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MECHANISMS OF HYPERLIPEMIA IN EXPERIMENTAL NEPHROSIS *

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Several hypotheses have been advanced to explain the hyperlipemia characteristic of the nephrotic syndrome. The work of Rosenman, Friedman and Byers (1) indicated that the liver fails to remove lipids from the circulation at a normal rate. It was suggested that a loss of lipoprotein lipase into the urine, together with a deficiency of unesterified fatty acids, leads to impaired lipid clearing, and accumulation of lipid in the serum (2). Heymann and colleagues (3), however, measured the rate of disappearance of labeled sodium laurate from the circulation and observed no difference between normal and nephrotic animals. Marsh and Drabkin (4) presented evidence that in severe experimental nephrotic syndrome there is a redistribution of lipid within the body and an associated mobilization lipemia, while in milder degrees of disease hyperlipemia is the result of increased hepatic lipogenesis (5).

We have attempted to determine the source of the excess lipid in the serum by labeling body fats with C^{14} before the injection of nephrotoxic serum and following the changes in the radioactivity of body fat compartments during the development of hyperlipemia. In addition, the rates of removal from serum of chylomicron lipid and of albumin-bound fatty acid were measured in established nephrosis.

METHODS

Production of nephrosis. Nephrosis was induced in rats by injection of rabbit antirat kidney serum prepared by the method of Heymann and Lund (6). The same lot of serum was used for all animals in each experiment. The dose was 0.4 ml. per Gm. of kidney (6), and was given in two equal injections 20 hours apart (animals sacrificed before 20 hours received only one injection of serum).

Isolation and measurement of total fatty acids and cholesterol. Liver and carcass were digested by refluxing

with ethanolic KOH (1 ml. 60 per cent KOH + 2 ml. ethanol per Gm. of tissue). To obtain the cholesterol, an aliquot of the digest was extracted three times with ether (1 volume). The pooled extracts were evaporated to dryness, the residue dissolved in acetone and cholesterol precipitated from solution with a fivefold excess of 0.5 per cent digitonin in 50 per cent ethanol. The precipitate was dissolved in glacial acetic acid and aliquots were taken for counting and for chemical determination (7). A standard curve was prepared with cholesterol digitonide.

The aqueous phase remaining after extraction of cholesterol was acidified and fatty acids were extracted with petroleum ether. The extracts were evaporated to dryness and made up to a known volume in ethanol. Fatty acids were determined gravimetrically in an aliquot of the ethanolic solution.

Serum. Two ml. aliquots were saponified with ethanolic KOH. Cholesterol was extracted with petroleum ether and purified by precipitation of the digitonide. The precipitate was dissolved in glacial acetic acid and aliquots were taken for chemical determination and counting as previously described.

The aqueous phase was acidified and fatty acids were extracted with ether. The ether was evaporated to dryness and the residue was dissolved in ethanol; fatty acids were determined on an aliquot of the ethanolic solution by oxidation with dichromate (8). Results were expressed in terms of palmitic acid. There was good agreement between the results of the chemical determination and direct weighing of fatty acids.

Serum unesterified fatty acids (UFA) were measured by the method of Gordon (9).

C^{14} chyle. This was prepared by the method of French and Morris (10), using tripalmitin-1- C^{14} . Animals received intravenously 1 ml. of chyle containing 36 mg. of lipid and 5×10^6 cpm.

Radioassay. Aliquots of the fatty acid and cholesterol fractions were plated onto aluminum discs with lens paper and counted in a windowless proportional flow gas counter. All samples were corrected for self-absorption from a curve constructed with C^{14} -labeled fatty acids which were isolated from the liver of a rat previously injected with sodium acetate-1- C^{14} .

Electrophoresis. Paper electrophoresis was carried out on Whatman paper No. 3 in veronal buffer of pH 8.6 and ionic strength 0.075, at room temperature. The dried strips were stained with bromphenol blue, scanned in an automatic integrating densitometer, then cut into 2 mm.

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segments which were placed in the flow counter for measurement of radioactivity. Starch block electrophoresis was performed by the method of Sehon, Harter and Rose (11). After separation of proteins the block was cut into segments of 0.5 cm. width, and the proteins eluted. The eluate was analyzed for protein (12) and for radioactivity.

Animals were adult male Sprague-Dawley rats weighing 225 to 250 Gm. at the onset of the experiments. When fed by gavage the diet used was 57 per cent carbohydrate, 24 per cent protein and 15 per cent fat; this was given in two daily feedings of 12 ml. each at 9 a.m. and 5 p.m. There was free access to water at all times. The animals were housed five to a cage, in a room maintained at a constant temperature of $78 \pm 1^\circ$ F. and relative humidity of 40 to 50 per cent.

RESULTS

A. Incorporation of acetate-1-C¹⁴ into lipids

In a preliminary experiment rats were injected intraperitoneally with carrier-free sodium acetate-1-C¹⁴ (6.6×10^6 cpm dissolved in 5 ml. water) and sacrificed at intervals thereafter. It was found that incorporation of radioactivity into fatty acids and cholesterol of serum, liver and carcass was maximal between two and seven hours after the injection. In subsequent experiments zero time was therefore taken as four hours after administration of labeled acetate.

