same extent to which SO per milligram of liver increased in this group. Thus, the initial drop in the enzyme system per milligram of liver at 10 days in group 1 is the maximum effect that the lack of dietary protein per se had on the system. Thereafter, the effects of partial inanition come

into play to cause an apparent increase in activity as shown by the parallel increase in groups 1 and 2.

When compared with the total proteins in the cell (fig. 2) SO was reduced by the 10th day in group 1 to 67% of normal. It then increased thereafter to the same level as that of the pair-fed controls at 72 days. Following repletion, the SO per milligram of nitrogen of group 1 returned rapidly towards normal. The SO of group 2 followed the same trends per milligram of nitrogen as for per milligram of liver. Thus in group 1 at 10 days and 30 days SO was lost at a faster rate than other liver proteins although the rate had begun to decrease by 30 days. Beyond 30 days, however, other liver protein was lost at a faster rate than the enzyme system, until at 72 days the ratio of activity to liver nitrogen was the same for group 1 and its pair-fed controls.

The results of SO per milligram of DNA are presented in figure 3. Since DNA is fairly constant per cell, these results are proportional to activity per cell. (See preceding paper [Williams, '61] for a discus-

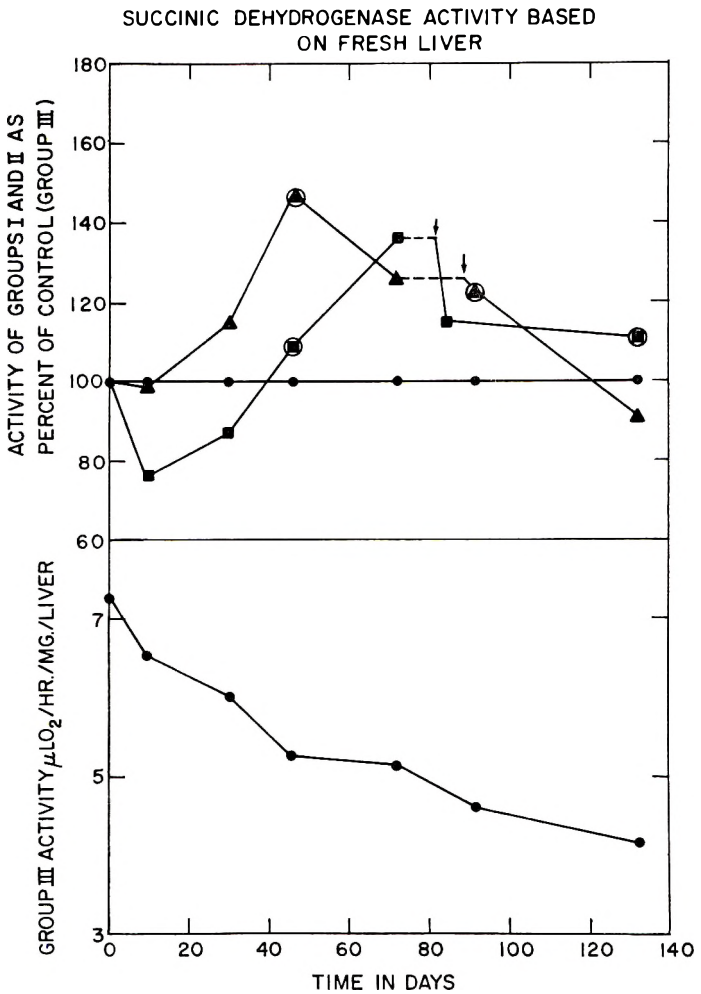

Fig. 4 Succinic dehydrogenase activity per milligram of fresh liver versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

sion of the constancy of DNA per cell under the conditions of the present studies.) After 10 days, the activity per milligram of DNA in group 1 fell to 40% of that of the ad libitum-fed controls. This was followed by an increase in activity up to 70% of normal at 72 days. In group 2 little change occurred in activity per cell; the apparent drop at 30 days is not statistically significant. Thus, although the number of cells per milligram of liver (see preceding paper) increased to 180% of normal in the pair-fed controls, the amount of SO per cell remained the same. This accounts for the continual rise in SO per milligram of liver for group 2: the concentration per cell remained the same while the number of cells per milligram of liver increased. In group 1 apparent synthesis of the enzyme system occurred between 30 and 46 days even though the animals were severely depleted of protein. This conclusion is upheld by the fact that the activity per milligram of DNA increased over that period, whereas the weight of each cell did not change. Following repletion, the SO per milligram

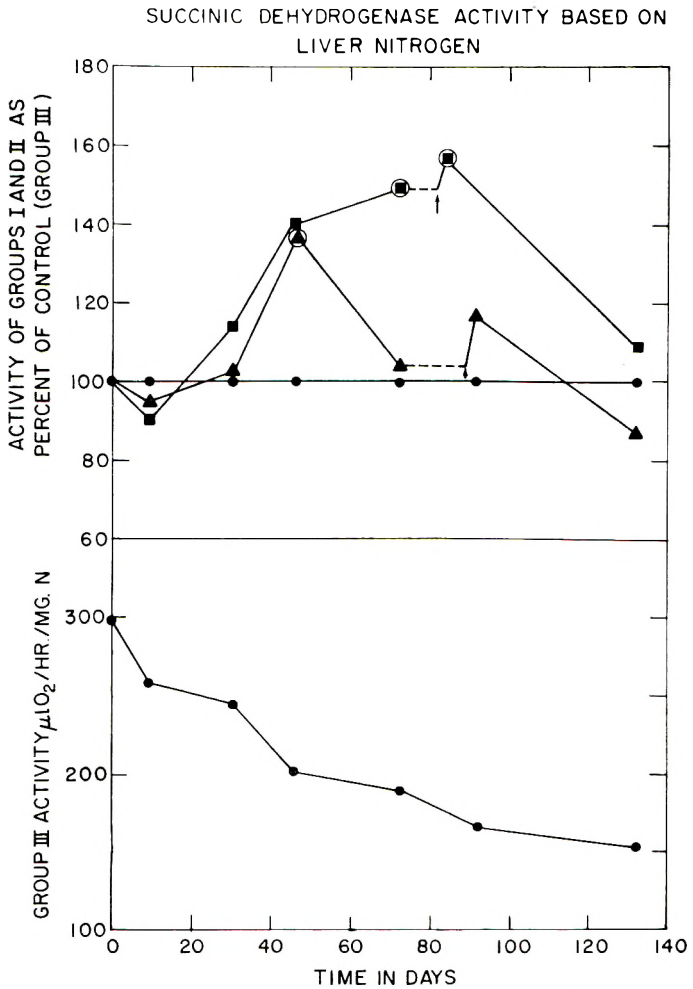

Fig. 5 Succinic dehydrogenase activity per milligram of liver nitrogen versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

of DNA in group 1 dropped and then returned to normal by 132 days.

Succinic dehydrogenase activity (SD). The results for the initial enzyme of the succinic oxidase system, succinic dehydrogenase, are presented in figures 4 to 6.[3] Based on fresh liver weight (fig. 4) the SD of group 1 followed the same pattern as the SO for that group. There was an initial drop at 10 days followed by an increase to above normal activity. The initial decrease in SD per milligram of liver, however, was not as great as the corresponding drop in SO per milligram of liver at 10 days. Following protein repletion, SD per milligram of liver decreased towards normal but even after 132 days it was still significantly higher than that of the pair-fed controls. The pair-fed controls showed an increase in activity for 46 days, followed by a decline at 72 days. Following repletion, the activity dropped only slightly at first. It returned to normal by 132 days, however.

With respect to other liver protein (fig. 5) the SD of groups 1 and 2 increased at the same rate for 46 days. Then the SD per

[3] See Addendum following text.

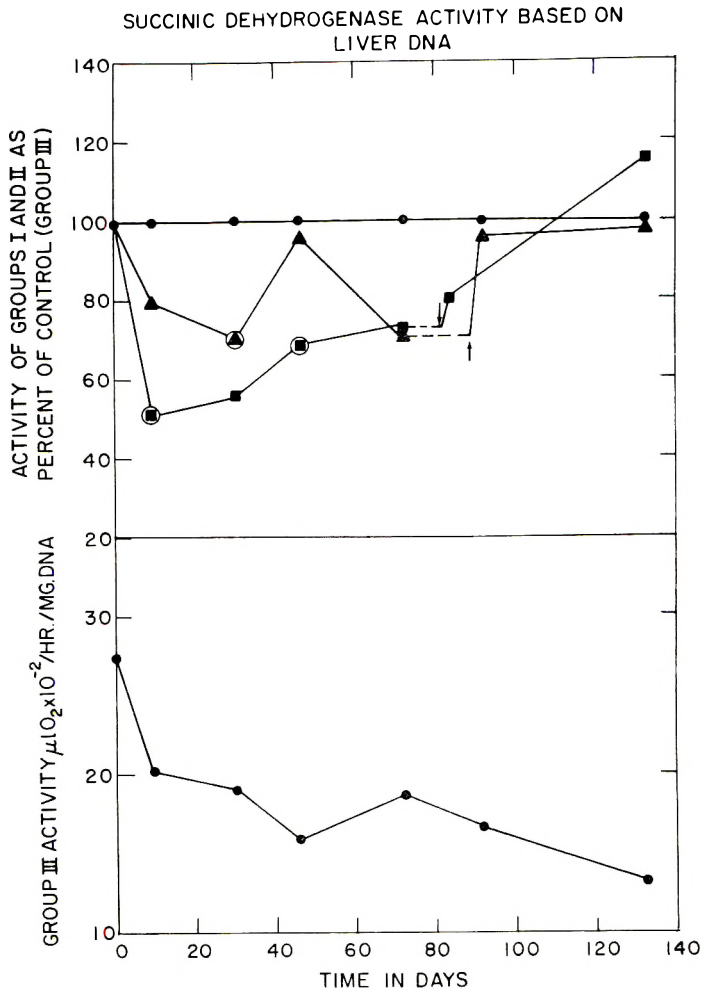

Fig. 6 Succinic dehydrogenase activity per milligram of liver DNA versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

milligram of nitrogen of pair-fed controls returned suddenly to normal while that of the protein-deficient group continued to rise. This behavior of the pair-fed controls is the most unusual among all the enzymes studied. At present there is no explanation for it, since no sudden upswing of liver nitrogen concentration, either per milligram of liver or per cell occurred at this point (see the preceding paper [Williams, '61]). Upon supplementation, the SD per milligram of nitrogen of both groups increased slightly over other liver proteins and then returned to normal.

Succinic dehydrogenase activity per milligram of DNA is presented in figure 6. As with SO per milligram of DNA in group 1, an initial loss in SD per milligram of DNA occurred followed by a gradual increase in activity. Again this probably reflects an actual synthesis of the enzyme from 10 to 46 days since no increase in average cell weight occurred during that period. A slight increase in activity occurred immediately following protein supplementation with a return to normal by 132 days. The SD per milligram of DNA of group 2 showed an initial decline until the 30th day. A sharp increase occurred at 46 days, which corresponds to the peak seen for the same point when SD was related to nitrogen per milligram of liver. The reason for this fluctuation is not clear. It may simply reflect a sudden adjustment of the animals over this interval to lowered food intake, since as the deficiency progressed group 1 ate less and less. Following supplementation the SD per milligram

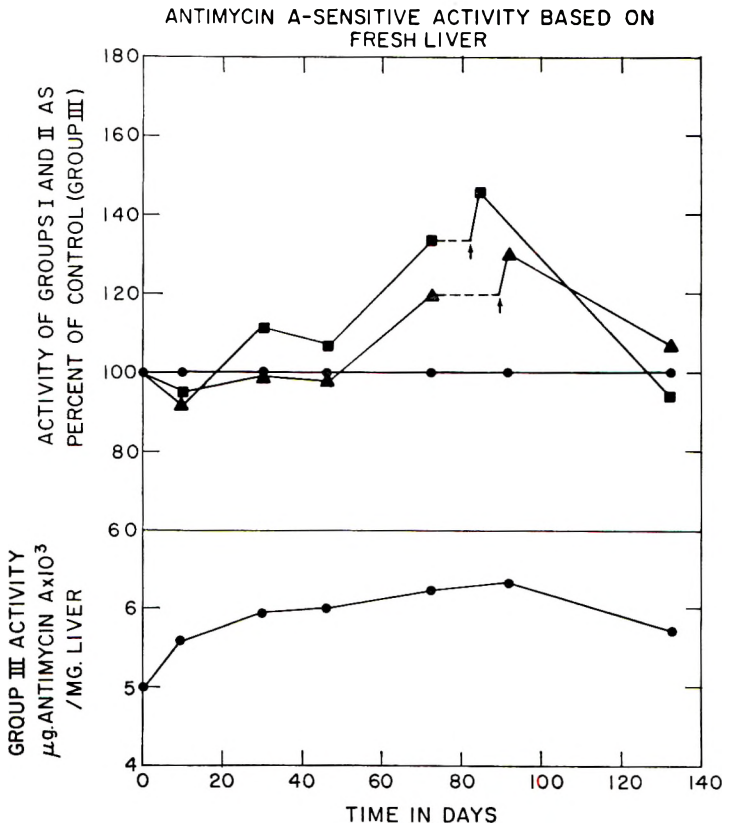

Fig. 7 Antimycin A-sensitive activity per milligram of fresh liver versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

of DNA of group 2 returned rapidly to normal.

Antimycin A-sensitive activity (AAS). The results for this activity, which is an indirect measure of the flow of electrons through cytochromes *b* and c_1, are presented in figures 7 to 9. It should be emphasized that AAS is not intended to be considered a measure of cytochrome *b* or cytochrome c_1 concentration but only an indication of the flow of electrons through these two cytochromes. The exact point of the antimycin A inhibition is not yet known except that it prevents the reduction of cytochrome c_1 without inhibiting the initial succinic dehydrogenase. Based on fresh liver weight (fig. 7) the AAS of groups 1 and 2 gradually increased throughout the depletion period. The AAS per milligram of liver of group 2 tended to be slightly below that of group 1 though the difference is not statistically significant. Following repletion, after a slight rise in both groups the activity per milligram of liver returned to normal.

With respect to other liver proteins (fig. 8), the AAS of group 1 increased rapidly above that of the pair-fed controls and leveled off after 30 days. Within two days after protein repletion, a large increase in activity of AAS per milligram of nitrogen over other liver proteins occurred, followed by a return to normal by 132 days. The AAS per milligram of nitrogen of the pair-fed controls did not change with respect to other liver proteins throughout the depletion period. After supplementation was begun, a slight rise occurred followed by a return to normal at 132 days. The response of AAS per milligram of nitrogen to partial

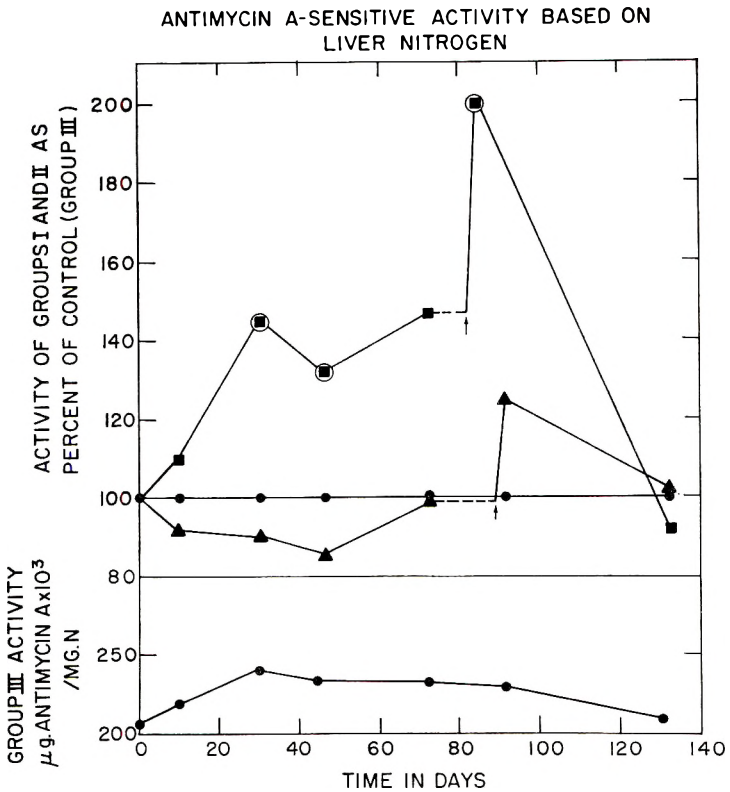

Fig. 8 Antimycin A-sensitive activity per milligram of liver nitrogen versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

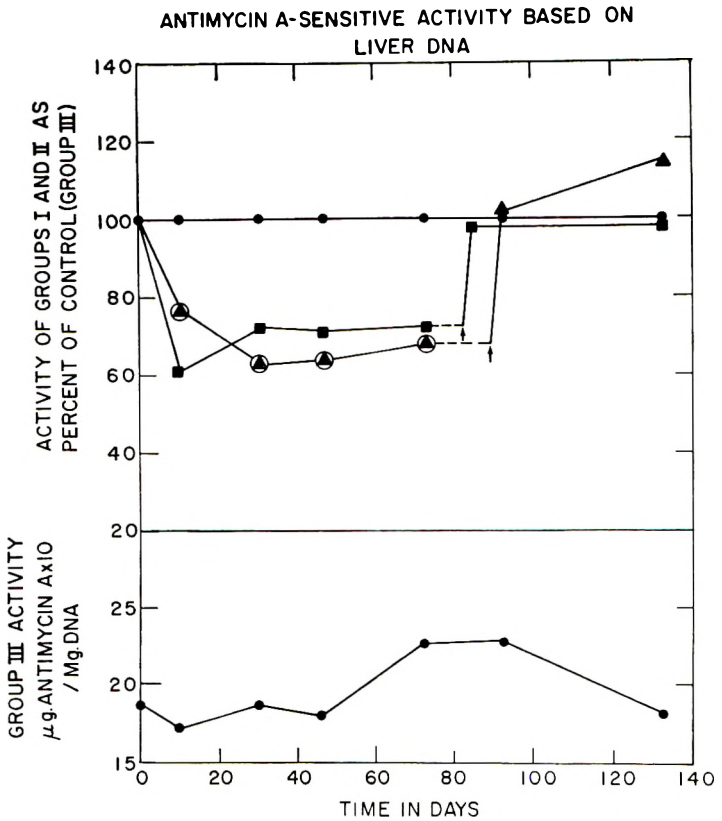

Fig. 9 Antimycin A-sensitive activity per milligram of liver DNA versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

inanition is quite different from the response of SD per milligram of nitrogen to that regimen. The latter enzyme was conserved more than other liver proteins for 46 days. From then on it was maintained at the same level with respect to other liver proteins as in the ad libitum-fed controls.

In figure 9 are presented the results for AAS per milligram of DNA. In both groups 1 and 2 a rapid and similar loss of this activity per milligram of DNA occurred. This decline leveled off after 10 days and remained constant until repletion began. No significant resynthesis of AAS per milligram of DNA occurred in group 1 as it did with SD per milligram of DNA after 10 days. Within two days after repletion was begun the AAS per milligram of DNA of groups 1 and 2 returned to normal and remained there.

Cytochrome c concentration. As shown in figure 10, cytochrome c per milligram of liver increased in groups 1 and 2 to the same extent. The maximum concentrations were reached after 10 days and remained the same thereafter until repletion was begun. The cytochrome c concentration per milligram of liver of both groups then gradually returned to normal. Thus, patterns followed by groups 1 and 2 for cytochrome c per milligram of liver were in general different from SO, SD, and AAS per milligram of liver.

Cytochrome c was retained much more tenaciously than other liver proteins during protein depletion as shown in figure 11. The pair-fed controls retained cytochrome c somewhat more strongly than other liver protein but not nearly to the same extent as in the protein deficient group. In both

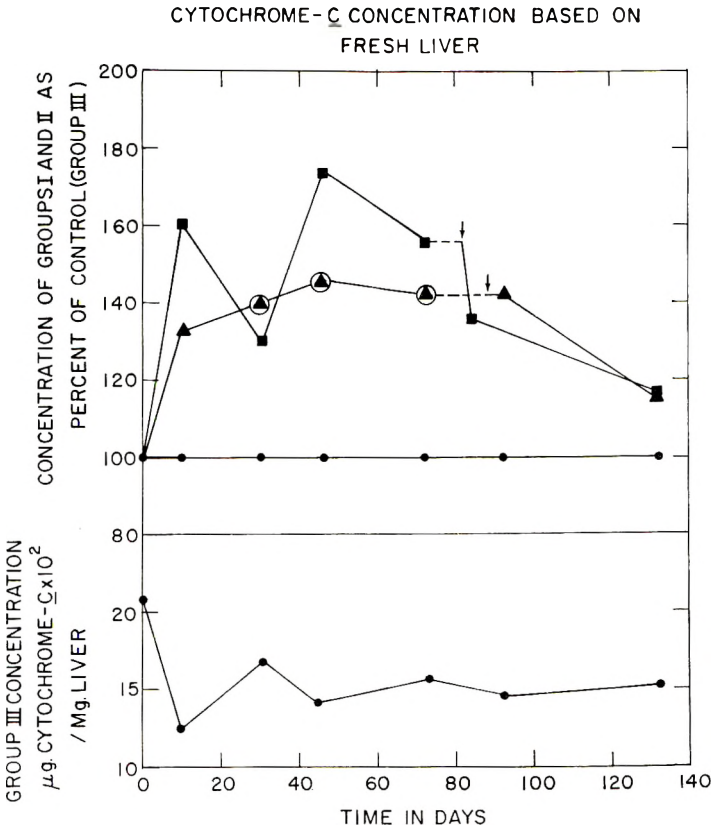

Fig. 10 Cytochrome *c* concentration per milligram of fresh liver versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

groups 1 and 2 the maximum change occurred by 10 days and remained the same thereafter for the depletion period. When repletion was begun, the cytochrome c per milligram of nitrogen of both groups increased slightly but were back to normal by the end of 132 days.

No change in cytochrome *c* concentration occurred per milligram of DNA (fig. 12), either in the protein deficient or the pair-fed control groups. Thus, although liver cellular nitrogen and weight changed markedly in these groups, the concentration of cytochrome c per cell remained remarkably constant.

Cytochrome oxidase activity (CO). The CO activity per milligram of liver of the protein-deficient rats decreased to 50% of normal after 10 days (fig. 13). By 46 days this activity had dropped to 25% of normal. From 46 to 72 days the activity increased slightly, but it could increase no further since the animals had become moribund at that point. Following supplementation, a rapid rise occurred after two days and normal activity per milligram of liver was reached by 132 days. The pair-fed controls showed no significant deviation from normal with respect to CO per milligram of liver throughout the whole study. Thus CO per milligram of liver showed a pattern that was entirely different from SO, SD, AAS, and cytochrome c per milligram of liver.

Based on liver nitrogen (fig. 14), the CO of the protein-deficient group was lost at a more rapid rate than other liver proteins. In fact this enzyme complex was

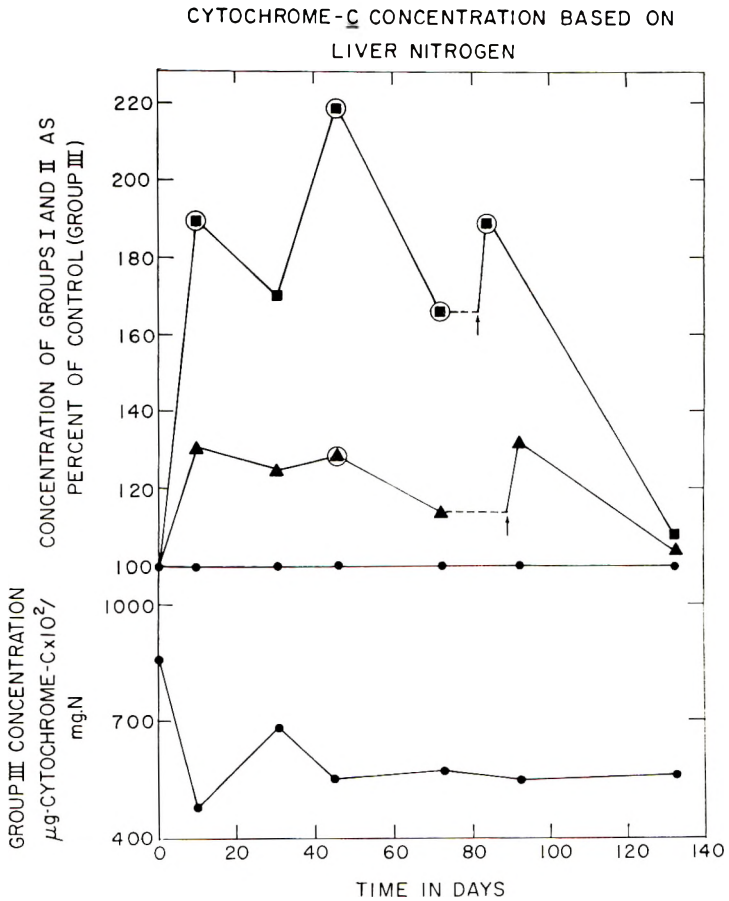

Fig. 11 Cytochrome c concentration per milligram of liver nitrogen versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

lost to a greater extent than any of the other enzymes of the succinic oxidase system. There was a slight tendency for the pair-fed controls to lose CO with respect to liver nitrogen, though this loss is not significant statistically. When protein was returned to the ration of group 1, the CO per milligram of nitrogen increased to normal after two days. This apparent complete return to normal after two days, however, must be interpreted with respect to the concentration of nitrogen per milligram of liver at that point (see preceding paper [Williams, '61]). Since the nitrogen concentration per milligram of liver decreased sharply after two days of protein supplementation, the apparently normal CO activity per milligram of nitrogen is both a reflection of the lowered nitrogen concentration and of slightly increased CO activity per milligram of liver.

In group 1, CO activity per milligram of DNA decreased to 15% of normal after 46 days of protein depletion (fig. 15). The partial inanition of the pair-fed controls, however, also caused a loss of CO per milligram of DNA to about 60% of normal. Therefore, the protein deficiency per se produced a maximum loss of CO to 25% of the corresponding pair-fed control value. Of all of the enzymes of the succinic oxidase system, CO is the enzyme lost

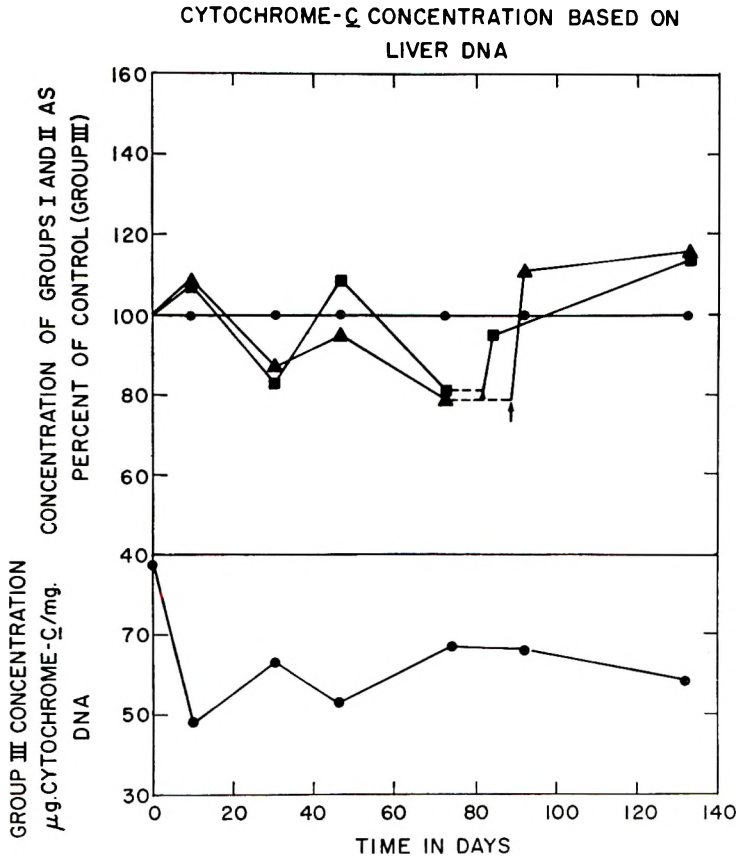

Fig. 12 Cytochrome c concentration per milligram of liver DNA versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

to the greatest extent from each cell during a protein deficiency. Following protein repletion, the CO activity per milligram of DNA of group 1 increased to 50% of normal after two days and finally returned to normal by the end of the study.

DISCUSSION

In 1947 Potter and Klug observed that, when young adult rats were fed a low-protein ration for 12 to 32 days, liver succinic oxidase activity based on dry liver weight decreased to 58% of normal. No attempt was made to follow the activity during the course of the experiment as in the present study; also a protein-free ration was not used. Wainio et al. ('53), who studied a variety of oxidative enzymes in the liver of protein-deficient and pair-fed rats, found that after about 50 days of protein depletion, succinic oxidase per milligram of nitrogen was slightly less than that of the ad libitum-fed controls and approximately 50% of the pair-fed controls. The pair-fed control activity tended to be slightly higher than the ad libitum-fed controls. In general their results are similar to those in the present paper at the 46-day point. A major discrepancy in the present results and those obtained by Wainio et al. for cytochrome oxidase exist, however. They found no change in activity of this enzyme in either the protein deficient rats or the pair-fed controls. Prigmore et al. ('55) observed that after 9 days of protein depletion of weanling rats, liver succinic oxidase activity per milligram of liver decreased to 60% of normal. Again only one

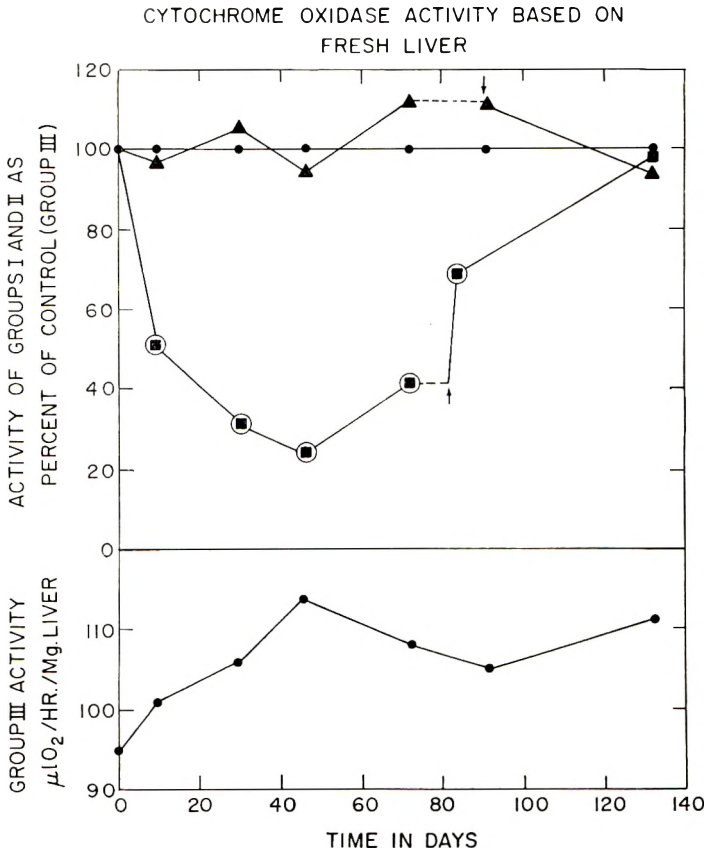

Fig. 13 Cytochrome oxidase activity per milligram of fresh liver versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

time interval was studied so that the increase of succinic oxidase activity observed after 30 days in the protein-deficient and pair-fed control rats in the present study cannot be compared with earlier work.

It is evident from the results that succinic oxidase activity followed a pattern that was quite different from any of its individual enzyme components with the exception of succinic dehydrogenase. Thus, succinic oxidase activity may appear normal in activity but still have many changes in the activities of its individual components. According to Potter ('59), SD is probably the rate-limiting step in the SO system. This in general holds true in the present study though there is by no means an exact correlation between SO and SD. The correlation is much more evident in group 1 than in group 2. Perhaps other effects come into play to influence the activity of the SO system. If the protein deficiency and partial inanition produced changes, for example, in the coupling between the various enzymes of the system, the activity of the total system might be expected to show changes in addition to those produced by variations in the enzyme protein concentrations. Such changes might not show up when the individual enzymes of the system were assayed. In any case, with the exception of SD, none of the other enzymes showed any similarity in pattern at all to the SO system.

Approximately 60% of the SO and SD and 85% of the CO activities per cell were

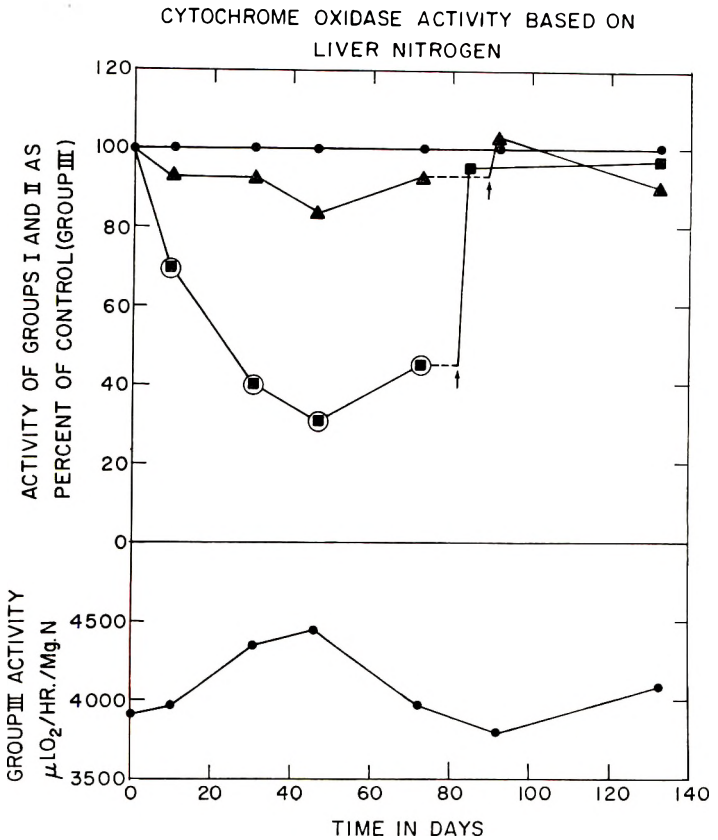

Fig. 14 Cytochrome oxidase activity per milligram of liver nitrogen versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

the maximum amounts of each enzyme that were lost in the protein-deficient group. Once these levels had been reached no further loss occurred. These results are reminiscent of the notion of labile and nonlabile stores of liver nitrogen. In the preceding paper it was seen that after 50% of the cellular nitrogen had been lost no further loss occurred. Thus, it appears that labile and nonlabile stores of SO, SD, and CO exist. This is not true, however, for AAS and cytochrome *c* per cell, both of which were insensitive to the protein deficiency.

The enzyme results in this paper point out the importance of including pair-fed controls when studying a protein deficiency. The partial inanition of the pair-fed controls produced changes in enzyme concentrations that might have been mistaken for the effects of a protein deficiency per se. For example, group 2 had lower enzyme activities per milligram of DNA than the ad libitum-fed controls except for cytochrome c. Based on activity per milligram of DNA the enzymes of the SO system most labile to partial inanition, in decreasing order of sensitivity, were CO = AAS > SD > SO > cytochrome c. The order for the enzymes most labile to the protein deficiency were CO > SO > SD > AAS > cytochrome *c*. Thus, although both protein deficiency and partial inanition produced loss of enzymes, the patterns of response to each regimen were entirely different. This holds whether the enzyme

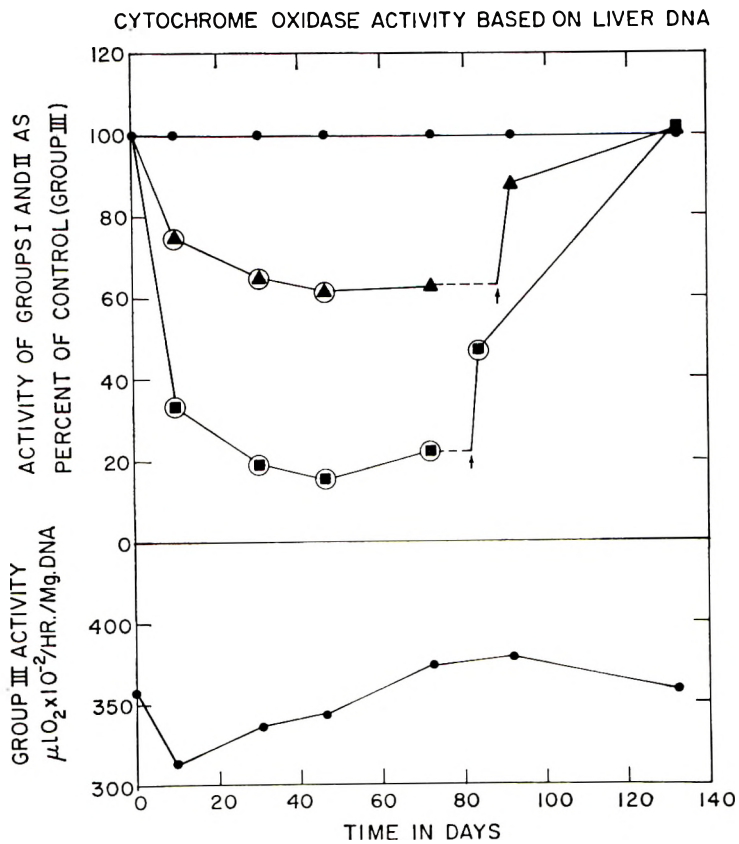

Fig. 15 Cytochrome oxidase activity per milligram liver DNA versus time of protein depletion and repletion. ■, protein-deficient group; ▲, pair-fed controls and ●, ad libitum-fed controls. The repletion regimens were begun at the arrows. The points for statistically significant differences ($P < 0.05$) between the protein-deficient rats and their pair-fed controls, and between the pair-fed controls and the ad libitum-fed controls, are encircled.

activities are expressed per milligram of liver, per milligram of nitrogen or per milligram of DNA.

Although a large proportion of CO was lost during the protein deficiency (80% was lost on a fresh liver basis), there was still enough left to maintain a high activity of SO. Therefore, CO is probably present in normal liver in very large excess. Electron transfer at the CO step is one of the points at which oxidative phosphorylation occurs. If there is not enough CO to meet the requirements for electron transport from all substrates of the citric acid cycle, this would be the point at which the most damage to high energy phosphate formation could occur. In the SO system as estimated in the present studies the only substrate involved is the succinate added to the flask. It is quite possible that in the protein-deficient animal not enough CO would be present to handle electron transport from all endogenous substrates.

In the present studies most of the enzyme changes in the succinic oxidase system occurring during protein deficiency were reversed by protein repletion. Many other metabolic processes, however, are dependent upon the high energy phosphate produced during the oxidation of succinate and other citric acid cycle intermediates. It is not yet known to what extent the abnormal enzyme patterns produced by the protein deficiency in the present study influence the production of high energy phosphate. If various metabolic processes which are dependent upon the production of high energy phosphate are disturbed, however,

it is quite possible that irreversible changes other than those reported in this and the preceding paper (Williams, '61) might take place. These questions are being studied at present.

SUMMARY

A timed study of changes in the succinic oxidase system and its component enzymes during a prolonged deficiency of dietary protein followed by repletion has been presented. Pair-fed and ad libitum-fed controls were included throughout. The data were calculated as activity or concentration per milligram of fresh liver, per milligram of liver nitrogen, and per milligram of liver deoxyribonucleic acid (DNA). The results are summarized as follows:

Based on fresh liver, the succinic oxidase activity (SO) of the protein-deficient rats dropped after 10 days to 50% of normal followed by an increase to above normal after 30 days. This activity in the pair-fed controls increased throughout the depletion period. The SO of both groups rapidly returned to normal after repletion. The succinic dehydrogenase (SD) of the protein-deficient group followed the same pattern as SO for that group. The SD of the pair-fed controls increased until 46 days and then decreased at 72 days. Both groups tended to return to normal following repletion; however, the SD of the protein-deficient group was significantly higher than that of the pair-fed controls even after 48 days of repletion. The antimycin A-sensitive activity (AAS) of both the protein-deficient and the pair-fed groups increased gradually and at the same rate throughout the depletion period. Following repletion, the AAS of neither group returned to normal at once; after 48 days of repletion, however, it returned to normal in both groups. Cytochrome c of both the protein-deficient and pair-fed groups increased above normal to the same extent and leveled off after 30 days of depletion. After repletion the cytochrome c of both groups gradually returned to normal. Cytochrome oxidase (CO) of the protein-deficient animals fell rapidly to 25% of normal. The CO of the pair-fed controls did not change throughout the study. Following repletion, the CO of the protein-deficient rats returned rapidly to normal.

Based on liver nitrogen, the SO of the protein-deficient and pair-fed groups followed about the same pattern as for SO per milligram of fresh liver. The SD of the protein-deficient group followed the same pattern as the SD per milligram of fresh liver for that group; the initial loss of activity of SD per milligram of nitrogen was not as great as SD per milligram of liver. The SD for the pair-fed controls increased until 46 days and then fell to normal by 72 days. Following repletion, the SD of both groups returned slowly to normal. The AAS of the protein-deficient group increased rapidly to well above normal by 30 days and leveled off. Following repletion a sharp further increase occurred with a return to normal after 48 days of repletion. The AAS of the pair-fed controls did not change throughout the study. Cytochrome c of the protein-deficient group reached 190% of normal after 10 days of depletion and remained at that level until repletion was begun. The cytochrome c of pair-fed controls increased to 130% of normal and remained there during depletion. Following repletion the cytochrome c of both groups slowly returned to normal. The CO of the protein-deficient and pair-fed groups followed the same patterns as for CO per milligram fresh liver.

Based on liver DNA, which is a measure of activity or concentration per liver cell, the SO of the protein-deficient rats dropped to 40% of normal after 10 days, which was followed by an increase for the rest of the depletion period. Following repletion, a decrease occurred after two days followed by a return to normal after 46 more days. The SO of the pair-fed controls remained nearly normal throughout the study. The SD of the protein-deficient group followed the same pattern as SO per milligram DNA for that group except that an immediate return to normal occurred after two days of repletion. The SD of the pair-fed group decreased to 70% of normal after 30 days, returned to normal at 46 days, and then fell to 70% of normal at 72 days. Repletion brought the SD of this group slowly back to normal. The AAS of both the protein-deficient and pair-fed groups fell to 60 to 70% of normal after 10 days and remained there throughout the depletion period. Repletion brought the AAS of both

groups immediately back to normal. No change in cytochrome c occurred in either group throughout the study. The CO of the protein-deficient rats fell to 15% of normal by 46 days. It fell to 60% of normal in the pair-fed controls. The CO of both groups rapidly returned to normal following repletion.

ACKNOWLEDGMENTS

The author wishes to thank Mrs. Esther Hurley for feeding and care of the animals and Mrs. Helen Hood for preparation of the diets used in these studies.

ADDENDUM

Since this manuscript was prepared, Singer and Lusty (Singer, T. P., and C. J. Lusty 1960 Permeability factors in the assay of mitochondrial dehydrogenases. Biochem. Biophys. Res. Comm., 2: 276) have reported that the use of phenazine methosulfate as electron acceptor for assaying succinic dehydrogenase in intact rat liver mitochondria does not give a true measure of the succinic dehydrogenase (SD) activity. They found that the mitochondrial membrane of undamaged mitochondria exhibits a permeability barrier toward phenazine methosulfate. If the membrane is damaged, for example by including calcium ions in the medium, this barrier may be eliminated. In the assay for SD reported in the present paper, the mitochondria were kept intact as much as possible. Thus, the SD activities obtained would be a function both of permeability of the membrane to phenazine methosulfate and concentration of SD within the mitochondrion. The relative effects of these two variables on the SD activity in these experiments is not known. However, unless the protein deficiency or partial inanition influenced the permeability of the mitochondrial membranes to the dye, the permeability effects should be the same in all groups. Thus the patterns of the response of SD to the dietary regimens might well be valid except that the magnitude of the absolute activities would be lower than theoretical. There is a definite possibility on the other hand that the dietary regimens themselves may produce changes in the permeability of the mitochondrial membranes. This problem and its possible effects on SD in protein deficiency are being investigated at present.

