TABLE V. Labeling in Epiphyseal Plate. Rats maintained for 10 days on stock diet containing 0.1% isotopic AAN.

| Substance isolated | $C^{14}$ conc./g fresh tissue Cts/min. | $\mu$M |
|---|---|---|
| Polysaccharide fraction* | 95 | .029 |
| Protein | 157 | .048 |

* Approximately 10 mg were obtained per g of plates.

pated in a variety of reactions was the finding of small but definite concentrations of isotope in glycogen and lipids of liver, 4 hours after injecting AAN-$C^{14}$ into rats. The glycogen accounted for 0.5%, and the lipids 0.6%, of the total $C^{14}$ in this organ.

*Summary.* Aminoacetonitrile, with $C^{14}$ in its cyanide group, was synthesized. When this compound was administered to weanling rats, the major portion of the radioactivity was eliminated in the urine, while a minor part appeared in respiratory $CO_2$. High initial $C^{14}$ concentrations were observed in various body tissues. The proportion of this $C^{14}$ due to unchanged nitrile decreased fairly rapidly with time. Labeling was found in tissue proteins, and to a lesser extent in liver lipids and glycogen. Also, in rats fed a diet containing isotopic aminoacetonitrile, radioactivity was detected in the sulfated polysaccharides of the enlarged epiphyseal plates of the bones. The presence of $C^{14}$ in glycine and serine of liver protein and in urinary creatinine, allantoin, and hippuric acid, indicated an appreciable conversion of the cyanide radical into a carboxyl group. However, the major portion of the $C^{14}$-containing metabolites was not identified.

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## Chronic Effect of Dietary Protein on Hypercholesteremia in the Rat.* (22804)

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A diet consisting of casein, lard and dextrin was found to be atherogenic in the rat(1). Variations in fat and cholesterol content of the diet seemed to have little influence on the consequent coronary arterial lesions(2). A reduction in quality or quantity of dietary protein has raised the serum cholesterol level and promoted atherogenesis in other experimental situations(3,4).

The serum cholesterol level of adult cholesterol-fed rats rises gradually over a period of months to reach a plateau, where it remains for at least another 3 to 6 months. The serum cholesterol after 1 or 2 months may not reflect the ultimate level to be reached when the animal has finally adapted to the diet. On the other hand, the great frequency of a chronic form of pneumonitis(5), which is virtually inescapable in the rat after the age of

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TABLE I. Dietary Variables Expressed as % by Weight.

| Series | First | | | | Second | | | Third | |
|---|---|---|---|---|---|---|---|---|---|
| Group | I | II | III | IV | V | VI | VII | VIII | IX |
| Casein | 18.75 | 25 | 12.5 | 7.5 | 18.75 | 9 | 7.5 | 40 | Purina |
| Gelatin | | | | 10.5 | | | | | lab |
| Lard | 30 | 15 | 15 | 15 | 30 | 15 | 15 | 15 | chow |
| Dextrin | 32.2 | 44 | 56.5 | 51 | 32.2 | 60 | 61.5 | 25.4 | |
| Cholesterol | .625 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | |

The constitution of supplementary salt mix, vitamin mix and other ingredients as well as their sources have been described previously(1,2). All groups received 0.625% choline chloride.

6 months, has led us to limit these studies to a 6-month period.

*Method.* Male weanling rats of the Sprague-Dawley stock were fed Purina Lab Chow until 3 months of age (250-300 g), when they were divided into dietary groups. They were fed and watered daily and weighed weekly; the food consumption was estimated daily. Each cage contained no more than 8 rats. Under seconal anesthesia and in the fasting state they were bled from the tail vein at 8 weeks, and by cardiac puncture when sacrificed at 25 weeks. Individual serum cholesterol determinations were done in duplicate by the method of Zlatkis(6) and serum phospholipid by a modification of the Fiske-SubbaRow technic(7) on aliquots of a 3:1 alcohol-ether extract of the serum. Gross autopsy of the sacrificed animals was performed, but histological examination was limited to frozen sections of the heart and liver, which were stained with Sudan III. The diets employed were variations from the original diet of Wissler, *et al.*(1), consisting of 30% lard and 18.75% casein. The level of lard was reduced to 15% in all cases, except for this original diet, and the amount of food offered was correspondingly increased. This lower fat level was introduced in order to minimize the incidence of pneumonitis and seemed to make no difference in the ultimate cholesterol level. An increase in level of cholesterol supplement from 0.31 to 0.625 or even 1.2% made no difference in the ultimate hypercholesteremia, though, as others have noted, the higher levels of dietary cholesterol accelerated the rise in serum cholesterol to that level(8). In each case, protein increments replaced dextrin, the fat level remaining constant. In 2 experiments the above pattern was followed in comparing various levels of casein from 25 to 7.5% and a combination of casein and gelatin as outlined in Table I. In a third experiment, using the diet described for group VIII, the rats were 15 months old at the onset of the experimental period, having been on a Purina Chow diet from the date of weaning and they were bled only after 6 months. In this experiment the rats in group VIII were compared with chow-fed animals of the same age. Lipoprotein concentrations of pooled serum of both groups were compared by the technic of Lewis, Green and Page(9) with the analytical ultracentrifuge.