B. Relation of lipid specific activities in liver, serum and depots during development of hyperlipemia

Animals were adapted to tube-feeding over a period of 14 days. Two hours after a regular feeding, they were injected intraperitoneally with 1 ml. of carrier-free sodium acetate-1-C¹⁴ in water (approximately 35×10^6 cpm). Four hours later (zero time), a group of five animals was sacrificed. The remainder was randomly divided into two groups, one of which was given anti-kidney serum to induce nephrosis, the other, saline. Feeding was continued on the previously established schedule. Subgroups of five or six animals from each group were sacrificed at intervals up to 72 hours after zero time. The sera, livers and carcasses, respectively, of each subgroup were combined and fatty acids and cholesterol extracted from each pool. The final subgroups of animals were sacrificed at 96 hours; in this case the extractions were carried out on individual nephrotic livers and sera, but on pooled carcasses.

Following injection of ant kidney serum, fatty acids and cholesterol increased markedly in serum (the differences between the regressions of lipid on time were significant at the 1 per cent level for both fatty acids and cholesterol), and moder-

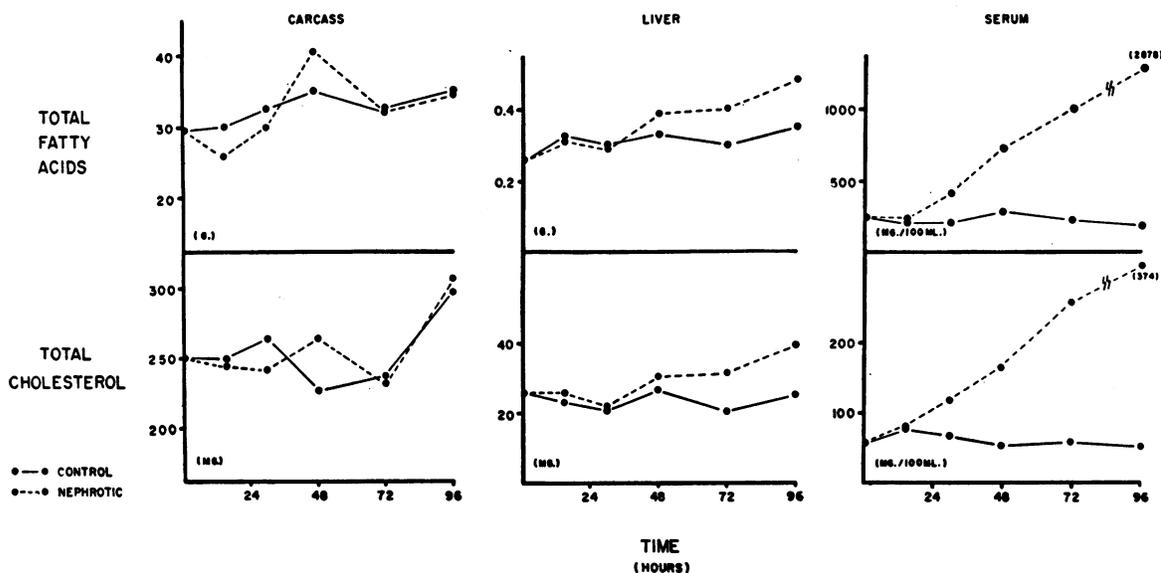


FIG. 1. CHANGES IN FATTY ACIDS AND CHOLESTEROL FOLLOWING INJECTION OF ANTIKIDNEY SERUM

Each point represents mean value of 5 to 6 animals. Carcass and liver values are for total lipid content; serum values are for concentration. (Solid line, control animals; broken line, nephrotic animals.)

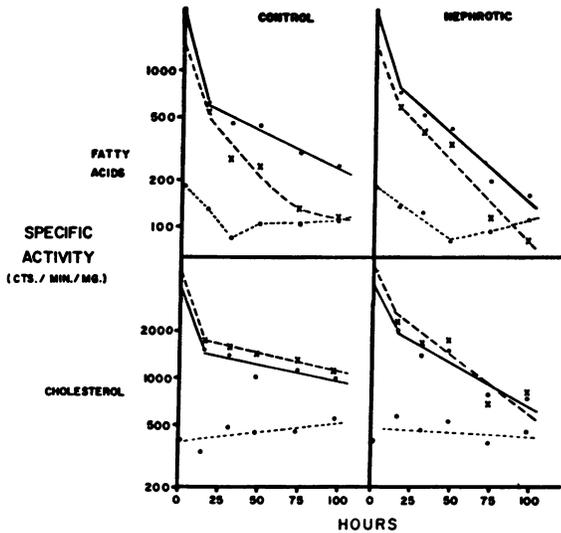


FIG. 2. CHANGES IN SPECIFIC ACTIVITY OF LIPIDS DURING DEVELOPMENT OF NEPHROTIC HYPERLIPEMIA

Animals were given acetate-1-C¹⁴ to permit labeling of endogenous lipids, and four hours later (Time 0 in figure) injected with ant kidney serum (·—· liver; — serum; ···· carcass).

ately in liver ($p < .01$ for fatty acids and $< .02$ for cholesterol); carcass lipids did not differ significantly from those of control animals (Figure 1). The time courses of specific activities (Figure 2) differed in normal and nephrotic animals.