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Amino Acid Balance and Imbalance

VI. GROWTH DEPRESSIONS FROM ADDITIONS OF AMINO ACIDS TO DIETS LOW IN FIBRIN[1]

U. S. KUMTA, L. G. ELIAS AND A. E. HARPER
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

Several amino acid imbalances induced by adding a small quantity of one or two amino acids to low-protein diets have been described. The addition of 0.25% to 1.0% of a mixture of methionine and phenylalanine (the second most limiting amino acids in fibrin) to diets containing 6% of fibrin as the protein source results in an amino acid imbalance that severely retards the growth of rats. This imbalance is corrected by a supplement of the most limiting amino acids, leucine, isoleucine, valine and histidine. (Deshpande et al., '58a,b; Kumta et al., '58; Harper and Kumta, '59; Kumta and Harper, '60a). Similarly, adding 0.36% of DL-threonine to diets low in niacin and containing 8 or 9% of casein as the protein source, depresses the growth rate of weanling rats and this is prevented by a supplement of either tryptophan or niacin (Hankes et al., '49; Henderson et al., '53; Koeppe and Henderson, '55; Morrison and Harper, '60; Morrison et al., '60). Growth retardations due to additions of the second most limiting amino acid have also been demonstrated in amino acid diets by Koeppe and Henderson ('55) and in diets composed almost entirely of rice by Deshpande et al. ('55) and Rosenberg et al. ('59).

These observations suggest that a delicate balance exists between the second and the most limiting amino acids in a low-protein diet. It is therefore important to ascertain how specific this relationship is. Morrison and Harper ('60) have tested the specificity of threonine in causing an imbalance in diets low in niacin and containing 8 or 9% of casein as the protein source. The severity of the imbalance was increased by adding mixtures of other amino acids along with threonine but the mixtures by themselves did not cause growth depressions if threonine was omitted.

Besides these effects of relatively small additions (less than 1.0%) of amino acids, imbalances can be consistently induced by adding fairly large amounts (3 to 10% of the diet) of unbalanced proteins or amino acid mixtures lacking one indispensable amino acid, to low-protein diets. (Harper, '58, '59; Salmon, '59; Kumta and Harper, '60a.) These imbalances differ, in one respect at least, from those of the former type in that the level of protein in the diet is not critical in demonstrating the growth depression (Henderson et al., '53; Kumta and Harper, '60a; Morrison and Harper, '60).

The present studies were stimulated by the observation (Kumta and Harper, '60b) that the addition of cystine and tyrosine, in contrast with the addition of methionine and phenylalanine, did not cause a depression in the growth rate of rats receiving a low dietary level of fibrin. This suggested that the effect of methionine and phenylalanine in inducing an imbalance in diets low in fibrin might be highly specific. Also, when groups of protein-depleted rats were fed equal amounts of the basal diet supplemented with 1% of various amino acid mixtures, the group ingesting the imbalanced diet containing methionine and phenylalanine showed a marked rise in blood urea. Rats fed a similar diet supplemented with the least limiting amino acids

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arginine and threonine, however, showed a similar increase in blood urea concentration.[2] This observation posed the question of whether all amino acid mixtures that cause a rise in blood urea concentration under these conditions also cause growth depressions. The results reported below indicate that additions of several amino acids and mixtures of amino acids will retard the growth of rats fed a diet containing 6% of fibrin.

EXPERIMENTAL

Male weanling rats of the Holtzman strain, weighing 40 to 50 gm were used in these experiments. The rats were housed in individual suspended cages and were fed the basal diet (described below) ad libitum. After a preliminary period of 4 to 5 days to allow the rats to adapt to the new environment, those which showed a progressive gain in weight were selected and separated into groups of 5 rats each. The average initial weights of the groups did not differ by more than one gram. These groups were then fed the various experimental diets ad libitum and were weighed at least twice weekly during the two-week experimental period.

The basal diet used in these experiments was essentially that described earlier (Kumta et al., '58). This contained fibrin, 6; salt mixture, 5 (Harper, '59); corn oil, 5; vitamin supplements,[3] 0.25 (Harper, '59); choline chloride, 0.15 and dextrin as the carbohydrate to make up 100%. Fat-soluble vitamins A, D and E were included in the corn oil to provide the following concentrations per 100 gm of diet: A, 400 I.U.; D, 40 I.U.; and E (α-tocopherol), 10 mg. All diets were refrigerated. Additions of amino acids as indicated in the tables of results were compensated for by adjusting the percentage of dextrin.

RESULTS

The object of the first experiment was to determine whether additions of each of the individual amino acids by themselves would cause growth depressions. Since in most of the previous experiments, methionine and phenylalanine were added at levels of 0.4 and 0.6%, respectively, in the diet, individual amino acids were added at these levels. The results are presented in table 1. Of the amino acids tested, leucine caused a severe depression (56%) in growth rate and each of the amino acids methionine, phenylalanine, threonine, isoleucine, histidine, arginine and tryptophan caused a moderate (15 to 25%) depression. Additions of cystine, valine, lysine and tyrosine were without effect.

TABLE 1

Effect on the growth of weanling rats of adding 0.4 or 0.6% of individual amino acids to diets containing 6% of fibrin

Amino acid added	Concentration	Weight gain
	%	*gm/two weeks*
—	—	32.8 ± 1.9[1]
DL-Valine	0.6	33.2 ± 2.7
L-Lysine	0.6	32.4 ± 1.5
L-Cystine	0.4	33.6 ± 2.2
L-Tyrosine	0.6	33.8 ± 3.9
DL-Methionine	0.4	27.4 ± 3.3
DL-Phenylalanine	0.6	25.6 ± 2.7
DL-Threonine	0.4	27.8 ± 2.2
DL-Isoleucine	0.6	28.0 ± 1.2
L-Histidine	0.6	26.4 ± 1.6[2]
L-Arginine	0.6	24.6 ± 3.9
DL-Tryptophan	0.6	20.4 ± 1.5[3]
L-Leucine	0.6	14.2 ± 2.0[3]

[1] Standard error of the mean for 5 rats.
[2] The difference between this group and the basal group showed a probability of less than 0.05.
[3] The difference between this group and the basal group showed a probability of less than 0.01.

Although neither methionine nor phenylalanine added alone severely retards growth, the addition of both together causes a depression of 45 to 50% in growth rate. To determine whether the growth-retarding effect was characteristic of methionine and phenylalanine, combinations of methionine with tyrosine and of phenylalanine with cystine and the hydroxy analogue of methionine were tested for their effect on the growth of weanling rats. The results presented in table 2 show that mixtures containing cystine or tyrosine did not cause any greater growth depression than that caused by each of the individual amino acids alone. The hydroxy analogue of methionine behaved like methionine. The combination of cystine with phenylalanine was without effect even though phen-

[2] Kumta, U. S., and A. E. Harper 1960 Some further observations on amino acid imbalance. Federation Proc., *19:* 12 (abstract).

[3] Some of the crystalline vitamins were kindly provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

TABLE 2

Effect of adding metabolites of methionine and phenylalanine to diets containing 6% of fibrin

Amino acid additions					Gain in weight
DL-Methionine 0.4%	DL-Hydroxy-methionine 0.4%	L-Cystine 0.4%	DL-Phenyl-alanine 0.6%	L-Tyrosine 0.6%	
					gm/two weeks
−	−	−	−	−	33.6 ± 3.3[1]
+	−	−	−	−	27.4 ± 2.1
+	−	−	+	−	17.4 ± 1.4[2]
+	−	−	−	+	26.4 ± 1.4
−	+	−	+	−	20.2 ± 1.8[2]
−	+	−	−	+	30.4 ± 1.4
−	−	+	+	−	36.0 ± 3.2
−	−	+	−	+	30.2 ± 2.5

[1] Standard error of the mean for 5 rats.

[2] The difference between this group and the basal group showed a probability of less than 0.01.

TABLE 3

Effect of adding various amino acids in combination with methionine or phenylalanine on the gain in weight of rats fed diets containing 6% of fibrin

Amino acid added in combination with 0.4% of methionine or 0.4% of phenylalanine[1]	Weight gain[2]	
	DL-Methionine	DL-Phenylalanine
	gm/two weeks	*gm/two weeks*
None	27.4 ± 2.7[3]	25.6 ± 2.1[4]
DL-Isoleucine	25.2 ± 3.5	24.6 ± 1.8[4]
DL-Valine	23.2 ± 1.1[5]	22.8 ± 1.3[5]
L-Leucine	9.0 ± 2.7[5]	5.6 ± 0.9[5]
L-Lysine	30.2 ± 2.3	29.0 ± 2.3
DL-Methionine	—	18.8 ± 2.0[5]
DL-Phenylalanine	17.4 ± 1.8[5]	—
L-Histidine	20.4 ± 1.7[5]	25.4 ± 1.8[4]
L-Arginine	24.8 ± 1.3[5]	22.0 ± 2.1[5]
DL-Threonine	26.0 ± 2.2[4]	19.2 ± 1.2[5]
DL-Tryptophan	18.4 ± 2.5[5]	19.8 ± 1.1[5]

[1] 0.6% of each amino acid.

[2] Rats fed a diet containing 6% of unsupplemented fibrin gained 32.8 ± 1.9 gm/two weeks.

[3] Standard error of the mean for 5 rats.

[4] The difference between this group and the basal group showed a probability of less than 0.05.

[5] The difference between this group and the basal group showed a probability of less than 0.01.

TABLE 4

Prevention of amino acid imbalance and antagonism due to addition of methionine plus leucine to diets containing 6% of fibrin

Group no.	Amino acid additions					Gain in weight	Amino acid intake		
	DL-Methio-nine 0.4%	L-Leucine 0.6%	DL-Iso-leucine 0.1%	DL-Valine 0.15%	L-Histi-dine 0.05%		Iso-leucine	Valine	Histi-dine
						gm/2 weeks	*gm*	*gm*	*gm*
1	−	−	−	−	−	32.8 ± 1.9[1]	1.96	2.1	1.12
2	+	−	−	−	−	27.4 ± 2.7	—	—	—
3	+	+	−	−	−	10.8 ± 0.8[2]	1.26	1.39	0.72
4	+	+	+	−	−	16.6 ± 0.06[2]	—	—	—
5	+	+	+	+	−	29.8 ± 3.5	2.13	2.52	1.36
6	+	+	+	+	+	36.4 ± 1.8	2.24	2.65	1.43

[1] Standard error of the mean for 5 rats.

[2] The difference between this group and the basal group showed a probability of less than 0.01.

ylalanine alone caused a slight growth depression (table 1).

Further studies were carried out to determine whether combinations of other amino acids would cause severe growth depressions. These fell into two series, one in which either methionine or phenylalanine was one member of the pair and another in which neither methionine nor phenylalanine was included. The results summarized in table 3 show that the addition of arginine, threonine, tryptophan or histidine in combination with methionine or phenylalanine caused a 30 to 40% depression in growth rate. A still greater depression was caused by the combination of leucine with methionine or phenylalanine.

The results in table 4 indicate that the severe growth-depressing effect of leucine is due to an antagonism between leucine and isoleucine and valine. Thus, although the addition of methionine plus leucine caused a 66% drop in growth rate, supplements of isoleucine and valine effectively prevented the growth depression attributable to leucine. Calculations of isoleucine, valine and histidine intakes from the total food intake figures for these groups indicate that the efficiency of utilization of these amino acids decreased (compare group 1 with 5 and 6) when a severe growth depression was caused by the addition of methionine and leucine.

When several amino acid mixtures containing neither methionine nor phenylalanine were tested, those consisting of the least limiting amino acids, arginine, threonine and tryptophan caused a 35 to 45% depression in growth rate (table 5), a depression of the same magnitude as that caused by the addition of second most limiting amino acids.

Since growth depressions due to additions of the least limiting amino acids appeared anomalous, attempts were made to correct them by adding lysine (Doermann, '44), valine (Gladstone, '39) and phenylalanine (Benton et al., '56) on the basis of structural antagonisms observed either in bacteria or in rats. Since an excess of arginine might increase the requirement for methyl groups, the effect of additional methionine was also studied. As the addition of tryptophan prevents the growth-depressing effects of arginine plus threonine in diets low in casein and niacin (Morrison and Harper, '60), the influence of tryptophan was also tested in the present experiment. As shown in table 6, none of these amino acid supplements prevented the growth depression due to the addition of arginine and threonine.

Since the amino acid imbalance induced by addition of methionine and phenylalanine was prevented only by the addition of a mixture of the most limiting amino acids (leucine, isoleucine, valine and histidine) its effects on the growth depression caused by addition of arginine plus threonine or threonine plus tryptophan was studied. The results of this experiment are presented in table 7. The growth-retarding effect of arginine plus threonine or threonine plus tryptophan was prevented by a low level of the mixture of most limiting amino acids (1X), but when 4 times this level was added, a growth depression was again observed. In contrast, the growth depression caused by methionine and phenylalanine was prevented by either level of

TABLE 5

Growth-depressing effects of least limiting amino acids when added to diets containing 6% of fibrin

Amino acids added				Gain in weight
L-Arginine 0.4%	DL-Threonine 0.6%	DL-Tryptophan 0.4%	L-Lysine 0.6%	
				gm/2 weeks
−	−	−	−	32.8 ± 1.9[1]
+	−	−	+	18.4 ± 2.8[2]
+	+	−	−	20.0 ± 1.8[2]
−	+	+	−	18.6 ± 2.2[2]

[1] Standard error of the mean for 5 rats.

[2] The difference between this group and the basal group showed a probability of less than 0.01.

TABLE 6

Attempts to prevent growth depression caused by addition of arginine and threonine to diets containing 6% of fibrin

Amino acid additions							Gain in weight
L-Arginine 0.6%	DL-Threonine 0.4%	L-Lysine 0.2%	DL-Tryptophan 0.1%	DL-Valine 0.2%	DL-Phenylalanine 0.2%	DL-Methionine 0.4%	
							gm/2 weeks
−	−	−	−	−	−	−	32.6 ± 3.4[1]
+	+						20.0 ± 1.8[2]
+	+	+					19.0 ± 2.2[2]
+	+		+				20.2 ± 3.1[2]
+	+		+		+		17.8 ± 1.0[3]
+	+	+			+		15.4 ± 1.3[3]
+	+			+	+		14.6 ± 1.0[3]
+	+					+	12.8 ± 1.9[3]

[1] Standard error of the mean for 5 rats.
[2] The difference between this group and the basal group showed a probability of less than 0.05.
[3] The difference between this group and the basal group showed a probability of less than 0.01.

TABLE 7

Influence of addition of the most limiting amino acids on the growth depressions produced by addition of the second most limiting amino acids and the least limiting amino acids in diets containing 6% of fibrin

Group no.	Amino acids added						Gain in weight
	DL-Threonine 0.4%	L-Arginine 0.6%	DL-Tryptophan 0.6%	DL-Methionine 0.4%	DL-Phenylalanine 0.6%	AAM[1]	
							gm/2 weeks
1	−	−	−	−	−	−	32.8 ± 1.9[2]
2	+	+	−	−	−	−	20.0 ± 2.7[4]
3	+	+	−	−	−	1X	30.6 ± 1.6
4	+	+	−	−	−	4X	24.8 ± 2.2[3]
5	−	−	−	−	−	4X	30.2 ± 3.9
6	+	−	+	−	−	−	20.0 ± 3.3[3]
7	+	−	+	−	−	1X	34.6 ± 2.6
8	+	−	+	−	−	4X	22.8 ± 2.2[3]
9	−	−	−	+	+	−	19.8 ± 1.2[4]
10	−	−	−	+	+	1X	45.6 ± 1.6[4]
11	−	−	−	+	+	4X	49.8 ± 3.8[4]
12	+	+	−	+	+	4X	51.2 ± 3.2[4]

[1] This contained in percentage: L-leucine, 0.1; DL-isoleucine, 0.1; DL-valine, 0.15; and L-histidine HCl, 0.05.
[2] Standard error of the mean for 5 rats.
[3] The difference between this group and the basal group showed a probability of less than 0.05.
[4] The difference between this group and the basal group showed a probability of less than 0.01.

the mixture of most limiting amino acids. However, when methionine and phenylalanine, now the most limiting amino acids, were added together with the higher levels of leucine, isoleucine, valine and histidine addition of arginine and threonine did not retard growth (group 12).

DISCUSSION

Amino acid imbalances due to the addition of a small quantity of the second most limiting amino acid or acids to low-protein diets have been observed frequently enough to suggest that the balance between the first and second most limiting amino acids in such diets is of unique nutritional significance. An imbalance is caused specifically by the addition of threonine, the second most limiting amino acid, to a niacin-deficient diet containing 9% of casein (Morrison and Harper, '60). In these experiments, however, using diets containing 6% of fibrin, additions of other amino acids and amino acid mixtures caused growth depressions. Thus, the postulated uniqueness of the relationship between the first

and the second most limiting amino acids in low-protein diets may be open to question.

On the other hand, in diets containing a low level of fibrin, 6 amino acids are very nearly equally limiting, whereas, in most studies of amino acid imbalances the diets have been such that two or three amino acids became limiting in stepwise fashion. When there is only a slight difference in the degree of deficiency of several amino acids, as in fibrin (Kumta and Harper, '60b), much less specificity in the creation of imbalances might be expected.

The failure of additions of cystine or tyrosine or both to behave like methionine and phenylalanine in causing growth depressions may be related to their inability to substitute for methionine and phenylalanine in stimulating the growth of rats fed a low-fibrin diet supplemented with the other limiting amino acids (Kumta and Harper, '60b). No explanation can be suggested for the failure of combinations of lysine with methionine or phenylalanine to depress the growth rate.

The severe growth-retarding effect of leucine, either alone or in combination with methionine or phenylalanine, appears to be a result of a leucine antagonism which differs from an amino acid imbalance in that it is specifically corrected by the addition of the structurally similar amino acids, isoleucine and valine, but not by a supplement of the most limiting amino acid in the diet (Harper et al., '55). In diets containing fibrin, isoleucine and valine are low and the addition of as little as 0.6% of L-leucine is apparently sufficient to cause such an antagonism (table 4). The severe growth-retarding effects of leucine in combination with methionine or phenylalanine may be attributed to the combined effects of an antagonism and an imblance.

The amino acid imbalances caused by mixtures of the least limiting amino acids appear to differ in some respects from those caused by mixtures of the second most limiting amino acids. The imbalance due to the addition of methionine and phenylalanine was corrected by supplementation with either 0.4 or 1.6% of a mixture of the 4 limiting amino acids, whereas those due to mixtures of the least limiting amino acids (arginine, threonine and tryptophan) were not corrected by the larger supplement. If all of the 6 most limiting amino acids were included in the diet, however, mixtures of arginine, threonine and tryptophan did not cause growth depressions. If, then, these 6 are considered together as the most limiting amino acid, the pattern resembles more closely that of typical imbalances which result in an increase in the need of an animal for the most limiting amino acids (Kumta and Harper, '60a).

SUMMARY

The question of the specificity of the second most limiting amino acids, methionine and phenylalanine, in causing an imbalance in diets containing 6% of fibrin has been examined. A variety of amino acids and combinations of amino acids caused growth depressions under the conditions of these experiments.

Among the growth depressions, that caused by leucine has been attributed to the antagonism between it and isoleucine and valine. Addition of leucine to the diet reduces the efficiency of utilization of isoleucine, valine and histidine.

Additions of certain combinations of the least limiting amino acids, arginine, threonine and tryptophan also caused growth depressions of the same magnitude as that caused by the addition of methionine and phenylalanine.

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The Effect of Different Levels of Ascorbic Acid in the Diet of Guinea Pigs on Health, Reproduction and Survival[1,2]

ORREA F. PYE, CLARA M. TAYLOR AND PRESENTACION E. FONTANARES[3]
Nutrition Laboratory, Teachers College, Columbia University, New York, N. Y.

The optimal level of ascorbic acid in the diet of the guinea pig throughout the life cycle has not been definitely established. Current data on the ascorbic acid requirement of the guinea pig have been summarized by Mannering ('49) and Reid ('58).

Uhl ('58) has reviewed some of the studies of guinea pigs in a discussion of human requirements for ascorbic acid, commenting upon the apparent wide difference between minimum adequacy and optimal intake. As stated in the review on ascorbic acid in Recommended Dietary Allowances (National Research Council, '58), there is evidence that intakes which provide protection from gross signs of scurvy may not be satisfactory for the preservation of optimum health through long periods of time or when the body is subjected to common forms of stress. As indicated in publication 589 (N.R.C., '58), although a daily supply of 0.5 mg of ascorbic acid will protect young guinea pigs against classical signs of scurvy, distinct gains in health have been demonstrated at higher levels. The fact that different dietary intakes of ascorbic acid produce a direct influence on tissue levels of the vitamin was again confirmed in a recent study of guinea pigs with surgical wounds by Abt et al. ('59).

Very little work has been done on the ascorbic acid requirement of the guinea pig for reproduction. Earlier studies of the effect of ascorbic acid on reproduction in the guinea pig have been reviewed by Russell and Leitch ('48). A number of the earlier workers commented on the need of the pregnant guinea pig for ascorbic acid and reported that abortion or resorption occurred when diets low in ascorbic acid were supplied. No quantitative studies were made, however. Kramer et al. ('33) observed that degeneration in the ovaries of female guinea pigs occurred unless they were given about 5 ml of orange juice or tomato juice daily. Although pregnancy was possible when 3 to 5 ml of orange juice was given daily, abortions and resorptions were common. It is likely that many of these diets were inadequate in other nutrients as well as in ascorbic acid.

Crampton and Bell ('47) investigated the effect on reproduction of different sources of ascorbic acid using groups of mature female guinea pigs who had already had two to three litters. The basal diet used was low in fiber, an essential for the guinea pig. In comparing ascorbic acid with orange juice, lemon juice, and fresh grass or hay, the grass was found to be superior in preventing hemorrhage and abortion. As Crampton and Bell emphasized, ascorbic acid was not the principal limiting factor in their diets. These workers estimated the requirement of ascorbic acid for reproduction to be 2 to 5 mg daily. In their study, the guinea pigs were not subjected to repeated pregnancies when receiving a measured level of ascorbic acid. Unpublished data from another laboratory[4] suggest that higher levels of ascorbic acid may be needed for the guinea pig to give maximum reproductive performance in re-

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[2] A preliminary report on some of these data was given at the Annual Meeting of the American Institute of Nutrition, Atlantic City, 1959.

[3] Present address: Department of Chemistry, Columbia University.

[4] Personal communication from Dr. C. A. Slanetz, Department of Animal Care, College of Physicians and Surgeons, Columbia University.

peated pregnancies with full protection of the female breeder.

Data on the length of survival in guinea pigs are few. Zilva ('36) reported prolonged survival when feeding 5 mg of ascorbic acid or less.

For the present study of health, reproduction and survival in guinea pigs, 4 levels of ascorbic acid, namely, 2, 4, 6 or 8 mg daily were fed throughout life to the breeders.

EXPERIMENTAL PROCEDURE

Healthy guinea pigs of mixed color strains, 4 weeks old, were obtained from commercial stock. Breeding lots consisting of one male and two female guinea pigs were established. Four groups of animals, with 16 to 19 lots in each group, were given daily supplements of 2, 4, 6 or 8 mg of ascorbic acid, respectively.

All the guinea pigs were fed a commercial pelleted rabbit ration.[5] This ration was held long enough (approximately two to three months) to render it essentially free of ascorbic acid before feeding to the guinea pigs. The pellets were checked periodically for ascorbic acid by either biological or chemical assay or both. In the biological tests on the stored pellets, the guinea pigs developed scurvy within a period of three weeks and died within approximately 4 weeks. Chemical tests on the stored pellets showed that they furnished a trace, to not more than 0.06 mg, of ascorbic acid per day.

The possibility of the loss of vitamins other than ascorbic acid from the pellets during holding was taken into account. Preliminary studies in our laboratory indicated that guinea pigs were able to grow well for about 8 weeks when fed the pelleted ration which had been held for three months and when sufficient ascorbic acid was also supplied. Since it was thought that larger amounts of vitamins would probably be needed during pregnancy than during growth, various supplements were added. Weekly supplements were given of halibut liver oil[6] providing 3000 IU of vitamin A and 50 USP units of vitamin D, α-tocopheryl acetate furnishing the equivalent of 1 mg per day, and 50 mg of liver concentrate powder.[7]

Since there was the possibility that vitamin factors interrelated in the metabolism of ascorbic acid, if given in too large amounts, might overshadow the effect of the differences between the levels of ascorbic acid, the amounts of the supplements given were maintained at relatively low levels. The daily dosage of ascorbic acid was administered by pipette and fed immediately after dissolving in distilled water. The breeding lots were kept throughout the experiment in all-metal cages on raised wire-mesh screens. Fresh water was always available to the guinea pigs. All sanitary precautions were taken including periodic steam sterilization of cages and other equipment. No litter material was permitted either before delivery or during the nursing period to avoid any possible contamination.

RESULTS AND DISCUSSION

Growth and general condition. At all 4 levels of ascorbic acid, the male and female guinea pigs grew well. Rate of growth for the first 25 to 30 weeks was comparable to that described by Reid ('58) for the growth of random-bred strains of healthy, well-nourished guinea pigs, maintained under sanitary conditions. After 25 to 30 weeks, until full growth was attained, both the male and female guinea pigs tended to exceed somewhat the rate of growth indicated by Reid.

The initial weights of the males at approximately 4 weeks of age averaged 346, 336, 335 and 335 gm, respectively, when daily supplements of 2, 4, 6 and 8 mg of ascorbic acid were started. At one year of age male weights averaged 1171, 1240, 1359 and 1299 gm for these respective levels.

Initial weights of the females averaged 328, 323, 330, 325 gm, respectively when daily supplements of 2, 4, 6 and 8 mg of ascorbic acid were started. At one year of age weights for the non-pregnant females fed these respective levels averaged 945, 880, 998 and 1060 gm.

The general appearance and condition of the guinea pigs was good at all levels

[5] Rockland.
[6] Haliver Oil, Abbott.
[7] Wilson, 1:20.

used. The color of the ears and toes, when not obscured by pigment, appeared pink. Weight tended to decline somewhat in the older guinea pigs although not quite as much at the 6- and 8-mg levels as at the two lower levels. It was noted at autopsy that fatty stores tended to be less in the guinea pigs receiving the lower levels. Also at autopsy some signs suggestive of mild, possibly chronic, scurvy such as slight hemorrhages in the muscles, about the knee joints, and in the intestinal tract were observed in a few of the older animals fed the 2-mg level of ascorbic acid.

Pregnancies. The number of productive females are shown in table 1 and includes only those females having at least one pregnancy in which young in different stages of development were identified.

At each level of ascorbic acid some females did not produce young in the period of a year. These females were eliminated from the study and are not included in the tabular counts presented in this report. The largest proportion of nonproductive females, 35%, was found among those receiving the 2-mg ascorbic acid level. Thirteen per cent of the females started at the 4-mg level of ascorbic acid, and 23% of those fed both the 6-and 8-mg levels were found nonproductive within one year. Hemorrhage associated with slight weight change was noted at least once in one female fed the 8-mg level and in 5 females receiving the 2-mg level. No hemorrhaging was noted at the 4- and 6-mg levels. Weight change without hemorrhage was noted in 4 of the females fed at the 8-mg level but was not noted in females receiving other levels.

The average reproductive record of the females receiving 8 mg of ascorbic acid daily was superior to that of all the other groups. As shown in table 1, the females fed the 8-mg level had 2.8 ± a standard error of 0.26 pregnancies per productive female. This was found to be significantly higher when the "t" test was applied to the difference between the means than the averages of 2.1 ± 0.25, 2.1 ± 0.26, and 1.9 ± 0.29, respectively, at the 6-, 4- and 2-mg levels. When the "t" test was applied to the differences between the means at the 2-, 4- and 6-mg levels, no statistical significance was found. Three pregnancies per female was the maximum per year except for one female fed the 8-mg level who had 4 pregnancies in the second year of life.

Thirty per cent of the females fed the 2-, 6-, and 8-mg levels and 23% of those receiving the 4-mg level were productive during the second year of life. Only a very few breeders (6 in all) were productive into the third year.

Young produced and survival of young. Table 2 shows the number of young produced, when using the different levels of ascorbic acid. The difference between the mean for the 8-mg level of ascorbic acid and that for the 2-mg level is highly significant as judged by the "t" test ($P < 0.01$). Also the differences between the 8-mg and the two intermediate levels are significant. In this respect the 8-mg level stands out clearly as there is no significance to

TABLE 1

Number of productive female guinea pigs and pregnancies when fed at different levels of ascorbic acid

Level of ascorbic acid	No. of productive females	Percentage of females having pregnancies						No. of pregnancies	
		Pregnancies						Total/ level	Av./ female
		1st	2nd	3rd	4th	5th	6th		
mg		%	%	%	%	%	%		
2	22(4)[2](4)[3]	100	50	23	9	9	5	43	1.9 ± 0.29[1]
4	27(3)[2](5)[3]	100	56	37	7	4	4	57	2.1 ± 0.26
6	26(1)[2](6)[3]	100	58	23	19	8	0	54	2.1 ± 0.25
8	29(2)[2](5)[3]	100	80	48	31	10	7	80	2.8 ± 0.26

[1] Standard error of the mean.

[2] Figures in parentheses indicate number of females which died during delivery of poorly formed or premature but identifiable young.

[3] Figures in parentheses indicate number of instances in which the young were not delivered before death of the female, but identified at autopsy.

TABLE 2

Number of young produced by guinea pigs fed different levels of ascorbic acid

Level of ascorbic acid	Total young	Live young	Young per female	Live young as percentage of 2-mg group
mg				%
2	90	45	4.1±0.75[1]	100
4	122	79	4.5±0.78	176
6	122	59	4.7±0.77	131
8	219	136	7.6±0.81	302

[1] Standard error of the mean.

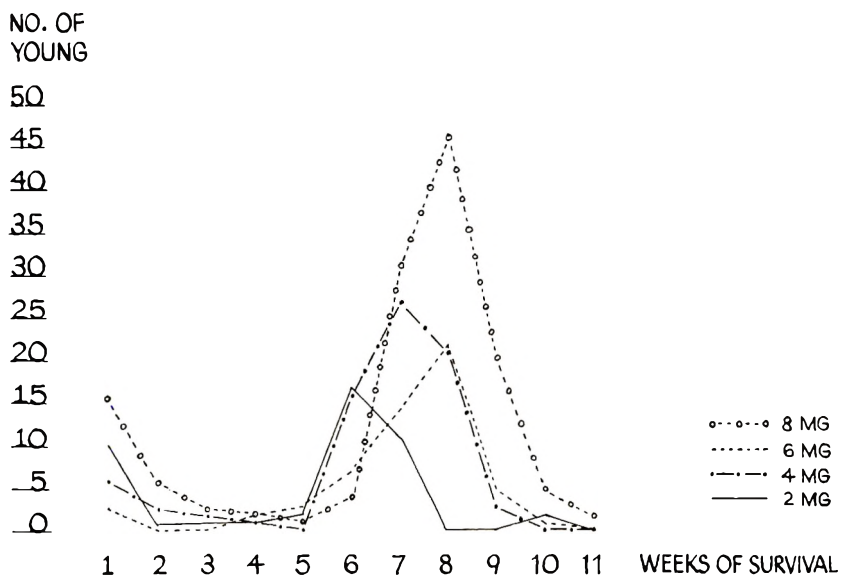

Fig. 1 Number and survival of young, using different levels of ascorbic acid (survival period without added ascorbic acid).

the differences between any of the three lower levels.

In figure 1 the length of the survival period in days for the live young produced by females when using the different levels of ascorbic acid is plotted. The period of survival was dependent on the storage of ascorbic acid at birth, the amount in the mother's milk, and the negligible amounts in the pelleted ration, since no added ascorbic acid was given to the young.

In figure 2 are shown the average total birth weights of the living young from each level of ascorbic acid. The weights were, respectively, for the 2-, 4-, 6- and 8-mg levels of ascorbic acid 182, 287, 222 and 415 gm.

Additional information on the weight of the young is given in table 3. The young were able to gain weight at every level of ascorbic acid for the first 4 weeks, perhaps due in large part to the supply of ascorbic acid from the mother's milk during the nursing period of about three weeks. But the surviving young from all levels had lost weight by the 6th week when they began to show definite signs of scurvy. In general, at 6 weeks, the young from mothers receiving the 8-mg level were superior in appearance to the young from other levels.

The average litter size was respectively 2.0, 2.4, 2.3 and 2.9 for the 2-, 4-, 6- and 8-mg levels of ascorbic acid.

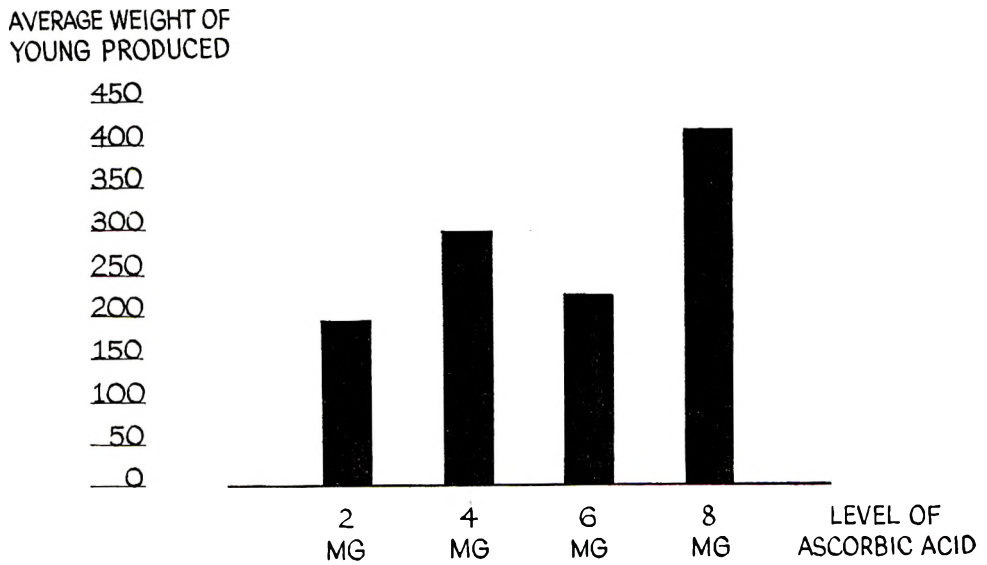

Fig. 2 Average total birth weight of living young, using different levels of ascorbic acid.

TABLE 3

Average weight of live young from guinea pigs fed different levels of ascorbic acid

Level of ascorbic acid	No. live young	Av. birth weight	Av. weight at 4 weeks	Av. weight at 6 weeks
mg		*gm*	*gm*	*gm*
2	45	87	254	214
4	79	100	308	272
6	59	100	302	284
8	136	102	311	293

TABLE 4

Productivity and survival of females receiving different levels of ascorbic acid

Level of ascorbic acid	Number		First pregnancy		Total no. of pregnancies	Av. length of reproductive period	Age at death
	Females	Young	Av. age	Dying			
mg			*days*	*%*		*days*	*days*
2	22	90	223	36	43	210	542±86[1](148–1680)[2]
4	27	122	186	22	57	211	647±93 (164–1484)
6	26	122	163	19	54	222	665±97 (152–1641)
8	29	219	162	17	80	264	420±50 (114–1209)

[1] Standard error of the mean.
[2] Figures in parentheses indicate range.

Survival of the productive females. The survival in days of the productive females receiving the different levels of ascorbic acid is given in table 4. Also, a summary of the productivity of the females at each level is included in this table.

In this study conditions of maximum stress of pregnancy were maintained as the females were permitted to breed early and as frequently as possible. The practice of postpartum mating was followed and as pointed out by Rowlands ('50) provided

for intensive breeding. Some mothers became pregnant just after delivery and thus were nursing young at the beginning of another pregnancy. The more productive females receiving the 8-mg level more frequently bore this double burden. The first pregnancy came earlier, on the average 163 and 162 days, respectively, for the females supplied with the 6-and 8-mg levels of ascorbic acid. Despite this earlier age, a smaller percentage of the females given the 8-mg level died as a result of this first pregnancy, only 17% compared with 36% for the females receiving the 2-mg level.

The average span of the reproductive period (the period between the approximate time of the beginning of the first pregnancy and the time of the last delivery) was longer for the females given the 8-mg level of ascorbic acid than for those at the other levels. The length of the period for the 8-mg level was found to be 26, 25 and 19% longer respectively than for that of the 2-, 4- and 6-mg levels.

Obviously the stress of pregnancy was much greater for the females supplied with the 8-mg level of ascorbic acid and this helps to explain the earlier age at death of these females compared with that of all other levels. That nature apparently favored the young at the expense of the mother was borne out by the facts that the total number of young produced by mothers receiving the 8-mg level was almost twice that of the young produced at any other level and that they survived longer.

At all levels of ascorbic acid a great deal of variation was observed among the individual females in the age at death. Hence, when the "t" test was applied to the differences between the mean age at death for the 4 levels of ascorbic acid no statistically significant differences among the groups were found.

Although the ration plus supplements proved fully adequate for growth, it may not have been entirely adequate for repeated pregnancies. Vitamin requirements for reproduction in the guinea pig are not well established. It is not known how much more liberal supplies of some factors may be needed for reproduction than for growth. Reid ('58) has summarized current knowledge of the requirements of the guinea pig for vitamins of the B complex, for example, and it is apparent that there are many gaps in our knowledge.

It must be kept in mind also that the guinea pigs in this study were maintained continuously on raised wire-mesh screens which kept coprophagy and refection at a minimum, possibly affecting the requirement for some of the vitamin factors.

No specific vitamin deficiency was identified at autopsy in the females supplied with the 8-mg level of ascorbic acid. A partial inadequacy of some factor or factors, however, may well have influenced the course of pregnancy and brought about premature death. Twenty-three of the 29 productive females receiving the 8-mg level died at delivery or directly following a pregnancy, a larger proportion than at any other level; and as pointed out earlier, a smaller percentage of the females receiving the highest level died in the first pregnancy. But in each succeeding pregnancy a larger proportion of the survivors were lost so that the females supplied with the 8-mg level were clearly at a disadvantage in terms of survival, having a larger number of pregnancies than any other group. It is obvious that the diet was not sufficiently adequate in some factors needed to support the stress of repeated pregnancies and the larger and more viable litters produced by females receiving the highest level. Whether still greater quantities of ascorbic acid and/or additional supplementary factors would have made a difference is not clear and points out the need for further research.

SUMMARY

Breeding lots consisting of one male and two female guinea pigs were fed a commercial ration essentially free of ascorbic acid. A total of 207 guinea pigs was divided into 4 groups (each group consisting of 16 to 19 lots) and given daily supplements of ascorbic acid per guinea pig, respectively, 2, 4, 6 or 8 mg. These levels of ascorbic acid were continued throughout the life of the breeders.

The 8-mg level of ascorbic acid was significantly superior to all other levels in terms of the number of pregnancies per female and in the number of total and living young. The females receiving the

8-mg level had a somewhat longer reproductive period. Average total birth weight of the young was greater and their period of survival tended to be longer.

Under the conditions of stress of pregnancy in this study, the average age at death of the females was respectively for the 2-, 4-, 6- and 8-mg levels, 542, 647, 665 and 420 days. There were no statistically significant differences between any of these averages because of the large variation in age at death of the individual females.

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Effect of Short-Term Feeding of Fish Oils and of Antioxidants on Plasma and Liver Cholesterol in the Rat

M. E. NIMNI,[1] A. E. MITTA, MARIA TROPAREVSKY AND A. TROPAREVSKY

Departamento de Biología y Medicina, Comisión Nacional de Energía Atómica, Buenos Aires, Argentina

The serum cholesterol level of the rat seems to be dependent to a great extent on the nature of the diet ingested (Portman and Stare, '59). When feeding cholesterol-free diets supplemented with various hydrogenated soybean oils, the highest cholesterol levels were found in the rats which consumed the diet containing the fats having higher iodine values (Swell et al., '55). Klein ('58) confirmed these findings by feeding diets with different levels of linoleic acid. He simultaneously found that the rats consuming the diets with a higher linoleic acid content showed a marked increase in their liver cholesterol esters. More recently, Okey et al. ('59) obtained similar results studying the deposition of cholesterol in liver during the feeding of fats of various degrees of unsaturation. On the other hand, Alfin-Slater et al. ('54) observed an increase in cholesterol esters in the liver, accompanied by fat infiltration, during the feeding of fat-free diets.

The property of fats of high iodine value to increase serum cholesterol in the rat under these circumstances, contrasts with the hypocholesterolemic effect exhibited by these same fats in man (Ahrens et al., '57), emphasizing the difference in behavior between species. Cholesterol levels of rats with dietary induced hypercholesterolemia, however, showed a decrease when one third of the dietary fat was substituted by fish oils. Linoleic acid was less efficient in this respect; palmitic and oleic acids had no effect.[2] A similar decrease in circulating cholesterol was observed in hypercholesterolemic rats which received a supplement containing the polyunsaturated fatty acids isolated from cod liver oil (Hauge and Nicolaysen, '59).

The experiments described, together with many others which appear in the literature, point out the difference in response exhibited by the rat under different physiological conditions when fed fats of varied degrees of unsaturation. At this stage it is hard to establish clearly the chemical groups responsible for these changes induced in the serum and liver cholesterol levels, and even more so, the mechanisms by which they occur. The iodine value of a fat per se does not, evidently, allow us to predict the effect it will produce when administered to the rat.

In an attempt to elucidate the mechanism for the decrease in serum cholesterol levels after the administration of dietary fish oils, the following experiment was undertaken.

EXPERIMENTAL

Male rats of the Wistar strain were used. They were housed in individual cages and their body weight and food consumption were measured regularly. A standard stock diet[3] (commercial animal feed) was used. By analysis, its percentage composition was as follows: protein, 19.3; total lipids, 6.8; carbohydrate, 51.2; ash, 6.2; fiber, 4.0; and water, 12.4. The high-carbohydrate diet was made up to contain (in per cent): casein, 20; corn starch, 72; corn oil, 3;

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[1] Present address: Department of Biochemistry and Nutrition, University of Southern California, Los Angeles 7, California.

[2] Peifer, J. J., and W. O. Lundberg 1959 Effect of unsaturated acids and fish oils on plasma and tissue lipides from hypercholestermic rats. Federation Proc., *18*: 300 (abstract).

[3] Especially manufactured by Molinos Rio de la Plata, Buenos Aires.

salt mixture (USP no. 5), 5; and choline·HCl, 0.2. The high-fat diet was made up to contain (in per cent): casein, 20; corn starch, 5; lard, 37; butter fat, 30; corn oil, 3; salt mixture (USP no. 5), 5; and choline·HCl, 0.2. These diets were supplemented with either sardine, tuna or shad oils, obtained from local fisheries. In these circumstances, a sufficient amount was added to reach a 20% concentration, replacing either carbohydrate or butterfat calories in order to maintain constant the protein levels. All diets were supplemented with a vitamin mixture.[4] The antioxidants used[5] in these experiments were N,N′-diphenyl - *p* - phenylenediamine (DPPD), 1,2 - dihydro - 2,2,4-trimethyl - 6 - ethoxyquinoline (Santoquin)[6] and butyl-4-methoxyphenol (BHT). The levels were similar to those which proved to be active in preventing dietary necrotic liver degeneration produced in rats by a vitamin E - deficient 30% Torula yeast ration (Schwarz, '58). The shad oil used (*Prochilodus lineatus*) had an iodine value of 87 and a saponification value of 192.