*Results.* Statistical comparisons (Table II) revealed a level of serum cholesterol in animals fed 12.5% casein for 25 weeks, which was significantly lower ($P<0.01$) than in those fed 25%, 9% or 7.5%. While the cholesterol level attained with 18.75% casein is not enough higher than that reached with 12.5% to achieve statistical significance, it is significantly lower ($P<0.01$) than that resulting from 25% casein or 9% casein in the diet. While it may not be permissible to compare directly the older animals in group VIII with the other groups, their serum cholesterol was so uniquely high for rats on an *ad lib.* diet that they are included here with their chow-fed controls, group IX. No such high levels of serum cholesterol have been observed by us in many other old animals fed the 18.75% casein diet for even longer periods (1,2).

The cholesterol/lipid phosphorus ratios became generally elevated in the first and third experiments where the higher levels of protein were used, and failed to rise significantly at the lower levels of protein fed in the second

TABLE II. Comparison between Mean Serum Cholesterol and Cholesterol/Lipid Phosphorus at Various Levels of Dietary Protein.

| Group | No. of rats | Diet: % casein | Diet: % gel | Wt in g: Initial | Wt in g: Final | Wt in g: Gain | Cholesterol (mg %) ± S.E.: 8 wk | Cholesterol (mg %) ± S.E.: 25 wk | Cholesterol/L. phosphorus (mg %) ± S.E.: 8 wk | Cholesterol/L. phosphorus (mg %) ± S.E.: 25 wk |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Exp. 1 | | | | | |
| I | 18 | 18.75 | | 285 | 435 | 150 | 121 ± 5.0 | 158 ± 7.1 | 24.1 ± .75 | 25.2 ± 1.07 |
| II | 23 | 25 | | 292 | 457 | 165 | 120 ± 3.0 | 224 ± 6.6 | 26.8 ± .70 | 41.9 ± 1.08 |
| III | 22 | 12.5 | | 290 | 413 | 123 | 148 ± 6.1 | 148 ± 5.7 | 25.8 ± 1.08 | 41.3 ± 1.57 |
| IV | 23 | 7.5 | 10.5 | 295 | 354 | 59 | 136 ± 3.4 | 178 ± 6.0 | 23.9 ± .66 | 34.9 ± 2.29 |
| | | | | | Exp. 2 | | | | | |
| V | 8 | 18.75 | | 323 | 506 | 183 | 155 ± 3.8 | 161 ± 5.6 | 23.4 ± .4 | 25.9 ± 1.38 |
| VI | 8 | 9 | | 302 | 400 | 88 | 142 ± 7.9 | 235 ± 9.1 | 23.2 ± 1.4 | 27.2 ± 1.40 |
| VII | 8 | 7.5 | | 307 | 330 | 23 | 121 ± 3.0 | 212 ± 29.5 | 21.4 ± .4 | 24.4 ± 2.60 |
| | | | | | Exp. 3 | | | | | |
| VIII | 10 | 40 | | 510 | 584 | 74 | | 490 ± 75.0 | | 32.5 ± 1.90 |
| IX | 9 | Chow | | 482 | 507 | 29 | | 162 ± 10.2 | | 21.2 ± .82 |

experiment—even though the serum cholesterol was equally elevated. In the first experiment the terminal ratios are significantly higher in groups II, III and IV than in group I ($P<0.001$), and that of group IV is significantly lower than groups II and III ($P<0.02$). The explanation for a greater disproportion between cholesterol and lipid phosphorus at 25% and 12.5% levels than is seen at 18.75% probably depends upon the fact that the percentage of lard was higher in the latter case(10). In the second experiment the phospholipid tends to rise more nearly parallel to the serum cholesterol, so that even with the higher cholesterol levels no statistically significant differences could be established in the ratios. Again in the third experiment where a very high casein level is fed and a phenomenally high serum cholesterol is seen, the cholesterol/lipid phosphorus ratio is elevated. It appears that the hypercholesteremia of high or adequate casein diets is accompanied by a dramatic increase in the cholesterol/lipid phosphorus ratio, which is not seen with the hypercholesteremia induced by the low levels.