At the end of the experimental period the animals were fasted for 8 hours and blood was extracted under ether anesthesia by way of the abdominal aorta. Livers were removed and maintained frozen until the time of analysis. Esterified and free cholesterol was determined using the method of Sperry and Webb ('50) and total cholesterol by the method of Abell et al. ('52).

RESULTS AND DISCUSSION

In table 1 are shown the values obtained when total serum and liver cholesterol was determined in rats fed a basal carbohydrate diet supplemented with sardine or tuna oils. Control serum cholesterol levels were determined by extracting a one-milliliter blood sample by cardiac puncture prior to feeding the experimental diets. Animals were then fed the stock diet for three more days and then fed the experimental diets over a 4-day period. From the results obtained it seems evident that the addition of fish oils to the diet significantly decreased the serum cholesterol level for each animal. Simultaneously an increase in cholesterol was noted in the liver. During the period in which the fish oil-supplemented carbohydrate diets were fed, the animals developed severe diarrhea and their growth was completely retarded. A cessation of growth induced by addition of fish oils to fat-free diets has recently been reported by Ershoff ('60).

These initial observations led to an investigation to attempt to gather information regarding the mechanism by which these changes occur and, in particular to investigate whether a relationship exists between the facility with which the fish oils undergo auto-oxidation and the changes induced in the cholesterol distribution. Therefore the next group of experiments was designed to investigate whether the same phenomenon occurred when a high-fat diet was supplemented with fish oil, and whether the addition of antioxidants to the diet would alter in any way the pattern of cholesterol distribution between serum and liver. The re-

[4] Composition of vitamin mixture per 100 gm of diet: vitamin A, 2000 U.S.P. units; vitamin D, 250 U.S.P. units; vitamin B_1, 125 U.S.P. units; and (in milligrams) vitamin B_2, 0.5; pyridoxine, 0.025; α-tocopherol, 0.5; Ca panthothenate, 0.25; niacinamide, 5; and vitamin B_{12}, 0.001.

[5] DPPD was generously supplied by B. F. Goodrich Chemical Company, Santoquin by Monsanto Chemical Company and BHT by Eastman Chemical Products, Inc.

[6] Monsanto Chemical Company, St. Louis.

TABLE 1

Effect of feeding a basal carbohydrate diet supplemented with sardine or tuna oils on the total serum and liver cholesterol levels of the rat[1]

Group	Supplement	No. of rats	Total serum cholesterol		Total liver cholesterol
			Initial	Final	
			mg/100 ml		*mg/100 gm*
1	None	6	—	—	252 ± 14[2]
2	Sardine oil, 20%	6	72.3 ± 2.7	54.6 ± 2.8	701 ± 62
3	Tuna oil, 20%	10	71.1 ± 4.6	63.5 ± 4.0	631 ± 55

[1] Experimental period: 4 days.
[2] Standard error.

sults obtained are summarized in table 2. The diets were fed over a 13-day period, and, in contrast with the previous set of experiments, all animals gained weight, although at a slower rate than the controls fed the stock diet. Santoquin and BHT additions induced a more pronounced growth retardation when included in the diet. All the animals which received a fish oil supplement in their diets showed a decreased level of circulating cholesterol ($P < 0.01$). This decrease was most marked in those animals which did not receive antioxidants simultaneously, and in all instances was due almost exclusively to a change in the esterified fraction. DPPD was the most efficient of the antioxidants tried, in counteracting the hypocholesterolemic effects of shad oil ($P < 0.01$). Santoquin was a little less efficient ($P < 0.01$), whereas BHT showed practically no protective effect.

The same pattern regarding the antioxidant effect is observed on the levels of liver cholesterol. A significant increase in liver cholesterol occurred in rats which received the fish oil supplements ($P < 0.01$). The addition of antioxidants seemed to counteract the cholesterol accumulation with the same order of efficiency as they reversed the changes in serum cholesterol. In the case of liver cholesterol, accumulation due to the ingestion of the fish oil diets was due to an increase in the free fraction. The degree of cholesterol deposition was lower in this case, using a high-fat basal diet, than in the first series of experiments where the supplemented diet was a high-carbohydrate diet. Histological studies of the liver of these animals failed to reveal any pathological alterations. The observation of the changes in the pattern of cholesterol distribution during the feeding of fish oil diets suggests a possible effect of these oils on the mechanism of cholesterol transport. The increased liver-free cholesterol fraction and the decrease in circulating esterified cholesterol could probably be accounted for by a decreased capacity of the liver to esterify cholesterol and thus render it available for mobilization. This functional impairment could be at least partially due to the toxic effects of the oxidation products derived from the polyunsaturated fats present in the fish oils.

TABLE 2

Effect of feeding a high-fat diet supplemented with 20% of shad oil (SO) and several antioxidants on the levels of serum and liver total and free cholesterol of the rat[1,2]

Group	Diet	Supplement	Average daily weight increase	Serum cholesterol		Total liver lipids	Liver cholesterol	
				Total	Free		Total	Free
			gm	*mg/100 ml*	*mg/100 ml*	*gm/100 gm*	*mg/100 gm*	
1	Stock diet	—	4.5	87.5 ± 1.1[3]	29.2 ± 0.3	4.9	262 ± 5	136 ± 2
2	High fat	—	4.1	86.1 ± 0.9	28.2 ± 0.2	6.1	279 ± 6	150 ± 5
3	High fat	20% SO	2.7	62.2 ± 0.8	26.0 ± 0.9	6.7	406 ± 9	257 ± 9
4	High fat	20% SO + 0.005% DPPD	2.4	71.7 ± 0.7	28.7 ± 0.5	7.5	344 ± 6	202 ± 4
5	High fat	20% SO + 0.25% Santoquin	1.8	68.4 ± 0.7	28.1 ± 0.2	7.1	371 ± 6	231 ± 4
6	High fat	20% SO + 0.5% BHT	1.1	64.9 ± 0.7	26.5 ± 0.5	6.8	411 ± 7	262 ± 5

[1] Experimental period: 13 days.
[2] Six animals/group.
[3] Standard error.

SUMMARY

The addition of different fish oils to basal carbohydrate or fat diets induced hypocholesterolemia in the rat. On the other hand these same diets increased the liver cholesterol levels. The observed hypocholesterolemia is a consequence of a decrease in the esterified fraction, whereas liver cholesterol increase was mainly due to an accumulation of free cholesterol.

The addition of N,N′-diphenyl-*p*-phenylenediamine and 1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline to high fat diets supplemented with 20% of shad oil tended to reverse the effects produced by the fish oil. It decreased the degree of hypocholesterolemia and the accumulation of liver cholesterol. Butyl-4-methoxyphenol, another antioxidant studied, was ineffective in this respect.

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Influence of Dietary Fats and Cholesterol on Tissue Lipids in Chickens[1,2]

LUCILE F. ADAMSON,[3] GERTRUDE K. LEEPER AND ERNEST ROSS
Departments of Foods and Nutrition and Poultry Science, Hawaii Agricultural Experiment Station, University of Hawaii, Honolulu, Hawaii

There is some belief in the thesis that essential fatty acids, in contrast with nonessential fatty acids, inhibit hypercholesterolemia and/or atherogenesis in experimental animals. Among the species which seem to stand out as an exception to this generalization is the chicken. Hypercholesterolemia and atherosclerosis are produced in chickens by feeding diets containing unsaturated oils and cholesterol, and these effects are enhanced by higher concentrations of dietary vegetable oils. This phenomenon has been observed in various laboratories. The apparent species difference in reaction to unsaturated fats, which is observed in chickens as compared with some other animals, is reviewed by Katz et al. ('58).

Recently, Hegsted et al. ('60b) published data showing an inverse relationship, with some exceptions, between the serum cholesterol concentrations and the iodine number of the dietary fat of cholesterol-fed chicks. These results contrast strongly with most previous reports of the effect of unsaturated fatty acids on cholesterolemia in chickens.

The work reported here was undertaken in a further attempt to clarify the question of whether serum cholesterol concentrations in the cholesterol-fed chicken are independent of the nature of the dietary fat. With this aim, two features in particular were introduced in the experimental plan on the theory that their absence in some previous studies might be responsible in part for the contradictory evidence: (a) a fat-free synthetic diet (with or without fat supplementation) was given to the chicks from the first day after hatching, thus avoiding the unknown effects of the fat from the commercial starter mashes which are usually fed for one or more weeks before the experimental diets are initiated; (b) the experimental diets were fed for varying periods up to 22 weeks, providing longer terms of observation than is usual in such experiments.

EXPERIMENTAL

Experimental plan. One hundred twenty-one one-day-old male chicks from the University of Hawaii strain of New Hampshires were divided into 11 groups of 11 birds each. One group of birds received a standard corn-soybean oilmeal ration containing approximately 3% of fat and 21% of protein. During a preliminary fat-depletion period of one week, all birds of the other 10 groups were fed a fat-free basal ration of the following percentage composition: fat-free casein, 20; gelatin, 8; cellulose, 2; sucrose, 58.5; DL-methionine, 0.3; choline chloride, 0.2; mineral mix, 6; vitamin mix, 5. The mineral mix supplied, in grams per kilogram of diet: $CaCO_3$, 15; K_2HPO_4, 9; Na_2HPO_4, 7.2; $Ca_3(PO_4)_2$, 14; NaCl, 8.8; $MgSO_4 \cdot 7HOH$, 5; ferric citrate, 0.4; $MnSO_4 \cdot 4HOH$, 0.42; KI, 0.04; $ZnCO_3$, 0.02; $CuSO_4 \cdot 5HOH$, 0.02; sodium molybdate, 0.10. The vitamin mix supplied, in milligrams per kilogram of diet: thiamine, 8; riboflavin, 8; Ca pantothenate, 20; niacin, 100; pyridoxine, 8; *d*-biotin, 0.3; folic acid, 3; vitamin B_{12}, 0.02; vitamin D_3, 0.02; α-tocopheryl acetate, 100; 2-methyl-1,4-naphthoquinone, 1; vitamin A, gelatin-coated (500 I.U./mg), 200; glucose,

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[3] Present address: Department of Pediatrics, School of Medicine, University of Missouri, Columbia, Missouri.

49,551.66. The ration was mixed mechanically.

At the end of one week, fat and cholesterol were substituted for part of the sucrose in the diets of some groups, as indicated in table 1. Silica was added along with the fat in amounts calculated to make the rations isocaloric with the fat-free basal diet. When both fat and cholesterol were added, the cholesterol was dissolved in the fat before further mixing. Ad libitum feeding was allowed for all diets. Two to 4 birds from each group were killed after 10, 16, and 22 weeks for observations of: liver cholesterol and liver lipid concentrations; iodine numbers of liver and storage lipids; the rate and degree of endogenous cholesterol synthesis; and the condition of the aortae. Because most of the animals in the fat-free groups (1 to 3) showed symptoms of respiratory infection, however, all survivors in groups 1 and 2 were sacrificed after 16 weeks and in group 3 after 10 weeks.

Blood samples for cholesterol determination were drawn from the wing veins of all surviving birds at 50, 70, 90, 113 and 155 days.

For the measurement of endogenous cholesterol synthesis, each chicken was injected intraperitoneally with 20 μc per kg of sodium acetate-2-C^{14} solution 8 hours before being sacrificed. The injections were made between 6 A.M. and 8 A.M. and the birds were caged for the next 8 hours in a hood with access to water but not to food. Blood samples (5 ml or less) for the measurement of cholesterol specific activity were drawn from the wing veins at two, 4 and 6 hours after the injection. An 8-hour blood sample was obtained when the bird was decapitated. The blood samples were centrifuged immediately after being drawn. The liver, the aorta, the contents of the gall bladder, and samples of fat from the rear of the abdominal cavity and from around the heart were removed from each chicken soon after it was killed. The aorta was placed in formaldehyde solution. All other samples were stored in sealed containers at 0° to 5°C for varying lengths of time (up to 6 months) until analyses could be carried out.

Analytical techniques. Serum total cholesterol concentration was determined by the method of Rosenthal and Jud ('58). A pooled sample of normal chicken serum was used as a control and was analyzed with each group of unknowns. As a check on the method, cholesterol concentrations of approximately 40 samples were determined by precipitation of the cholesterol with digitonin and reaction of the dried digitonides with the iron reagent.

TABLE 1

Composition of experimental rations

Group no.	Ingredients				
	Premix[1]	Sucrose	Silica	Cholesterol	Fat
	%	%	%	%	%
0[2]	—	—	—	–	3 (corn-soy)
1	61	34	—	–	—
2	61	34	—	1	—
3[3]	61	34	—	–	—
4	61	27	4	–	3 (corn oil)
5	61	—	19	–	15 (corn oil)
6	61	—	19	–	15 (vegetable shortening)[4]
7	61	—	19	–	15 (butterfat)[5]
8	61	—	19	1	15 (corn oil)
9	61	—	19	1	15 (vegetable shortening)
10	61	—	19	1	15 (butterfat)

[1] Fat-free basal ration (see text) from which 34/58.5 of the sucrose was withheld during mixing.

[2] This group received a corn-soybean oil meal starter mash.

[3] This ration also contained 0.5% of crude (65%) methyl linoleate.

[4] A partially hydrogenated vegetable shortening made from cottonseed oil.

[5] Separated from melted butter and washed repeatedly with warm water.

The results were found to check with the values obtained without digitonin precipitation. This precaution was deemed necessary when it was found that the liver vitamin A concentrations of the chickens were extremely high (as much as 6000 I.U./gm). Vitamin A has been reported to cause false high results in cholesterol determinations by the Zak method (Kinley and Krause, '58). Digitonin precipitation was used for all liver cholesterol determinations.

Abdominal and pericardial fat samples were chopped and extracted in the Goldfisch extraction apparatus. Livers were chopped, weighed, and homogenized in 95% EtOH; the homogenates were stored at 0° to 5°C until needed. For extraction of liver lipids, an aliquot of the homogenate equivalent to 5 to 10 gm of liver was filtered through an extraction thimble. When the precipitate was well drained, it was extracted with diethyl ether for three hours on the Goldfisch apparatus. The ether extract was then added to the ethanol filtrate and made up to a convenient volume. Aliquots of this extract were taken for determinations of total lipid concentration, cholesterol concentration, iodine number and cholesterol specific activity. The aliquots for total lipids and iodine numbers were washed three or more times with water before further treatment. Iodine numbers were determined by standard methods.

For the measurement of cholesterol specific activity, an aliquot of ethanol-ether extract of serum or liver which was calculated to contain approximately 4 mg of cholesterol was taken. It was saponified by heating for 30 minutes at 70° to 80°C with 4 drops of 10N KOH. The extract was then neutralized with glacial acetic acid. A calculated 50% excess of digitonin solution (1% in 50% EtOH) was added and the sample was allowed to stand overnight or longer. The precipitate was then separated by centrifugation and successively washed with hot 50% ethanol, acetone, and ether. It was then resuspended in approximately 2 ml of ether and poured into a weighed, aluminum, cupped planchet. Evaporation of the ether left the precipitate evenly distributed on the planchet. The planchet was reweighed and radioactivity was measured in a gas flow counter. Corrections were made for counter efficiency and for self-absorption. Results were calculated in terms of disintegrations per minute per milligram of cholesterol, using a factor of 4 for conversion from digitonide to cholesterol.

For measurement of biliary cholesterol radioactivity, bile samples were diluted with 50% EtOH and saponified with KOH. The saponification mixture was then extracted repeatedly with petroleum ether. The extract was washed with water and evaporated to dryness. The residue was redissolved in 50% EtOH and digitonides were precipitated and treated as above.

RESULTS

Growth and mortality. The effects of the different diets on growth and morbidity during the first 6 weeks will be discussed more fully in another paper.[4] Growth data for the full 22-week experimental period are presented in table 2. The mean body weights of the groups receiving 15% of corn oil with 1% of cholesterol (group 8) and the 3% fat stock diet (group zero) were consistently higher than those observed in the other groups. Growth with the fat-free diets (groups 1 to 3) was less than in the groups receiving dietary fat. Because of the smallness of the groups, the wide variability, and the greater disease incidence in some groups, the significance and the causes of these differences are uncertain.

More striking than the growth difference was the difference in disease incidence. The spores of the *Aspergillus* fungi are widely distributed in nature, particularly in warm damp climates. Vigorous healthy birds ordinarily withstand considerable exposure occurring under normal conditions (Biester and Schwarte, '58). In the present experiment a high percentage of the birds maintained with the fat-free diets (groups 1 to 3) showed symptoms of aspergillosis with a high mortality. Because birds were killed at intervals after 6 weeks, no data on mortality can be given for the period between 6 and 22 weeks. The disease incidence was so high in the groups without dietary fat, however, that all of the surviving birds in group 3 were sacrificed after 10 weeks and those remaining in groups 1 and 2 were killed after 16

[4] Ross and Adamson, in preparation.

TABLE 2

Body weights

	Diet	Weight after diet fed 7 weeks		No.[2]	Weeks fed diet									
					7		10		13		16		22	
		No.	$\bar{x}$[1]		Range	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$
			gm		*gm*		*gm*		*gm*		*gm*		*gm*	
0	Stock (3% fat)	10	808 ± 99	4	593–745	812	1310–1563	1450	1896–2180	2021	2620–2792	2682	2986–3409	3159
1	Fat-free basal (FFB), no additions	9	610 ± 50	4	593–745	640	948–1130	1036	—	—	—	—	—	—
2	FFB + 1% cholesterol	6	649 ± 69	3	589–701	648	1082–1214	1158	—	—	—	—	—	—
3	FFB + linoleate	9	632 ± 117	–	—	—	—	—	—	—	—	—	—	—
4	FFB + 3% corn oil	11	674 ± 120	4	531–812	738	1170–1523	1370	1856–2212	1965	2099–2477	2240	2699–3100	2910
5	FFB + 15% corn oil	10	795 ± 172	4	417–921	706	1000–1482	1323	1633–2156	1906	2190–2421	2323	2260–2994	2617
6	FFB + 15% vegetable shortening	8	741 ± 87	3	724–834	768	1257–1617	1445	1700–2210	1922	2320–2730	2557	2579–3200	2973
7	FFB + 15% butterfat	9	741 ± 87	3	608–761	704	1262–1383	1327	1661–1882	1775	2282–2670	2431	2687–3000	2889
8	FFB + 15% corn oil + 1% cholesterol	9	888 ± 99	4	761–959	870	1465–1693	1605	1892–2180	2026	2320–2787	2564	2995–3418	3149
9	FFB + 15% vegetable shortening + 1% cholesterol	11	750 ± 118	4	675–797	739	1206–1543	1358	1566–2053	1771	2119–2860	2352	2489–3310	2742
10	FFB + 15% butterfat + 1% cholesterol	9	738 ± 113	4	674–796	738	1251–1491	1364	1700–2051	1841	1863–2360	2154	2848–3259	3012

[1] Mean body weight ± standard deviation for all birds in each group.

[2] Groups consisting only of those birds fed diets for full 22 weeks.

weeks. In contrast with groups 1 to 3, most of the birds receiving dietary fat appeared healthy even when maintained with the diets for 22 weeks.

Serum cholesterol concentrations. Serum cholesterol concentrations in each group after receiving the experimental diets for 7, 10, 13, 16 and 22 weeks are shown in table 3. Because birds were sacrificed at intervals, the composition of each group changed with time. For purposes of comparing the change of cholesterol concentration with time, values are also given for a smaller group within each of the groups receiving fat. These smaller groups are of constant composition and consist only of those birds which were maintained with the diets for the full 22-week period.

The mean serum cholesterol concentrations for the fat-free-plus-cholesterol group and all of the cholesterol-free groups (zero to 7) were quite similar. When the wide ranges within some of the groups and the changes with time are taken into consideration, neither the amount (zero, 3 or 15%) nor the kind (corn oil, vegetable shortening or butterfat) of fat appeared to exert a consistent influence on the serum cholesterol concentrations in the absence of dietary cholesterol.

With each of the diets containing both fat and cholesterol (groups 8 to 10), serum cholesterol concentrations were markedly increased. These increases were augmented to a maximum value after 13 or 16 weeks when the group means were between two and three times those seen on the corresponding cholesterol-free diets. In these groups also, there were no consistent differences in the effects of corn oil, vegetable shortening and butterfat on serum cholesterol concentrations.

A decrease in serum cholesterol concentrations after 13 or 16 weeks was observed in each of the groups receiving dietary fat (groups 4 to 10) and in almost all of the individuals within the groups with one exception: the group of birds fed the cholesterol-free 15% of corn oil diet (group 5) maintained a constant mean serum cholesterol concentration throughout the period from 7 to 22 weeks.

Liver lipids. Liver cholesterol and liver total lipid concentrations are given in table 4. At 10 weeks, the liver cholesterol was higher in the two birds fed the vegetable shortening diet (group 6) than in the birds receiving the other diets containing fat but no cholesterol (groups zero, 4, 5, and 7). Thereafter, the concentrations in the latter groups increased until, at 22 weeks, liver cholesterol concentrations were equivalent in all of the cholesterol-free diet groups.

Liver cholesterol concentrations at 10 weeks in birds fed diets containing both fat and cholesterol (groups 8 to 10) were two to 5 times as great as in those fed the corresponding cholesterol-free diets. With two of the three test fats, this difference in effect of the diets with and without cholesterol increased progressively with time so that, at 22 weeks, liver cholesterol concentrations in groups 8 and 9 (corn oil plus cholesterol, and vegetable shortening plus cholesterol) were approximately 11 times those of their control groups (5 and 6). Values for group 10 (butterfat plus cholesterol) at 22 weeks were still only three times those of group 7 and at both 10 and 22 weeks, liver cholesterol concentrations were markedly lower with dietary butterfat and cholesterol than with corn oil or vegetable shortening and cholesterol. At all ages, liver cholesterol showed much greater intragroup variation in group 8 than in groups 9 or 10.

Liver cholesterol concentrations in the fat-free, fat-free-plus-linoleate, and fat-free-plus-cholesterol groups were slightly higher than in groups fed fat without cholesterol but were much lower than in groups fed both fat and cholesterol.

Liver total lipid concentrations were approximately equal in all groups at 10 and 16 weeks. After 22 weeks, however, the liver lipids in the groups receiving both fat and cholesterol were two to three times those of the other groups. As was the case with liver cholesterol, the liver total lipid storage was less with butterfat and cholesterol than with corn oil or vegetable shortening and cholesterol.

The increased liver cholesterol concentrations in groups 8 to 10 preceded by at least 13 weeks the increases in the liver total lipids.

Iodine numbers of tissue lipids. The iodine numbers of lipids from three sites

TABLE 3

Serum cholesterol concentrations

Diet		Weeks fed diet														
		7			10			13			16			22		
		No.	Range	$\bar{x}$	No.	Range	$\bar{x}$	No.	Range	$\bar{x}$	No.	Range	$\bar{x}$	No.	Range	$\bar{x}$
		mg/100 ml			*mg/100 ml*			*mg/100 ml*			*mg/100 ml*			*mg/100 ml*		
0	Stock (3% fat)	10[1]	121–176	145	10	91–176	140	8	109–164	140	8	80–129	100	–	—	—
		4[1]	121–176	152	4	110–170	143	4	109–164	132	4	80–129	102	4	76–110	93
1	Fat-free basal, no additions	3	125–149	134	5	82–191	126	2	110–124	117	3	113–206	153	–	—	—
		—	—	—	—	—	—	–	—	—	–	—	—	–	—	—
2	Fat-free basal + 1% cholesterol	3	106–128	115	3	110–139	128	2	146–147	147	1	—	134	–	—	—
		—	—	—	—	—	—	–	—	—	–	—	—	–	—	—
3	Fat-free basal + linoleate	4	131–177	154	8	110–165	145	–	—	—	–	—	—	–	—	—
		—	—	—	—	—	—	–	—	—	–	—	—	–	—	—
4	Fat-free basal + 3% corn oil	8	108–193	161	10	108–180	144	8	108–172	145	5	112–163	135	–	—	—
		2	144–151	148	2	129–163	146	2	145–161	153	2	144–163	154	2	88–97	93
5	Fat-free basal + 15% corn oil	9	131–171	152	9	105–184	151	7	126–211	154	7	122–168	137	–	—	—
		4	131–162	143	4	130–169	145	4	126–150	139	4	124–168	143	4	98–228	141
6	Fat-free basal + 15% vegetable shortening	7	124–176	148	8	113–196	144	6	132–219	159	5	124–170	139	–	—	—
		3	130–176	149	3	130–148	139	3	138–158	151	3	132–170	147	3	97–119	105
7	Fat-free basal + 15% butterfat	9	116–156	140	9	106–187	146	7	105–244	181	7	106–154	134	–	—	—
		3	123–156	137	3	106–166	133	3	105–207	156	3	119–142	130	3	102–125	110
8	Fat-free basal + 15% corn oil + 1% cholesterol	9	177–413	282	9	162–409	268	7	188–370	290	7	130–351	265	–	—	—
		4	177–325	259	4	193–279	245	4	188–336	260	4	179–346	266	4	125–195	164
9	Fat-free basal + 15% vegetable shortening + 1% cholesterol	11	158–440	282	10	165–339	259	9	172–503	322	8	155–338	263	–	—	—
		4	158–336	265	4	228–339	287	4	196–474	317	4	204–338	288	4	155–253	219
10	Fat-free basal + 15% butterfat + 1% cholesterol	9	142–273	212	9	125–300	188	6	155–430	265	4	176–496	334	–	—	—
		4	142–273	195	4	134–205	168	4	155–430	278	4	176–496	334	4	109–265	189

[1] For diets zero, 4 to 10, mean values for all surviving birds within the group at each age are given in the upper row. The values in the second row for these diets are for groups consisting only of those birds which were fed the diet for the full 22-week period.

TABLE 4

Liver lipid concentrations

	Diet	Lipid	Weeks fed diet: 10			16			22		
			No.	Range	$\bar{x}$	No.	Range	$\bar{x}$	No.	Range	$\bar{x}$
0	Stock (3% fat)	Cholesterol[1]	2	4.73–4.82	4.78	4	3.86–4.82	4.44	4	6.19–9.90	7.34
		Total lipid[2]	2	4.10–4.84	4.47	4	3.67–4.93	4.15	4	5.55–8.36	6.51
1	Fat-free basal (no additions)	Cholesterol	2	7.41–8.31	7.86	3	4.33–9.58	6.71	–	—	—
		Total lipid	2	4.07–4.61	4.34	3	4.21–4.99	4.69	–	—	—
2	Fat-free basal + 1% cholesterol	Cholesterol	2	7.82–13.06	10.44	2	9.22–15.62	12.42	–	—	—
		Total lipid	2	4.69–4.98	4.84	2	4.76–5.85	5.31	–	—	—
3	Fat-free basal + linoleate	Cholesterol	7	3.38–9.02	5.60	–	—	—	–	—	—
		Total lipid	7	3.47–8.49	5.23	–	—	—	–	—	—
4	Fat-free basal + 3% corn oil	Cholesterol	2	4.06–4.80	4.43	3	4.30–5.91	5.35	4	6.10–8.12	6.73
		Total lipid	2	4.11–4.41	4.26	3	3.95–4.56	4.20	4	6.03–8.15	6.94
5	Fat-free basal + 15% corn oil	Cholesterol	2	3.61–4.16	3.89	3	3.11–8.15	5.76	4	5.05–9.13	6.77
		Total lipid	2	4.63–4.71	4.67	3	4.54–5.75	5.02	4	5.49–7.17	6.20
6	Fat-free basal + 15% vegetable shortening	Cholesterol	2	5.96–8.85	7.41	2	5.33–8.90	7.12	3	6.95–8.21	7.77
		Total lipid	2	4.54–4.98	4.76	2	3.98–4.74	4.36	3	5.00–6.02	5.54
7	Fat-free basal + 15% butterfat	Cholesterol	2	3.79–5.01	4.40	3	4.14–4.71	4.49	4	4.06–11.25	7.39
		Total lipid	2	4.20–6.34	5.27	3	3.48–4.85	4.24	4	3.01–6.95	4.82
8	Fat-free basal + 15% corn oil + 1% cholesterol	Cholesterol	2	13.58–21.08	17.33	3	28.41–98.22	58.14	4	36.42–136.96	68.01
		Total lipid	2	5.28–6.07	5.68	3	3.16–8.22	5.91	4	17.71–23.33	21.57
9	Fat-free basal + 15% vegetable shortening + 1% cholesterol	Cholesterol	2	17.78–19.27	18.53	4	8.18–19.35	11.44	4	63.46–104.99	81.56
		Total lipid	2	5.74–6.10	5.92	4	3.51–6.51	5.16	4	13.13–28.56	18.62
10	Fat-free basal + 15% butterfat + 1% cholesterol	Cholesterol	2	7.30–10.36	8.83	1	—	17.27	4	7.95–32.33	21.60
		Total lipid	2	5.31–5.87	5.59	1	—	6.39	4	7.37–16.53	10.59

[1] Mg/gm of liver.

[2] Per cent wet weight of liver.

are shown in table 5. In the table, data from all three ages studied are pooled because there were no apparent differences with age in any group.

Although there was considerable variation within groups, the iodine numbers of liver lipids of all groups were similar despite the wide differences in dietary fat saturation. Liver lipids were considerably more unsaturated than the dietary fat in the case of the groups fed vegetable shortening or butterfat. In contrast with the liver lipids, the iodine numbers for storage fats from both sites were almost the same as for the dietary fat in all groups receiving 15% of fat, with or without cholesterol. Iodine numbers of storage fat in groups zero and 4—the two groups receiving only 3% of fat—were lower than those of the dietary fat and were equivalent to those observed when feeding the fat-free and the vegetable-shortening diets. The presence or absence of dietary cholesterol was without consistent influence on the iodine numbers of tissue fats.

Endogenous cholesterol synthesis. The specific activities of serum, liver, and bile cholesterol 8 hours after injection of sodium acetate-2-C^{14} are shown in table 6. Synthesis was almost completely inhibited by those diets containing both 15% of fat and 1% of cholesterol (groups 8 to 10). This inhibition is apparently somewhat less thorough with dietary vegetable shortening or butterfat than with corn oil.

Biliary cholesterol with appreciable specific activity was observed in several individual birds of groups 8, 9 and 10 accompanied by serum and liver cholesterol of very low activity. This may be an indication that cholesterol synthesis was not always highly inhibited by the fat-plus-cholesterol diets, but that the mixing of the newly synthesized cholesterol with the serum and the liver cholesterol was restricted. An alternative and more probable explanation is that the radioactive biliary "cholesterol" was in fact not cholesterol but a closely related digitonin-precipitable com-

TABLE 5

Iodine numbers of tissue lipids

	Diet	Diet fat	Liver lipids		Storage fat A[1]		Storage fat B[2]	
		I no.	N	$\bar{x}$[3]	N	$\bar{x}$[3]	N	$\bar{x}$[3]
0	Stock (3% fat)	129	10	146±21	10	60±10	10	54±8
1	Fat-free basal (no additions)	—	5	108±37	4	67±10	2	55±3
2	Fat-free basal + 1% cholesterol	—	4	105±12	4	85±55	3	60±6
3	Fat-free basal + linoleate	—	7	99±20	8	61±4	8	50±11
4	Fat-free basal + 3% corn oil	108	9	126±24	9	59±15	6	57±12
5	Fat-free basal + 15% corn oil	108	9	125±33	9	92±15	7	97±15
6	Fat-free basal + 15% vegetable shortening	77	7	112±13	5	67±4	6	59±8
7	Fat-free basal + 15% butterfat	36	9	137±37	9	51±7	7	44±9
8	Fat-free basal + 15% corn oil + 1% cholesterol	108	9	111±27	7	118±24	7	105±8
9	Fat-free basal + 15% vegetable shortening + 1% cholesterol	77	10	113±33	10	74±9	9	69±5
10	Fat-free basal + 15% butterfat + 1% cholesterol	36	7	89±13	6	51±3	6	41±10

[1] Fat from around the upper heart.

[2] Fat lining the posterior abdominal cavity.

[3] Mean iodine value ± standard deviation. Data obtained after 10-, 16-, and 22-week feeding periods showed no differences and are here combined.

TABLE 6

Specific activity of cholesterol 8 hours after C^{14}-acetate injection

	Group		Weeks fed diet								
			10			16			22		
			No.	Range	$\bar{x}$	No.	Range	$\bar{x}$	No.	Range	$\bar{x}$
0	Stock diet (3% fat)	Serum	2	310–438[1]	374	4	268–463	355	4	53–368	207
		Liver	2	655–902	779	4	636–852	697	4	163–1237	679
		Bile	2	868–881	875	4	587–1365	832	4	607–1779	968
1	Fat-free basal (no additions)	Serum	2	117–233	175	2	59–60	60	–	—	—
		Liver	2	114–212	163	2	82–88	85	–	—	—
		Bile	1	—	94	1	—	97	–	—	—
2	Fat-free basal + 1% cholesterol	Serum	2	97–185	141	2	69–81	75	–	—	—
		Liver	2	106–228	167	2	57–77	67	–	—	—
		Bile	2	115–205	160	–	—	—	–	—	—
4[2]	Fat-free basal + 3% corn oil	Serum	2	235–507	371	1	—	179	3	37–52	43
		Liver	2	415–965	690	1	—	308	4	90–188	121
		Bile	2	418–878	648	1	—	198	4	52–533	241
5	Fat-free basal + 15% corn oil	Serum	2	268–538	403	3	88–132	104	4	13–159	86
		Liver	2	546–992	769	3	176–225	201	4	27–374	203
		Bile	2	427–809	618	3	180–228	199	4	77–391	214
6	Fat-free basal + 15% vegetable shortening	Serum	2	101–365	233	1	—	332	3	53–99	78
		Liver	2	163–526	345	1	—	483	3	117–182	142
		Bile	1	—	202	1	—	276	3	181–380	294
7	Fat-free basal + 15% butterfat	Serum	2	275–428	352	3	42–173	118	2	50–54	52
		Liver	2	764–1212	988	3	84–260	195	4	91–176	118
		Bile	2	282–571	427	1	—	608	3	52–192	135
8	Fat-free basal + 15% corn oil + 1% cholesterol	Serum	2	10–15	13	3	2–9	5	4	3–8	6
		Liver	2	5–13	9	3	2–10	5	4	1–10	5
		Bile	2	6–20	13	2	7–16	12	4	9–46	28
9	Fat-free basal + 15% vegetable shortening + 1% cholesterol	Serum	2	11–12	12	4	5–29	11	4	7–17	13
		Liver	2	10–15	13	4	4–36	14	4	1–4	3
		Bile	2	41–45	43	3	18–168	113	4	1–224	78
10	Fat-free basal + 15% butterfat + 1% cholesterol	Serum	2	17–37	27	–	—	—	4	9–32	19
		Liver	2	29–49	39	–	—	—	4	7–43	18
		Bile	2	20–40	30	–	—	—	4	40–489	219

[1] Disintegrations/minute/milligram of cholesterol.

[2] Because of poor physical condition, the birds of group 3 were killed without radioactive acetate injection.

pound which was excreted in the bile when the synthesis of cholesterol was inhibited.[5]

In the groups receiving fat but no cholesterol (zero, 4 to 7), the specific activity of both the liver and bile cholesterol tended to be appreciably higher than that of the serum cholesterol. With the fat-free diets, however, (groups 1 and 2), serum, liver, and bile cholesterol were labeled to about the same extent.

Cholesterol synthesis with the fat-free and fat-free-plus-cholesterol diets was appreciable even after 16 weeks, but was only one-third to one-half as great as when the diets contained fat without cholesterol. In diets containing fat, it is doubtful whether the amount of fat (groups zero, 4, 5) or the kind of fat (groups 5, 6, 7) influenced the degree of synthesis. The tendency of endogenous cholesterol synthesis to decrease with age is clearly shown in all groups except those in which synthesis was strongly inhibited.

The rate of labeling of serum cholesterol over an 8-hour interval (not shown in the tables) was practically constant regardless of the diet and the age of the bird. Expressed as percentage of the 8-hour value, the average specific activities of serum cholesterol for groups zero to 7, inclusive, were 64% at two hours, 88% at 4 hours and 94% at 6 hours.

Atherogenesis. Aortae from the birds of each group were examined by an experienced pathologist. No abnormalities were observed in any of the specimens, despite the prolonged hypercholesterolemia exhibited by some of the birds.

DISCUSSION

In this experiment, neither high-cholesterol intake from a fat-free diet nor high-fat intake from a cholesterol-free diet caused hypercholesterolemia. When, however, diets containing both fat and cholesterol were fed, serum cholesterol concentrations were approximately doubled, reaching a maximum at 13 or 16 weeks. The unexplained decrease in serum cholesterol concentrations which occurred in all groups after the 13th or 16th week (with the single exception of the 15% of corn oil group) was presumably not due to any nutritional inadequacy because it occurred in birds fed the standard chicken ration (group zero) as well as in the groups fed the experimental diets. It may represent a normal change as the bird approaches sexual maturity. A similar spontaneous decrease in hypercholesterolemia of cholesterol fed cockerels at 20 weeks of age was reported by Katz et al. ('55).

The groups receiving no dietary cholesterol exhibited fairly constant serum cholesterol concentrations. In the groups given high-fat, high-cholesterol diets, however, continuing changes were observed during a period of many weeks without dietary changes. These long-term changes with time, if not peculiar to the present experiment, illustrate the fallibility of conclusions which may be drawn on the basis of short-term or single-term experiments. Thus, had the present experiment been terminated at any time between the 7th and 11th weeks, the data would have indicated that butterfat had an anticholesterolemic effect and that corn oil and vegetable shortening did not, whereas quite different conclusions would have been drawn on the basis of the 16-week or the 22-week data.

Our results are in agreement with those from several other laboratories (see review by Katz et al., '58) in showing that the degree of cholesterolemia in cockerels is not influenced by the degree of saturation of the dietary fat, either in the presence or the absence of dietary cholesterol. These results are not in agreement with those of Hegsted et al. ('60b), who report a direct correlation in cholesterol-fed chicks between degree of dietary fat saturation and serum cholesterol concentration. The feeding period used by these authors, however, was short (three to 7 days of supplying a commercial starter mash followed by the experimental diets for three weeks). Their atypical results may also be in part due to the fact that their results were obtained from very young chicks, between three and 30 days of age. The blood and liver lipids of chicks have been reported to be very high at hatching with as much as 9.7% of the liver being cholesterol. Liver and se-

[5] The presence of such newly synthesized digitonin precipitable compounds in rat liver and intestine after the inhibition of cholesterol synthesis by MER-29 has been demonstrated by MacKenzie and Blohm (Federation Proc., *18:* 417, 1959).

rum cholesterol concentrations drop rapidly during the first month of life to reach adult levels at approximately 36 days. Thus, serum cholesterol was reported to be 302, 296, 126, and 110 mg per 100 ml on the first, 5th, 15th, and 36th day of life, respectively (Entenman et al., '40). Therefore, the period before 36 days of age may be an inappropriate one for the study of the effect of exogenous factors on the cholesterol economy of the chick.

The iodine numbers observed for the dietary fats agree with previously reported values. The parallelism of the iodine numbers of dietary and abdominal fats after feeding high-fat diets confirms previous similar observations by Cruickshank ('34) and by Hegsted et al. ('60a). This parallelism was not maintained in the present experiment, however, when diets containing only 3% of fat were fed. This may be interpreted as an indication that 3%, in contrast with 15%, corn oil diets did not provide excess unsaturated fatty acids in amounts adequate to dominate the composition of the storage fat. Liver lipids, even when the high fat diets were used, failed to reflect the iodine numbers of the dietary fat.

In the groups receiving both dietary cholesterol and fat, the liver cholesterol and total lipid concentrations were greater with dietary corn oil or vegetable shortening than with butterfat. Whether this difference would have persisted with a longer feeding period is unknown. However, these results are in agreement with similar observations which have been published with reference to rats (Best et al., '58; Klein, '59; Okey et al., '57, '59). In each of the studies mentioned, increased unsaturation of dietary fat resulted in increased liver cholesterol and/or total lipid storage. The increases of liver cholesterol seen here when corn oil or vegetable shortening diets were supplemented with cholesterol were proportionately much greater than the corresponding increases in serum cholesterol: a tenfold change in liver concentration was accompanied by only a twofold increase in the serum. This observation serves as a reminder that although, for practical reasons, cholesterol concentrations are measured more frequently in blood than in any other tissue, these blood concentrations do not necessarily reflect the changes which are of most significance.

SUMMARY

The effects in New Hampshire cockerels of dietary fats and cholesterol on serum and liver cholesterol concentrations, liver lipid concentration, the degree of saturation of tissue fats, and endogenous cholesterol synthesis were measured after 10-, 16- and 22-week feeding periods.

Serum cholesterol concentrations were increased only when both fat and cholesterol were fed. The increase was not inhibited by unsaturated dietary fat.

Cholesterol, when fed with corn oil, vegetable shortening, or butterfat, caused greatly increased liver cholesterol and (subsequently) increased liver lipid concentration; the increases were greatest with dietary corn oil and least with butterfat.

The iodine number of the abdominal fat reflected that of the dietary fat when the diet contained 15% of fat but not when only 3% of fat was fed. Iodine numbers of liver lipids were independent of dietary fat saturation.

Endogenous cholesterol synthesis decreased with age and appeared to be independent of the degree of saturation of dietary fat; it was strongly inhibited by diets containing both fat and cholesterol.

No atherosclerotic changes were observed.

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Effect of Feeding Alcohol-Soluble and Alcohol-Insoluble Soybean Phosphatides on Plasma Lipids and on Atheromatosis in Rabbits

E. VAN HANDEL[1]
Stuart, Florida

Serum cholesterol and phospholipid levels are closely related (Adlersberg et al., '56). When the serum cholesterol level can be influenced, phospholipids usually follow the same pattern, both in humans (Ahrens, '57) and in animals (Hirsch and Nailor, '56); and infusion of phospholipids leads to hypercholesterolemia (Byers and Friedman, '56). In the atherosclerotic aorta, both cholesterol and phospholipids accumulate (Buck and Rossiter, '51). In the rabbit, aortic plaque phosphatides turn over rapidly and this may constitute an attempt of the artery to rid itself of accumulated cholesterol (Zilversmit et al., '54). It therefore seemed of interest to investigate the effect on hypercholesterolemia and atherosclerosis of addition of phospholipids to diet. In the rabbit, there is no difference in effect between liquid and hydrogenated vegetable oils, added to rabbit chow, with or without cholesterol supplementation, either on plasma cholesterol levels or on atherogenesis (Van Handel and Zilversmit, '57, '59). Therefore, the cholesterol-fed rabbit seems to be a good test animal for studying effects of phospholipid supplementation, as any effect would not likely be due to slight differences in the fatty acid makeup of the particular phosphatide preparation. In the present experiment, alcohol-soluble (rich in lecithin) and alcohol-insoluble (rich in inositides and phytosphingolipids) fractions of soybean phosphatides have been used in an attempt (a) to retard the onset of atherosclerosis, and (b) to resolve aortic plaques previously induced by cholesterol-feeding.

MATERIAL AND METHODS

New Zealand White rabbits were used in both experiments. In the first experiment all animals received a daily ration of 90 gm of rabbit chow, 5 gm of hydrogenated oil and 0.25 gm of cholesterol. The ration of one group was supplemented with 5 gm of alcohol-soluble soybean phospholipids, that of a second group with 5 gm of alcohol-insoluble soybean phospholipids, whereas the ration of the control group was completed with an additional 5 gm of hydrogenated oil (table 1). Littermates of three-month-old rabbits were distributed at random among the three dietary groups of 15 to 16 animals each. Blood was taken monthly from the central artery of the ear. Food was withdrawn 16 to 20 hours prior to blood sampling. All animals were sacrificed after receiving this atherogenic diet for 120 days.

In the second experiment, three-month-old rabbits were made atherosclerotic by feeding chow supplemented with 0.5% of cholesterol dissolved in 1% of hydrogenated vegetable shortening. After receiving this atherogenic diet for 100 days, the animals were returned to regular chow for 40 days, after which littermates were randomly distributed among three dietary groups of 12 animals each. The animals then received 90% of chow mixed with 5% of hydrogenated oil, and with either 5% of alcohol-soluble, 5% of alcohol-insoluble soybean phospholipids, or with an additional 5% of hydrogenated oil (table 1). The animals received these diets for 140 days and were then sacrificed, 6 months after cessation of cholesterol feeding. Animals were housed in individual cages in an air-conditioned room at 25 ± 2°C. Cages were placed in racks at random as

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[1] Present address: Florida State Board of Health, Entomological Research Center, Vero Beach, Florida.

TABLE 1
Composition of diets

	Commercial rabbit chow[1]	Margarine oil[2]	Soybean phosphatides	
			Alcohol-soluble[3]	Alcohol-insoluble[3]
	%	%	%	%
Experiment 1, atherogenic diet		(Containing 2.5% of cholesterol)		
Control	90	10	—	—
Soluble	90	5	5	—
Insoluble	90	5	—	5
Experiment 2, therapeutic diet		(Containing no cholesterol)		
Control	90	10	—	—
Soluble	90	5	5	—
Insoluble	90	5	—	5

[1] Purina Rabbit Chow, Ralston-Purina Company, St. Louis.
[2] Margarine oil was a semisolid partly hydrogenated vegetable oil, iodine value 100.
[3] Alcohol-soluble and alcohol-insoluble soybean phosphatides (Centrophil SM and IM) were generously supplied by the Central Soya Company, Inc., Chicago.

to litter, sex and diet. Food records showed that of 100 gm of food offered each day, an average of less than 5 gm was left in the feeding jars.

Plasma cholesterol was determined according to Abell et al. ('52) and phospholipids by the method of Bartlett ('59) in a chloroform-methanol extract. Animals were killed by intracardiac air injection. The aorta was removed completely from the arch to the bifurcation, and cleaned of all extraneous tissue. Its wet weight was recorded and photographs were taken on color reversal film[2] using an electronic flash. By projecting the resulting color transparencies on white paper of uniform thickness, the diseased areas and circumference could be accurately traced with pencil and cut out with scissors. The ratio of diseased to total aorta was determined by weighing the cut out areas. These are presented as percentage of diseased intimal area. Subsequently, aorta was ground in a mortar with methanol after addition of a small amount of sand. Then, one volume of chloroform was added and, after several days in contact with the solvent, the dissolved lipids were filtered into a 50-ml volumetric flask and the filter paper was washed with chloroform so that the final ratio of methanol to chloroform was 1:2. Total cholesterol was determined by evaporating the solvent, dissolving the lipids in 2 ml of acetic acid and adding 4 ml of acetic acid anhydride-sulfuric acid mixture 20:1 (Van Handel, '59). Free cholesterol was determined as the digitonide by the method of Sperry and Webb ('50).

In the first experiment, after feeding the diet for the third month, droppings from all rabbits were collected for 5 days and pooled for each of the three groups. Lipids were extracted by grinding with methanol and chloroform in a Waring blender, followed by extraction in a Soxhlet with chloroform. The extract was washed with water, and organic phosphorus in the chloroform layer was calculated as phospholipid. Another portion of the washed chloroform extract was dried, saponified with alcoholic KOH and the sterols extracted with petroleum ether. Liebermann-Burchard positive digitonin precipitable material was determined according to Sperry and Webb ('50), using cholesterol as a standard. Statistical calculations are based on the method of Dunnet ('55) by which the effect of the two phospholipid diets could be compared with the control diet.

RESULTS

Experiment one. All animals were in good health during the experiment. A number of rabbits were lost after the first month due to accidents during blood sampling. A few others were sacrificed after the third month to check for the progression of atherosclerosis. Individual values for total cholesterol and phospholipid contents of aortas and their percentage dis-

[2] Kodachrome, Eastman Kodak Company, Rochester, New York.

TABLE 2

Aorta analysis and percentage of diseased intimal area of rabbits fed various diets

Litter and sex	Weight	Total cholesterol	Total phospholipids	Diseased intimal area
	gm	*mg*	*mg*	*%*
		Hydrogenated vegetable oil + cholesterol		
2-M	1.51	16.1	10.2	64.2
2-F	1.65	33.5	14.7	75.8
3-F	2.81	75.0	27.0	70.8
4-M	0.96	10.4	6.6	18.7
5-F	1.12	14.6	7.9	34.4
6-F	1.14	23.4	10.2	53.8
7-F	1.15	17.6	8.5	40.7
8-M	0.99	7.8	6.1	33.2
9-F	1.13	13.2	7.6	20.6
10-M	0.96	10.8	6.2	17.6
10-F	0.71	6.9	4.8	19.3
11-F	1.47	33.3	15.2	61.4
Av.	1.30	21.8	10.4	42.5
S.E.[1]		5.5	1.8	6.1
		Alcohol-soluble phospholipids + cholesterol		
1-M	1.66	16.2	7.0	32.2
1-F	1.12	6.5	6.0	18.2
2-M	1.25	11.1	7.6	33.6
3-M	1.68	21.8	11.2	39.3
3-F	1.68	34.0	14.6	43.6
5-F	1.49	31.0	13.7	54.8
6-F	1.12	9.6	6.3	20.0
8-M	1.13	5.3	6.2	27.7
9-F	0.95	11.2	6.7	22.8
9-M	1.44	17.0	9.7	31.6
11-F	1.43	35.2	11.7	54.9
Av.	1.36	18.1	9.1	34.4
S.E.		3.3	0.96	4.0
P		>0.05	>0.05	>0.05
		Alcohol-insoluble phospholipids + cholesterol		
2-M	1.03	11.5	8.0	38.6
2-F	1.00	5.4	5.6	17.9
3-F	1.28	19.8	10.0	20.5
3-F	2.20	40.5	18.0	41.7
4-F	1.16	6.4	5.0	15.4
5-M	1.42	33.4	10.7	56.0
5-F	1.40	18.8	9.5	45.6
6-M	0.85	1.4	3.3	0.0
7-F	1.25	20.0	11.0	68.7
8-M	1.01	2.8	4.7	25.7
8-F	1.16	23.3	10.5	47.1
9-F	1.10	17.1	7.0	34.3
9-M	1.11	8.1	6.7	31.0
10-F	1.29	13.4	8.2	36.2
11-M	0.99	1.4	4.1	0.0
Av.	1.22	14.9	8.2	31.9
S.E.		3.0	0.96	5.0
P		>0.05	>0.05	>0.05

[1] Standard error.

eased area are presented in table 2. The photographic score (percentage of diseased area) was a very good parameter for the total aortic cholesterol concentration ($r = 0.79$ for the pooled 38 rabbits and $P < 0.0001$).

The average monthly weights, cholesterol, cholesterol/phospholipid ratio of plasma and the total amount of cholesterol per aorta at autopsy are presented in figure 1. The alcohol-soluble fraction (rich in lecithin) had no effect on levels of plasma cholesterol and phospholipids. The alcohol-insoluble fraction (rich in inositides and sphingolipids) caused less elevation of both plasma cholesterol and phospholipids than the control diet ($P < 0.05$) except in the last month, although a difference still persisted ($P = 0.10$). When all the plasma lipids during the 4-month period were compared so that every rabbit that was sacrificed after 4 months enters 4 times in the calculation, the difference as compared with the control both for plasma cholesterol and for phospholipid levels was highly significant ($P < 0.01$).

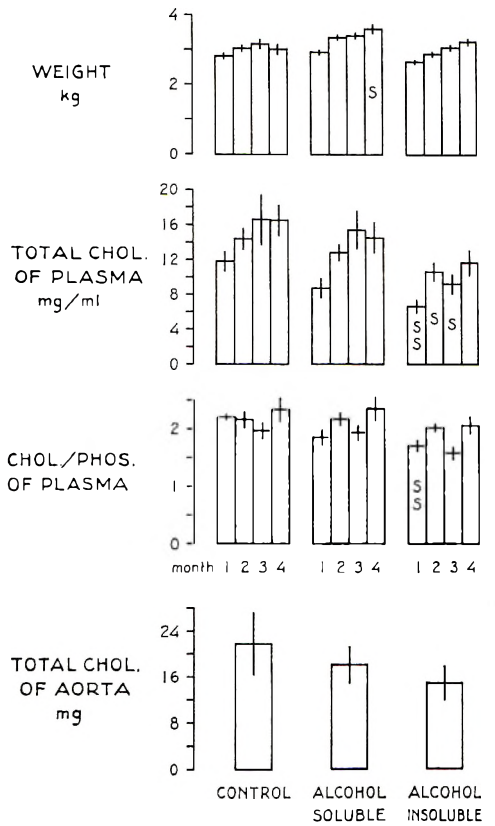

Fig. 1 Monthly weight, plasma cholesterol, cholesterol-phospholipid ratios and total aortic cholesterol contents of rabbits fed hydrogenated vegetable oil (control), alcohol-soluble and alcohol-insoluble soybean phosphatides. The vertical line at the top of each bar is two standard errors in length. S in the bar indicates: different from control with $P < 0.05$; SS in the bar indicates: different from control with $P < 0.01$.

Inspection of the analysis of the aorta (table 2) shows that by all parameters, the means for the alcohol-soluble diet were lower than for the control diet, and the means for the alcohol-insoluble diet were lower than for the alcohol-soluble diet. The difference between the alcohol-insoluble diet and the control diet, however, was still too small to be statistically significant. One animal in the control group exhibited extreme atherosclerosis, whereas two of the 15 animals in the alcohol-insoluble group showed no lesions. Omitting these three extreme values, there was no appreciable difference in the means between the control and the alcohol-insoluble group, thus explaining the insignificance of the apparent difference. The 38 plasma cholesterol levels were compared for each month with the total aortic cholesterol contents found at autopsy. The correlation coefficients for the first, second, third and 4th month were 0.54 ($P < 0.001$); 0.24 ($P > 0.1$); 0.16 ($P > 0.1$) and 0.26 ($P = 0.1$), respectively.

Fecal analysis showed that from 5 gm of daily intake of phospholipids, virtually nothing (much less than 1%) could be recovered from feces. Although the daily excretion of sterol appeared elevated from 75 mg in the control diet to 105 mg in the phospholipid diets, the "excess" over normal rabbits (70 mg per day) fed plain chow amounted to no more than 35 mg per day or only 12% of the daily cholesterol intake. The sterols determined in this way yielded maximum Liebermann-Burchard color within a few minutes after addition of sulfuric acid-acetic acid anhydride mixture. Undoubtedly, therefore, "fast-acting" dehydrocholesterol was present. Chromatographic separation of the sterol fraction on silicic acid impregnated glass paper, using 10% chloroform in petroleum ether as an eluant revealed at least 6 sterols in

TABLE 3

Percentage of diseased intimal area and aortic lipids of rabbits first made atherosclerotic, then fed hydrogenated oil (control), alcohol-soluble and alcohol-insoluble phospholipid-containing diets

	Diseased area	Cholesterol/ aorta	Cholesterol/ gm of aorta	Free cholesterol
	%	*mg*	*mg*	%
Control	64.0 (12)[1]	39.4	21.8	57.1
S.E.[2]	5.3	3.8	1.7	2.6
Soluble	65.7 (11)	37.7	18.7	60.6
S.E.	6.1	6.4	2.5	1.8
P[3]	>0.05	>0.05	>0.05	>0.05
Insoluble	71.8 (12)	48.8	23.7	60.7
S.E.	5.5	8.6	2.5	1.1
P	>0.05	>0.05	>0.05	>0.05

[1] Figures within parentheses indicate number of rabbits.
[2] Standard error.
[3] P indicates probability that the difference from the control is due to chance.

the digitonin soluble fraction and at least two sterols in the precipitable fraction. One of the latter had the R_f value of cholesterol and the other showed a larger value. As determinations were made on pooled stool specimens during only a 5-day period, the data do not permit a firm conclusion regarding phospholipids and cholesterol excretion. Furthermore, small amounts of unabsorbed plant sterols from the commercial phospholipid preparations may also account for the slightly elevated sterol excretion in the phosphatide groups.

Experiment two. In this group, only terminal plasma lipids were determined, 180 days after cessation of cholesterol feeding. Cholesterol averaged 2.0 mg per ml for all three diets and phospholipids 2.5, 2.3, and 2.2 mg per ml for the control, soluble and insoluble diets, respectively. The effect on aortic lipids is presented in table 3. No beneficial effect of either phosphatide fraction on aortic atherosclerosis could be observed.

DISCUSSION

The results suggest that dietary plant phosphatides are not important in the prevention or therapy of experimental rabbit atheromatosis, although the alcohol-insoluble fraction diminished hypercholesterolemia. It is possible that further fractionation would have yielded products with more pronounced hypocholesterolemic effect. Lack of knowledge of the source and composition of many phosphatide preparations used in experiments makes it somewhat difficult to compare these results with those of other investigators.

Addition of phosphatides to the diet of hypercholesterolemic patients lowers blood cholesterol (Steiner and Domanski, '44; Pottenger and Krohn, '52; Morrison, '58) or has little effect (Delevett and Bruger, '48; Gertler and Oppenheimer, '54). In these studies with humans, it remains to be determined how much of the effect, if demonstrated, was due to unsaturated fatty acids in the phospholipid molecule. Kesten and Silbowitz ('42) found that soybean phosphatides, when added to the diet of cholesterol-fed rabbits, lessens the incidence of atheromatosis. Moses ('54) found inositol phosphatide (somewhat similar to the alcohol-insoluble fraction) to be without effect.

This study was not designed to show regression of aortic lesions; that would have required sacrifice of groups of animals at different time intervals. It showed, however, that the two phosphatide preparations have no more lipid-resolving effect than hydrogenated vegetable oil. The very severe lesions and very high cholesterol content (average more than 40 mg per aorta) 6 months after cessation of cholesterol feeding suggest that difficult barriers have to be overcome to remove arterial lipid deposits. Lesions in the iris of the eye were photographed immediately after cessation of cholesterol feeding and 6 months later. Slow, but definite, regression took place in about 40% of the animals. In the other animals, regression could not

be observed with certainty, although the contours of the lesions became more diffuse.

Reversal of advanced aortic lesions in rabbits has, in general, been unsuccessful (McMillan et al., '55; Beher et al., '56; Miller et al., '59; Friedman and Byers, '59; Constantinides et al., '60). The only long-term study suggestive of reversal of aortic lipidosis (Anitschkow, '28) showed that 500 to 800 days after cessation of cholesterol feeding, lipid deposits were still present but more persistent in the abdominal portions and around intercostal arteries than in the arch.

Failure to affect atherogenesis by long-term feeding of phospholipid seems to be at variance with Friedman et al. ('57), who reported that infusion of a brain phosphatide emulsion partially resolves existing plaques. The excess hypercholesterolemia reported by these authors, however, also failed to materialize. Obviously, only a very small portion of ingested phospholipids is absorbed unaltered. Whereas 250 mg of cholesterol per day increased the total phospholipid pool of plasma from about 200 to about 1000 mg, 5 gm of alcohol-soluble phospholipids (containing about 3 gm of lecithin) had no effect on plasma phospholipids, and a daily intake of 5 gm of the alcohol-insoluble fraction *reduced* the total phospholipid pool about 30%.

A significant correlation existed between plasma and aortic cholesterol only during the first month. In subsequent months plasma cholesterol appeared to become a very unreliable parameter for the degree of atherosclerosis, an observation also made in a previous experiment (Van Handel and Zilversmit, '59). It seems as though early hypercholesterolemia primes atherogenesis and once the process has begun, progression no longer depends on the elevation of the plasma cholesterol level. If this phenomenon parallels the human situation, it is entirely possible that once atherosclerosis has developed, lesions will progress in spite of any successful therapy of hypercholesterolemia.

SUMMARY

With rabbits fed cholesterol-supplemented chow, equicaloric amounts of the following were compared for their effect on atherogenesis: hydrogenated vegetable oil, alcohol-soluble and alcohol-insoluble soybean phosphatides. After 4 months, no difference in atherogenesis was noted among animals of the three groups; the alcohol-insoluble phosphatides caused less hypercholesterolemia and less hyperphospholipemia than alcohol-soluble phosphatides or vegetable oil.

In a second experiment, rabbits were made atherosclerotic by cholesterol feeding. Subsequently the animals were fed chow, supplemented with alcohol-soluble, alcohol-insoluble phosphatides or with hydrogenated vegetable oil. Six months after cessation of cholesterol feeding, lesions and lipid deposits were equally severe in all three groups.

ACKNOWLEDGMENT

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Glycine Need of the Chick Fed Casein Diets and the Glycine, Arginine, Methionine and Creatine Interrelationships[1]

HOWARD N. WATERHOUSE[2] AND H. M. SCOTT
Department of Animal Science, University of Illinois, Urbana, Illinois

The arginine requirement of the growing chick has been extensively investigated in recent years (Almquist, '47; Almquist and Merritt, '50; Wietlake et al., '54; Fisher et al., '56b; Snyder et al., '56; Hogan et al., '57; Krautmann et al., '57).

There are discrepancies in the quantitative arginine requirement reported for the growing chick that appear to be influenced by the composition of the diet. When supplying a casein-glucose diet (20% protein), the chick's arginine requirement is approximately 1.7%, whereas with a corn-soya diet the requirement is nearer 1.1% of the diet (Synder et al., '56; Krautmann et al., '57).

Almquist et al. ('40) demonstrated that chicks require a dietary source of glycine for optimal growth and in a subsequent report, Almquist and Mecchi ('40) showed that acetates and creatine improved the growth-promoting ability of a refined diet of the casein-brewers' yeast-fishmeal (water-washed) type. Supplemental glycine and acetate had little effect on muscle creatine whereas supplemental creatine materially increased the creatine content of muscle tissue. The interaction of these supplements was not investigated. Following these observations, Hegsted et al. ('41), using a casein-brewers' yeast basal diet, reported that arginine was more effective than either glycine or creatine in enhancing chick growth; the glycine-arginine combination resulted in still better growth. Supplemental creatine was superior to all other treatments in elevating muscle creatine. The data were interpreted as indicating that supplemental creatine spared glycine and arginine to the extent that they would be used for creatine synthesis. In addition to confirming these results, Almquist et al. ('41) demonstrated further that glycocyamine was capable of increasing both growth and the level of muscle creatine. It was postulated that the growth-promoting effect of creatine could be explained on the basis of its having spared glycine and arginine.

The glycine requirement of the chick appears to be equally as variable as has been indicated for arginine. Fisher et al. ('55, '56a) have shown that the glycine requirement of 1.5% reported by Almquist and Mecchi ('42) is not adequate for optimum growth and feed utilization of fast-growing chicks. In experiments designed to study the amino acid adequacy of casein for chick growth, Wietlake et al. ('54) concluded that the response to supplemental glycine was slight in comparison with that resulting from either supplemental arginine or creatine. When the basal diet contained adequate arginine, supplemental creatine was without effect. The evidence indicated that creatine could replace most of the supplemental arginine required for maximum growth. In a subsequent report from the same laboratory, Hogan et al. ('57) submit evidence that creatine exerts a sparing effect on the requirement for both arginine and glycine. Creatine, however, could replace only a small amount of the supplemental arginine required to maximize growth. This is consistent with the demonstration by Fisher et al. ('56b) that arginine is constantly being diverted to the synthesis of

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[2] Present address: General Mills, Inc., Pet Food Nutrition Laboratory, Indianola, Iowa.

creatine even when the diet is supplemented with adequate creatine.

More recently Savage and O'Dell ('60) have reported that glycine not only improves chick growth at all levels of arginine but spares the requirements for arginine as well. Creatine was found to spare the requirement for glycine. Moreover, both creatine and guanidoacetic acid were shown to spare arginine. Edwards et al. ('58) had previously reported this sparing action of creatine and guanidoacetic acid.

Briggs et al. ('42) have shown that chicks fed a casein diet respond to supplemental sulfur amino acids (cystine) only when arginine and glycine are present in adequate amounts. In contrast Fisher et al. ('56a) have concluded that the effect of supplemental methionine was minor when it was added alone or in combination with arginine and glycine to a casein diet.

It was the purpose in this series of experiments to investigate the arginine-glycine, glycine-creatine and glycine-methionine relationships when chicks were fed a purified diet containing casein as the sole intact protein.

In the present paper, evidence will be presented to demonstrate that glycine does not "spare" arginine, creatine does not "spare" glycine, and the growth response to supplemental sulfur amino acids depends on the concentration of supplemental glycine when chicks are fed casein as the source of protein.

EXPERIMENTAL

Crossbred 7-day-old male or female chicks from a NH ♂ × Columbian ♀ mating were used throughout this series of experiments. For the first 7 days, they had been fed a corn-soya diet and were weighed on the 7th day, wingbanded and distributed according to weight into lots of 10 birds each, that contained birds with the same range in initial weight. The birds were housed in electrically-heated battery brooders with raised wire floors, and were supplied ad libitum with their respective diets and water for the period from 7 to 21 days of age. Feed consumption and weights were recorded weekly.

A casein diet containing 20% of protein was selected for this series of experiments although it has been shown by Scott et al. ('57) that the protein requirement of the chick fed a purified diet of the casein-glucose type is approximately 36%. A 20% protein diet was chosen with the thought that the interrelationships under study could best be demonstrated at a suboptimal level of protein in the diet.

The composition of the basal diet is given in table 1. It is a casein-glucose diet, adequately fortified with vitamins and minerals. Analysis for nitrogen by the macroKjeldahl method showed it to contain 19.5% of crude protein. By calculation it contained approximately 0.8% of arginine and 0.4% of glycine (Block and Weiss, '56).

RESULTS

In order to adequately test the interrelationship of arginine and glycine,[3] a factorial experiment with 5 levels of arginine and 5 levels of glycine was used. The diets were fed to quadruplicate groups of 5 female chicks per treatment. In table 2 are shown the 7- to 21-day gain data for experiment 1.

Each addition of arginine improved chick gains at all increments of glycine with the following exception: at the 4.0% level of supplemental glycine, no further beneficial growth response was noted with supplemental levels of arginine in excess

TABLE 1

Composition of basal diet

	%
Glucose	71.03
Casein, crude	22.20
Salts[1]	5.27
Corn oil, refined	1.00
DL-Methionine	0.30
Choline Cl	0.20
Vitamins[2]	+
Penicillin, 11 mg/kg diet	+
Total	100.00
Per cent protein (calculated)	20.0
Per cent protein (determined)	19.5

[1] Illinois Salts 59A as a percentage of the total diet. $CaCO_3$ (USP), 2.166; KH_2PO_4, 1.050; $CaHPO_4 \cdot 2H_2O$ (USP), 0.940; NaCl, 0.800; $MgSO_4$, 0.250; $FeSO_4 \cdot 7H_2O$, 0.30; $MnSO_4 \cdot H_2O$, 0.20; $ZnCO_3$, 0.010; $CuSO_4 \cdot 5H_2O$, 0.002; KI, 0.001, $NaMoO_4 \cdot 2H_2O$, 0.001. (All reagent chemicals were used except as noted.)

[2] See Snetsinger and Scott ('58).

[3] The authors are indebted to Dr. Laurent Michaud of Merck Sharp and Dohme, Rahway, New Jersey, for a generous supply of glycine.

of 0.4% (1.2% total). Growth increased progressively with each increment of glycine at each level of arginine. The gain to feed ratios, not shown here, were improved with increasing levels of both arginine and glycine. Statistical analysis of the data indicated that the main effects of both arginine and glycine were highly significant ($P < 0.01$), whereas the interaction was not significant.

It would appear from these data that the growth response to glycine decreased as the level of arginine approached optimum and it seemed desirable to determine whether the chick's need for glycine would continue to decrease at even higher levels of arginine than those used in experiment 1 and thus offer conclusive evidence of arginine having been spared by supplemental glycine. Therefore, experiment 2 (table 3) was designed to determine the arginine level at which the response to supplemental glycine would be minimal.

Again, a factorial experiment with 5 levels of arginine and 4 levels of glycine was used. Each diet was fed to triplicate groups of 10 male chicks each. The data in table 3 show that although growth was maximal at the 1.0% supplemental level of arginine (1.80% total arginine) at all levels of glycine, the arginine requirement was no greater than 1.6 to 1.7% of the diet in either the absence or presence of supplemental glycine. These data indicate clearly that glycine continues to increase chick gains even when the arginine levels are in excess of the requirement and hence the hypothesis that the chick's need for glycine would decrease at arginine levels in excess of the arginine requirement, was not substantiated. Again the gain to feed ratios improved with each increment of glycine at all levels of arginine.

Statistical analysis for this experiment shows that the main effects of both arginine and glycine, as well as the interaction between the two, were highly significant ($P < 0.01$).

The increased chick gains to increments of glycine at suboptimal levels of arginine in experiment 1, in view of the concept of the first limiting amino acid, could be interpreted as evidence that glycine has a true "sparing" effect for arginine. The continued growth response to supplemental glycine at arginine levels in excess

TABLE 2

Effect on chick growth of supplementing a casein diet with arginine and glycine (exp. 1)

Glycine	Supplemental levels of Arginine				
	0.0%	0.1%	0.2%	0.4%	0.8%
%	*gm*	*gm*	*gm*	*gm*	*gm*
0.0	46[1]	70	92	134	169
0.5	53	79	99	141	184
1.0	64	92	104	147	184
2.0	64	97	119	155	189
4.0	66	110	134	191	196

[1] Average gain (gm) from 7 to 21 days of quadruplicate groups of 5 female chicks per treatment with an average initial weight of 68 gm.

TABLE 3

Effect on chick growth of supplementing a casein diet with arginine and glycine (exp. 2)

Glycine	Supplemental levels of Arginine				
	0.4%	0.7%	1.0%	1.3%	1.6%
%	*gm*	*gm*	*gm*	*gm*	*gm*
0.0	109[1]	158	164	167	164
1.0	142	170	156	175	187
2.0	148	170	189	188	183
4.0	173	192	198	198	196

[1] Average gain (gm) from 7 to 21 days of triplicate groups of 10 male chicks per treatment with an average initial weight of 64 gm.

of the arginine requirement in experiment 2 would, however, strongly suggest that glycine was not "sparing" arginine but had an essential independent role of its own.

In the next phase the interrelationship of glycine and creatine on a casein-glucose diet was investigated. The experiment described here was conducted with a casein diet (table 1) supplemented with a level of arginine (0.9% supplemental) considered to be adequate for this diet. Thus any response to creatine could not be attributed to its having "spared" arginine.

The experimental plan and results of experiment 3 are recorded in table 4. It can be observed that 0.5% of supplemental creatine hydrate increased growth, both in the absence and presence of supplemental glycine. No further improvement in growth rate or feed utilization was noted, however, following additional increments of creatine hydrate either in the presence or absence of supplemental glycine. Chick growth increased progressively with increments of glycine at all levels of creatine hydrate and maximal growth occurred at the highest increment of glycine at each level of creatine hydrate. Statistical analysis for this experiment shows that the main effects of both glycine and creatine were highly significant ($P < 0.01$), but no significant interaction existed between these two sources of variation. The response to creatine observed in experiment 3 is in the presence of an arginine level (1.7% total) shown in experiment 2 to be adequate for this diet. The evidence is clear that even though chick growth increased with only the first increment of creatine hydrate, it did not "spare" glycine nor did higher levels of creatine influence the growth response to supplemental glycine.

The plan and results of experiment 4, designed to determine the relationship between glycine and sulfur amino acids on a casein-glucose diet, are given in table 5. The basal diet used in this experiment was

TABLE 4

Effect on chick growth of supplementing a casein diet with glycine and creatine (exp. 3)

Glycine	Supplemental levels of Creatine hydrate			
	0.0%	0.5%	1.0%	1.5%
%	*gm*	*gm*	*gm*	*gm*
0.0	179[1]	203	193	206
1.0	198	210	212	220
2.0	202	215	221	220
4.0	215	227	229	229

[1] Average gain (gm) from 7 to 21 days of triplicate groups of 10 male chicks per treatment with an average initial weight of 73 gm.

TABLE 5

Effect on chick growth of supplementing a casein diet[1] with glycine and DL-*methionine (exp. 4)*

Glycine	Supplemental levels of DL-Methionine			
	0.0%	0.2%	0.4%	0.8%
%	*gm*	*gm*	*gm*	*gm*
0.0	175[2]	174	176	147
	(0.55)[3]	(0.56)	(0.56)	(0.54)
1.0	185	189	188	185
	(0.59)	(0.60)	(0.61)	(0.61)
2.0	181	196	193	194
	(0.58)	(0.61)	(0.62)	(0.62)
4.0	198	199	211	210
	(0.65)	(0.68)	(0.70)	(0.71)

[1] As described in table 1. The modified basal diet calculated to contain 1.7% total arginine (0.9% supplemental), 0.4% glycine and 0.7% sulfur amino acids.

[2] Average gain (gm) from 7 to 20 days of triplicate groups of 10 male chicks per treatment with an average initial weight of 75 gm.

[3] Gain to feed ratios.

the same as that used in experiment 3 and contained 0.9% of supplemental arginine (1.7% total), but the supplemental DL-methionine was omitted from the basal diet.

In the absence of supplemental glycine, supplemental methionine did not improve chick growth. Indeed, a marked growth depression, accompanied by a lowered feed intake, was noted at the 0.8% level of supplemental methionine. No increase in chick growth was noted with additions of supplemental methionine at the 1.0% level of supplemental glycine. At this level of glycine, 0.8% of supplemental methionine did not depress chick growth. In the presence of 4.0% of supplemental glycine, the two highest levels of supplemental methionine increased chick growth. Supplemental glycine progressively increased the weight gain of chicks at all levels of supplemental methionine with maximum gains occurring at the 0.4% level of supplemental methionine. This evidence strongly suggests that in the presence of adequate glycine, the unsupplemented basal diet is deficient in sulfur amino acids. In the presence of adequate glycine, the optimum level of sulfur amino acids appears to be in the range of 0.9 to 1.1% (0.2 to 0.4% of supplemental DL-methionine). The analysis of variance of this experiment shows that only the main effects of glycine and the interaction of glycine and methionine were significant ($P < 0.01$).

DISCUSSION

The significant growth increases to supplemental arginine at levels in excess of 1.2% of total arginine with the casein-glucose diet used in these experiments substantiates the observations of Wietlake et al. ('54), Fisher et al. ('56a) and Hogan et al. ('57), that for optimum growth this diet requires high levels of arginine. The growth response to glycine additions in excess of the 1.0% supplemental level (1.40% total) and the continued improvement in growth rate with each additional increment of glycine also agrees with the view expressed by Fisher et al. ('55) to the effect that chicks fed a casein diet can tolerate glycine levels in excess of 4.0%. In view of the low muscle creatine levels of young chicks reported by Fisher et al. ('55), the role of arginine and glycine, in part at least, in the formation of muscle creatine is undoubtedly an important one.

The evidence from experiments 1 and 2 does not explain why the arginine needs of the chick are accentuated when they are fed casein protein. Klain et al. ('59) have reported that the arginine requirement was 2.06% of the diet when chicks were fed a crystalline amino acid mixture simulating the amino acid composition of 30% protein from casein. The data of these workers suggest that the amino acid pattern in casein is primarily responsible for the high arginine requirement rather than the presence of factors in other proteins that enhance the utilization of arginine as proposed by Krautmann et al. ('57) and Edwards et al. ('58). This evidence would also refute the theory suggested by O'Dell et al. ('58) that a fast absorption rate of free crystalline arginine permits catabolism of arginine by kidney arginase and, therefore, is responsible for an apparently higher arginine requirement when free crystalline arginine is added.

The response to 0.5% of supplemental creatine hydrate in experiment 3 is difficult to explain in terms of creatine having "spared" arginine, since the level of 1.7% of total arginine was demonstrated in experiment 2 to be adequate for this diet. It is very unlikely that the chick converts creatine to arginine in any appreciable quantity and under the dietary conditions imposed would seem unnecessary. The growth response to supplemental creatine both in the presence and absence of supplemental glycine would suggest that creatine is not "sparing" glycine but is functioning entirely independently of glycine.

The growth response to supplemental sulfur amino acids, only in the presence of added glycine and adequate arginine in experiment 4, is in agreement with the observations of Briggs et al. ('42). It is conceivable that the response to supplemental methionine in the presence of adequate glycine and arginine is a reflection of the role that methionine plays in the formation of muscle creatine. The sulfur amino acid requirement observed here (approximately 1.0% of the diet) is greater than the requirement proposed by the National Research Council ('54) for a

20% protein diet (0.8% total sulfur amino acids).

Although the ability of glycine to overcome the toxic effects of 0.8% of supplemental DL-methionine was demonstrated, the mechanism involved remains obscure. The gain to feed ratio of this group of chicks (table 5) was almost identical to the other lots receiving lesser amounts of methionine but the same amount of glycine. This would indicate that the growth depression was due primarily to a reduced feed intake rather than to a decrease in efficiency of feed utilization.

SUMMARY

The arginine-glycine interrelationship on a casein-glucose diet of approximately 20% of crude protein was studied in factorial experiments. These two amino acids, added either singly or in combination, were demonstrated to increase growth over that observed in chicks fed the unsupplemented basal diet. Chick growth continued to increase with increments of glycine at arginine levels in excess of the arginine requirement established for this diet and the favorable effect of glycine was shown to be independent of the arginine concentration in the diet.

The glycine-creatine and glycine-methionine interrelationships were examined on the casein-glucose basal diet in the presence of adequate arginine. Chick growth increased significantly when 0.5% of creatine hydrate was added to the diet at all levels of glycine. Chick growth increased progressively with each increment of glycine at all levels of creatine and was shown to be independent of the creatine concentration in the diet. The response to supplemental DL-methionine (0.2 to 0.4%) was shown to be dependent on the presence of supplemental glycine. Supplemental glycine alleviated the growth depression induced by 0.8% of supplemental DL-methionine.

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Effects of Nutrition on Growth, Lifespan and Histopathology of Mice with Hereditary Muscular Dystrophy[1]

D. L. COLEMAN AND W. T. WEST
Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine

Since the development, at the Roscoe B. Jackson Memorial Laboratory, of a stock of mice (129 *Re-Dydy*), segregating for animals afflicted with a form of hereditary progressive muscular dystrophy, much data has been published with respect to the development and progression of both gross (Michelson et al., '55; Baker et al., '58) and histological lesions (Michelson et al., '55; West and Murphy, '60). The similarity of many of the gross symptoms in this syndrome to the disease pattern which develops as a result of vitamin deficiency has been emphasized (Baker et al., '58; Tubis et al., '59). It has been shown by Tubis et al. ('59) that vitamin therapy with large doses of all known vitamins, starting as early as 5 days after birth, had little or no effect in eliminating the dystrophy per se or in diminishing other symptoms such as eye lesions, abnormal muscle reflexes or poorer growth of bone and other nonmuscle tissues. Since the dystrophic animal had been found to eat, on the average, as much or more food per day per unit weight as normal controls, it was further suggested by the latter authors that this mutant mouse may be unable to utilize vitamins and other nutrients at the cellular level and thus suffers from a hereditarily-induced deficiency. The present study deals with the effects of lipid and protein quality on growth rate, lifespan, and histopathology in the dystrophic mouse.

MATERIALS AND METHODS

Both dystrophic (*dydy*) and normal (*Dydy*) control mice were obtained at weaning from the colony (129 *Re-Dydy*) maintained at the Roscoe B. Jackson Memorial Laboratory by ovarian transplantation, as described by Stevens et al. ('57). For the lifespan studies these weanling dystrophic and normal mice were separated into groups of 5 or 6 and supplied immediately with the diets to be studied. Diets 1[2] and 2[3] were commercial mouse chows, and diet 3[4] was essentially the semisynthetic mouse ration recommended by Fenton and Carr ('51) with the exception that the casein content was reduced from 30 to 20% and the difference made up by an additional 7% of sucrose and 3% of nonnutritive cellulose,[5] the latter to prevent diarrhea. Diets 4 to 6 were all modifications of diet 3 with one ingredient increased in each case at the expense of sucrose. Diet 4 thus contained 50% of casein, diet 5, 20% of corn oil and diet 6, 1.5% of glycine. The commercial chows were finely ground and all diets were placed in food cups in the bottom of the cage to allow easy access to the food by the dystrophic mice. Food and water were given ad libitum, and the animals were weighed once a week. Weight gains and time of death were recorded.

In addition a total of 55 weanling dystrophic mice representative of both sexes received the experimental diets and were sacrificed after various periods, ranging from 5 to 51 weeks on these regimes. All

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[1] Supported by a grant from the Muscular Dystrophy Associations of America.
[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis. Percentage composition: protein, 25; fat 6.0; fiber, 4.5; nitrogen-free extract, 47.5.
[3] Old Guilford Mouse Breeder Diet, Emory-Morse Company, Guilford, Connecticut. Percentage composition: protein, 17; fat, 11; fiber, 1.9; nitrogen-free extract, 56.2. This diet is manufactured essentially according to the Morris formulation (Morris, '44).
[4] Diet 3, percentage composition: casein, 20; corn oil, 5; Wesson salts, 5; Alphacel, 3; sucrose, 67; plus adequate amounts of all known vitamins (Fenton and Carr, '51).
[5] Alphacel, Nutritional Biochemicals, Cleveland, Ohio.

animals were killed with ether and the selected muscle samples (calf, thigh, lumbar, thoracic and brachium) fixed in a solution of two parts 10% formalin and one part 10% trichloracetic acid. After decalcification with 5% formic acid, cross and/or longitudinal samples of the above muscles were embedded in paraffin, sectioned at 6 to 7 μ, and stained with hematoxylin and eosin or by the periodic acid Schiff method after diastase digestion and counterstained with hematoxylin and orange G.

RESULTS

The growth responses of "normal" (*Dydy*) control mice to the various diets are shown in table 1. Differences among the responses to the 6 diets were minor. More rapid growth was obtained with diets 2 and 4, while diet 1 appeared to be somewhat inferior with respect to producing animals of maximum size. The heterozygous (*Dydy*) normals used here, and the homozygous (*DyDy*) normals are indistinguishable phenotypically and exhibited the same growth responses under the different dietary regimens although abnormal muscle pathology has been demonstrated, in some instances, in older heterozygotes which were maintained with diet 1.[6]

The dystrophic animals at first grew at the same rate as the normal animals when using a given diet. As the disease became more severe, however, growth was slowly retarded until a point of maximum weight was reached, usually between 8 and 12 weeks. After this point the dystrophic animals gradually lost weight until the time of death. In table 2 are summarized the data obtained for dystrophic mice fed each of the 6 diets. For purposes of comparison these data can be divided into three main groups. Animals fed diet 1 were characterized by poor growth and a rapid course of the disease. These animals at all stages presented a much poorer appear-

TABLE 1

Cumulative weight gains of normal female mice (129-Re-Dydy) fed various diets[1]

Diet		Weeks fed diet		
		4	8	12
		gm	*gm*	*gm*
1[2]		4.2	6.4	7.0
2[3]		3.2	7.5	10.1
3	Basal	3.3	6.3	8.5
4	Casein, 50%	5.0	8.8	10.5
5	Fat, 20%	3.7	6.3	9.2
6	Glycine, 1.5%	4.0	6.3	9.5

[1] Mice were supplied with experimental diets at weaning (4 weeks) and weighed between 14 and 15 gm.

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

[3] Old Guilford Mouse Breeder Diet, Emory-Morse Company, Guilford, Connecticut.

[6] West, W. T., unpublished data.

TABLE 2

Response of dystrophic mice to various diets[1]

Diet	Sex	No.	Average weight gain weeks after weaning		Maximum gain	Per cent of normal growth at time of maximum gain	Average survival time in days
			4	8			
			gm	*gm*	*gm*	%	
1[2]	♂	15	5.7	—	5.7 (4)[3]	75	64
1	♀	20	4.4	5.6	5.6 (8)	87	94
2[4]	♂	18	6.3	7.0	7.4 (12)	73	153
2	♀	10	4.0	6.1	6.7 (12)	78	156
3 Basal	♂ and ♀	21	6.3	7.9	8.4 (12)	70	176
4 Protein, 50%	♂ and ♀	14	5.1	7.1	7.8 (12)	71	170
5 Fat, 20%	♂ and ♀	18	6.2	7.4	7.4 (8)	71	158
6 Glycine, 1.5%	♂	21	6.9	8.9	9.1 (11)	96	165
6 Glycine, 1.5%	♀	18	5.0	6.7	7.7 (12)	81	180

[1] Both normal and dystrophic mice were supplied with experimental diets at weaning (4 weeks).

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

[3] Figures in parentheses indicate number of weeks after weaning.

[4] Old Guilford Mouse Breeder Diet, Emory-Morse Company, Guilford, Connecticut.

ance than even the animals with the most advanced dystrophy fed other diets. The average survival time for male animals (64 days) was significantly ($P < 0.001$) lower than that of the female mice (94 days). Animals of both sexes died much sooner than dystrophic animals fed any of the other diets (64 to 94 vs. 153 to 180 days). Female dystrophic mice receiving diet 1, as well as having an increased lifespan, also grew somewhat better than male dystrophics when compared with the normal control mice fed the same diet.

Animals fed diets 2 to 5 form another group which is characterized by a much longer lifespan (153 to 176 days). In these instances there were no significant differences noted between males and females. Grossly the symptoms appeared less severe in these animals. Growth was considerably better with these diets than it was with diet 1, not because of a more rapid growth rate but rather because the period of growth was extended. The maximum weight was not obtained until 8 to 12 weeks with these diets compared with 4 to 8 weeks for diet 1.

Diet 6 (high glycine) was unique in that it markedly improved the growth rate in the dystrophic animals so that at the time at which maximum weight was reached there was little difference between the normal and the dystrophic mice. This observation was even more striking when it is considered that these animals at weaning weighed on the average 25% less than their normal controls. Dystrophic animals maintained with this diet were in all cases more vigorous than those fed the other diets and in some instances the dystrophic animal was recognized only with difficulty. Further, these dystrophics were well groomed and did not exhibit the usual scruffiness of coat nor eye lesions which have been described. Neither lifespan nor time of achieving maximum size, however, was influenced by this improved diet (diet 6) when compared with animals raised with diets 2 to 5.

Since the lesions observed in the dystrophic mice raised with diets 2 to 6 showed little or no significant variation with respect to the particular diet fed, no attempt is made in this report to evaluate on a histological basis minor differences among these latter diets. The lesions observed when feeding diets 2 to 6, however, differed considerably from those previously described in dystrophic mice reared with a standard commercial ration[7] (West and Murphy, '60) in two important respects: the status of coagulation necrosis of segments of muscle fibers and the subsequent regenerative activity.

Coagulation necrosis in dystrophic mice raised with these special diets (fig. 1) differed from that observed in dystrophic mice raised with the commercial diet (diet 1, fig. 2) solely in the degree of involvement of the muscles by this process. This form of necrosis, though still a component of the observed histological lesions, is substantially reduced in amount in the dystrophic mice receiving all of the special diets. The descriptive morphology (figs. 1 and 2) and age distribution of this lesion are similar in both groups of animals. The lesion occurs to greatest extent in animals ranging from 4 to 8 weeks of age (West and Murphy, '60). Invasion of the coagulum by macrophages and polymorphonuclear leukocytes occurs, with subsequent phagocytosis and elimination of the necrotic material. It is at this stage that regeneration of muscle normally begins.

The incidence of regenerative activity is low in dystrophic animals raised with diets 2 to 6 as compared with that found in dystrophics reared with the commercial ration[8] (West and Murphy, '60), as might be expected from the reduced amount of necrosis. Unexpectedly, however, this regenerative activity is usually atypical in appearance, a finding which is difficult to explain. Occasionally, normal regenerative activity, as described by West and Murphy ('60) may be observed; this consists of proliferation of muscle nuclei with investing sarcoplasm that have survived the degenerative process, the attainment by these cells of a basophilic sarcoplasm and spindle-shape characteristic of myoblasts, and the subsequent appearance of band-like fibers with central nuclei, basophilic sarcoplasm and peripheral myofibrils. For the most part, regenerative activity with diets 2 to 6 is atypical, however, in that the formation of myoblast-like cells is accom-

[7] See footnote 2.
[8] See footnote 2.

panied by the appearance of a number of mononucleate round cells with basophilic cytoplasm (fig. 3). Sometimes, only these rounded cells may be observed with no evidence of spindle cell formation (fig. 4). The band-like fibers with myofibrils, described above, are rarely seen, and connective tissue infiltrates the segment of muscle fiber showing the atypical regenerative activity. Frequently, phagocytosis of the coagulum is accompanied by a connective tissue proliferation resulting in fibrous replacement of the necrotic segment of muscle fiber without evidence of previous regenerative activity (fig. 5).