Except for occasional pneumonitis in the older animals of the third experiment, all animals were quite healthy until sacrifice, and gave no evidence of disease on gross autopsy. Histological studies of the heart revealed no coronary artery lesions in any of the groups excepting group VIII where 3 out of 9 animals exhibited subintimal fat staining atheromatous lesions identical to those previously reported in younger rats after one year on the diet fed group I(1,2). Serum lipoprotein concentrations were examined in this group and there was a higher level of faster rising lipoprotein fractions with a lower concentration of high density lipoproteins than in the pooled sera from chow-fed controls (Table III).

*Discussion.* The serum cholesterol level of the rat is apparently quite sensitive to the sustained protein level in the diet. When gelatin is added to a low casein allowance, hypercholesteremia tends to be less marked (group IV compared to Group VII) though not significantly different from that seen with an equivalent amount of protein derived solely from casein (group I vs. group IV). There is no significant elevation of the cholesterol/lipid phosphorus ratio in the animals on the low casein intake, as compared with those on moderate or higher levels of casein and comparable fat intake; hence, while we may

TABLE III. mg % Lipoproteins Determined on Pooled Sera of 21-Month-old Rats Fed 40% Casein or Chow for Preceding 6 Months.

| Diet | –S 1.21* 0–20 | 20–40 | 40–70 | 100–400 |
|---|---|---|---|---|
| 40% casein | 95 | 33 | 65 | 152 |
| | 79 | 40 | 57 | 160 |
| Chow | 144 | 38 | 15 | 65 |
| | 169 | 43 | 27 | 96 |

* Flotation rate in $10^{-13}$ cms/sec. of macromolecules in NaCl-KBr medium of density = 1.21.

postulate an amino acid imbalance in the former case, we would anticipate a different mechanism in the latter.

Fillios and Mann(3) have shown that a hypercholesteremia develops in rats fed a methionine deficient diet, and were able partially to correct the abnormal elevation of serum cholesterol by methionine supplementation. A casein diet of 7½% is suboptimal in methionine and permits the development of a fatty liver which may be partially corrected by further protein or methionine supplement (11).

Higher levels of serum cholesterol have been observed in mice fed high casein diets (80%) than in their litter mate controls fed a low casein (10%) diet(12). Other workers have reported a reduction in hypercholesteremic response with increasing levels of dietary casein up to 60%(13). In this case, however, supplemental cholate was included in the diet, thus masking any choleretic effect of the dietary protein.

The possibility must be considered that it is decrease in carbohydrate rather than increase in protein that is responsible for the hypercholesteremia induced in groups II and VIII. The actual amount of dextrin supplied in each diet is given in Table I, and it can be seen that the percentage supplied in group VIII was almost as much as in groups I and V, so that it seems doubtful that the marked elevation in serum cholesterol in group VIII could be due primarily to the low dextrin level, even though Portman and co-workers (14) have demonstrated a difference between corn starch and simpler sugars in choleretic potential and their effect on experimental hypercholesteremia.

The group of rats fed 40% casein developed a higher cholesterol level than heretofore reported in rats on an *ad lib.* diet free of thiouracil or cholic acid supplements. This correlates with a decrease in alpha lipoprotein and an increase in lower density lipoproteins. The development of coronary atheromatous lesions required a shorter period of time than has been noted previously even in rats of the same age(1,2).

*Summary.* Chronic dietary experiments in rats have revealed that months are necessary to evaluate the ultimate level of serum lipids resulting from sustained feeding of various levels of dietary protein. Quantitative changes in dietary protein can influence the levels of serum cholesterol and cholesterol/lipid phosphorus ratio. Reduction or elevation of dietary casein beyond a modest range (12-18%) will lead to ultimate elevation of serum cholesterol and phospholipid under conditions herein described. The insignificant change in cholesterol/lipid phosphorus ratio at low levels of dietary casein as opposed to its elevation at higher levels suggests that different mechanisms are operating in these two situations. Unusually high levels of serum cholesterol were achieved in old rats fed 40% casein. Coronary arterial atheromatous lesions developed in one-third of these animals, but in none of the other groups in the 6-month period.

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