DISCUSSION

Semisynthetic rations (diets 3 to 6), known to contain high quality protein and lipid, consistently resulted in increased lifespan and extended the period of growth in dystrophic animals when compared with the results obtained with dystrophic mice maintained with a commercial laboratory chow (diet 1). A similar increase in lifespan and improvement in overall health was also noted when these animals were maintained with a commercial mouse breeder ration (diet 2) of the Morris formula (Morris, '44). Although many of the gross dystrophic symptoms also seemed to be alleviated, in no case was there any remission or cure of the dystrophic condition as observed histologically. Since diet 1 is manufactured according to a closed formulation, the actual ingredients are unknown; thus the specific factor or factors involved in improving diets 2 to 6 with respect to the nutrition of dystrophic mice cannot be evaluated at this time. Previous experiments have indicated that the addition of either 6% of casein or 1% of glycine, but not 5% of corn oil, had the effect of increasing growth and improving health (Coleman and Ashworth, '60). This would suggest that the protein quality in diet 1 was not optimal for nutrition of these mice possibly because of unavailability or imbalance of certain amino acids, particularly those such as methionine, arginine and glycine which have been shown to be critical in experimental dystrophies (Nesheim et al., '60).

The addition of 1.5% of glycine to the basal ration (diet 3) had the unique effect of temporarily increasing the growth rate of these dystrophic mice over that of the normal controls. It had been shown previously that the addition of 1% of glycine to diet 1 also markedly increased growth and at the same time increased the activity of the enzyme glycine transamidinase in the kidneys of these dystrophic mice to the level of activity normally associated with dystrophic mice maintained with diets 2 or 3 alone (Coleman and Ashworth, '60). A large increase in the amount of creatine excreted in the urine of dystrophic mice was also associated with a shift from diet 1 to diet 3 (Kandutsch and Russell, '58). Prolonged survival of dystrophic mice treated with 17 α-ethyl-19-nortestosterone, an anabolic agent which augments the synthesis of creatine markedly, has recently been reported (Dowben, '59). These observations suggest a relationship between the rate or amount of creatine synthesis and lifespan in dystrophic mice.

The growth promoting ability of glycine in the dystrophic mice is of further interest when one considers the unusual metabolism of glycine in muscular dystrophy resulting from E-avitaminosis. Although the concentration of free amino acids in urine and muscle is normally increased in this condition, the relative concentrations of glycine and in some cases its precursor serine are considerably diminished (Tallan, '55; Diehl, '59). Also the incorporation of 1% of glycine into the vitamin E-deficient diets has been shown to eliminate the reduction of tissue phosphatase normally observed in dystrophic rabbits (Smith and Nehorayan, '58).

The specific reasons for the changes in gross symptomatology and in the histologic lesions of dystrophic mice reared with diets 2 to 6 as compared with dystrophic animals raised with diet 1 are not apparent from the present data. At this time, the best rationale lies in the fact that higher quality diets such as diets 2 to 6 have increased nutritive value, thus tending to reduce stresses such as inanition, specific nutritional deficiencies or disturbances, and lowered resistance to infectious diseases, which may result directly or indirectly from diets of insufficient or lower grade nutritive value. This is most evident when it is considered that muscular dystro-

phy itself is a wasting disease accompanied by cachexia and muscular atrophy and that it is essentially a disease in which the catabolic-anabolic ratio is gradually increased.

The improvement in the gross symptoms of muscular dystrophy and the decrease in the incidence of coagulation necrosis can be explained on the basis discussed above. However, the apparently detrimental effect on regenerative activity (its atypical form and its absence in many post-necrotic segments of muscle fibers) is contrary to what would be expected from this explanation. But if it is postulated that coagulation necrosis in dystrophic mice reared with the better regimes (diets 2 to 6) occurs only in fibers which have reached a well advanced stage of the dystrophic process, this effect on regenerative activity may be explained by the hypothesis advanced by Walton and Adams ('56) that a muscle fiber in an advanced stage of atrophy or irreversible degeneration, from any cause, loses its ability to regenerate.

The data presented here would suggest that diet 1 may not be adequate for the nutrition of mice suffering from hereditary progressive muscular dystrophy and draws attention to the importance of the nutritional status when studying mice afflicted with this disease.

SUMMARY

Semisynthetic rations known to contain high-quality protein and lipid, resulted consistently in increased lifespan and extended the period of growth in strain 129 mice suffering from hereditary, progressive muscular dystrophy, when compared with results obtained with dystrophic mice maintained with a commercial laboratory chow (diet 1). A similar increase in lifespan and improvement in overall health was noted when dystrophic animals were reared with a certain commercial mouse breeder ration of the Morris formula. The addition of 1.5% of glycine to the basal semisynthetic ration temporarily increased the growth rate of dystrophic mice over that of the normal controls. Although many of the gross symptoms appeared to be alleviated, and certain histopathological lesions of mouse dystrophy were altered, there was no remission or cure of the dystrophic condition as observed histologically. Histopathologically, all the improved diets gave the same effects, with no detectable variation in respect to the particular diet or to sex. There was a decrease in the incidence of coagulation necrosis of muscle fiber segments and in the subsequent regenerative activity as compared with these same processes observed in dystrophic animals fed diet 1. Further, much of the regenerative activity was atypical at the cellular level, and, not infrequently, the necrotic zone of a fiber showed connective tissue replacement, without any evidence of previous regenerative activity. The data presented draw attention to the importance of nutritional status when studying strain 129 mice afflicted with hereditary, progressive muscular dystrophy.

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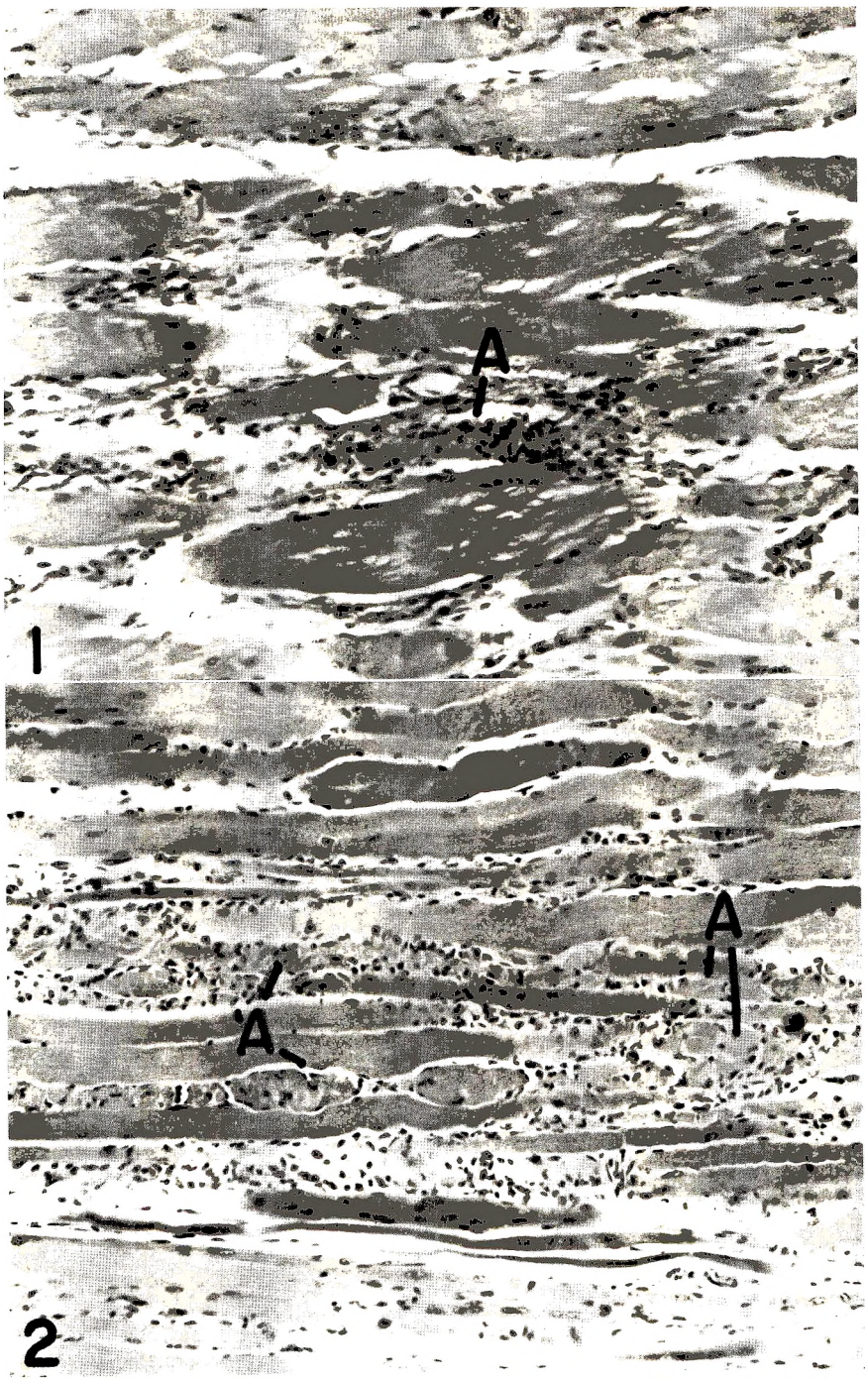
PLATE 1

EXPLANATION OF FIGURES

1 and 2 Representative sections of musculature showing the incidence of coagulation necrosis (A) in 9-week dystrophic mice. Figure 1, from a mouse reared with an improved diet (diet 6), shows only one necrotic fiber segment, whereas figure 2, from an animal reared with an insufficient diet (diet 1), shows several such segments. Note that the morphology of the coagulated areas are essentially the same. Both sections from brachial musculature. H & E. × 175.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1
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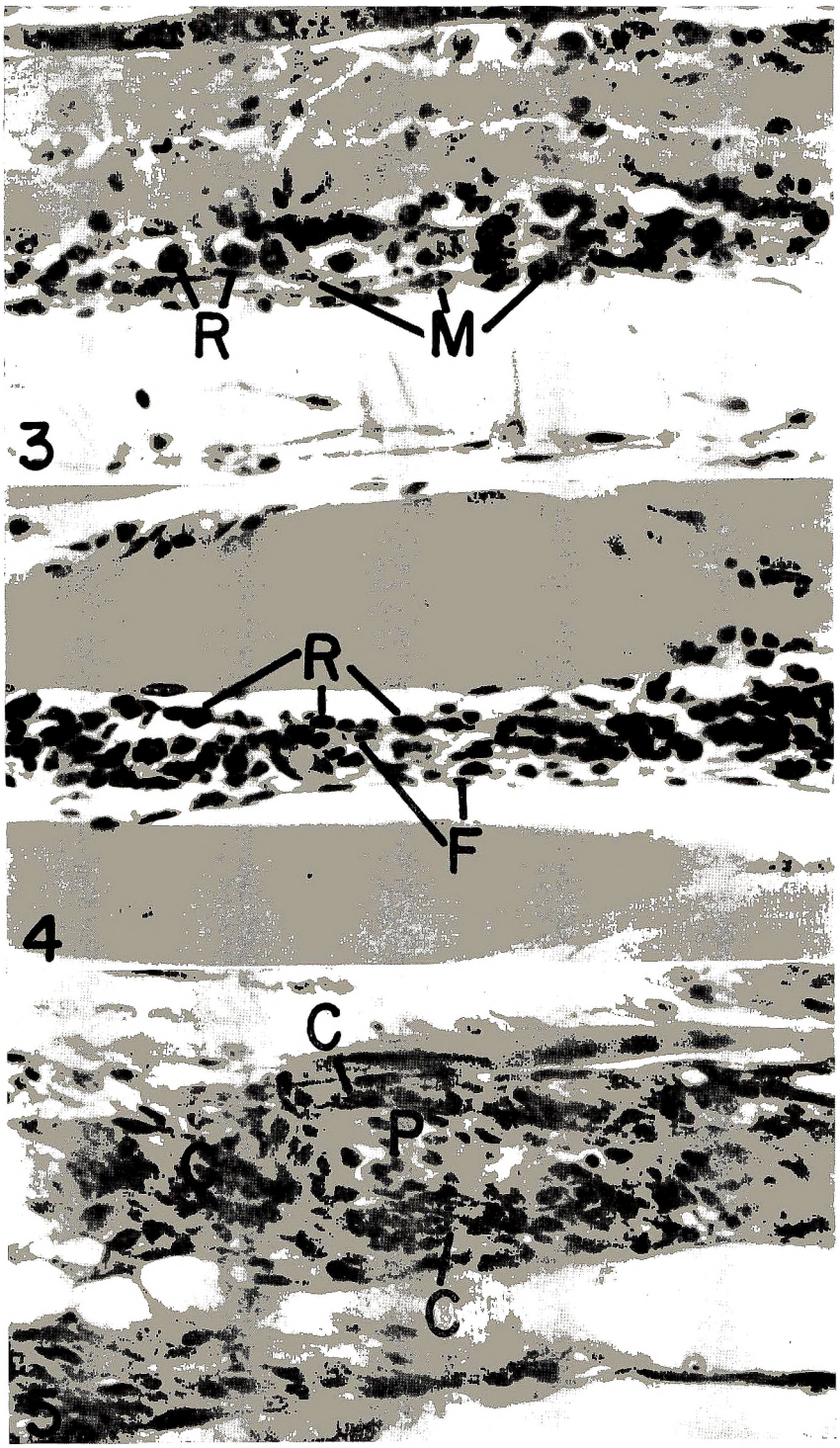
PLATE 2

EXPLANATION OF FIGURES

3 Atypical regenerative activity in muscle of an 11-week dystrophic mouse reared with an improved diet (diet 3). Note the appearance of atypical, mononucleate, basophil round cells (R), probably of muscle origin, along with the more normal myoblast-like cells (M) typical of early stages of regeneration of muscle in the mouse. H & E. × 500.

4 Atypical regenerative activity in muscle of a 13-week dystrophic mouse reared with an improved diet (diet 3). In this case only the atypical, mononucleate round cells (R) and a few fibroblasts (F) can be seen. No myoblast-like elements are present. H & E. × 500.

5 Phagocytosis (P) and connective tissue replacement (C) of a coagulated segment of muscle fiber in a 13-week dystrophic mouse reared with an improved diet (diet 3). Note the absence of regenerative activity in the affected area. PAS, hematoxylin and orange G, after diastase digestion. × 500.


R
M
3
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5

Fatty Acid and Lipide Distribution in Egg Yolks from Hens Fed Cottonseed Oil or *Sterculia foetida* Seeds[1]

ROBERT JOHN EVANS, J. A. DAVIDSON AND SELMA L. BANDEMER
Departments of Agricultural Chemistry and Poultry Science, Michigan State University, East Lansing, Michigan

Hens fed rations containing crude cottonseed oil produce eggs that develop viscous pink whites and very large, fluid salmon-colored or large thick brown yolks after 6 months or more of cold storage (Sherwood '28). Eggs from hens fed *Sterculia foetida* oil develop similarly (Masson et al., '57). Schaible and Bandemer ('46) showed that the discoloration was caused by diffusion of iron through the vitelline membrane from the yolk into the white where it formed the pink iron-conalbumin complex and diffusion of conalbumin from the white into the yolk where it reacted with iron. An increase in the permeability of the vitelline membrane was indicated.

Lorenz ('39) believed that the substance in crude cottonseed oil that causes "pink-white" egg discoloration is the same as the one that gives the Halphen reaction or something very closely related to it. Sterculic acid from *S. foetida* oil and malvalic acid from certain species of Malva give the Halphen reaction and cause "pink-white" egg discoloration (Masson et al., '57; Shenstone and Vickery, '59). Sterculic and malvalic acids are characterized by a cyclopropene group (Nunn, '52; Macfarlane et al., '57), which is believed to be responsible for the Halphen reaction (Faure, '56) and "pink-white" egg discoloration (Shenstone and Vickery, '59).

We believed that if sterculic acid is deposited as such in the egg, it should be possible to determine the amount of sterculic acid present and, by fractionation of the yolk lipides, to determine which lipide fraction contains the sterculic acid. Lorenz et al. ('33) found that the lipide extracted from eggs produced by hens fed cottonseed oil gave the Halphen test. We decided to follow the Halphen reactive substance in the eggs of hens fed *S. foetida* and cottonseed oils, also, in the hope that the location of these substances in the egg might shed some light on the mode of their action in producing egg discoloration. The fatty acid distribution in total egg yolk lipides and various lipide fractions were also determined in order to see whether the feeding of cottonseed oil, which contains a high percentage of linoleic acid, would increase the proportion of linoleic acid in egg yolk fatty acids.

METHODS

Three groups of 12 Single Comb White Leghorn hens were housed in laying cages. The percentage composition of the basal diet was: ground corn, 34.5; ground oats, 20.0; wheat bran, 15.0; flour middlings, 10.0; dehydrated alfalfa (15% protein), 3.0; meat scrap (50% protein), 3.0; dried milk, 2.0; menhaden fish meal (60% protein), 2.5; soybean oil meal (44% protein), 2.5; ground oyster shell flour, 5.0; steamed bone meal, 1.5; salt, 0.6; and fish oil (3000 units of vitamin A and 400 units of vitamin D), 0.4. This ration contained 4.1% of lipide. Group 1 hens received the basal ration only. Group 2 hens received the basal ration to which had been added 2.5% of crude cottonseed oil[2] at the expense of the complete ration. Group 3 hens received the basal ration to which had been added one pound of ground

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[2] Crude cottonseed oil was kindly furnished by Dr. Phillip D. Aines of Buckeye Cotton Oil Division.

S. foetida seeds[3] to 150 pounds of ration. The *S. foetida* seeds added 0.167% of oil to the ration.

One dozen eggs were saved from each group after the hens had been on experiment for two weeks. Yolks and whites of the eggs were separated. The yolks were well mixed and dried by lyophilization. Duplicate 10.0-gm samples of dried yolks were extracted three times by shaking with 50-ml portions of chloroform-methanol (1:1) at room temperature for 30 minutes each time. The solvent was evaporated in a stream of nitrogen and the residue dissolved in chloroform-methanol (98:2) and made to a volume of 100 ml.

The Halphen reaction was carried out as described by Jamieson ('43) except that 1-ml portions of carbon disulfide containing 1% of sulfur and amyl alcohol were used rather than 5-ml portions; 20 ml of yolk extract were taken and the solvent removed by evaporation under a stream of nitrogen before the carbon disulfide and amyl alcohol were added. The mixture was slowly heated at 130°C and kept at that temperature for 30 minutes.

To determine the fatty acid distribution, 20-ml aliquots of yolk lipide solution were evaporated to dryness under nitrogen, and the oil was saponified by mixing with 10 ml of ethanol containing 1.5 gm of potassium hydroxide and allowing to stand overnight at room temperature. The resulting soaps were dissolved in water and acidified with sulfuric acid to convert them to the free fatty acids. The fatty acids were extracted with peroxide-free ether and converted to methyl esters with diazomethane. The fatty acid methyl esters were separated on a diethylene glycol succinate (DEGS) column in an Aerograph Model A-90-C gas-liquid chromatographic instrument operated at 200°C with a helium flow rate of 89 ml per minute. The Aerograph was equipped with a Weston 1 millivolt single point recorder.

Lipides were separated into three fractions by the procedure of Rhodes ('58). Twenty-five milliliters of the chloroform-methanol (98:2) solution of lipide were applied to a chromatographic column of 10 gm of silicic acid.[4] Neutral fats were eluted with chloroform-methanol (98:2), cephalins with chloroform-methanol (70:30), and lecithins with methanol-chloroform-H_2O (75:20:5). Solvents were removed by heating under a stream of nitrogen, the last traces of solvent and water were removed under a vacuum in a desiccator, and the dry lipides were weighed.

Lipides were also separated into 6 fractions by the procedure of Barron and Hanahan ('58). Hydrocarbon, sterol ester, triglyceride, sterol, di- and monoglyceride, and phospholipide fractions were obtained. Hydrocarbons were eluted with hexane,[5] sterol esters with 15% of benzene in hexane, triglycerides with 5% of diethyl ether in hexane, sterols with 20% of diethyl ether in hexane, mono- and diglycerides with diethyl ether, and phospholipides with methanol-chloroform-water (75:20:5).

RESULTS AND DISCUSSION

Methyl sterculate when run through the gas-liquid chromatogram using a DEGS column gives a characteristic peak which emerges soon after the methyl linoleate peak (fig. 1). The methyl sterculate peak differs in position from the methyl linolenate peak. Yolks of normal eggs contained no measurable quantities of fatty acids the methyl esters of which emerged after methyl linoleate. No sterculic acid methyl ester peak was observed in any of the gas-liquid chromatograms of the fatty acid methyl esters of egg yolk oils or lipide fractions obtained in this study. In most cases no peaks were detectable after that of the methyl linoleate. Even with the chromatographic column heavily loaded with fatty acid methyl esters and the sensitivity increased 8 times for the portion of the curve following methyl linoleate, only two weak peaks past methyl linoleate were obtained and neither corresponded with methyl sterculate in position. If sterculic acid was present in any of the egg oils, it was there in amounts too small to be detected by the method of chromatography used, and the method would detect any sterculic acid over 0.4% of the total fatty acids in the yolk. Oil from eggs of hens

[3] *Sterculia foetida* seeds were kindly furnished by Dr. Quentin Jones, U.S.D.A., Beltsville.

[4] Acid Silicic, Mallinckrodt A. R., suitable for chromatographic analysis by the method of Ramsey and Patterson.

[5] Skelly-Solve B, Skelly Oil Company.

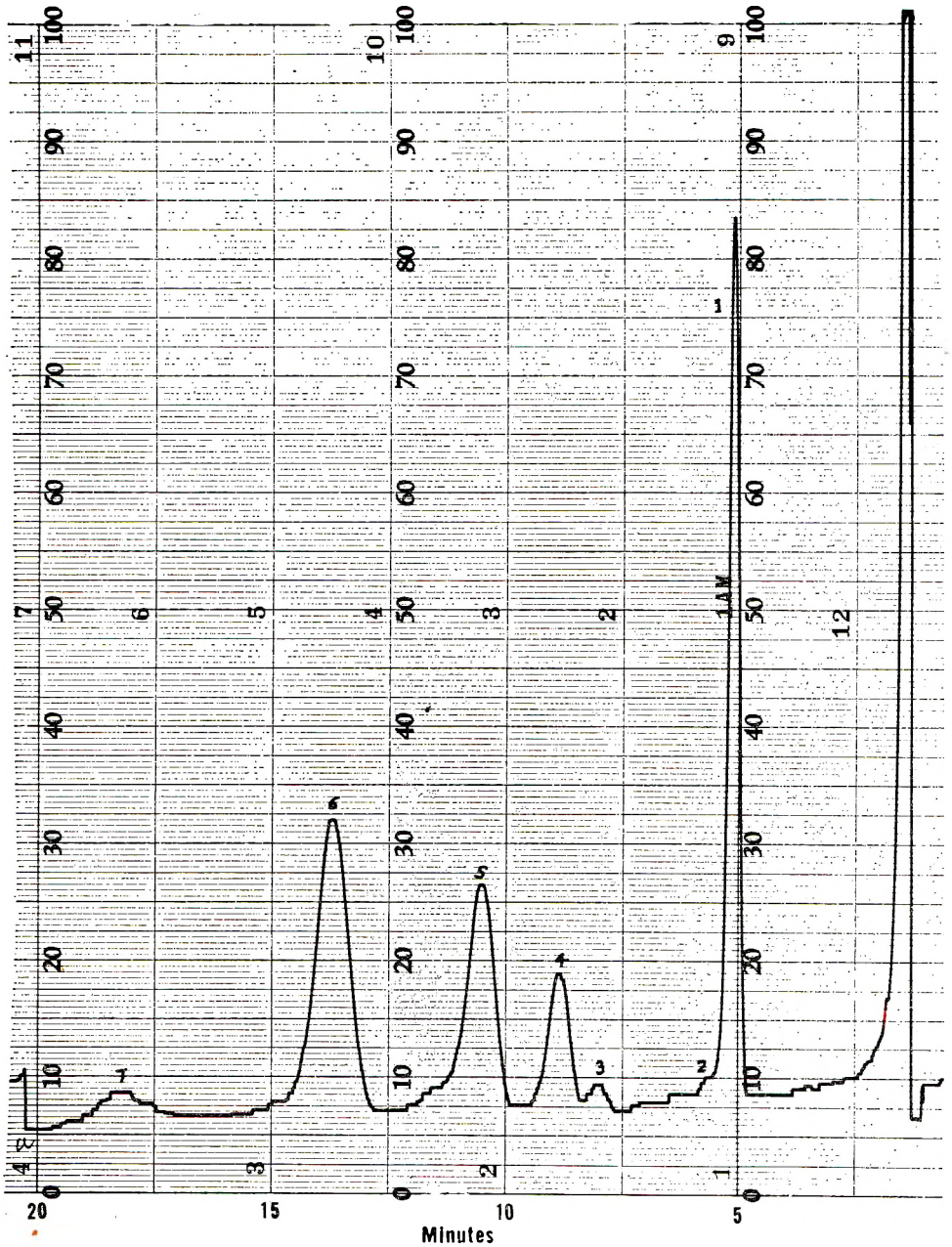

Fig. 1 Gas-liquid chromatogram of methyl esters of fatty acids from *Sterculia foetida* seed fat. Peaks are as follows: 1, palmitate; 2, palmitoleate; 3, stearate; 4, oleate; 5, linoleate; 6, sterculate; and 7, an unknown fatty acid methyl ester associated with sterculate, perhaps a C_{20} cyclopropene fatty acid.

fed *S. foetida* oil gave a positive Halphen reaction, but oil from eggs of the other hens did not. Either the Halphen reaction was much more sensitive for the detection of sterculic acid than gas-liquid chromatography, or sterculic acid was changed to some other Halphen reactive compound when deposited in the egg or *S. foetida* oil contains some substance other than sterculic acid that gives the Halphen reaction and is deposited in the eggs. Both *S. foetida* oil and cottonseed oil gave positive Halphen reactions (table 1).

The presence of 2.5% of cottonseed oil or of 0.17% of *S. foetida* oil in the ration did not cause any marked change in the distribution of neutral lipides, cephalins, or lecithins (table 2) or of hydrocarbons, sterol esters, triglycerides, sterols, mono- and diglycerides, or phospholipides (table 2) in egg yolk oils. Although the percentages of sterol esters and mono- and diglycerides appeared to be smaller in the oil of eggs from hens fed cottonseed or *S. foetida* oils, the amounts involved were so small that the differences are of doubtful significance. The procedure of Barron and Hanahan ('58) gave a slightly higher percentage of phospholipides in oils of eggs from hens fed *S. foetida* oil than in oils of eggs from hens fed cottonseed oil, but the procedure of Rhodes ('58) did not.

The only fractions that gave positive Halphen reactions were the neutral lipides obtained by the procedure of Rhodes ('58) and the triglycerides obtained by the procedure of Barron and Hanahan ('58) (table 3) from oils of eggs produced by

TABLE 1

Fatty acid distribution in oils from basal ration, cottonseed oil, and Sterculia foetida seeds

Fatty acid	Fatty acid composition		
	Basal ration oil	Crude cottonseed oil	*Sterculia foetida* oil
	%	%	%
Myristic	1.7	1.0	0.2
Palmitic	16.6	27.9	25.8
Palmitoleic	2.7	1.0	1.2
Unknown no. 1			0.2
Stearic	3.2	2.3	1.8
Oleic	27.4	17.0	8.8
Linoleic	45.1	50.9	19.0
Linolenic	2.2		
Unknown no. 2			3.6
Sterculic			32.6
Unknown no. 3			6.8
Eicosenoic	1.2		
Halphen reaction	−	+	+

TABLE 2

Lipide distribution in egg oils from hens fed different rations

	Ration fed		
	Basal	Cottonseed oil	*S. foetida* seed
	%	%	%
Method of Rhodes ('58)			
Neutral lipides	69.3	69.8	69.9
Cephalins	21.4	21.6	21.4
Lecithins	9.0	8.6	8.8
Method of Barron and Hanahan ('58)			
Hydrocarbons	0.5	0.5	0.5
Sterol esters	0.3	0.2	0.1
Triglycerides	63.8	63.5	63.7
Sterols	5.6	5.8	5.8
Mono- and diglycerides	1.2	0.8	0.6
Phospholipides	28.6	28.2	29.3

TABLE 3

Fatty acid distribution in egg oils and oil fractions from hens fed different rations

Ration fed	Fatty acids						Saturation			Halphen reaction
	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Saturated acids	Monoenoic acids	Dienoic acids	
	%	%	%	%	%	%	%	%	%	
Egg oils										
Basal	0.4	29.6	4.0	10.4	38.1	17.5	40.4	42.1	17.5	—
Cottonseed oil	0.5	29.1	2.0	22.2	25.6	20.6	51.8	27.6	20.6	—
Sterculia seed	0.5	32.0	1.9	26.3	18.4	20.9	58.8	20.3	20.9	+
Neutral lipides										
Basal	0.7	28.7	4.3	8.6	40.7	17.0	38.0	45.0	17.0	—
Cottonseed oil	0.8	29.5	2.1	22.7	25.8	19.2	53.0	27.9	19.2	—
Sterculia seed	1.0	33.5	2.1	26.7	17.4	19.4	61.2	19.5	19.4	+
Triglycerides										
Basal	0.6	29.8	4.3	8.9	39.6	16.8	39.3	43.9	16.8	
Cottonseed oil	0.8	29.4	2.0	22.4	26.2	19.3	52.6	28.2	19.3	
Sterculia seed	1.0	33.5	1.7	27.4	17.2	19.2	61.9	18.9	19.2	+
"Sterols"										
Basal	0.3	13.2	3.7	10.9	48.3	23.6	24.4	52.0	23.6	
Cottonseed oil	0.7	12.0	2.2	28.6	30.6	26.0	41.3	32.8	26.0	
Sterculia seed	0.5	15.0	2.0	36.4	20.7	25.5	51.9	22.7	25.5	—
Di- and monoglycerides										
Basal	0.7	23.5	4.8	11.8	39.9	19.3	36.0	44.7	19.3	
Cottonseed oil	0.6	22.5	2.8	24.7	26.5	22.9	47.8	29.3	22.9	
Sterculia seed	0.4	21.8	1.8	42.0	15.3	18.7	64.2	17.1	18.7	—
Cephalins										
Basal	0.0	34.0	2.3	21.7	25.1	17.0	55.7	27.4	17.0	—
Cottonseed oil	0.0	33.2	1.5	23.1	21.5	20.8	56.3	23.0	20.8	—
Sterculia seed	0.0	34.8	2.6	20.7	18.6	23.3	55.5	21.2	23.3	—
Lecithins										
Basal	0.6	38.2	3.9	14.3	26.4	16.6	53.1	30.3	16.6	—
Cottonseed oil	0.0	37.0	3.4	15.8	24.0	19.9	52.8	27.4	19.9	—
Sterculia seed	0.0	40.5	3.0	13.2	21.4	22.2	53.7	24.4	22.2	—

hens fed *S. foetida* seed. Sterculic acid or some other cyclopropene fatty acid thus appears to be concentrated in the triglyceride fractions of the egg yolk. It may be present, therefore, either in combination with other fatty acids in the triglyceride molecule or as free sterculic acid or other cyclopropene acid because the free fatty acids are present in this fraction (Barron and Hanahan, '58).

The fatty acid distribution in the oils of eggs from hens fed cottonseed oil or *S. foetida* seeds was quite different from that in the oil of eggs from hens fed the basal ration (table 3). Linoleic acid content was slightly higher in oil of eggs from hens fed *S. foetida* seeds or cottonseed oil than in eggs of hens fed the basal ration, but the saturated fatty acid content was much higher and the monoenoic acid content lower. Oil of eggs from hens fed *S. foetida* seeds contained less than half as much of the monoenoic acids and nearly one-and-one-half times as much of the saturated fatty acids as oil of eggs from hens fed the basal ration. The extracted oil of eggs from hens fed *S. foetida* seeds was solid at room temperature whereas that from hens fed the basal ration was liquid. Changes in fatty acid distribution in lipides of eggs from hens fed cottonseed oil were not as great as those in lipides of eggs from hens fed *S. foetida* seed.

Sterculic acid was the principal fatty acid in the *S. foetida* seeds fed and made up about one third of the fatty acids in the oil (table 1). The *S. foetida* seeds added only 0.17% of fat to the basal ration, which already contained 4.1% of fat. Linoleic acid accounted for 45.1% of the fatty acids in the basal ration (table 1). The addition of *S. foetida* seeds did not increase appreciably the linoleic acid content of the ration, but the linoleic acid content of the egg oils was increased from 17.5 to 20.9%. Cottonseed oil feeding also increased linoleic acid in the egg oil to 20.6%, but this increase could have been caused by the linoleic acid added to the ration by the cottonseed oil.

Neither cottonseed oil nor *S. foetida* seeds added significant amounts of stearic acid to the hen's ration (table 1), but both increased the stearic acid content in the egg yolk fatty acids (table 3). Feigenbaum and Fisher ('59) fed 10% levels of several oils to laying hens and analyzed the egg oils for fatty acid distribution by the alkaline isomerization method. Both cottonseed and safflower oils increased linoleic and saturated fatty acid contents of egg fat, but cottonseed oil gave the larger increases even though safflower oil contained more linoleic acid. On the other hand, Wheeler et al. ('59) obtained large increases in linoleic acid content of eggs from feeding up to 30% of safflower oil in the hen's ration, but only slight increases in saturated fatty acid content as determined by alkaline isomerization and no increases when measured by the gas-liquid chromatographic method. Ten per cent of cottonseed oil in the ration increased stearic acid content of the eggs, but not to the extent observed in the present experiment. Wheeler et al. ('59) did not describe their cottonseed oil, so it is not known whether it was a crude or refined oil or what the content of Halphen-reactive fatty acids was.

Evans et al. ('60) fed various fractions of cottonseed oil fatty acids to laying hens. Eggs produced by these hens were stored for nearly 12 months and then observed for pink-white discoloration. The yolks were extracted and the saponified oils analyzed for fatty acids by the gas-liquid chromatographic procedure. Although the data for egg discoloration were not reported, eggs from hens fed the fraction of cottonseed fatty acids that was soluble in acetone at −60°C developed pink whites on storage and this fraction of oil gave a strong Halphen reaction. The egg oil contained 49.8% of saturated fatty acids compared with 41.3% in oil of eggs from hens receiving very little supplemental lipide. The fraction of cottonseed fatty acids that was precipitated at −60°C but soluble at −50°C added about the same amount of lipide to the ration, and contained the same percentage of linoleic acid, but it gave only about half as strong a Halphen reaction. Eggs produced by hens fed this ration developed slightly pink whites on storage and the egg oil contained 45.6% of saturated fatty acids. Oil from fresh eggs produced by laying hens fed *S. foetida* seeds in the present experiment gave a positive Halphen reaction, but eggs from hens fed cottonseed oil in the present ex-

periment or cottonseed fatty acid fractions in the earlier experiment did not. *S. foetida* seeds at the level fed must, therefore, have added more Halphen reactive compounds to the ration than the cottonseed oil or any of the cottonseed fatty acid fractions did. Hens fed the rations containing *S. foetida* seeds also produced eggs with a higher proportion of saturated fatty acids in them than hens fed cottonseed oil or any of the cottonseed fatty acid fractions.

The principal constitutents of the neutral lipide fraction of egg oil were the triglycerides (table 2). The fatty acid distribution was similar in the triglyceride fraction to that in the neutral lipide fraction (table 3). Triglycerides of eggs from hens fed *S. foetida* seeds contained more of the saturated fatty acids and linoleic acid and less of the monoenoic acids than those of eggs from hens fed the basal ration.

Only a very small sterol ester fraction was obtained (table 2). When the fatty acid methyl esters were separated on the gas-liquid chromatographic column, the peaks were so small they were difficult to evaluate; therefore, the data obtained are not presented in tabular form because they are not very reliable, but they do indicate a distribution of each of the egg yolk fatty acids in the sterol esters. Saturated acid content was higher (50% palmitic acid where hens were fed cottonseed oil) and monoenoic acid content lower in sterol esters of oils of eggs from hens fed *S. foetida* seeds or cottonseed oil than in those of eggs from hens fed the basal ration. Sterol ester fatty acids of eggs from hens fed the basal ration also contained 12.1% of a fatty acid, the methyl ester of which emerged after methyl linoleate and was probably a monounsaturated C_{20} acid. This acid was not present in sterol esters of eggs from hens fed *S. foetida* seeds or cottonseed oil.

Some fatty acids were also present in the hydrocarbon fractions, which may have been contaminated with sterol esters, but here again such small quantities were involved that the results obtained were inconclusive and are not presented.

According to Barron and Hanahan ('58), free sterols are eluted with 15 or 20% of ether in hexane, diglycerides by 30% of ether in hexane, and monoglycerides by 90 to 100% of ether in hexane. In the present study, 20% of ether in hexane was used to elute free sterols, and ether was used to elute both di- and monoglycerides. Possibly the 20% ether extracted part or all of the diglycerides as well as sterols. The glyceride fraction may have been largely monoglyceride. The free sterol fraction was contaminated with fatty acids, and the fatty acid distribution is given in table 3. Fatty acid distribution in the di-and monoglyceride fraction is presented in table 3. Feeding *S. foetida* seeds or cottonseed oil changed the fatty acid distribution in egg oil "sterol" and "glyceride" fractions in the same way as it did in the triglyceride fraction. There was less palmitic acid and more oleic acid in the "sterol" fraction than in the other two fractions. The "glyceride" fraction contained less palmitic acid and more stearic acid than the triglyceride one.

Saturated fatty acids did not increase in the cephalin or lecithin fractions of eggs from hens fed *S. foetida* seeds or cottonseed oil (table 3). Monoenoic acids decreased and dienoic acids increased, however. The phospholipide fatty acids were composed roughly of 55% of saturated fatty acids and 45% of unsaturated ones. This is a lower percentage of saturated and a higher percentage of unsaturated acids than reported by Evans and Bandemer ('60) and supports the observations of Rhodes ('58) and Tattrie ('59) that half of the fatty acids of the egg phospholipides are saturated and half unsaturated. Hawke ('59) found 59.7% of the fatty acids in cephalin and 42.5% of those in lecithin from eggs of New Zealand hens to be saturated. Hawke and Lea ('53) observed that fatty acids of egg yolk phospholipides were more unsaturated than the corresponding glycerides, but the data presented here indicate that the composition of the phospholipides was more constant than that of the glycerides and that the nature of the hen's diet would determine whether the phospholipides or the glycerides are the more unsaturated. Both cephalin and lecithin contained more palmitic acid than stearic, and the lecithins contained nearly twice as much palmitic as stearic. The data of Choudhury and

Reiser ('59) do not agree with the present data because both percentage of unsaturated fatty acids and degree of unsaturation of egg yolk phospholipides were increased when they fed 20% of safflower oil, but the percentage of saturated fatty acids in the triglycerides was not changed; only the dienoic acids increased at the expense of the monoenoic.

Besides the pink-white and salmon-yolk discoloration that occurs when eggs from hens fed crude cottonseed oil or sterculic acid are stored, several other defects in the eggs have been observed. Reiser ('50) and Naber and Morgan ('57) observed decreased hatchability of eggs from hens fed cottonseed oil. Shenstone and Vickerey ('59) described the yolks of eggs from hens fed sterculic or malvalic acids as follows: "From the time of laying the cold yolks had a firm, resilient or rubbery nature which was often maintained when the yolk was warmed to 20°C. After further storage the yolks became more plastic or pasty and the cold yolks remained spherical when placed on a flat plate." The present experiment helps to explain some of the above defects. Sterculic acid or cottonseed oil feeding increased the saturated fatty acids in the egg yolks (table 3) and this increased the solidification point of the egg oil. Yolks of eggs at refrigeration temperature would thus be less fluid than normal yolks and would have the plastic consistency observed by Shenstone and Vickery ('59). The increased proportion of saturated fatty acids or the presence of the "Halphen" acid in the egg yolks may have caused decreased egg hatchability. Two things occur when eggs from hens fed crude cottonseed oil are stored. There is an increased permeability of the vitelline membrane allowing the migration of water and egg white proteins from the white to the yolk (Evans et al., '59) and some reaction occurs that causes the release of iron from the yolk protein so that it can react with the conalbumin in the white and yolk to produce the pink-white and salmon-yolk discoloration. The results of the present experiment do not indicate whether the increased permeability of the vitelline membrane and the release of iron are related or whether they are caused by the "Halphen" acid in the yolk, the increased percentage of saturated fatty acids, or some other as yet unknown change occurring in the egg yolk.

SUMMARY

Laying hens were fed a ration containing 4.1% of fat, either unsupplemented, or supplemented with 2.5% of crude cottonseed oil or 0.67% of *Sterculia foetida* seeds. Eggs from each group were used. Egg yolk lipides were fractionated on silicic acid columns, and each lipide fraction was analyzed for fatty acid distribution by gas-liquid chromatography.

No differences in distribution of the various lipide fractions were observed among eggs from the three groups of hens. The yolk lipides contained approximately 64% of triglycerides, 21% of cephalins, 9% of lecithins, and 6% of mono- and diglycerides, sterols, and sterol esters.

The substance giving the Halphen reaction was concentrated in the triglyceride fraction of the egg yolk lipides in eggs from hens fed *S. foetida* seeds. No measurable amount of sterculic acid was detected in the yolk lipides. Yolk oil from eggs of hens fed crude cottonseed oil did not give a positive Halphen reaction.

Feeding either *S. foetida* seeds or crude cottonseed oil to laying hens caused them to lay eggs with an increased proportion of saturated fatty acids and a decreased proportion of monoenoic acids. Over 50% of the fatty acids in eggs from hens fed cottonseed oil or *S. foetida* seeds were saturated. The linoleic acid content was also slightly increased. The saturated fatty acid content of the phospholipides was fairly constant at about 55%, but the dienoic acids increased from 18 to 24% and the monoenoic acids decreased from 28 to 20% of the total fatty acids. Larger changes took place in the fatty acid distribution in the trigylcerides, where the saturated acids increased from 39 to 62%, and the monoenoic acid decreased from 44 to 19% of the total fatty acids in the case of hens fed *S. foetida* seeds. Linoleic acid increased from 17 to 19%.

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The Metabolism of Fats

I. EFFECT OF DIETARY HYDROXY ACIDS AND THEIR TRIGLYCERIDES ON GROWTH, CARCASS, AND FECAL FAT COMPOSITION IN THE RAT[1]

E. G. PERKINS, J. G. ENDRES AND F. A. KUMMEROW
Armour and Company, Food Research Division,[2] Chicago, Illinois and Department of Food Technology, University of Illinois, Urbana, Illinois

Modifications of the body fat composition by means of dietary fatty acids have served previous workers as a means of studying fat metabolism. Fatty acids have been labeled by elaidinization (Barbour, '33), or with deuterium (Cavanaugh and Raper, '39). The feeding of an excess amount of unsaturated fatty acids has also been used as a means of studying fatty acid metabolism by Feigenbaum and Fisher ('59), Spadola and Ellis ('36), Longenecker ('39a,b) and Beadle and associates ('48).

Castor oil, which has as its chief component 12 - hydroxy - 9 - octadecenoic acid (ricinoleic acid) has been reported to be about 98% digestible in the rat (Paul and McCay, '42). In another study, rats were fed for several weeks a diet containing 48.11% of castor oil. No catharsis occurred and the animals grew well. About 7% of the fatty acids of depot fat were shown to be ricinoleic acid (Stewart and Sinclair, '45). These authors investigated only the storage of ricinoleic acid in the rat; the effects of the high dietary ricinoleic acid intake upon the general fatty acid composition of the rat was not reported.

It was the purpose of the present study to investigate the influence of ingested ricinoleic acid, 12-hydroxystearic acid and their corresponding triglycerides on rat growth and their effects on carcass fat composition when compared with the effects of a commercial hydrogenated shortening and corn oil. The influences of these dietary fats on the saturated and unsaturated fatty acids of rat carcass fat are reported here.

EXPERIMENTAL

A basal diet containing 12% of fat was used in all experiments. The details of the basal diet preparation, and the composition of the fat and water-soluble vitamin mixtures have been published previously (Perkins and Kummerow, '59). The test fat represented 10% and fresh cottonseed oil 2% of the 12% of fat. The diets were kept stored under nitrogen and refrigerated to prevent fat deterioration. Groups of 5 animals each were kept in single cages, weighed on alternate days, and arbitrarily restricted to the same amount of food intake as those fed corn oil. Male weanling rats were maintained with the test diet for 59 days. Samples of the feces were collected and frozen. After the test period the animals were sacrificed, the livers removed, weighed, the carcass frozen and stored in a deep freeze until required.

The total carcass fat of the rats was obtained by digestion and extraction of the digested carcass according to the procedure of Johnston et al. ('58). The rat fat so obtained was stored in the cold under nitrogen until required. Rat feces samples were pooled within groups, hydrolyzed with 6 N ethanolic hydrochloric acid solution for 12 hours at 25°C; the acid was removed and the hydrolyzate extracted for 4 hours with acetone in a Soxhlet extractor. The acetone extract was dried and solvent removed by distillation; final traces

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[2] 1425 West 42nd Street, Chicago.

of solvent were removed under vacuum. The fecal fat so obtained was stored under nitrogen in the cold.

Aliquots of the fat samples were converted to their corresponding methyl esters prior to gas-liquid chromatographic analysis as follows. Approximately 1 gm of the fat to be esterified, 25 to 30 ml of methanol (anhydrous), and 50 to 100 mg of dry sodium methoxide were added to a small flask; the contents of the flask were refluxed for 45 minutes, poured into a separatory funnel and 100 ml of water added. The material was then extracted twice with petroleum ether (Skellysolve F), the extracts combined, washed with water and dried over sodium sufate. Solvent was removed under vacuum on a rotary evaporator. Duplicate samples of methyl esters were prepared from all fats, and stored under nitrogen in the cold until analyzed.

The Wijs iodine values were determined in duplicate on each fat sample according to the official A.O.C.S. method. Acetyl values were determined according to the method of Ogg et al. ('45).

The fresh corn oil diet supplement was obtained locally and was a light-colored refined corn oil. Corn oil fatty acids were prepared from fresh corn oil by saponification of the oil with potassium hydroxide in 95% ethanol; regeneration of the acids from the soaps by acidification with dilute hydrochloric acid, and extraction with petroleum ether. The extracts were washed several times with water to remove all mineral acid, and dried over anhydrous sodium sulfate. Solvent was removed by distillation with the aid of steam heat; last traces of solvent were removed under vacuum. The fresh corn oil fatty acids thus prepared were stored under nitrogen in the cold until used. Ricinoleic acid was prepared from purified triricinolein in a similar manner and was also stored in the cold until required.

The shortening used in these experiments was also obtained locally and was a hydrogenated animal-vegetable fat shortening. Hydrogenated castor oil fatty acids[3] were obtained commercially and were used without further purification. Triricinolein was prepared by the exhaustive extraction of castor oil with petroleum ether. After 10 extractions of one kilogram of castor oil with one-liter portions of petroleum ether, a triricinolein concentrate was obtained which was found to be about 97% pure (% OH = 5.04; theory = 5.47). This material was used without further purification.

Gas-liquid chromatographic analysis of fatty acid methyl esters. The gas chromatographic analyses of the fats obtained in this experiment were carried out using an instrument constructed in our laboratory, and a thermal conductivity detector system. A 4-foot, ¼ inch internal diameter copper column was packed with 60% 60 to 80 mesh chromasorb[4] which was impregnated with 40% by weight of a succinic anhydride-diethylene glycol polyester as the stationary phase. The general method for preparing this type of a column has been published by Orr and Callen ('59). Columns prepared in this manner were stable up to 210°C and provided well-defined separations of saturated and unsaturated compounds in the fatty acid methyl ester series, but were unable to separate the isomeric unsaturated 16 and 18 carbon fatty acid methyl esters.

Analyses were usually run at 190 to 205°C with a helium pressure of 11 pounds per square inch, which resulted in a flow rate of about 60 ml of gas per minute. Under these conditions a curve was obtained for a plot of retention time vs. chain length for a standard mixture of fatty acid methyl esters (fig. 1). A standard mixture was analyzed periodically as a check of column condition, although retention times varied little from day to day.

For a typical analysis of fatty acid methyl esters, 1 to 5 µl of the ester were injected into the column with a micro syringe. The analysis required from 30 to 60 minutes to complete, depending upon the operating column temperature selected. Identification was based on comparison of the retention time of the esters with those of authentic samples. The composition of each mixture analyzed was calculated on the basis of the area under each peak, as found by the triangulation method.

[3] Emery Industries, Cincinnati, Ohio.

[4] 60–80 mesh Chomasorb, W, F. & M. Scientific Corp., New Castle, Delaware.

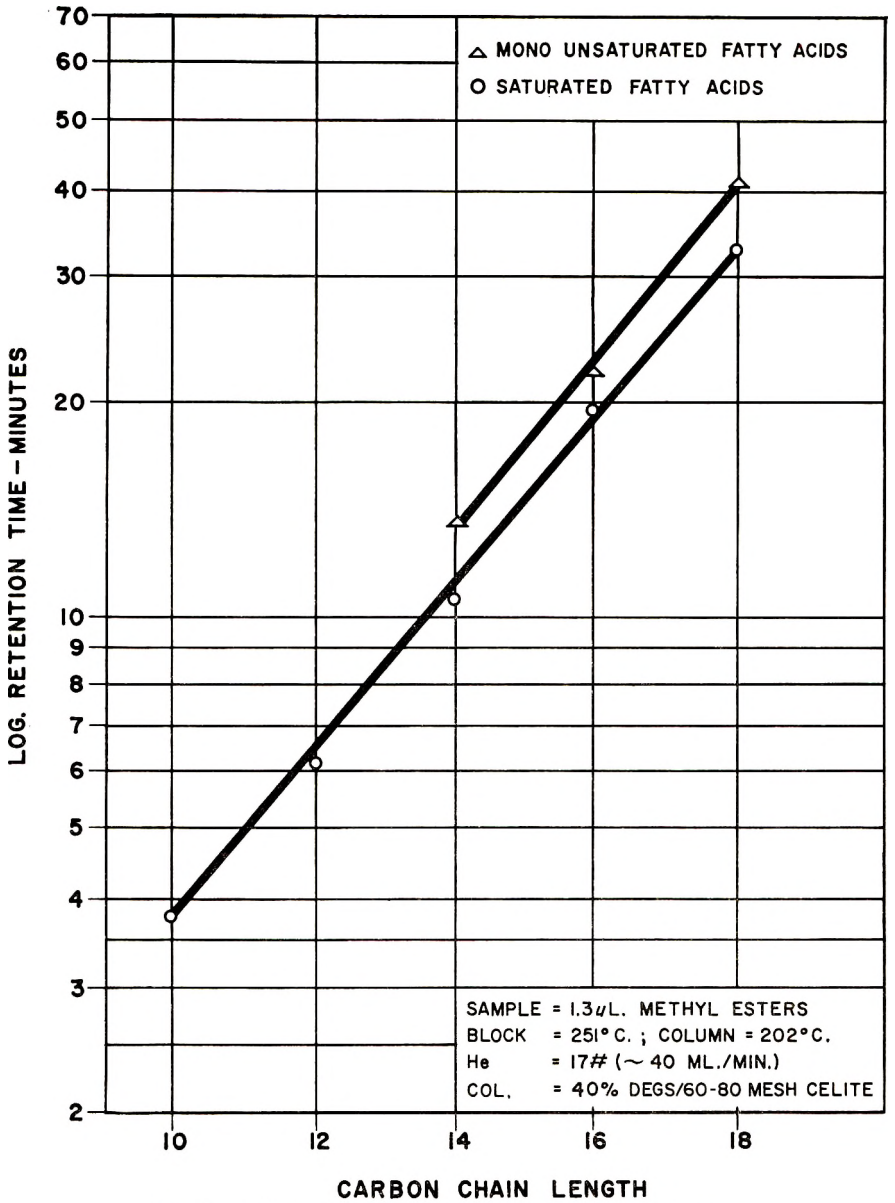

Fig. 1 Effect of carbon chain length on retention time.

RESULTS AND DISCUSSION

Biological response. Rats fed fats containing hydroxyl groups gained slightly less weight than those fed shortening or fresh corn oil (table 1). Rats fed diets containing 10% of a commercial shortening or fresh corn oil weighed between 190 to 207 gm at the termination of the growth study; those fed hydroxylated fats at the same level weighed from 157 to 199 gm. Statistically significant differences were found in the growth of animals fed ricinoleic acid and hydrogenated castor oil fatty acids when compared with those fed fresh corn oil, whereas those animals fed ricinoleic acid in the form of the triglyceride gained as much weight as those fed fresh corn oil diets.

TABLE 1

Comparison of biological response of the rat to various ingested dietary fats

Diet supplement	Changes in weight in 59 days	Growth ratio[1]	Liver—% of body weight
	gm		
Fresh corn oil	204.2 ± 20.3[2]	1.00	3.54 ± 0.41
Fatty acids from fresh corn oil	207.3 ± 9.8	1.01	3.33 ± 0.14
Triricinolein	199.0 ± 4.5	0.97	4.07 ± 0.33
Ricinoleic acid	177.0 ± 0.3[3]	0.85	4.21 ± 0.36[3]
Hydrogenated castor oil acids	172.5 ± 1.2[3]	0.83	3.31 ± 0.14
Commercial shortening	191.0 ± 4.3	0.93	3.20 ± 0.17

[1] Weight gain with test fat/weight gain with control fat, fresh corn oil.
[2] Standard error of the mean.
[3] Statistically significantly different from the fresh corn oil group at the 95% level.

TABLE 2

Comparison of dietary supplement fatty acid composition

Fatty acid component	Fresh corn oil	Fatty acids from fresh corn oil	Triricinolein	Ricinoleic acid	Hydrogenated castor oil fatty acids	Commercial shortening
	%	%	%	%	%	%
Linoleic	54.4	54.4	—	—	—	10.1
Oleic	28.8	28.8	—	—	—	39.9
Stearic	2.3	2.3	3.0	3.0	10.9	17.5
Hexadecenoic	—	—	—	—	—	3.5
Palmitic	13.0	13.0	—	—	5.1	23.7
Myristic	0.2	0.2	—	—	—	3.5
Lauric	0.4	0.4	—	—	—	1.1
Hydroxy acids	—	—	97.0	97.0	84.3	—
Iodine value	126.0	124.0	81.6	85.2	0.4	49.5

The degree of utilization of the ingested fats, as measured by the ratio of weight gain of animals fed the test fat to those fed fresh corn oil, was 83.5 and 85.8% for ricinoleic acid and hydrogenated castor oil fatty acids, respectively, and that of triricinolein was 97.0%. Livers were slightly enlarged, as were the livers of those fed triricinolein. Statistically significant differences in liver enlargements were observed in livers of animals fed ricinoleic acid when compared with those fed fresh corn oil and fresh corn oil acids; but no significant differences were observed in the livers of rats fed either ricinoleic acid or hydrogenated castor oil fatty acids. The differences in growth response caused by ingestion of hydroxy acids as a source of dietary fat may be attributed to the fact that fatty acids are more poorly absorbed than triglycerides (Deuel, '55). It is possible that this effect was therefore reduced to a minimum when the animals were fed the hydroxy acids as the triglycerides, which were obviously metabolized quite readily by the growing rat, as evidenced by the weight gain of the animals fed this diet. These results are in agreement with those of Stewart and Sinclair ('45) who found that about 2% of the ingested ricinoleic acid was excreted by the rat. Increase in liver weight may have resulted from an attempt by the animal to compensate for the substitution of foreign fats in its diet by an increased rate of metabolism to produce from a foreign fat or dietary protein or carbohydrates, or combination of these, fats of a more normal structure that it may specifically require.

Effect of dietary fat on body and fecal fatty acid composition. Fresh corn oil and fresh corn oil fatty acids fed as dietary supplements had high linoleic, oleic and palmitic acid contents, whereas the commercial shortening used had a low linoleic acid content and a high oleate, stearate and palmitate content (table 2). The purified

hydroxylated materials fed were ricinoleic acid, and triricinolein, which had stearic acid as the main impurity (about 3%); and hydrogenated castor oil fatty acids, consisting of about 84% of 12-hydroxystearic acid and the remainder mostly of stearic acid (table 2).

Analysis of the fatty acid composition of the carcass fat of rats fed fresh corn oil and the corresponding fatty acids (table 3) disclosed a high linoleic, oleic and palmitic acid content. A small amount of hexadecenoic acid was also deposited. The composition of feces fat of animals fed fresh corn oil and fresh corn oil fatty acids was slightly different than the composition of carcass fat of rats fed these diets. When animals were fed fresh corn oil, the composition of feces fat exhibited a marked increase in stearic acid and a decrease in linoleic acid content, when compared with that of carcass fat. Animals fed fresh corn oil fatty acids had very similar carcass and feces fat composition, except for stearic acid which increased from 2.4 to 8.3% and myristic acid, which decreased from 2.5 to 0.6%. The increase in stearic acid excretion can be explained by postulating a preferential excretion of the high melting point fatty acids, so as to give a body fat having a fairly low melting point—near the animals' body temperature. The decreased excretion of linoleic acid may be a result of utilization by the animal of linoleic acid and its conversion to oleic acid or to saturated fatty acid having a lower melting point such as palmitic acid. The hydrogenation of unsaturated acids to saturated acids (Hilditch and Pedelty, '40) and their conversion to shorter chain homologues has been demonstrated by Schoenheimer and Rittenberg ('37) using deuterium-labeled fatty acids. No significant amounts of hydroxy acids were noted in the carcass fats of animals fed diets containing fresh corn oil and fresh corn oil fatty acids. In all of the feces fats examined, relatively large amounts of hydroxylated material were found but the amounts of material available were insufficient to allow an exact determination of the hydroxy fatty acid content.

Ingestion by the rat of a commercial hydrogenated shortening low in linoleic acid content, and high in stearic, palmitic, oleic, and hexadecenoic acids (table 2) was used to determine the effects of this type of fat on carcass and fecal fat composition (table 3). The composition of carcass fat was similar to that of the ingested fat except that the stearic acid content had been reduced by about 75%, and the hexadecenoic acid content increased about 100%. Levels of linoleic acid remained essentially identical to that of the ingested fat; the oleic acid content increased somewhat, possibly again at the expense of stearic acid. The fecal fat composition differs markedly from that of the carcass fat and also from the fecal fat of rats fed fresh corn oil. The major portion of the fatty acids found in the feces consisted of oleic, stearic, and palmitic acids, with only a small amount of linoleic and hexadecenoic acids being excreted.

Ingestion of hydroxy acids both in the form of their triglycerides and as the free fatty acids caused marked changes in both carcass and fecal fat composition when compared with the effects of corn oil and shortening (table 3). Animals fed triricinolein, ricinoleic acid and hydrogenated castor oil fatty acids had approximately the same carcass and feces fat composition, except that animals fed ricinoleic acid excreted more linoleic acid than those fed other sources of hydroxy acids. Levels of oleic acid in the carcass fat of rats fed esterified and unesterified hydroxy acid diets were very high, and conversely, levels of linoleic acid were low. In all animals the oleic acid levels ranged from 41 to 45%, and those of linoleic acid from 8.6 to 10.0%. Since the animals did not receive these acids as major dietary components, it must be concluded that they and other acids of rat carcass fat were formed from the ingested 12-hydroxy fatty acids, or by resynthesis from acetate derived from carbohydrate and protein. Oleic acid and linoleic acid could have been formed from the hydroxy acids by a series of dehydration, hydrogenation steps or by synthesis from acetate formed by degradation of the hydroxy acids. Since a low level of linoleic acid was deposited, and since approximately double the amount of linoleic acid was excreted as found in the carcass fats, it is probable that a certain level of linoleic acid is required by the rat in its

TABLE 3

The influence of dietary fat on the carcass and feces fatty acid composition of the rat

Fatty acid component	Dietary Supplements											
	Fresh corn oil		Fresh corn oil fatty acids		Tricricinolein		Ricinoleic acid		Hydrogenated castor oil acids		Commercial shortening	
	Carcass	Feces	Carcass	Feces	Carcass	Feces	Carcass	Feces	Carcass	Feces	Carcass	Feces
	%	%	%	%	%	%	%	%	%	%	%	%
Linolenic	0.2 ± 0.5[1]	—	0.8 ± 0.8	—	—	—	0.1 ± 0.2	—	—	—	0.4 ± 0.5	—
Linoleic	36.1 ± 2.3	17.9	32.9 ± 3.8	40.4	10.0 ± 1.1	17.7	8.6 ± 1.1	26.9	9.3 ± 1.1	17.2	9.9 ± 1.0	3.6
Oleic	31.9 ± 1.3	34.3	33.2 ± 1.2	38.4	45.3 ± 2.0	27.6	41.8 ± 1.4	25.7	42.3 ± 4.0	24.6	47.1 ± 2.1	27.3
Stearic	2.9 ± 0.8	17.8	2.4 ± 0.7	8.3	3.5 ± 0.6	16.2	3.9 ± 0.5	11.7	4.4 ± 0.8	25.1	4.0 ± 0.6	21.9
Hexadecenoic	5.1 ± 0.0	8.8	6.0 ± 0.6	—	11.7 ± 1.9	—	14.1 ± 1.5	3.2	10.4 ± 0.5	—	8.9 ± 1.4	3.6
Palmitic	21.4 ± 2.4	25.6	22.4 ± 1.0	11.6	26.6 ± 0.1	27.9	28.8 ± 2.4	24.1	30.2 ± 3.2	30.4	24.9 ± 3.6	39.3
Myristic	1.6 ± 0.7	10.2	2.5 ± 0.5	0.6	2.6 ± 0.1	5.1	2.6 ± 0.3	3.6	2.4 ± 0.1	1.6	3.1 ± 0.0	3.8
Tetradecenoic	0.1 ± 0.0	—	0.6 ± 0.2	—	0.6 ± 0.2	—	0.3 ± 0.2	—	0.1 ± 0.1	—	0.2 ± 0.1	—
Lauric	0.3 ± 0.5	2.8	0.6 ± 0.1	0.6	0.4 ± 0.2	5.7	0.4 ± 0.1	4.9	0.4 ± 0.1	1.0	0.7 ± 0.4	0.5
Hydroxy acids	1.5 ± 1.0	2.5[2]	—	2.5[2]	7.5 ± 1.0	—	5.5 ± 0.7	3.5[2]	6.1 ± 3.6	3.7[2]	2.2 ± 0.5	3.8[2]
Iodine values	99.7 ± 3.7	103.0	99.7 ± 1.4	103.7	71.5 ± 4.0	65.5	68.5 ± 2.9	85.7	66.4 ± 1.3	83.6	69.9 ± 1.4	62.2

[1] Standard error of the mean.

[2] Reported as percentage of hydroxyl for feces fats only.

carcass fat composition. This same observation has been made for stearic acid, except that the ratio of excreted to deposited fat is about 5 to one; levels of oleic acid were reversed and resulted in a ratio of one to two, or about one half as much oleic acid was deposited as was excreted. The level of palmitic acid deposited and excreted remained almost equal.

The very high oleic acid content of the carcass fat of animals fed the esterified and nonesterified hydroxy acid containing diets and the low linoleic acid content of the same fats could be explained by the formation of monoenoic acids from the ingested hydroxy acids and subsequent formation of dienoic acids from monoenoic acids. This suggestion is in agreement with the evidence presented by Lennartz and Bloch[5] who demonstrated that 9(10)-hydroxy stearic acid formed monoenoic acids when incubated with yeast cells under anaerobic conditions. If monoenoic acids can be formed from saturated hydroxy fatty acids acting as intermediates, it would seem reasonable that hydroxy octadecenoic acids (namely, ricinoleic acid) may act as intermediates in the synthesis of dienoic acids. The observation, however, that animals fed ricinoleic acid and triricinolein had very similar oleic and linoleic acid levels (42 and 9%, respectively) when compared with the animals fed 12-hydroxy-octadecanoic acid indicates that ricinoleic acid is probably not a useful intermediate for the synthesis of dienoic acids.

Deposition of hydroxy acids in the carcass fat has been shown by the present study to be on the order of 5 to 7% of the total carcass fatty acids. Our results are in agreement with the work of Stewart and Sinclair ('45) who showed that the rat deposited about 7% of hydroxy acids when fed a diet containing about 40% of hydroxy acids in the form of ricinoleic acid.

The results obtained in the present study indicate that the rat, when fed a metabolizable fat source, in this case hydroxy acids, will seek to form a body fat having a composition which is normal with respect to melting point and glyceride structure. The presence of considerable amounts of hexadecenoic acid in the carcass fats of animals fed hydroxy acids and its near absence in the feces fats remains to be explained. It appears that this acid is preferentially deposited in the carcass fats of animals fed hydroxy acid-containing diets. The presence of this acid in the fats of animals fed fresh corn oil and fresh corn oil fatty acids was substantially lower (60 to 75%), except when hydroxy acid was fed as a dietary component. Hexadecenoic acids may have been formed through resynthesis from acetate followed by dehydrogenation or by dehydration and rehydrogenation of an octadecanoic acid fragment. This could occur in the following manner. A two-carbon fragment could be removed from 12-hydroxyoctadecanoic acid, to give 10-hydroxyhexadecanoic acid. This acid could be specifically dehydrated to give the normally occurring 9-hexadeceneoic acid. A similar series of reactions could be applied to ricinoleic acid to give the same end product.

SUMMARY

Six groups of weanling rats were kept for 8 weeks in individual cages and fed adequate diets which contained 2% of cottonseed oil and 10% of the following fats: (1) corn oil; (2) the fatty acids of corn oil; (3) triricinolein; (4) ricinoleic acid; (5) the hydrogenated fatty acids of castor oil; and (6) commercial hydrogenated shortening. All animals were restricted to the same amount of daily food intake as those fed fresh corn oil and samples of feces collected for lipid analysis. At the end of the test period the animals were sacrificed, the livers removed, carcass and livers digested in dilute hydrochloric acid, lipids extracted, converted to methyl esters and subjected to gas-liquid chromatographic analysis. The results indicated that dietary hydroxy acids are deposited and influence the character of the normal mixed fatty acid composition of the carcass fat; and that both saturated and unsaturated hydroxy fatty acids are converted to monoeneoic acids in the rat. A larger amount of oleic acid and hexadecenoic acid seemed to be deposited and a preferential excretion of stearic and linoleic acids seemed to occur in animals fed a source of hydroxy

[5] Lennartz, W. J., and K. Bloch 1960 Hydroxystearic acids and the biosynthesis of unsaturated fatty acids. J. Biol. Chem., 235: PC 26.

fatty acids in comparison with those fed a source of linoleic acid. The significance of hydroxy fatty acid deposition on the characteristics of body fat composition were discussed and possible mechanisms to account for these results suggested.

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Effect of Various Oils and Fats on Serum Cholesterol in Experimental Hypercholesterolemic Rats

RAGNAR NICOLAYSEN AND ROLV RAGÅRD
Johan Throne Holst's Institute for Nutrition Research, University of Oslo, Blindern, Norway

In previous papers (Hauge and Nicolaysen, '59a,b) a method for the study of cholesterol metabolism in rats was presented, with some results indicating that cod liver oil had a distinctly stronger cholesterol-depressive effect than linoleic acid. Since then a number of experiments have been conducted in this laboratory, using this method. Various marine oils have been compared with vegetable oils. Egg yolk fat as well as whole egg yolk have been compared with vegetable oils, and we have tested a synthetic arachidonic acid. The results are presented below.

EXPERIMENTAL

Briefly, the original method was as follows. The pregnant mothers were transferred to the experimental diet (10% of hydrogenated coconut oil, 1% of cholesterol) about two weeks before the birth of litters was expected. Following 70 days of feeding such a diet, hypercholesterolemia and signs of essential fatty acid deficiency developed in the offspring. These 70-day-old rats were then used for the assay of the oils in a two-week test; the cholesterol-depressive effect was measured in each rat.

This procedure was used in a number of the experiments to be presented. In other experiments, however, it was found necessary to use long-term prophylactic tests; the actual procedure will be described in the appropriate sections.

In the pre-experimental treatment, we have now found it more desirable to delay feeding the mothers the diet free of polyunsaturated fatty acids until about the day of birth of new litters, which then develop normally in the following weeks. Previously, a number of the newborn died following the pre-treatment of the mothers for about two weeks, and such a postponement of the dietary treatment is therefore of definite advantage. In most instances the development of the essential fatty acid deficiency has been regular, and the rats have stopped growing at the age of about 70 days. In some instances rats continued to grow, and occasionally the fatty acid deficiency developed much earlier, resulting in rats unsuitable for a proper assay.

The oils containing high amounts of polyunsaturated fatty acids were administered daily by pipette. When fats with a low content of such acids have been tested, however, the fats were incorporated in the diet. The 10% of fat and the 1% of cholesterol in the diet were maintained by removing from the diet amounts of coconut fat and cholesterol equivalent to those provided by the supplements. Such a procedure was of special importance when the fat from egg yolk and the whole yolk were to be tested. In the last instance the protein content of the diet was also adjusted to the original level.

The arachidonic acid ethyl ester[1] was a synthetic product. The following specifications are given by the producers: "Rotation = N_D^{20} = 1.4778. Alkaline isomerization according to Herb and Riemenschneider (J. Am. Oil Chem. Soc., 1952, 29, 456) gave the value of 106.2%. The product is stabilized with 0.05% butylated hydroxy toluene. Purity = 96–99%."

The menhaden oil[2] was subjected to gas chromatographic analyses.[3]

The results of two types of analysis of this oil were as tabulated on the following page.

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[1] Kindly supplied by Hoffman-LaRoche and Company, Basel.

[2] Kindly supplied by Marine Chemiurgics.

[3] The authors are grateful to E. H. Ahrens, Jr., for the gas chromatographic analysis.

	Percentage of total fatty acids				
	Dienoic	Trienoic	Tetra-enoic	Penta-enoic	Hexa-enoic
Gas chromatography	3.6	3.2	5.5	16.7	4.1
Alkali isomerization (this laboratory)	5.9	4.6	5.8	14.5	8.9

CHEMICAL METHODS

The cholesterol method of Hauge and Nicolaysen ('58) was used. Because one batch of acetyl chloride delivered to us caused difficulties, we now accept only batches specifically controlled by the manufacturers. We have also subjected the method itself to considerable control. In the laboratory the Beckman Photoelectric C Colorimeter is subjected to repeated controls every day by the use of a stable salt solution, as recommended by Beckman. (Cuvettes should be filled and stoppered before work with the instrument to avoid corrosion by the HCl vapors of the mixture.) Two cholesterol standards were run with each series of analyses, and simultaneously a human serum sample was analyzed. Thus any systematic error is eliminated. On several occasions we controlled the values obtained with those obtained by other methods.

The Carr and Drekter ('56) method was used in this laboratory independently by two workers, and results identical to the $ZnCl_2$ method were achieved. Also we received series of human sera from two sources. The Finnish group under P. Rome, who collaborates with Ancel Keys and uses his method, sent two series of samples and Dr. B. Josephson of Stockholm sent three series. Dr. Josephson uses the method of Pearson et al. ('53) which, in the hands of Holmgård ('57), gave good agreement with the Bloor and the Sperry, Schoenheimer method. In all instances the agreement between the results were within a few per cent, and no systematic difference was observed.

The cod liver oil was separated into the fatty acid portion and the unsaponifiable portion following the procedure of the USP XV ('55). The alkali isomerization procedure of Herb and Riemenschneider ('53) was used and the calculations were made according to the method of Hammond and Lundberg ('53).

The test with the dehydrated yolk was performed with the fat extracted in a 6-hour run in a Soxhlet extractor. The fat content of the yolk was 52% with 5 mg of P per gm of extract. The extraction methods of Fillerup and Mead ('53) as modified by Hauge ('59) or that of Holman and Hayes ('58) resulted in slightly lower values for fat and P, respectively. This is of minor importance for our purposes, however, since the result with the whole yolk is the more important one.

The oils used were fresh, and were stored in deepfreeze with all precautions taken to avoid oxidation. It appeared that one batch of cod liver oil which had been stored for about a year showed a very slight reduction in its content of penta- and hexaenoic acids.

RESULTS

Two-week curative tests. In table 1 are shown the results of a number of tests. Frequently two or more marine oils were tested simultaneously with one or two of vegetable origin. In most cases about 200 rats were used in such a series. As before, the rats were divided according to serum cholesterol level on zero day, and by sex. The variability of serum cholesterol within litters was considerable, and interlitter variability was observed as well. We have found it impossible to use littermates only.

The following samples were run in the same tests:

Cod liver oil (b) and menhaden oil.
Cod liver oil (e), corn oil (a) and three marine body oils: herring, mackerel and sardine.
Corn oil (b) and fat extracted from dehydrated egg yolk.
Arachidonic acid and cod liver oil (c).

Cod liver oil (a) and soybean oil represent the pooled results of three separate tests (table 1). The results were subjected to

TABLE 1

The change in serum cholesterol following 2 weeks' daily administration of marine oils, soybean oil, corn oil, arachidonic acid and egg yolk[1]

Sample tested	Daily dose			Depression of serum cholesterol[2]	No. of rats	
	Oil	+[3]	++[4]		Female	Male
	mg	*mg*	*mg*	*% of zero day value*		
Cod liver oil (a)	20	1.2	4.7	23	4	6
	40	2.3	9.4	37	37	34
	80	4.6	18.8	59	24	27
Cod liver oil (b)	40	2.3	9.4	61 ± 5[5]	13	14
	80	4.6	18.8	60 ± 4	13	14
Cod liver oil (c)	40	3	8.4	43 ± 8	8	9
	80	6	16.8	48 ± 7	8	9
Cod liver oil (d)	40	3	8.4	44 ± 5	24	20
	80	6	16.8	58 ± 5	24	22
Cod liver oil (e)	40	2.3	9.4	57 ± 8	13	12
	80	4.6	18.8	68 ± 4	12	13
Menhaden oil	40	4.9	8.3	44 ± 5	13	14
	80	9.8	16.6	52 ± 6	13	14
Herring body oil	40	6.4	7.4	37 ± 8	12	13
	80	12.8	14.8	55 ± 5	12	13
Mackerel body oil	60	7.6	7.8	42 ± 8	13	12
	120	15.2	15.6	46 ± 7	12	13
Sardine body oil	40	4.1	9.1	36 ± 8	13	13
	80	8.2	18.2	56 ± 6	13	12
Soybean oil	20	12.2		16	5	6
	40	24.4		15	15	17
	80	48.8		36	25	27
Corn oil (a)	40	22.6		17 ± 12	12	12
	80	45.1		41 ± 5	12	13
Corn oil (b)	40	22.6		8 ± 16	13	13
	80	45.1		19 ± 11	12	14
Arachidonic acid	20	20		4 ± 15	8	9
	40	40		26 ± 9	8	9
	80	80		40 ± 9	6	9
	% in diet					
Egg yolk fat	2.35	23.6	2.1	1 ± 15	12	13
	4.7	47.2	4.2	37 ± 6	12	14
Whole egg yolk	4.5	23.0	2.1	27 ± 10	11	12
	9.0	46.0	4.2	45 ± 8	11	12

[1] Diet: 10% of hydrogenated coconut oil, 1% of cholesterol.
[2] The zero day average serum cholesterol values were the same as earlier, between 300 and 400 mg per 100 ml.
[3] + indicates sum of di-, tri-, and tetraenoic acids.
[4] ++ indicates sum of penta- and hexaenoic acids.
[5] S.E. of the mean.

statistical analysis (Bliss, '51).[4] The result was the following:

Relative potency of cod liver oil/soybean oil:

Males, 238% (90% confidence interval, 170 to 402%)
Females, 198% (90% confidence interval, 132 to 405%)

Despite the considerable number of animals used, the method as employed here allows only an approximate evaluation of the relative potencies. On the other hand, the results of all the comparisons of marine oils with vegetable oils support the con-

[4] Statistical analysis performed by Egede Larsen.

clusions that marine oils, with about 25 to 30% of polyenoic acids have a potency per unit weight which is double that of vegetable oils with about 60% of linoleic acid. This would imply that the polyenoic acids of marine oils are 4 times as potent in depressing serum cholesterol as the linoleic acid in vegetable oils.

The two tests with egg yolk fat and with the whole egg yolk gave results in proportion to the content of linoleic acid.

The arachidonic acid ethyl ester depressed serum cholesterol, and since this is a synthetic product, the result is important from the point of view that no doubt can remain about the cholesterol-depressive effect of the polyenoic fatty acids. On the other hand, the percentage of depression is lower than expected, but the method does not allow for more than 2:1 comparison. The acid may, in reality, have a potency that is double that which the present results indicate. The possibility exists, however, that pure fatty acids singly may have less effect than a combination of several.

Four-week curative tests. In some of the series, blood was sampled after two and 4 weeks. The results of these tests are shown in table 2. It appears that supplying the egg yolk fat for 4 weeks, as well as corn oil (b), was advantageous from the point of view that the depressive effects became clearer and more in line with the results achieved in general.

The results with the two fractions of the cod liver oil indicate that the cholesterol depressive effect is not in the unsaponifiable fraction but in the fraction containing the fatty acids. As was the case with arachidonic acid, the fatty acids of the cod liver oil were less effective than expected. The growth rate was just as good with arachidonic and cod liver oil fatty acids as with cod liver oil.

Long-term prophylactic tests. In a preliminary experiment in which the butter and margarine were tested over a two-week period, no consistent correlation between serum cholesterol concentration and the content of the polyenoic acid consumed was observed. It was decided, therefore, to

TABLE 2

The change in serum cholesterol following 28 days' administration of various fats and oils[1]

Sample tested	Daily dose			Depression of serum cholesterol	No. of rats	
	Oil	+[2]	++[3]		Female	Male
	mg	*mg*	*mg*	*% of zero day value*		
Cod liver oil (d)	40[4]	2.3	9.4	53 ± 5[5]	24	20
	80[4]	4.6	18.8	62 ± 4	24	22
Cod liver oil fatty acid (d)	40[6]	2.2	7.0	22 ± 11	24	19
	80[6]	4.4	14.0	43 ± 6	24	20
Cod liver oil (d) unsaponifiable fraction	40[7]			5 ± 11	23	20
	80[7]			0 ± 9	24	18
Corn oil (b)	40[4]	22.6		19 ± 16	13	12
	80[4]	45.1		45 ± 10	13	12
Herring body oil	40	3.1	4.8	18 ± 15	12	13
	80	6.2	9.5	34 ± 12	13	13
	% in diet					
Egg yolk fat	2.4[4]	22.8	2.1	13 ± 12	12	13
	4.8[4]	45.6	4.2	40 ± 7	12	13

[1] Diet: 10% of hydrogenated coconut oil, 1% of cholesterol.
[2] + indicates sum of di-, tri-, and tetraenoic acids.
[3] + + indicates sum of penta- and hexaenoic acids.
[4] These samples were the same as in table 1 and serum cholesterol was thus analyzed on the 14th and the 28th day.
[5] S.E. of the mean.
[6] The sample given by pipette contained 90% of fatty acids and 10% of coconut oil.
[7] The sample given by pipette consisted of 2.5 gm of unsaponifiable fraction and 217.5 gm of coconut oil, i.e., the equivalents of 40 and 80 mg of cod liver oil were given.

use a different type of test, and this resulted in the data shown below.

The fats were supplied in the diet at 5 and 10% levels. The total fat content of the diet was, without exception, 10%. At the 5% level, the diets thus contained 5% of the hydrogenated coconut fat and 5% of the sample.

The three margarines used correspond to Norwegian margarines, number 1 being a summer margarine; number 2, a winter margarine; and number 3 what is called "vegetable" margarine. The composition of these samples was as follows: margarines 1 and 2 contained about 70% of hydrogenated marine fats, (mixture of 30/32° and 38/40° m.p.), and 20 to 25% of coconut oil, the rest being a mixture of vegetable oils. Margarine number 3 contained 50% of coconut oil, 25% of hydrogenated vegetable oil, and 25% of a mixture of vegetable oils. The calculated daily intake of polyenoic fatty acids was made on the basis of a series of accurate food intake measurements of other female rats fed the same diets and of the same age and body weight. They consumed 11 to 14 gm of food daily. The males consumed somewhat more food. The relevant point, however, is the correlation between the serum cholesterol level and the intake of polyenoic fatty acids.

The results are given in table 3. It appears that even the average value for 20 rats indicates considerable and irregular variation, although it is clear that the serum cholesterol throughout is lower in the high polyenoic acid groups. The results have been pooled and the average of the 4 averages for each group is given in the right hand column.

The 10 values resulting are next reproduced in figure 1. This type of treatment of the results gives a highly significant correlation between the serum cholesterol average for the 11-week period of observation and the amount of polyenoic acids in the diet. In this instance the type of polyenoic acid was linoleic acid only.

The results of another type of long-term experiment are shown in table 4. It appears also in this series that the cod liver oil is more potent than soybean oil. On the other hand, it is characteristic in this series for the serum cholesterol to be reduced with time, and for the female rats receiving the soybean oil to show a final value of serum cholesterol at about the same level as the rats fed cod liver oil. It is tempting to suggest an adaptation in the

TABLE 3

Serum cholesterol values in long-term experiments with various fats[1,2]

Sample	Level in diet	Polyenoic acids[3]	Serum cholesterol				
			No. of weeks fed diet				Av. of 4 values
			3	5	7	11	
	%	*mg/day*	*mg/100 ml*				*mg/100 ml*
Butter	5	9.0	319	297	352	324	323
	10	18.0	237	244	257	258	249
Margarine 1	5	12.5	288	340	307	228	291
	10	25.0	257	266	287	204	253
Margarine 2	5	21.0	336	349	327	296	327
	10	42.0	246	256	271	228	251
Hydrogenated coconut fat + soybean oil[4]		24.4	195	264	238	240	235
		48.4	186	213	153	158	178
Margarine 3	5	45.5	168	191	214	206	195
	10	90.0	142	152	205	160	165

[1] Diet: 10% of fat, 1% of cholesterol.

[2] The number of rats in each group was 10 males and 10 females at the start; however, in some groups one or two died before the end of the experiment.

[3] For estimated daily intake of polyenoic acids, see text.

[4] Following food intake measurement, soybean oil was mixed in the fat to give the estimated values for polyenoic fatty acids (see text).

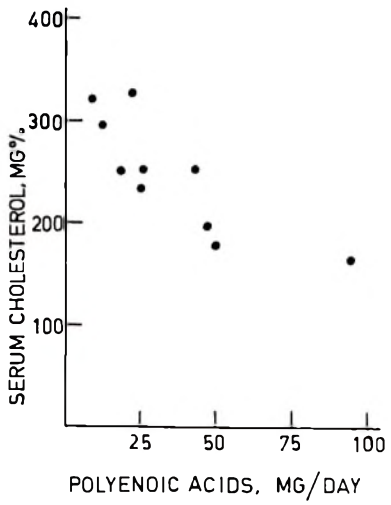

Fig. 1 Average serum cholesterol levels in rats given 10% of fat of various types in the diet over 11 weeks.

course of time. In view of the irregular curves shown for the animals in table 3, however, such a contention is not fully justified. The two experiments seen together are also valuable from the point of view that they indicate the difficulties encountered in work with serum cholesterol. In our preliminary work (Hauge and Nicolaysen, '59a,b) the depression of liver cholesterol was also studied; however, it was observed that liver cholesterol depression showed no more consistency than serum cholesterol.

DISCUSSION

The fact that marine oils have a stronger cholesterol-depressive effect than vegetable oils in the type of experiments used is well established by the experimental results. The various types of experiments, specifically those with arachidonic acid and the fatty acids from cod liver oil, indicate that the factor active in the depression of serum cholesterol is the fatty acid part of the oils. Under the present circumstances the activity of the marine fatty acids (nearly all penta- and hexaenoic acids) is about 4 times that of linoleic acid when given by mouth in small doses, or 5 to 10% of the total fat intake. The statistical work presented above indicates that the method is not well suited for a two- or three-point factorial assay. From this point of view it is discouraging that an assay with the aid of more than 100 animals will not allow a differentiation of more than a 2:1 potency.

The reason for this seems to be that the level of cholesterol in the serum (and also in the liver of our rats, see Hauge and Nicolaysen, '59a,b) is quite variable. This fact, by now also well established in man, may explain some apparently contradictory results reported by others, and this may be offered as a tentative explanation, although not explicitly stated in the part of the discussion to follow.

Recently other workers have presented results on the effect of marine oils in rats. Dam et al. ('59) observed that cod liver oil was a stronger anticholesterol agent than linseed oil when given in the diet of rats at a 10% level. De Groot and Reed ('59) found that cod liver oil had a stronger anticholesterolemic effect than corn oil, both

TABLE 4
Serum cholesterol values in long-term prophylactic test[1,2]

	No. of rats	Serum cholesterol		
		52 days	76 days	90 days
		mg/100 ml	*mg/100 ml*	*mg/100 ml*
Cod liver oil, 29.3% polyenoic fatty acids	5 male	109 ± 11[3]	131 ± 11	111 ± 5
	15 female	117 ± 4	114 ± 5	94 ± 5
Soybean oil, 61.1% polyenoic fatty acids	8 male	231 ± 25	129 ± 9	110 ± 5
	15 female	166 ± 17	136 ± 10	122 ± 6

[1] Diet: 10% of hydrogenated coconut oil, 1% of cholesterol, plus 2% of the coconut oil on day the litter was born.

[2] According to food intake measurements in these female rats when 90 days old, the daily intake of polyenoic fatty acids was about 75 mg for the females fed cod liver oil and about 150 mg for those fed soybean oil.

[3] S.E. of the mean.

oils given at a 10% level in the diet. When next they fed the corresponding amount of the unsaponifiable fraction of cod liver oil to the rats, no anticholesterolemic effect was obtained; the effect remained with the fatty acids. In our work, amounts corresponding to only 40 and 80 mg of cod liver oil were given. These two series seen together lead to the same conclusion with regard to the active factor which depresses serum cholesterol in rats. The conclusion of Wood and Bieley ('60) following work with chickens, is to the contrary. In experiments with 4 animals in each group they obtained results indicating that in Ling cod liver oil, unsaponifiable fraction, an anticholesterolemic factor is present, the fatty acids having no effect. In another publication, Wood and Bieley ('60b), work with 20 chickens in each group is presented. The period of experiment was 13 days with a diet containing 1% of cholesterol. Ten per cent of corn oil in such a diet resulted in the value for serum cholesterol of 729 ± 219 mg per 100 ml, whereas the value following 10% of tallow in the diet was lower. Marine oils resulted in lower values; however, the range of variability was disturbing, as is apparent from the standard error of the mean.

Recent work in man with arachidonic acid, egg yolk and polyenoic acid-enriched egg yolk[5] has also resulted in some discrepancies, which warrant a brief report of the pertinent observations. Worne et al. ('58) gave 4 gm of arachidonic acid daily for 90 days to 6 men and the serum cholesterol was reduced by about 30%. Keys et al. ('59) gave 4 gm of arachidonic acid to 6 men daily for 13 days, but, in their experiment, serum cholesterol increased initially, and was reduced only slowly in the post-experimental period. The early work of Messinger et al. ('50) with 10 yolks daily given to men, resulted in a remarkable increase in serum cholesterol, and others have also found that yolks will increase serum cholesterol in man. However, simultaneously Gordon et al. ('58) and Horlick and O'Neil ('58) published work in man with ordinary and enriched egg yolks, respectively. Gordon et al. ('58) observed no effect of the enriched yolk, but the result of Horlick and O'Neil ('58) in their two subjects was as follows:

	Serum cholesterol	
	Ordinary egg yolk	Enriched egg yolk
	mg per 100 ml	
Subject 1	200	100
2	175	125

In a recent study by Horlick and O'Neil ('60), the experiment was repeated, although with some modifications. However, the enriched yolk did not bring about any serum cholesterol depression. The authors suggest that other factors in the yolk may have strong hypercholesterolemic effects.

The results obtained with egg yolk and the fat extracted from it in our work in rats indicate clearly that under such circumstances no unknown factor is needed to explain what happened.

The results of Messinger et al. ('50) were remarkable in that 3.75 gm of cholesterol in the yolk given to the subjects resulted in much greater serum cholesterol elevation than the 10 gm of crystalline cholesterol emulsified in cream. It may be that the cholesterol in the yolk is a form more readily absorbed than otherwise occurs. For many years it has been accepted that variations in the daily intake of cholesterol, as they occur in the diets consumed in the Western part of the world, have little influence on the serum cholesterol level. It is not unexpected, but valuable, that Beveridge et al. ('60), in experiments on man, observed that some cholesterol in the diet gave higher blood cholesterol levels than none. On the other hand, it appears from the results that 1600 mg of cholesterol per 950 Cal. gave definitely lower serum cholesterol values than 800 mg per 950 Cal. It is not out of place to recall that unpredictable variability in serum cholesterol levels calls for care in the interpretation of results.

It is becoming increasingly clear that serum cholesterol is not subjected to any good homeostatic control. Levere et al. ('58) have given numerous examples of the difficulties encountered in the evaluation of cholesterol-depressive agents in man. In our laboratory we followed 20

[5] Egg yolks were "enriched" by feeding groups of hens a diet rich in linoleic acid-containing oils.

men (engaged in moderate bodily activity in a factory) over a year with fortnightly serum cholesterol analyses. Our results fully support the observations of Levere et al. In fact, one of our men had a minimal value of 250 mg per 100 ml of serum cholesterol and a maximal value of 500.

Alfin-Slater and Jordan ('60) observed in patients given safflower oil that only 50% of them reacted with reductions in serum cholesterol.

The authors question whether it is warranted to generalize and to apply without doubt results achieved in one species to another. In other words, can we apply some biochemical results obtained from rats to man? It has been reported that the chain length of the fatty acids is of importance. Malmros and Wigand ('59) report that lauric acid (the C_{12} acid of coconut oil) had an extraordinary hypercholesterolemic effect in rabbits. Hashim et al. ('60), following experiments in man, reported that the fraction of coconut oil below the C_{12} acids had very little hypercholesterolemic effect when compared with corn oil. In our experiments with butter, margarines and coconut fat, fully hydrogenated, the polyenoic fatty acid content dominated any such effects.

Marine oils have been used in a number of experiments on man (Bronte-Stewart et al., '56; Keys et al., '57; Malmros and Wigand, '57; Ahrens et al., '59). Worne and Smith ('58) worked with concentrates of marine penta- and hexaenoic acids in man. In all instances the marine oils and the concentrates had very strong anticholesterolemic effects, at least as strong as vegetable oils. Although lively discussions have gone on for years, it appears safer to say that the data so far obtained in man do not allow any statistically valid comparison. It will remain a matter of opinion whether results in rats presented here can be applied in human biochemistry and dietetics. However, so far they appear to warrant the conclusion that marine oils per unit of weight of polyenoic acid content are about 4 times as potent as vegetable oils in depressing serum cholesterol in rats.

SUMMARY

A two-week curative assay in rats made hypercholesterolemic by a diet (Hauge and Nicolaysen, '59) containing 10% of hydrogenated coconut fat and 1% of cholesterol was used in a number of comparisons of the anticholesterolemic effect of various sources of polyunsaturated fatty acids.

A synthetic arachidonic acid and the fatty acids from cod liver oil gave a distinct depressive effect on serum cholesterol. The unsaponifiable fraction of cod liver oil, in amounts corresponding to what gives nearly maximal serum cholesterol depression following administration of the whole cod liver oil, gave no depression.

It is therefore concluded that the polyenoic fatty acids are largely responsible for the anticholesterolemic effect observed. The fatty acids did not give the full effect of the cod liver oil, however, it is possible, therefore that the medium in which the fatty acids are administered will also prove to be of influence.

The cholesterol-depressive effect of marine oils such as cod liver oil and various fish body oils corresponded fairly well to their content of polyunsaturated fatty acids, chiefly of the penta- and hexaenoic type. Following a number of comparisons of marine oils and vegetable oils such as soybean oil and corn oil, it was calculated on the basis of the content of polyenoic fatty acids that the marine fatty acids had about 4 times the cholesterol-depressive effect of linoleic acid when given in their natural media.

The fat of egg yolk, and the whole egg yolk were used in two experiments in which the type of fat was the one variable. The cholesterol-depressive effect corresponded fairly well to the content of polyenoic fatty acids, chiefly linoleic acid. This result does not support the view that egg yolk contains an unknown factor which will increase serum cholesterol.

In a long-term prophylactic test with butter, three types of margarine of varied fat-base and with soybean oil in hydrogenated coconut fat, it was found that the serum cholesterol correlated well with the content of polyenoic fatty acids in the fats, irrespective of the variation in the type of fatty acids in the fat.

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Dietary Magnesium, Calcium, and Vitamin B_6 and Experimental Nephropathies in Rats: Calcium Oxalate Calculi, Apatite Nephrocalcinosis[1]

S. N. GERSHOFF AND S. B. ANDRUS
Department of Nutrition, Harvard School of Public Health and the Department of Pathology, Harvard Medical School, Boston, Massachusetts

Studies from this laboratory, of human beings, cats and rats have shown that endogenous oxalate excretion is inversely related to the amount of vitamin B_6 in the diet (Gershoff et al., '59, '59a; Gershoff and Faragalla, '59). It was shown in the cat and man that amounts of vitamin B_6 apparently adequate to sustain normal health and growth do not necessarily insure minimal endogenous oxalate excretion. We (Andrus et al., '60) have also observed the development of renal calcium oxalate calculi in vitamin B_6-deficient rats, accompanied by obstructive sequelae similar to those seen in humans.

Cramer's ('32) original description of nephrocalcinosis produced by magnesium deficiency has been confirmed though not extensively documented. In the absence of analytic data, it is assumed that such calcium deposits consist of apatite. The only report of calcium oxalate calculi induced by experimental means not involving the feeding of oxalates, other than the above, was made by Hammarsten ('37). She observed calcium oxalate calculi in rats fed natural diets which she considered magnesium-deficient, since the addition of magnesium protected against stone formation. Hammarsten's stone-producing diets contained 44 mg of magnesium per 100 gm of diet. In this and many other laboratories, purified diets containing approximately 40 mg of magnesium per 100 gm have been used for years without producing calcium oxalate calculi. It is possible (Andrus et al., '60) that Hammarsten's diets were suboptimal with respect to vitamin B_6. Gershoff and Prien ('60) have observed significantly less urinary citrate in patients with histories of recurrent oxalate calculi than in normal individuals. In the present study the effect of dietary vitamin B_6 and magnesium on the urinary excretion of citric acid and other metabolites in rats has been studied as well as the deposition of oxalate in the urinary tract. Since oxalate is usually deposited as its calcium salts, the effect of varying dietary calcium in stone-producing diets has also been studied. Intrarenal deposition of apatite was also evaluated.

EXPERIMENTAL AND RESULTS

Magnesium studies

In these studies weanling male Charles River CD rats were used. The diet fed in the first experiment consisted of the following (expressed as percentages): casein, 15; sucrose, 72.7; corn oil, 4; cod liver oil, 1; glycine, 3; salts IV (Hegsted et al., '41), 4; and choline, 0.3. Each 100 gm of diet was supplemented with the following (in milligrams): thiamine, 0.4; riboflavin, 0.8; niacin, 4; Ca pantothenate, 2; folic acid, 0.1; menadione, 0.1; biotin, 0.02; and vitamin B_{12}, 0.005. When used, 0.4 mg of pyridoxine was added per 100 mg of diet.

Two groups of 6 rats were pair-fed this experimental diet with and without vitamin B_6. After 15 days a 24-hour urine collection was obtained from each rat and analyzed for citric acid (McArdle, '55). On the evening of the 21st day, food was taken away from the rats. On the morning of the 22nd day, the animals were pair-fed

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TABLE 1

The effect of dietary vitamin B_6 on urinary citric acid in rats[1]

Diet	Days on experiment	Average weight	Citric acid
		gm	*mg/24 hours*
−Vitamin B_6	15	67	2.1 ± 0.3[2]
+Vitamin B_6	15	85	19.2 ± 1.9
−Vitamin B_6	21	70	2.5 ± 0.8[3]
+Vitamin B_6	21	90	20.5 ± 1.8

[1] Diet contained 550 mg of calcium and 40 mg of magnesium per 100 gm.
[2] Standard error of the mean.
[3] Collections made on 22nd day while rats were fed nitrogen-free diet.

nitrogen-free diets with and without vitamin B_6 and another 24-hour urine collection was obtained for citric acid analysis. The results of this study presented in table 1 show that vitamin B_6-deficient rats excreted markedly less citric acid than their pair-fed controls and that the urinary citric acid in these animals is not affected by short-term removal of nitrogen from their diets.

The diets used in the second experiment were essentially the same as those above, except that 72.75% of sucrose and 3.75% of Jones and Foster salt mixture ('42) minus its $MgSO_4$ component were used. To this basic diet were added pyridoxine at zero, 0.1, and 0.4 mg per 100 gm and magnesium at 20, 40, and 400 mg per 100 gm, in the form of MgO. Nine groups of 6 rats each were fed these diets so as to represent all combinations of concentrations of magnesium and pyridoxine. The diets were fed ad libitum for 6 weeks.

On the 16th day, the rats fed zero and 0.4 mg of vitamin B_6 per 100 gm of diet were placed in metabolism cages in groups of three, and two successive 24-hour urine collections were made. Thus, for each of these 6 dietary groups, 4 samples were available for analyses of citric acid, oxalic acid (Powers and Levatin, '44) and xanthurenic acid (Glazer et al., '51). The weight changes observed during the experimental period are shown in table 2. Among the groups lacking vitamin B_6, the best growth was obtained with the highest concentration of magnesium, whereas in rats receiving 0.4 mg of vitamin B_6 per 100 gm of diet, growth was retarded at this high level of magnesium.

TABLE 2

The effect of dietary vitamin B_6 and magnesium on urinary metabolites and growth in rats[1,2,3]

No. of rats	4	5	6	6	6	6	6	5	6
Vitamin B_6, mg/100 gm diet	0	0	0	0.1	0.1	0.1	0.4	0.4	0.4
Mg, mg/100 gm diet	20	40	400	20	40	400	20	40	400
Citric acid excretion	4.2 ± 1.1[4]	6.4 ± 0.8	29.6 ± 4.8				12.5 ± 1.4	16.4 ± 1.9	18.8 ± 3.0
Oxalic acid excretion	0.68 ± 0.10	0.71 ± 0.17	0.95 ± 0.08				0.39 ± 0.01	0.51 ± 0.01	0.44 ± 0.04
Xanthurenic acid excretion	1.59 ± 0.36	0.92 ± 0.30	0.76 ± 0.21				0.33 ± 0.06	0.42 ± 0.08	0.23 ± 0.02
Weight gain, gm/6 weeks	11 ± 2	9 ± 6	24 ± 3	91 ± 9	80 ± 6	82 ± 7	175 ± 14	183 ± 4	133 ± 11

[1] Dietary calcium, 600 mg/100 gm.
[2] All excretions expressed as mg/100 gm of body weight/24 hours.
[3] All values include the standard error of the means.
[4] Urine collections were made on the 16th day.

The low urinary citrate excretion of vitamin B_6-deficient rats can be markedly raised by increasing the magnesium content of the diet (table 2). Oxalate excretion was significantly higher in animals deficient in vitamin B_6 than in those receiving the vitamin. Magnesium supplementation was not effective in lowering the oxalate excretion of vitamin B_6-deficient rats and, in fact, the highest levels of oxalate were observed in vitamin B_6-deficient rats receiving 400 mg of magnesium per 100 gm of diet. Differences in oxalate excretion were not, however, significant by statistical test. As expected, vitamin B_6-deficient rats excreted considerably more xanthurenic acid than rats receiving the vitamin. This was particularly true for the rats receiving the low-magnesium diets. The differences in xanthurenic acid excretion among the vitamin B_6-deficient groups in this experiment are not statistically significant. In repeating part of this work with two other groups of 6 rats, similar differences which were statistically significant ($P < 0.02$) have been observed. Vitamin B_6-deficient rats fed diets containing 20 and 400 mg of magnesium per 100 gm of diet excreted 1.63 ± 0.12 and 0.94 ± 0.17 mg of xanthurenic acid per 100 gm of body weight per day, respectively.

At the end of the 6-week experimental period, all surviving rats of experiment 2 were decapitated and prepared for morphologic study. For the purpose of these studies, additional groups of 10 to 11 animals each had been fed the diets containing zero and 0.4 mg of vitamin B_6 per 100 gm and the three levels of magnesium for 6 weeks. The urinary tract was exposed and examined at all levels under a dissecting microscope. The kidneys were sectioned transversely and somewhat obliquely so as to include the renal papillae intact. The distribution and extent of all crystalline deposits were recorded. Papillary apical oxalate incrustations were measured directly under the dissecting microscope. As these deposits at this stage are generally thin surface plaques, only the areas covered by the incrustations were measured with no attempt to determine their thickness. Deposits of apatite when present were seen as fine stippling of the cortex, most marked at the corticomedullary junction. These can generally be distinguished from parenchymal deposits of calcium oxalate by their slightly yellowish cast as contrasted with the dead white appearance of the latter. More certain differentiation between the two types of parenchymal calcium deposits was achieved by alizarin red S staining of either fresh or formalin-fixed kidney slices and examination of the wet preparation under the dissecting microscope. By this technique only the apatite is stained. When present, parenchymal deposits of apatite or calcium oxalate were graded under magnification on a one to 4 + basis. Samples of any critical crystalline deposits were generally picked from the fresh specimens and examined under polarized light. Representative specimens of both apatite and calcium oxalate were also studied with x-ray diffraction techniques. Kidney blocks were fixed in 10% neutral formalin for histologic examination (paraffin embedding and Alcian blue-hematoxylin-eosin). All paraenchymal crystalline deposits were again graded visually on a microscopic basis.

The urinary tract pathology is presented in table 3. Calcium oxalate was seen only in those groups receiving no vitamin B_6. Within those groups there was an inverse relationship between the level of dietary magnesium and the extent of calcium oxalate deposition. This was particularly prominent when comparing 40 mg of magnesium (the usual level) and 400 mg per 100 gm of diet. This relationship was true not only of papillary apical incrustations and all sequelae, but also of intratubular oxalate deposition proximal to the apex of the renal papillae. At the lowest concentration of dietary magnesium there frequently resulted large amounts of oxalate high in the nephron, a phenomenon rarely seen except in trace amounts at the 40 mg per 100 gm level in this and other experiments. Except for this more proximal extension of oxalate deposits seen in the vitamin B_6-deficient animals supplied with the lowest magnesium intake, the histologic appearance of the kidneys was generally similar to the published description of renal lesions in vitamin B_6 deficiency in rats (Andrus et al., '60). Inflammation was observed somewhat more frequently

TABLE 3

Incidence of calcium oxalate and apatite lesions in relation to dietary levels of vitamin B_6 and magnesium in rats[1]

No. of rats	15	15	16	6	6	6	16	15	17
Vitamin B_6, mg/100 gm diet	0	0	0	0.1	0.1	0.1	0.4	0.4	0.4
Mg, mg/100 gm diet	20	40	400	20	40	400	20	40	400
Calcium oxalate lesions									
Papillary apical incrustations	12	11	1	0	0	0	0	0	0
Mean area of incrustations[2]	46	35	2	0	0	0	0	0	0
Parenchymal deposition	10	11	2	0	0	0	0	0	0
Extent of parenchymal deposits[3]	1 to 2+	trace	trace	0	0	0	0	0	0
Ureteral concretions	5	6	0	0	0	0	0	0	0
Hydroureter	7	9	5	0	0	0	0	0	0
Bladder concretions	9	6	0	0	0	0	0	0	0
Gross hematuria	2	3	0	0	0	0	0	0	0
Apatite lesions									
Parenchymal deposition	1	0	0	0	1	0	13	4	0
Extent of parenchymal deposits[3]	trace	0	0	0	trace	0	1 to 2+	trace to 1+	0

[1] Dietary calcium, 600 mg/100 gm.
[2] One unit of area = 0.04 mm².
[3] Microscopic evaluation.

in the present study. This feature showed no correlation with magnesium intake. Frequently, increased numbers of neutrophilic polymorphonuclear leukocytes were seen in the dilated capillaries of the papillary apex adjacent to oxalate deposits. No specific changes were seen in those groups receiving the highest level of magnesium, other than those above. X-ray diffraction photographs prepared from papillary apical incrustations revealed strong lines indicative of calcium oxalate monohydrate.

In contrast with oxalate, apatite was seen almost exclusively in those groups receiving 0.4 mg of vitamin B_6 per 100 gm of diet, only two animals with trace amounts being seen in other groups. Within the three groups of animals receiving 0.4 mg of vitamin B_6 per 100 gm of diet, there was again a prominent inverse relationship between the dietary level of magnesium and the extent of crystalline deposits. Histologically the apatite deposits were most pronounced at the corticomedullary junction and resembled descriptions of the renal lesions seen in magnesium deficiency in rats (Hellerstein et al., '57). X-ray diffraction studies carried out on this crystalline material were strongly suggestive of apatite, though due to the small quantities the photographic lines lacked definition, a frequent difficulty with analysis of apatite.

Calcium studies

The basic diets used in experiments 3 and 4 were essentially the same as those used in experiment 2. Two-and-one-quarter per cent of Jones and Foster salt mixture minus its calcium and magnesium salts were used. The different concentrations of calcium and magnesium used were provided by $CaCO_3$ and MgO supplements. None of the diets was supplemented with vitamin B_6. Owing to difficulties in procurement of the CD strain of Charles River rats, a brief comparative study of this strain and the Charles River SD rats was made in experiment 3. Two groups of the CD strain and two groups of the SD strain, each group numbering 6 rats, were fed the above diet containing 40 mg of magnesium and either 600 or 1200 mg of calcium per 100 gm of diet. At the end of the experimental period, in this case 31 days, the urinary tracts of the rats were studied as described above.

The results obtained are summarized in table 4. Although the numbers and sizes of primary papillary apical oxalate incrus-

TABLE 4

Comparative incidence of calcium oxalate lesions in two strains of vitamin B_6-deficient rats in relation to dietary levels of calcium[1]

No. of rats	6	6	6	5
Ca, mg/100 gm diet	600	600	1200	1200
Strain	SD	CD	SD	CD
Mean weight gain, gm/31 days	23±2	9±2	15±2	11±3
Calcium oxalate lesions				
Papillary apical incrustations	2	5	4	5
Mean area of incrustations[2]	24	49	21	35
Parenchymal deposition	0	6	6	5
Extent of parenchymal deposits[3]	0	trace	1 to 2+	2 to 3+
Ureteral concretions	1	2	4	4
Hydroureter	2	5	6	5
Bladder concretions	1	2	5	5
Gross hematuria	0	2	1	0

[1] Dietary magnesium, 40 mg/100 gm. No apatite deposition was seen in this experiment.
[2] One unit of area = 0.04 mm^2.
[3] Microscopic evaluation.

tations are not significantly different, the incidence of stones in the ureters and bladder was higher in the groups receiving the higher concentration of calcium. This difference was supported by the distinctly greater size of such stones in these groups as well as more marked degrees of hydroureter. As concluded previously (Andrus et al., '60), it is assumed that concretions at lower levels of the urinary tract of such animals originate through fragmentation of renal papillary apical incrustations. Perhaps the most striking difference was the marked increase in intratubular calcium oxalate deposits as seen microscopically at the higher calcium intake. This was seen both as a more proximal extension of papillary incrustations into the terminal collecting ducts as well as scattered foci at much higher levels of the nephron. The differences in the extent of oxalate deposition in the two strains of rats would appear to be quite distinct, the SD strain of Charles River rats being relatively more resistant to the development of the renal lesions as well as maintaining greater growth with vitamin B_6-deficient diets as compared with the CD strain. Microscopically, the renal lesions resembled those seen in experiment 2. No apatite was seen in any of the groups.

In view of the enhancing effects of low dietary magnesium on calcium oxalate deposition in vitamin B_6 deficiency, a 4th experiment was carried out in which dietary calcium levels were varied in the face of vitamin B_6 deficiency and a very low magnesium intake. Three groups of 10 SD rats each were placed on such a regimen with the following calcium levels: 200, 480, and 960 mg per 100 gm of diet. In all three groups magnesium was supplied at the level of 5 mg per 100 gm. The animals were sacrificed in this instance at 30 days. The urinary tract was treated as above. The data obtained are presented in table 5. At this very low magnesium intake, all animals showed papillary apical incrustations despite the fact that this experiment was of only 30 days' duration. The incidence and amount of oxalate deposition both at the papillary apex and within the parenchyma of the kidney were particularly impressive when the figures are compared with those of the preceding experiment, despite the higher calcium levels in the latter. With increasing calcium intakes there was a marked increase in the amount of parenchymal calcium oxalate as in the preceding experiment and, in addition, there was a significant increase in the size of the papillary apical incrustations.

Sequelae in the lower urinary tract showed no apparent differences. Intratubular deposition of apatite was also markedly increased by the very low magnesium intake of this as contrasted with the preceding experiment and appeared very directly related to the dietary level of calcium. Histologically, the lesions of the renal papillae in this experiment showed certain unusual features not seen previ-

TABLE 5

Incidence of calcium oxalate and apatite lesions in relation to dietary levels of calcium in rats fed diets deficient in vitamin B_6 and low in magnesium (5 mg/100 gm)

No. of rats	10	9	10
Ca, mg/100 gm diet	200	480	960
Mean weight gain, gm/30 days	10±2	2±2	−2±2
Calcium oxalate lesions			
Papillary apical incrustations	10	9	10
Mean area of incrustations[1]	62	149	172
Parenchymal deposition	0	9	10
Extent of parenchymal deposits[2]	0	trace to 1+	2 to 3+
Ureteral concretions	8	8	6
Hydroureter	6	8	5
Bladder concretions	6	7	4
Gross hematuria	5	2	7
Apatite lesions			
Parenchymal deposition	3	8	10
Extent of parenchymal deposits[2]	trace	1 to 2	2 to 3

[1] One unit of area = 0.04 mm².
[2] Microscopic evaluation.

ously. These changes, which are attributed to the very low dietary level of magnesium, included a clearly interstitial location of many of the papillary oxalate deposits as well as prominent inflammation and necrosis. These lesions will be documented elsewhere. It should be noted that the extent of these latter changes was directly related to the dietary calcium level.

DISCUSSION

Urinary calculi are a major human health problem throughout the world. In the United States and England the inorganic part of a majority of renal stones is composed either of pure calcium oxalate or calcium oxalate mixed with apatite (Prien, '49; Hodgkinson, '58). There does not appear to be a significant difference, however, in the urinary oxalate or calcium excretion of people with and without histories of renal oxalate lithiasis (Hodgkinson, '58; Gershoff and Prien, '60; Dempsey et al., '60).

This suggests that differences in the solvent properties of the urine of patients with renal oxalate calculi may be of importance in the etiology of their disease. In the present study, the feeding of high levels of magnesium provided marked protection against oxalate deposition in vitamon B_6-deficient rats fed diets supplemented with glycine, a precursor of endogenous oxalate. This protection was not accompanied by a detectable decrease in oxalate excretion. Considerably more oxalate was excreted by vitamin B_6-deficient rats than by their controls at all levels of magnesium intake. It appears likely that increasing the dietary magnesium may increase the solvent characteristics for oxalate of the urine of vitamin B_6-deficient rats.

Miller et al. ('58) have shown that calcium oxalate is more soluble in urine than water, and that citric acid is particularly effective in increasing the solubility of oxalate. In the present work, a marked decrease in urinary citrate, not related to caloric or nitrogen intake, was observed in vitamin B_6-deficient rats. This decrease in citrate excretion was completely prevented by feeding high-magnesium diets. Andrus et al. ('60) have shown that acidification of urine, a process which decreases citrate excretion, enhances oxalate deposition in the kidneys of vitamin B_6-deficient rats. In the present study no significant differences in pH were observed in urines from animals fed the various diets of experiments 1 and 2. It appears that the protective effect of high-magnesium diets is at least in part related to its effect on urinary citrate levels. This is of particular interest in view of the decreased citric acid excretion of patients with renal oxalate stone disease (Gershoff and Prien, '60).

It is a well known fact that rats and most other species of animals excrete increased amounts of xanthurenic acid when

deficient in vitamin B_6. In this work less xanthurenic acid was excreted by vitamin B_6-deficient rats receiving the high levels of magnesium although xanthurenic acid excretion was still considerably higher than that observed in rats receiving vitamin B_6 irrespective of the magnesium level. Just how magnesium functioned biochemically in the present study is not clear. Magnesium is essential, however, for many of the reactions of intermediary metabolism and it appears possible that enzyme systems in vitamin B_6-deficient rats may be more sensitive to the dietary level of magnesium than in animals receiving the vitamin.

Until recently, nutritionists have almost universally been concerned with problems of undernutrition rather than overnutrition. It is apparent that in populations receiving diets high in dairy products there is no marked increase in diseases associated with high-calcium intakes. Since most renal calculi contain calcium, however, it is logical to ask whether high-calcium intakes are not possibly deleterious to individuals with a tendency towards kidney stone formation. Henneman ('59) has pointed out that in some patients, kidney stone formation (type of stone not specified) can be related to excessive milk consumption. The omission of milk from the diets of these patients resulted in decreased urinary calcium excretion and generally in a cessation of stone formation.

In experiments 3 and 4 of this study, rats were fed vitamin B_6-deficient diets with a commonly-used level of magnesium and with very low levels of magnesium since low-magnesium diets enhance oxalate stone formation. Calcium levels in these two experiments varied from 0.2 to 1.2%, variations which cannot be considered very radical. The complete Jones and Foster and Salts IV salt mixtures ordinarily provide 0.6 and 0.55% of calcium, respectively, when used as 4% of the diet. In both experiments 3 and 4, there was a marked increase in the amount of calcium oxalate deposited in the urinary tract when the highest levels of calcium were fed. This was particularly true in experiment 4. It should be stressed that diets containing 0.96 or 1.2% of calcium would not be ordinarily considered as containing excessive amounts of calcium, since this is less calcium than is present in most commercial laboratory chows. The results of this study indicate that under circumstances favorable to the production of kidney disease associated with deposition of calcium oxalate, low-calcium diets may be advisable. Renal apatite deposition in rats maintained with diets low in magnesium was similarly enhanced at the above levels of calcium intake.

The requirements for a nutrient consist of the dietary level of the nutrient which will prevent clinical, histological, and biochemical deficiency signs in a variety of physiological states. This is often made difficult by uncertainty as to what the deficiency signs are. Consequently, estimates of nutrient requirements are often based primarily on growth and balance data. Although thousands of nutrition studies with rats have been conducted, very few of them have involved critical examination of kidneys for crystalline deposits. Various estimates of the growing rat for magnesium have made (in mg per 100 gm of diet): 6 (Medes, '26); 5 (Tufts and Greenberg, '38); 20 (Kunkel and Pearson, '48); and 25 (Hegsted et al., '56). These studies showed that magnesium requirements can be changed by alterations in dietary calcium levels, temperature, and by gestation and lactation. The feeding of atherogenic diets increases the magnesium requirements of rats (Hellerstein et al., '57). This same group (Hellerstein et al., '60) has also reported calcium deposits in the kidneys of rats receiving nonatherogenic diets containing 24 mg of magnesium per 100 gm. In experiment 2 of this study, 13 of 16 rats fed diets containing 0.4 mg of vitamin B_6 and 20 mg of magnesium per 100 gm showed renal apatite deposition. When the magnesium content was raised to 40 mg per 100 gm, the incidence of apatite deposition dropped to 4 out of 15 animals. Raising the dietary magnesium to 400 mg per 100 gm prevented all apatite deposition. This is in general agreement with the accumulated experience of this particular laboratory over the past several years, during which time careful scrutiny of all fresh kidney tissue for crystalline deposits has been maintained. The usual control purified diet employed has contained 40 mg

of magnesium and 550 mg of calcium, supplied as 4% of Salts IV, and 0.4 mg of vitamin B_6 per 100 gm. Thus, in experiments of 6 weeks' duration, 17% of rats fed these purified diets have shown from a trace to small amounts of renal apatite deposition. The use of 4% of Jones and Foster salt mixture instead of Salts IV has resulted in no differences in the renal deposition of either apatite or calcium oxalate. The former mixture contains 46 mg of magnesium per 100 gm and a slightly higher level of calcium, namely 600 mg per 100 gm.

In experiments 1 and 2 of this work, best growth in vitamin B_6-deficient rats was obtained when 400 mg of magnesium per 100 gm was fed. This probably resulted from the protective effect of the high-magnesium diets upon renal oxalate deposition and its sequelae. When diets containing adequate vitamin B_6 were fed, some growth inhibition was observed on the highest magnesium diets. Thus, it would appear that the magnesium requirement for growing rats is probably greater than 40 mg and less than 400 mg per 100 gm of diet.

It should be pointed out that vitamin B_6 deficiency exerts a protective effect upon the renal apatite deposition of diets low in magnesium. In contrast with the incidence of such deposits in animals maintained with 20 mg of magnesium and 0.4 mg of vitamin B_6 per 100 gm, noted above as 13 out of 16 rats, removal of vitamin B_6 from the diet decreased the incidence to one out of 15. At the 40 mg per 100 gm dietary level of magnesium, our accumulated experience from comparable experiments shows that in the face of vitamin B_6 deficiency only two of 63 animals have shown small amounts of renal apatite. This again is in sharp contrast with the figure of 17% noted above when vitamin B_6 is present at the 0.4 mg per 100 gm concentration. This protective action of vitamin B_6 deficiency on renal apatite deposition may simply be a function of the impaired growth and decreased food consumption attendant upon the vitamin deficiency. At very low levels of dietary magnesium, however, renal apatite deposition may become extensive even in the face of vitamin B_6 deficiency, as seen in experiment 4.

SUMMARY

The effects of varying dietary levels of vitamin B_6, magnesium and calcium were studied in rats, in terms of the urinary excretion of various metabolites and of the deposition in the urinary tract of calcium oxalate and apatite. Primary renal deposits of oxalate with secondary obstructive sequelae involving the lower urinary tract occurred in vitamin B_6-deficient rats, accompanied by increased excretion of oxalic and xanthurenic acids and by a marked decrease in citric acid excretion. The feeding of diets high in magnesium (400 mg per 100 gm) markedly reduced the deposition of oxalate in vitamin B_6-deficient rats although they remained hyperoxaluric. The urinary citrate, however, returned to normal levels or above, whereas the excretion of xanthurenic acid was lowered. The efficacy of high concentrations of dietary magnesium in decreasing renal oxalate deposition in vitamin B_6 deficiency appeared related to change in the solvent characteristics of the urine.

Varying the dietary calcium level from 0.2 to 1.2%, in conjunction with vitamin B_6 deficiency, demonstrated a direct correlation between dietary calcium and the extent of oxalate deposition, an effect that became more pronounced when combined with low dietary magnesium.

Distinct differences could be observed between two strains of white rats as to relative sensitivity to vitamin B_6 deprivation, judged both by weight gain and extent of renal oxalate deposits.

Renal deposition of apatite is correlated inversely with the dietary level of magnesium and directly with the level of dietary calcium. At the commonly used magnesium intake of 40 mg per 100 gm, such deposits were seen in minimal amounts in a quarter of the animals. At this magnesium concentration, deficiency of vitamin B_6 protected the animals against apatite deposition. The magnesium requirement of weanling white rats, as judged by the occurrence of renal apatite deposits, would appear to be in excess of 40 mg per 100 gm.

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The Adverse Effect of Raw Whale Liver on the Breeding Performance of Female Mink[1]

D. W. FRIEND AND E. W. CRAMPTON
Department of Nutrition, Macdonald College (McGill University)
Province of Quebec, Canada

The use of from 5 to 10% of raw liver in mink diets is generally recommended. Schaefer et al. ('47) using purified diets, showed a need by growing foxes and mink of unidentified factors present in raw liver or fresh milk. In dry diets used for mink, Kifer and Schaible ('55) used 5% each of liver meal and dried skim milk powder. Ontko and Phillips ('58) found that the addition to a basal semipurified diet of 10% of fresh liver daily, reduced from 62 to 37% the mortality rate of the pups from bitches to which the basal diet was fed.

Overfeeding of liver, however, may be harmful. The vitamin A content and the toxicity of polar bear and of seal liver have been discussed by Rodahl and Moore ('43). That the concentration of liver vitamin A can be influenced by species was demonstrated by the values given by Moore ('53) shown below.

Storage of vitamin A in the liver of various animals

Species	*I.U./gm*
Pig	100
Cow	150
Sheep	600
Sperm whale	4,400
Polar bear	20,000

The richness of whale's liver was explained by Kon and Thompson[2] on the basis of the preformed vitamin present in the krill, or shrimps, used as feed. Nieman and Klein Obbink ('54) described investigations which confirmed that, independent of other toxic factors in fish oils, an overdose of vitamin A caused a specific hypervitaminosis.

Symptoms of hypervitaminosis A in mink were recorded by Helgesbostad ('55) as anorexia, bone changes with decalcification and spontaneous fractures, loss of fur, cramp and local hyperesthesia of the skin.

Evidence has been presented by Giroud and Martinet ('54) and by Berdjis ('58) for the adverse effect on productivity in the pregnant rat, of excess vitamin A.

The observations reported herein, were derived from the results of a comprehensive feeding trial designed to evaluate the suitability of some Newfoundland marine products, including whale liver, for feeding to female breeding mink.

EXPERIMENTAL PROCEDURE

The feeding trial was conducted at the Government Experimental Mink Ranch in Newfoundland. A total of 144 Pastel mink (113 kits and 31 two-year-old adults) were allocated at random to individual pens[3] within the feed lots, a few days before mating started.

During mating, the animals within each lot were given daily an equal opportunity to mate. The normal ranch practice of "second mating" was avoided intentionally. Under this latter system, the female mink in estrus, is mated during each of two successive receptivity periods; the second mating being attempted 8 days after the date of the first successful mating.

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[1] Research conducted in cooperation with the Department of Mines and Resources, Government of Newfoundland, and supported by a grant from the Canada Department of Agriculture for extramural research.

[2] Kon, S. K., and S. Y. Thompson 1949 Preformed vitamin A in northern krill. Biochem. J., 49: PC XXXI.

[3] The pens were of conventional design consisting of a wire cage to which was attached a wooden nest box.

This practice would presumably have reduced the percentage of non-pregnancies,[4] but because not all mink will take a second mating, the possible dietary effect on pregnancy would have been confounded with mating practice. For this test a system of "double mating," within the same receptivity period, was adopted. A different male was put to a female the day after her first successful mating in an attempt to overcome any incidence of recondite male sterility.

The experimental rations fed from the time that mating started, were prepared daily by mixing together the ingredients according to the prescribed formulae (see fig. 1). The marine products, namely, whale meat, whale liver, codfish fillet waste, whole codfish, were stored frozen until required for ration preparation. The cereal mixture was a product commercially prepared for mink feeding.

The criterion by which the effects of the different rations was judged, was the number of successfully mated mink in each feed lot which whelped one or more living kits.

[4] Friend, D. W., and E. W. Crampton 1960 Observations on the effect of four mating routines for female mink. Canad. J. Comp. Med. Vet. Sci., 24: 3.

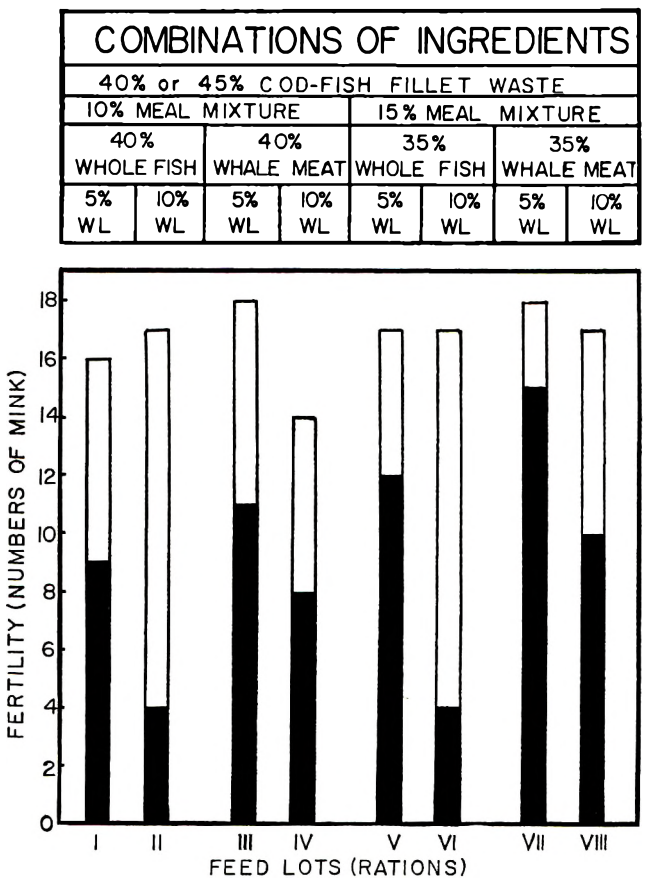

Fig. 1 Comparison of the number of mink whelped with the number mated per feed lot. Each bar represents the number of successfully mated mink, and the shaded portion the number of them which whelped. The codfish fillet waste was composed of the bony skeleton, the fleshy flank and fillet trimmings, the fins and some skin. Whole fish refers to whole codfish (head and viscera removed), and WL to whale liver. When 5% of whale liver was used, 5% of codfish fillet waste was added to the ration as a "filler." The cereal was a commercially-prepared mixture for mink feeding.

TABLE 1

Calculation of chi-square in a 2 × 2 table for the independence of fertility in mink on the level of whale liver in the ration fed

Level of whale liver in ration	Fertility			
	% Whelped		% Not whelped	
	Observed (O)	Expected (E)	Observed (O)	Expected (E)
%				
5	68	54	32	46
10	40	54	60	46
Totals	108		92	
Chi-square (with 1 degree of freedom) $= S[(O-E)-\frac{1}{2}]^2/E = 14.7$[1]				

[1] Significant at the 1% level of probability.

RESULTS AND DISCUSSION

Represented graphically in figure 1 is the fertility of the groups of successfully mated mink fed similar rations containing either 5 or 10% of whale liver. Although all young born appeared normal, there was a tendency towards lowered fertility when 10% of whale liver was used in the rations tested. The difference in fertility was shown, by calculating chi-square according to the method of Snedecor ('56), to be significant at the 1% level of probability; the statistical analysis is presented in table 1.

Normally, it would not seem reasonable to question the use of as much as 10% of liver in mink rations. The whale liver, however, by analysis contained 480,400 I.U. of vitamin A per 100 gm of dry matter and the amount (in rounded numbers) of the vitamin ingested daily per mink, computed from average daily intakes of "hamburg," was for lots with 5% of whale liver, 14,300 I.U., and with 10% of whale liver, 27,700 I.U. per day.

The requirements of vitamin A for breeding mink have not been experimentally determined. Rosenberg ('45) suggests, however, that minimum requirements are probably the same per kilogram of body weight for all mammals, and amount to 20 I.U. of vitamin A per kg of body weight for maintenance, and 60 I.U. for reproduction. At this level a 2- to 3-pound pregnant mink would require about 60 I.U. of vitamin A per day.

Table 5 of the NRC Mink Feeding Standard ('53) proposes a concentration of 117 I.U. of vitamin A in 0.44 pound (wet) of hamburg. A breeding mink in our tests averaged to eat 0.5 pound of hamburg. On this basis the necessary vitamin A intake might to estimated as $0.5 \div 0.44 \times 117 = 132$ I.U. per day. Thus the vitamin A ingested in the 10% liver lots was from 230 to 460 times the requirement, depending on which value for the requirement is used. Thus it is postulated that under the conditions of this feeding trial, in which whale liver was used, quantities of vitamin A might have been ingested by the mink, sufficient to have provoked hypervitaminosis A. Because the test rations were fed only from the time of mating to whelping, and not for any preliminary period before, the "toxicity" if present, possibly induced only the reproductive manifestations observed.

SUMMARY AND CONCLUSIONS

In an experiment in which rations containing codfish and whale products were fed to breeding mink, increasing to 10% from 5% the quantity of whale liver in the ration resulted in a significantly greater number of unsuccessful pregnancies. It is postulated that the quantity of vitamin A derived from the higher level of whale liver was sufficient to have provoked a hypervitaminosis A, which adversely affected the reproductive ability of the female mink.

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Lipemia and Experimental Hypertension in Rats

J. B. ALLISON, P. F. BRANDE, R. W. WANNEMACHER, JR., L. P. PARMER AND J. H. LEATHEM[1]
Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey

Hypertension has been produced in rats by combining unilateral nephrectomy with administration of desoxycorticosterone acetate (DCA), and increased intake of sodium chloride (Prado et al., '47; Selye and Stone, '48). Hypertrophy of the heart, liver and kidney with damage to the kidney and cardiovascular system were associated with the resulting hypertension (Skelton, '55). Recently Omae and Masson ('59) reported a rise in serum cholesterol in rats with an increase in blood pressure. The following experiments are a part of a study involving the effects of hypertension upon serum proteins and lipids in rats fed various diets. Previous experiments had demonstrated that a lipemia developed in rats and dogs which were depleted in body proteins. This lipemia could be corrected completely by repletion with casein but not with high intakes of wheat gluten (Allison and Wannemacher, '59, '60). Thus, in these experiments involving hypertension, the dietary protein was varied by feeding casein or wheat gluten while one group of rats was fed a protein-free diet.

EXPERIMENTAL

Adult female rats, weighing about 170 gm, were divided into 4 groups, with 6 to 7 animals per group. In three of the groups the rats were unilaterally nephrectomized, injected with 2.5 mg of DCA per day and given 1% of sodium chloride in the drinking water. One group was fed 18% of casein, another 40% of wheat gluten, and a third received a protein-free diet. The 4th group of normal control rats was fed 18% of casein. After 35 days all of the animals were sacrificed.

In another experiment, weanling animals were divided into two major groups, one receiving 18% of casein and the other was fed a diet containing 40% of wheat gluten. After the experimental diet had been supplied for 28 days, each of the two groups of young animals was divided into 4 subgroups. One subgroup received no treatment, another was unilaterally nephrectomized, a third was injected subcutaneously with 2.5 mg of DCA per day and the 4th was unilaterally nephrectomized, injected with 2.5 mg of DCA per day and given 1% of sodium chloride in the drinking water. The rats were continued on this treatment for another 28 days, then sacrificed.

The composition of the diet was described previously (Allison et al., '54). This diet contained (in per cent): casein, 18; sucrose, 13.4; dextrose, 20.2; dextrin, 15.9; lard, 23.2; salt mixture, 4; agar, 3.3; and a mixture of vitamins. When wheat gluten replaced casein at a level of 40%, the two diets were kept isocaloric by reducing sucrose and dextrose. Similarly, the protein-free diet was isocaloric with the others, the protein being replaced by carbohydrate. The percentages of protein were selected so that the gain in body weight would be approximately the same over the experimental period. Food intake and body weights were recorded throughout the experiment. Systolic blood pressure was determined on each rat by the cuff method using a photoelectric cell to indicate the flow of blood through the leg (Kersten et al., '47).

Serum proteins were separated by paper electrophoresis. The proteins were stained with an alcoholic solution of bromphenol blue and lipoproteins were stained by the oil red O method of Jencks et al. ('55). The serum was extracted with a mixture of two parts chloroform and one part meth-

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[1] Supported by a grant from the New Jersey Heart Association.

anol. This extract was divided into two portions, one of which was used for determination of serum phospholipid phosphorus by the method of Youngburg (Hawk et al., '54), and the remainder of the extract was used to determine serum cholesterol by the chromatographic method of Wycoff and Parsons ('57).

RESULTS

The effects of unilateral nephrectomy, administration of DCA and sodium chloride in the mature animals upon blood pressure are illustrated in figure 1. The blood pressure in the normal control rats fed casein, remained unchanged during the experimental period of 5 weeks. The blood pressures of the animals fed casein but nephrectomized and treated with DCA and sodium chloride increased linearly during this same period. The blood pressures of the animals fed wheat gluten were statistically higher than those fed casein at two, three and 4 weeks ($P < 0.01$, 0.01 and 0.02, respectively). For the first two weeks, blood pressure rose also in the depleted animals which were nephrectomized and treated with DCA and sodium chloride but then the pressure dropped rapidly to control values. This fall in blood pressure in the depleted rats may be associated in part with a fall in plasma albumin and volume and a decreased effectiveness of DCA in the depleted rat (Leathem, '57).

The hypertrophy of the liver, heart and kidney which was associated with the hypertension is illustrated in table 1. The average weight of the kidney in the animals fed wheat gluten was statistically greater than in the animals fed casein. These results suggest that wheat gluten does increase the stress, possibly through excess

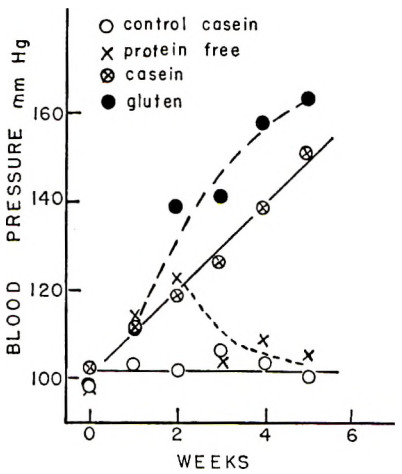

Fig. 1 Blood pressure in rats with unilateral nephrectomy and administration of desoxycorticosterone acetate (DCA) and of sodium chloride.

of an imbalance in the pattern of amino acids (Harper and Kumta, '59).

The plasma albumin decreased in the adult rats with high blood pressure (see fig. 2), decreases which are associated, at least in part with kidney damage that accompanies the hypertension (Skelton, '55; Omae and Masson, '59). Although the plasma globulins of the hypertensive animals did not change in concentration, the lipid migrating electrophoretically with the $alpha_1$ globulin increased significantly in animals fed the protein-free and casein diets, but was most marked in rats fed the wheat gluten. Even the lipid migrating electrophoretically with the beta globulin was increased markedly in animals fed the wheat gluten. It has been suggested that the lipemia associated with depletion in protein reserves is the result, at least in part, of decreased activity of oxidative

TABLE 1

Average organ weights in rats unilaterally nephrectomized, given DCA[1] and sodium chloride (groups 1, 2, 3) and in normal control (group 4)

Group	Dietary protein	Liver	Heart	Kidney
		gm	*gm*	*gm*
1	None	6.43 ± 0.58[2]	0.55 ± 0.03	0.91 ± 0.09
2	Wheat gluten	10.50 ± 0.68	0.97 ± 0.07	2.55 ± 0.18
3	Casein	9.18 ± 0.70	0.93 ± 0.05	1.82 ± 0.10
4	Casein	7.41 ± 0.34	0.68 ± 0.01	1.49 ± 0.10

[1] Desoxycorticosterone acetate.

[2] Standard error.

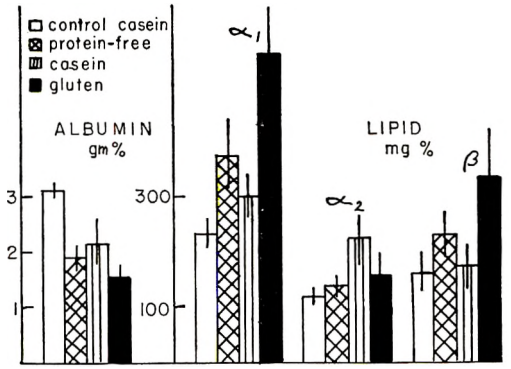

Fig. 2 Plasma albumin and the lipid migrating electrophoretically with the α- and β-globulins. The lines through the bars record standard errors.

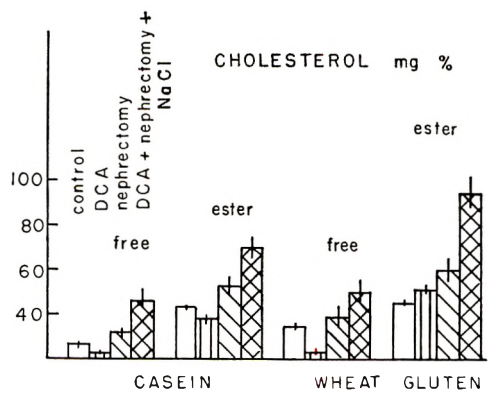

Fig. 4 Serum cholesterol in young rats under various treatments.

enzyme systems and transport mechanisms (Allison and Wannemacher, '59, '60). Such a lipemia was observed in depleted dogs when the fat content of the diet was relatively high and of the more saturated type suggesting an overloading of these depleted enzyme systems. Possibly similar damage in the hypertensive rats may be discovered, a possibility that is under investigation.

The increase in cholesterol and the phospholipid phosphorus in the depleted rats and in the hypertensive rats are illustrated in figure 3. The P values are less than 0.01 as compared with normal controls for all of these increases. The increases were most marked in the cholesterol ester and phospholipid phosphorus in hypertensive rats fed the wheat gluten diet. Similarly the cholesterol ester and phospholipid phosphorus remained elevated in dogs previously depleted in body proteins but repleted by feeding wheat gluten (Allison and Wannemacher, '60).

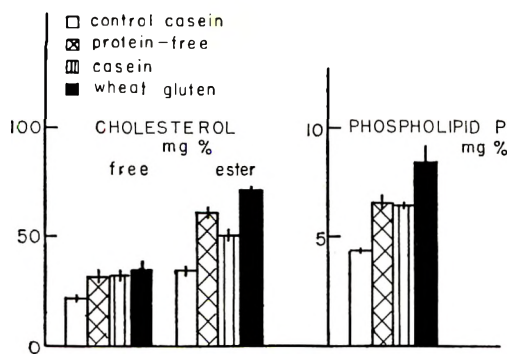

Fig. 3 Serum cholesterol and phospholipid phosphorus in normal control rats fed casein diet and in hypertensive animals.

The effects of hypertension upon the young rats were similar to those described for the older animals. The data in figure 4 record the increase in serum cholesterol in the experiments involving the weanling rats. These results illustrate the tendency for cholesterol ester to be higher in animals fed wheat gluten and also the tendency for cholesterol to rise in animals with unilateral nephrectomy but without other treatment. The terminal blood pressures of the rats with unilateral nephretcomy and given DCA plus sodium chloride were approximately 150 mm Hg with no significant difference between the groups fed wheat gluten or casein. The blood pressures of the other groups illustrated in figure 4 were normal.

Nath et al. ('59) produced hypercholesterolemia in rats by feeding a diet containing 25% of hydrogenated coconut oil, 1% of cholesterol and 5% of cholic acid. Serum cholesterol was higher when casein was included in the diet than when wheat gluten replaced the casein. Furthermore, as wheat gluten was varied from 10 to 68.5% there was a progressive lowering of serum cholesterol. The authors have suggested that this lowering of serum cholesterol may be associated with unsaturated fatty acids in the lipid portion of the wheat gluten.[2] The response of cholesterol to the hypertension stress in rats fed wheat gluten differs from the response associated with this dietary hypercholesterolemia. These

[2] Harper, A. E., personal communication.

different results emphasize the importance of the physiological state of the animal as well as the importance of the diet upon the metabolism of cholesterol.

SUMMARY

Hypertension was induced in unilaterally nephrectomized rats by administration of desoxycorticosterone acetate and 1% of sodium chloride in the drinking water. The hypertensive rats developed hypertrophy of the heart, liver and kidney with a reduction in serum albumin. Serum cholesterol, phospholipid and the lipid migrating electrophoretically with alpha and beta globulins increased in the hypertensive rats. Hypertrophy of the kidney and the increase in serum lipids were greater in animals fed wheat gluten than in those fed casein. There was a small increase in serum cholesterol in rats with normal blood pressure but with unilateral nephrectomy. Hypertension was not produced in rats fed the protein-free diet although increases in serum lipids were associated with depletion in body proteins.

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Relation of Serum Tocopherol Level to Nitrogen Retention, Creatine, Creatinine and Free Alpha Amino Acid Nitrogen Excretion in Premature Infants[1]

LULA O. LUBCHENCO, JOSEPH BUTTERFIELD, JENS BERGSTEDT, FRANK A. IBBOTT AND DONOUGH O'BRIEN[2]
Department of Pediatrics, University of Colorado Medical Center, Denver, Colorado

The low serum tocopherol which is a characteristic of the premature infant fed a partially skimmed cow's milk formula, is known to be associated with a tendency to *in vitro* hemolysis of the red blood cells on exposure to hydrogen peroxide (Mackenzie, '54). This observation raises the possibility that these infants may be deficient in this vitamin, a supposition that is strengthened, although tenuously, by the finding of creatinuria and α-amino-aciduria in both premature infants receiving a relatively high protein intake (Norton et al., '54; Woolf and Norman, '57; Marples, '42; O'Brien et al., '60) and in tocopherol-deficient experimental animals (Hove and Harris, '47; Dinning et al., '56; Hurley and Williams, '55). There is, however, a significant difference in protein metabolism between these two groups in that, in the tocopherol-deficient animals, there is a negative nitrogen balance; whereas the premature infants, over the period when they exhibit a low serum tocopherol, are in active positive nitrogen balance, though perhaps not maximally so.

The purpose of this study, then, was to determine the effect of tocopherol administration, as judged by serum tocopherol levels, on nitrogen retention, creatine, creatinine and free α-amino nitrogen excretion in premature infants and to assess from the results whether there is any indication for routine tocopherol supplementation of the diet of these infants in the first three months of life.

SUBJECTS AND METHODS

Thirteen premature infants, whose birth weights were less than 1500 gm and who had received no supplements of α-tocopherol during their hospital stay, were studied as follows.

Approximately two weeks prior to discharge from the hospital, when the serum tocopherol levels are usually at their lowest, a control balance period of 5 days was carried out and was followed by a second 5-day balance period during which an oral supplement was administered of 10 to 20 mg of α-tocopheryl acetate[3] per 24 hours. In 5 of the infants, a third 5-day balance, also during tocopherol supplementation, was obtained immediately after the second period; and in two of these, the tocopherol supplementation amounted to 50 mg per 24 hours. The amount of vitamin E administered was in all cases in excess of the 2.5 to 5.0 mg per kg per 24 hours known to overcome the *in vitro* hemolytic tendency of the red blood cells shown by premature infants with low serum tocopherol levels (Mackenzie, '54). The data obtained on these infants was compared with that from 12 premature infants of similar weights supplemented with α-tocopherol and studied continuously from one week after birth to discharge from the nursery.[4]

The feedings consisted of a partially skimmed milk and carbohydrate mixture[5]

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[2] John and Mary Markle Foundation Scholar in Medical Sciences.
[3] Kindly supplied by Hoffman-LaRoche Inc., Nutley, New Jersey.
[4] Lubchenco, L. O. and R. Y. Ting, in preparation.
[5] Dalactum, kindly supplied by Mead Johnson and Co., Evansville, Indiana.

ad libitum. Nitrogen in the food, urine and stool was determined by a microKjeldahl procedure, and total and preformed creatinine in the urine by the Jaffé reaction after ether extraction of interfering chromogens (Taussky, '56). Total α-amino nitrogen in the urine was determined by a copper method (O'Brien and Ibbott, '60) after absorption and elution from a Dowex 50 column. Serum α-tocopherol levels were determined by the oxidimetric color reaction with ferric chloride and α, α-dipyridyl (Quaife et al., '49).

RESULTS AND DISCUSSION

In table 1 are shown the data concerning the levels of serum tocopherol, before and at the end of the study periods, on nitrogen retention and on the excretion coefficients of creatine, creatinine and free α-amino nitrogen. The birth weights ranged from 630 to 1361 gm; and the subjects were 34 to 79 days old at the beginning of the study periods, at which time their weights ranged from 1410 to 2360 gm. Serum tocopherol levels at the beginning of the study were 0.04 to 0.54

TABLE 1

Nitrogen balance data in premature infants with low serum tocopherol levels

Infant no.	Age (days) at start of study	Mean weight during study	Period[1]	Serum tocopherol	Nitrogen retention	Creatinine excretion	Creatine excretion	Total α-amino nitrogen excreted
				mg/100 ml	*mg/kg/ 24 hours*	*mg/kg/ 24 hours*	*mg/kg/ 24 hours*	*mg/kg/ 24 hours*
1	75	2135	1	0.19	565	9.6	2.4	12.7
	82	2290	2	2.37	535	13.7	2.7	14.9
	88	2475	3	—	436	14.2	3.6	16.8
2	57	1525	1	0.08	282	13.3	1.4	—
	63	1650	2	0.23	411	18.6	0.1	—
3	64	2440	1	0.15	490	13.9	0.1	14.6
	69	2625	2	0.99	458	11.7	2.4	14.7
	74	2820	3	0.68	277	12.2	2.1	15.1
4	60	2215	1	0.10	406	9.5	0.7	19.3
	65	2390	2	0.31	396	10.5	1.8	12.3
5	69	2295	1	0.31	553	13.2	1.3	22.2
	76	2465	2	3.68	500	14.9	1.2	23.2
	81	2740	3	—	446	13.4	0.3	14.2
6	62	1940	1	0.16	475	8.3	1.4	11.2
	67	2145	2	0.99	425	9.1	2.6	22.2
7	45	2000	1	0.23	380	7.6	0.2	14.5
	49	2130	2	1.90	374	7.6	0.05	14.6
8	35	1945	1	0.36	455	11.4	2.1	—
	40	2090	2	2.30	463	10.6	2.9	—
9	44	1835	1	0.27	499	10.4	2.8	—
	51	2080	2	3.85	539	12.3	0.2	—
10	34	2025	1	0.54	397	13.2	1.4	—
	40	2185	2	1.43	455	14.0	0.7	11.1
11	58	1425	1	0.26	388	15.4	—	16.9
	66	1640	2	1.95	549	13.4	4.0	18.2
	79	2045	3	5.49[2]	255	13.5	2.5	13.0
12	47	1465	1	0.23	384	8.2	2.2	11.7
	54	1575	2	2.79	430	11.5	2.3	13.8
	71	2085	3	3.80[2]	394	14.2	3.4	14.7
13	63	2030	1	0.04	363	5.2	6.1	11.7
	72	2275	2	0.71	346	6.2	6.4	12.7

[1] Period 1, no supplemental tocopherol in diet; periods 2 and 3, during tocopherol supplementation, 10 to 20 mg/24 hours.

[2] Supplement of tocopherol, 50 mg/24 hours.

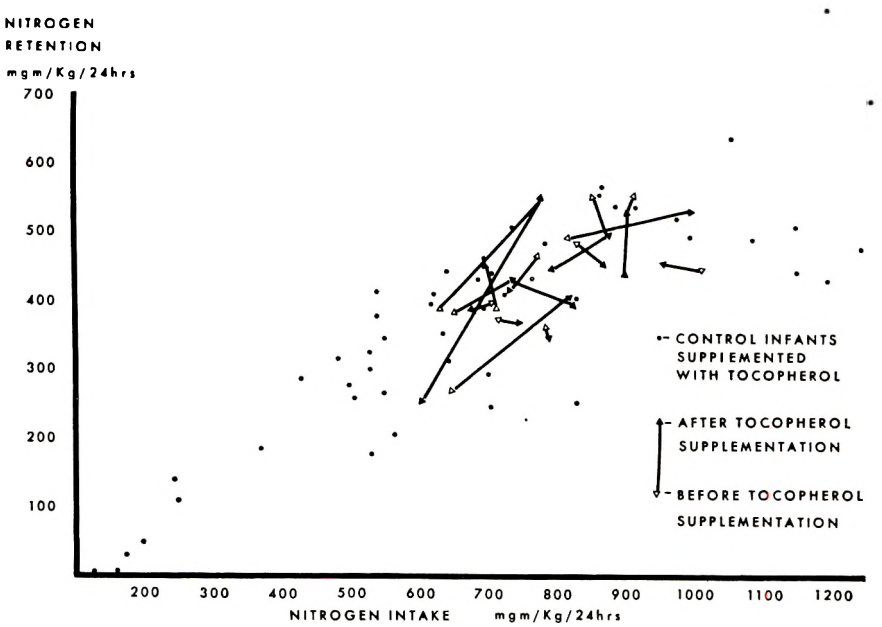

Fig. 1 Nitrogen intake and nitrogen retention of infants with low serum tocopherols.

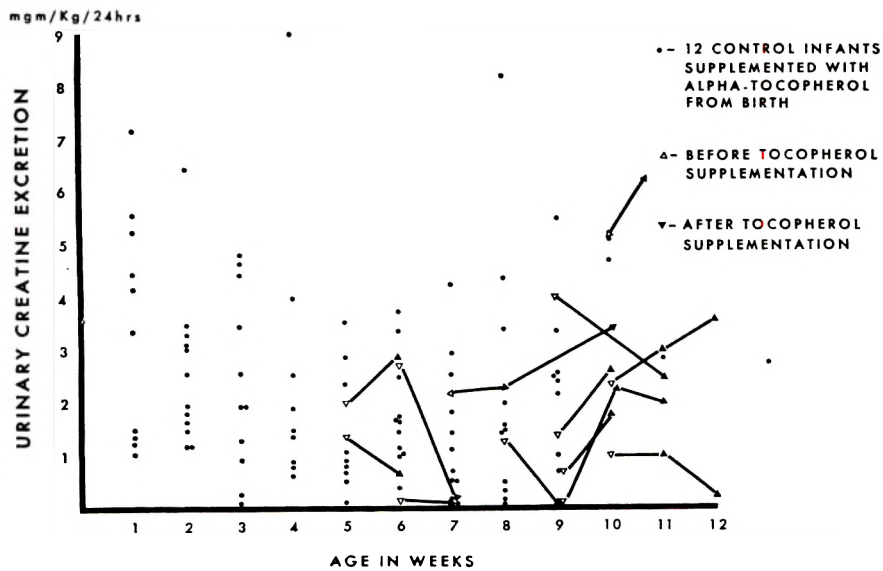

Fig. 2 Effect of tocopherol supplementation on the urinary creatine excretion in premature infants.

mg per 100 ml, the lowest levels ocurring in the infants with the smallest birth weights. All of the infants demonstrated some rise in the levels of α-tocopherol in the serum after 5 days of the supplementation; but two were not elevated over 0.5 mg per 100 ml, the level above which red cells are resistant to hemolysis after exposure to hydrogen peroxide (Mackenzie, '54). Final levels ranged from 0.23 to 5.49 mg per 100 ml.

The nitrogen intake and nitrogen retention of the infants from the control periods before tocopherol supplementation and

during the ensuing study periods after supplementation are also graphically shown in relation to data from a control group of infants whose diet had been supplemented with α-tocopherol since birth in figure 1. Nitrogen retention was not altered by the administration of α-tocopherol, nor were the values for nitrogen retention at any time significantly different from those in infants supplemented from birth. Tocopherol supplementation was also without apparent effect on the urinary excretion coefficients of creatine, both absolutely and as a proportion of total creatinine. As shown in figure 2, 6 of the 13 infants showed a decrease in urinary creatine excretion after administration of tocopherol; but in the rest, there was a variable increase. Such fluctuations were within the range of normal for the premature infant (Marples, '42; Bergstedt et al., '60). Finally, no significant differences were noted in α-amino nitrogen excretion between the two periods (table 1). In a small number of cases, the individual amino acid pattern was studied before and after tocopherol administration and the patterns were similar in both instances.

SUMMARY AND CONCLUSIONS

In a group of premature infants, aged 24 to 79 days, fed an evaporated milk formula without tocopherol supplementation, serum tocopherol levels ranging from 0.04 to 0.54 mg per 100 ml were observed. Tocopherol supplementation raised the serum tocopherol levels, but was without significant effect on nitrogen retention or upon the excretion coefficients of creatine, creatinine or α-amino nitrogen.

These results suggest no indication for special dietary tocopherol supplementation in the premature infant in the first trimester of life.

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Various Natural and Modified Bile Acids in Cholesterol Metabolism[1]

OSCAR W. PORTMAN[2] AND DOROTHY BRUNO
Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

It has been observed consistently that rats fed diets containing cholesterol or cholic acid have slightly higher serum cholesterol levels than those fed unsupplemented diets. When cholesterol and cholic acid are fed together, the serum cholesterol levels are dramatically increased, the values being proportional to the logarithm of dietary cholic acid concentration over a fairly wide range (Hegsted et al., '57; Portman, '60).

In an attempt to elaborate the mechanism of the cholic acid-induced hypercholesterolemia, a number of studies comparing cholic acid with other bile acids have been carried out (namely, Member et al., '44; Swell et al., '53). These reports indicated that taurocholic and glycocholic acids are more active than cholic acid in rats. Another study (Portman, '60) indicated that certain reported bacterial conversion products of cholic acid, namely, deoxycholic, 7-ketodeoxycholic, 12-ketolithocholic, and dehydrocholic acids, were not as active as cholic acid in elevating the serum cholesterol level of rats. On the other hand, Howe et al. ('60) observed that taurocholic acid was less active than cholic acid in elevating the serum cholesterol level of mice. Beher and co-workers ('60) have studied the effect of several bile acids and have emphasized the low serum cholesterol values in mice fed hyodeoxycholic acid. In addition, Howe et al. ('60) have emphasized the effectiveness of hyodeoxycholic, chenodeoxycholic, and lithocholic acids in counteracting the hypercholesterolemic effect of cholic acid in mice.

In view of the demonstration by Bergström ('59) that the conversion of cholesterol to bile salts in the liver is dependent on the level of a bile acid "feedback" via the portal vein, it is possible that the effects of the various antagonistic bile salts may be mediated through an interference with the feedback signal, perhaps by interfering with the absorption of endogenous taurocholate (in the rat).

The present report involves the evaluation in rats of a number of naturally occurring and substituted bile acids for their effects on serum and liver cholesterol concentrations, serum β-lipoprotein concentrations, and weight gains. The metabolism of 3α-, 7α-, 12α-triformoxycholanic acid-24-C^{14}, which was completely inactive in the feeding trials in elevating the serum cholesterol level, was studied. An evaluation of the effect of feeding diets containing triformoxycholanic acid to Cebus monkeys was also made.

EXPERIMENTAL

Male albino rats weighing 200 ± 10 gm were selected. They were caged individually and given water ad libitum. The animals were control-fed; this was essentially ad libitum feeding, since there was no difference in food intake with the relatively low levels of dietary bile acids which were used. The percentage composition of the basal diet was as follows: casein, 12.0; sucrose, 73.0; hydrogenated vegetable fat,[3] 10.0; salts (Hegsted et al., '41), 4.0; inositol, 0.1; choline, 0.2; *p*-aminobenzoic

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[2] This study was made during the tenure of an Established Investigatorship of the American Heart Association.
[3] Spry, Lever Bros. Company, New York.

TABLE 1
Bile acids evaluated in this study

Trivial name	Chemical name	Source
Cholic acid	3α, 7α, 12α-trihydroxycholanic	Eastman Organic Chemicals, Rochester, New York
Taurocholic acid	Taurine conjugate (a)	Cortese and Bashour ('37)
Cholic acid + taurine (2%)		Eastman Organic Chemicals: Nutritional Biochemicals Corporation
Deoxycholic acid	3α, 12α-dihydroxycholanic	General Biochemicals, Inc. Chagrin Falls, Ohio
Lithocholic acid	3α-hydroxycholanic	Mann Research Laboratories, Inc. New York
Apocholic acid	3α, 12α, $\Delta^{8(14)}$-dihydroxycholenic	Boedecker ('20)
Triformoxycholanic acid	3α, 7α, 12α-triformoxycholanic	Cortese and Bashour ('37)
Diformoxycholanic acid	3α, 12α-diformoxycholanic	Modification of Cortese and Bashour ('37)
Triformoxycholanyl chloride	3α, 7α, 12α-triformoxycholanyl chloride	Cortese and Bashour ('37)
Triacetoxycholanic acid	3α, 7α, 12α-triacetoxycholanic	Bergstrom et al. ('53)
Very unsaturated acid	Unknown—apocholic preparation	Boedecker ('20)
Cholanic acid	Same	Mann Research Laboratories, Inc.
3 β-Hydroxy bisnor Δ^5 cholenic acid	Same	Mann Research Laboratories, Inc.
Methyl cholate	Methyl-3α, 7α, 12α-trihydroxy-cholanate	Transmethylation with H_2SO_4-MeOH

acid, 0.1; cholesterol, 0.45; and standard supplements (Portman and Mann, '55) of fat and water-soluble vitamins. Cholesterol was excluded from some of the diets.

Bile acids were included at 0.0098 moles per kg of diet (equivalent to 0.40% of cholic acid). The trivial names, chemical names, and the source or method of synthesis of the bile acids which were evaluated are listed in table 1. The structural formula for the parent bile acid, cholanic acid, is illustrated in figure 1. Rats were fed the experimental diets for 21 days. They were weighed twice weekly and at the termination of experiments.

The rats were decapitated and samples of serum and liver were obtained. Aliquots of both fractions were taken, homogenized in chloroform:methanol (2:1), incubated at 40°C for 30 minutes, brought to the mark and filtered through fast filter paper into culture tubes with teflon lined caps. Liver and serum lipids were chromatographed on silicic acid columns, by a

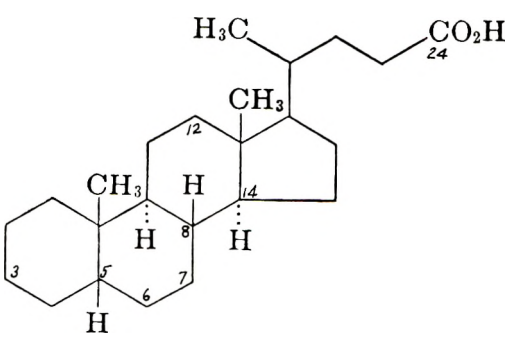

Fig. 1 The structural formula of cholanic acid, the parent compound to those bile acids evaluated in this study. Positions 3, 5, 6, 7, 8, 12, 14 and 24 which were involved in the various substituted compounds considered, are indicated.

method previously described (Portman et al., '59). Free and ester cholesterol determinations were performed on the appropriate fractions by a modification of the method of Abell et al. ('52). Serum β-lipoproteins were separated and analyzed

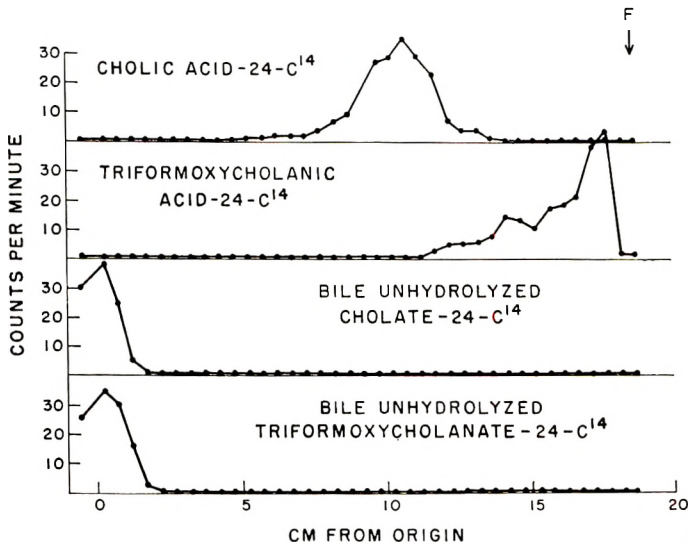

Fig. 2 Paper radiochromatographs of cholic acid-24-C^{14}, triformoxycholanic acid-24-C^{14}, unhydrolyzed bile from a rat given cholate-24-C^{14} and bile from a rat given triformoxycholanate-24-C^{14}. The chromatographic system was that of Sjövall ('54). The moving phase was isopropyl ether: *n*-heptane, 80:20 (v/v).

in certain cases by the ultracentrifugal method of Gofman et al. ('49). Bile acids were separated in certain instances by paper chromatography using the procedures of Sjövall ('54).

Four Cebus monkeys were also used to evaluate the effect of one of the bile acids, 3α-, 7α-, 12α-triformoxycholanic acid, which gave interesting results in the rat experiments. The serum cholesterol levels of these monkeys varied between 120 and 600 mg per 100 ml at the time of initiation of bile acid feeding, depending on the previous diet. Serum cholesterol determinations were measured weekly.

RESULTS

Effect of bile acids in diets containing cholesterol. In table 2 are shown mean values for total serum cholesterol, serum β-lipoproteins, total and free liver cholesterol, liver weight, and 21-day weight gain for the first experiment in which 0.45% of cholesterol and a variety of bile salts were included in the diet.

Three of the bile acid supplements resulted in mean serum cholesterol values which were not significantly elevated above those observed in the rats fed the basal (cholesterol-containing) diet without bile acids added. These were lithocholic and triformoxycholanic acids, and triformoxycholanyl chloride. Diformoxycholanic, triacetoxycholanic, and the highly unsaturated bile acids produced serum cholesterol values which were slightly higher than those of the first group ($P \leq 0.1$). At the other end of the spectrum, cholic acid, taurocholic acid, methyl cholate, and cholic acid fed in a diet containing 2% of taurine produce very high mean terminal serum cholesterol levels which statistically were indistinguishable. Intermediate mean serum cholesterol values were observed in the rats fed deoxycholic and "apocholic" (3α-, 12α-dihydroxy $\Delta^{8(14)}$-cholenic) acid.

Mean total liver cholesterol concentrations ranged from 7.5 mg per gm of wet tissue for the group fed triformoxycholanic acid to 50.6 mg per gm for the group fed cholic acid. The values for the other groups were distributed in the same general order as were the serum values. The liver free-cholesterol values of the various groups were not strikingly different, since most of the elevations in the liver cholesterol concentrations were in the ester fractions.

Effect of dietary bile acids and mixtures of bile acids. Since triformoxycholanic

TABLE 2

The effect of feeding a variety of bile acids at 0.0098 moles/kg of diet (equivalent to 0.4% of cholic acid) on the serum cholesterol and β-lipoprotein concentrations, liver weight, liver free and ester cholesterol concentrations and 21-day weight gain[1]

Supplement	Serum total cholesterol	β-Lipoproteins[2]					Liver weight	Liver cholesterol		Weight gain
								Total	Free	
	mg/100 ml	*mg/100 ml*					*gm*	*mg/gm*		*gm*
None[3]	110 ± 13.6[4]	0	0	0	0	24	11.88	7.6	2.2	67
Triformoxy-cholanic	113 ± 5.5	0	0	0	0	119	14.63	7.5	2.4	75
Triformoxy-cholanyl-Cl	118 ± 10.6	0	0	0	0	100	12.66	8.5	2.3	55
Lithocholic	118 ± 9.4	0	0	0	0	119	13.35	10.1	2.6	71
Diformoxy-cholanic	139 ± 7.2	0	0	10	100	124	13.20	13.9	2.8	67
Triacetoxy-cholanic	145 ± 16.5	0	0	2	52	119	14.75	11.2	2.7	62
Very unsaturated acid	166 ± 16.5	0	0	17	119	48	15.55	27.4	3.2	63
Apocholic	236 ± 11.8	0	0	14	157	171	15.76	36.4	3.6	82
Deoxycholic	262 ± 27.2	0	0	28	188	112	15.03	30.7	3.2	62
Taurocholic	325 ± 17.9	0	0	31	262	176	15.61	46.7	3.7	63
Cholic	334 ± 19.6	0	0	40	252	169	16.39	50.6	3.8	79
Cholic + 2% taurine	336 ± 31.2	0	0	31	236	131	15.38	43.9	3.8	66
Methyl cholate	377 ± 27.3	0	0	28	242	248	15.39	46.0	3.8	60

[1] All diets contained 0.45% of cholesterol.

[2] β-Lipoprotein concentrations list, respectively, the concentrations (mg/100 ml of serum) of the Sf 0–12, 12–20, 21–35, 35–100, and 100–400 fractions. The concentrations are based on refractive indices determined for human β-lipoproteins.

[3] Five rats/group.

[4] Values are means ± standard errors of the means.

acid seemed to be devoid of the serum- and liver-cholesterol-elevating properties characteristic of cholic acid in diets containing cholesterol, this compound was further evaluated in a second experiment. Triformoxycholanic acid was fed in cholesterol-containing and cholesterol-free diets, and was also fed in combination with cholic acid. In this trial two other bile acids, cholanic acid and 3 β-hydroxy bisnor Δ^5–cholenic acid were evaluated in diets containing cholesterol. The results are indicated in table 3.

The rats fed cholesterol-free diets with either cholic acid or cholic acid plus triformoxycholanic acid had higher serum and liver cholesterol values, whereas those fed diets containing triformoxycholanic acid were not different in these respects from the unsupplemented controls.

Among the rats fed cholesterol, the group fed cholic acid again had the highest concentrations of serum and liver cholesterol. The serum concentrations of the groups fed triformoxycholanic or cholanic acids were not statistically different from the values for the controls not fed bile acids. The group fed 3 β-hydroxy bisnor Δ^5–cholenic acid had a lower mean serum cholesterol value than the control group ($P = 0.10$). The group fed equal molar concentrations of cholic and triformoxycholanic acids (two times the molar concentration of bile salts fed to the other groups) had a lower mean serum cholesterol concentration than the group fed

TABLE 3

The effect of feeding different bile acids at 0.0098moles/kg of diet (equivalent to 0.4% of cholic acid) in cholesterol-free and cholesterol-containing diets[1,2]

Bile acid supplement	Cholesterol supplement	Serum total cholesterol	β-Lipoproteins					Liver weight	Total liver cholesterol	Weight gain
		mg/100 ml	*mg/100 ml*					*gm*	*mg/gm*	*gm*
None	0	80 ± 1.8[3]	0	0	0	0	296	11.20	2.1	70
Cholic	0	111 ± 5.9			—			11.90	5.0	55
Triformoxycholanic	0	91 ± 8.9			—			12.60	2.3	80
Cholic + triformoxycholanic	0	107 ± 7.6			—			13.60	3.8	59
Cholic	+	499 ± 41.0	0	0	88	536	224	14.30	39.0	63
Triformoxycholanic	+	127 ± 11.4	0	0	31	0	228	13.70	5.5	78
Cholic + triformoxycholanic	+	328 ± 15.9	0	0	38	266	133	14.00	23.9	56
Cholanic	+	142 ± 14.1			—			14.20	10.4	19
3 β-OH Bisnor Δ^5-cholenic	+	108 ± 11.2			—			10.80	7.1	51
None	+	134 ± 10.2	0	0	0	0	228	11.90	12.2	59

[1] In those cases where two bile acids were fed in the same diet, 0.0098 moles of each acid were included/kg of diet.
[2] Footnotes of table 2 also apply to this table.
[3] Values are means ± standard errors of the mean.

cholic acid alone ($P = 0.01$). The liver cholesterol values formed a distribution similar to that observed for the serum cholesterol. The liver cholesterol values of the triformoxycholanic groups, however, were lower than those seen in the unsupplemented control group.

The rats fed cholanic acid gained less weight than those in the other groups. There was no significant difference between the mean weight gains in any of the other groups. The sera of all of the rats in the cholanic acid group were of a yellow-green color in contrast with the appearance of all other sera.

Triformoxycholanic acid in Cebus monkeys. Since triformoxycholanic acid appeared to be, of the bile acids tested, one of the least active in elevating the serum and liver cholesterol concentrations of rats, and, in fact, inhibited the effect of cholic acid, further evaluation of this acid was made in Cebus monkeys. Triformoxycholanic acid was fed to three monkeys at 0.0049 moles per kg of diet and to one monkey at 0.0098 moles per kg (the concentration used in the rat diets). The mean pretreatment serum cholesterol values for the monkeys administered the lower concentration of bile acid were 356, 271, and 116 mg per 100 ml; the mean treatment values were 259, 241, and 117 mg per 100 ml. The mean pretreatment and treatment values for the monkey given the higher concentration of dietary triformoxycholanic acid were 670 and 636 mg per 100 ml, respectively. Although a further evaluation of this substituted acid at higher concentrations seemed to be justified, there was no clear-cut effect on Cebus monkeys at the concentrations used.

Absorption and biliary excretion of triformoxycholanic acid-24-C^{14}. Two rats weighing about 350 gm whose bile ducts were cannulated, each received into the upper duodenum one milligram of labeled triformoxycholanic acid (neutralized with NaOH) in 0.6 ml of H_2O. One rat was similarly treated with cholic acid-24-C^{14}.

The cumulative excretion of labeled material via biliary fistulas at 5, 24 and 48 hours for each of the experimental animals is shown in table 4. These data indicate that after the administration of C^{14}-labeled triformoxycholanic acid, large amounts of labeled material are rapidly excreted in the bile. These results indicate that the acid is absorbed from the gastrointestinal tract and then excreted into the bile, although they do not indicate whether there was prior deformoxylation in the intestine or liver. There also is no indication as to whether the labeled acid was conjugated.

Nature of the labeled biliary products. Figure 2 illustrates the paper radiochromatographs (procedure according to Sjövall et al. ['54]) obtained for cholic acid-24-C^{14}, triformoxycholanic acid-24-C^{14}, bile from the radiocholate-treated rat, and bile from a radiotriformoxycholanate - treated rat. The triformoxy acid is markedly less polar than cholic acid. Radioactivity in the two rat biles remained at the origin as is consistent with conjugated acids in this system. Unfortunately, alkaline hydrolysis used for hydrolyzing conjugated bile salts will also hydrolyze the formoxy groups. Therefore, definite identification of the conjugated bile salt in the bile of the rats treated with triformoxycholanic acid-24-C^{14} must await the characterization of taurocholic and synthetic taurotriformoxycholanic acids in other chromatographic systems.

DISCUSSION

Very little definitive information is available about the mechanisms involved in the induction of a pronounced hypercholesterolemia by feeding cholesterol and cholic acid to rats. When either component is fed alone the serum cholesterol level is increased about 25% above the level of the unsupplemented controls. The liver cholesterol ester fraction is increased one-hundred-fold or more when both components are fed together, whereas the feeding of cholesterol without cholic acid results in a smaller and slower accumulation of liver cholesterol ester. The feeding of cholic acid alone causes a relatively small increase in liver cholesterol concentrations.

Clearly, cholic acid is not completely specific in the above respect. Taurocholic and glycocholic acids, as well as methyl cholate, are at least as active as cholic acid. This study, and others (Howe et al., '60), however, indicate that there is a wide range of serum- and liver-cholesterol- potentiating activities of various other natural and substituted bile acids.

The activity of the bile acids is not a simple function of polarity. Apocholic acid and the very unsaturated preparation obtained in the synthesis of apocholic acid should have polarities similar to that of cholic acid, but they possess only intermediate serum- and liver-cholesterol-potentiating activity. One property characteristic of those bile acids which had minimum serum- and liver-cholesterol-elevating properties was the absence of or esterification of the 12 α-hydroxy grouping. The acids in this study which fulfill this criterion were cholanic, lithocholic, 3 β-hydroxy bisnor Δ^5-cholenic, triformoxycholanic, diformoxycholanic and triacetoxycholanic acids. All of the acids which contained a 12 α-hydroxy grouping had some cholesterol-elevating activity. It was also shown in a previous study (Portman, '60) that dehydrocholic and 12-ketolithocholic acids, which have a carbonyl group

TABLE 4

Excretion of labeled material into the bile after the administration of one milligram of triformoxycholanic acid-24-C^{14} or one milligram of cholic acid-24-C^{14} (both neutralized with NaOH) in 0.6 ml of H_2O[1]

Exp. no.	Acid used	Cumulative excretion		
		0–5 hours	0–24 hours	0–48 hours
		%	%	%
1	TFC[1]-24-C^{14}	41.7	85.3	85.3
2	TFC-24-C^{14}	29.8	61.4	78.6
3	Cholic-24-C^{14}	19.2	57.5	69.0

[1] TFC(3 α-, 7 α-, 12 α-triformoxycholanic acid-24-C^{14}) was prepared from cholic acid-24-C^{14} by a modification of the method of Cortese and Bashour ('37).

at position 12, also have some serum-cholesterol-elevating activity.

The studies of Beher et al. ('60) and of Howe et al. ('60) lend weight to the apparent importance of position 12 in the structure of bile acids which elevate the serum cholesterol. Their studies have shown that hyodeoxycholic (3α-, 6α-dihydroxy) and chenodeoxycholic (3α-, 7α-dihydroxy) did not elevate the serum cholesterol level of mice. On the other hand, Howe et al. ('60) reported that the acetylation of the 3- and 7-hydroxy groups of methyl cholate, leaving the 12-hydroxy group unsubstituted, resulted in an inactive compound. It, therefore, remains to be demonstrated whether the other hydroxyl groups contribute to the activity related to the critical 12 position.

The present study agrees with that of Howe et al. ('60), using mice, that methyl cholate is as effective as cholic acid in promoting increased serum cholesterol levels. Since hydrolysis of the methyl ester bond in the gut is, at least, a possibility, it will be necessary to test other substituents in the side chain before concluding that the nature of the side chain does not influence the serum-cholesterol-elevating properties of cholic acid.

This study indicates that the inactivity of triformoxycholanic acid is apparently not related to decreased absorption of the compound. Similarly, cholanic acid, which is also inactive, has been shown by Sjövall and Åkesson ('55) to be absorbed from the gut. Also, Lindstedt and Norman ('56) have not observed striking differences in the turnover rates of a variety of labeled bile acids.

A number of other substituted bile acids are being prepared for dietary evaluation in order to gain a more complete picture of those components of the cholic acid molecule which influence the serum cholesterol level. These include a variety of 12-hydroxy acids, compounds with side chain substitutions, and compounds with ring hydroxy substitutions (namely, amino for hydroxy substituted compounds). If the most active groupings can be established, it will then be possible to investigate the most appropriate substitutions for the development of an active serum-cholesterol-depressing agent.

SUMMARY

A variety of bile acids was included in the diet of rats at 0.0098 moles per kg diet to evaluate their effect on the serum and liver cholesterol concentrations and on serum β-lipoprotein concentrations. A wide range of values was observed. Triformoxycholanic acid, triformoxycholanyl chloride, lithocholic acid, cholanic acid and 3 β-hydroxy Δ^5-cholenic acid did not elevate the serum cholesterol level. The remaining 9 regimens resulted in varying degrees of elevation of serum and liver cholesterol values, but none was more active than cholic acid. Triformoxycholanic acid partially inhibited the action of cholic acid when both compounds were fed together. Triformoxycholanic acid was without significant effect on the serum cholesterol level of Cebus monkeys. Three α-, 7α-, 12α-triformoxycholanate-24-C^{14} was rapidly absorbed from the intestine and excreted into the bile as a conjugated (but uncharacterized) bile acid. The evidence suggesting that the configuration at position 12 is crucial to the serum- and liver-cholesterol-elevating properties of certain bile acids was discussed.

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