(a) *Fatty acids.* In normal rats, serum specific activity fell rapidly to the level of carcass specific activity; in plotting log specific activity against time, the line of best fit was found to be curvilinear, represented by the equation $Y = 2.9398 - 0.0169X + 0.0000807X^2$, with significant deviation from linearity ($p < .05$). The liver specific activity fell rapidly during the first 15 hours, then more slowly in an exponential manner with a rate constant (K)¹ of $-.0046$. In nephrotic rats after 15 hours the liver specific activity fell more rapidly than normal ($K = -.0086$) in a curve almost parallel to that of serum ($K = -.0110$). The serum specific activity in all nephrotic animals at 96 hours was lower than that of the pooled carcass fatty acids. The carcass specific activity in both groups showed an initial fall, then a plateau.

(b) *Cholesterol.* In both normals and nephrotics, following an initial rapid decline, the specific activities of serum and liver fell in a parallel manner, but more rapidly in the latter group; the rate constants for serum and liver, respectively, were $-.0024$ and $-.0020$ in the normals and $-.0064$ and $-.0053$ in nephrotics. Changes in carcass specific activity were not marked in

¹ K = change in log specific activity per hour.

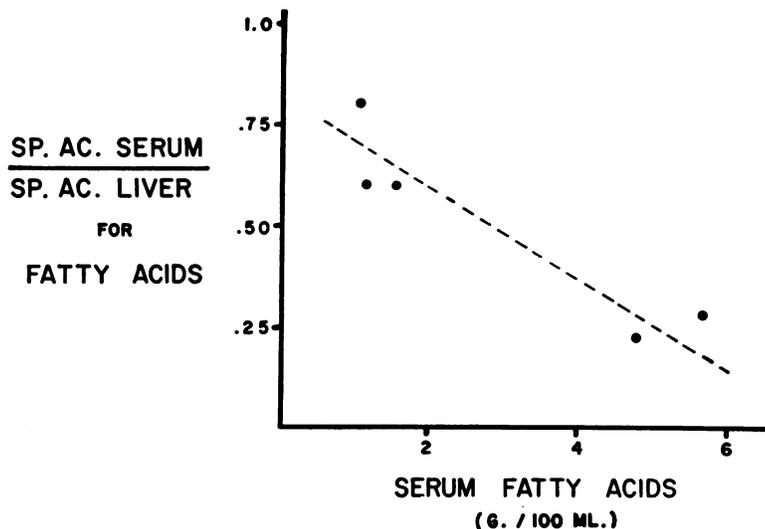


FIG. 3. RELATION BETWEEN DEGREE OF HYPERLIPEMIA AND RATIO:

$$\frac{\text{SPECIFIC ACTIVITY SERUM FATTY ACIDS}}{\text{SPECIFIC ACTIVITY LIVER FATTY ACIDS}}$$

Values are from nephrotic rats 96 hours after injections of ant kidney serum.

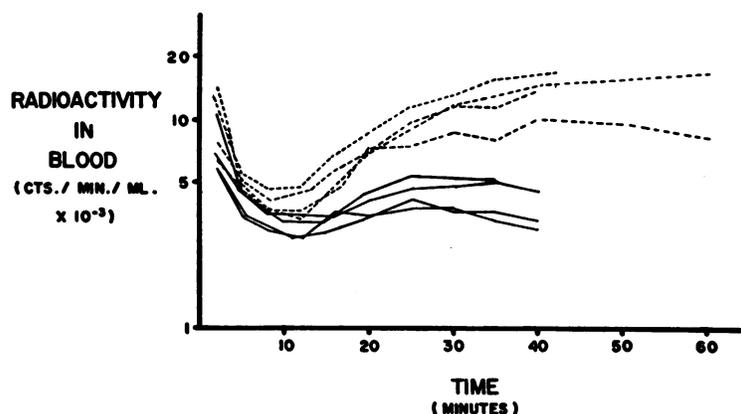


FIG. 4. RADIOACTIVITY IN WHOLE BLOOD FOLLOWING INJECTION OF PALMITIC ACID-1-C¹⁴ BOUND TO SERUM ALBUMIN (Solid line, control animals; broken line, nephrotics.)

either case, and the rate constants did not differ significantly from zero.

In nephrotic rats sacrificed at 96 hours there appeared to be an inverse relationship between concentration and specific activity of serum fatty acids. To overcome the influence on specific activity of variations in initial incorporation of C¹⁴ into lipids, the ratio: specific activity serum fatty acids/specific activity liver fatty acids was plotted against serum lipid concentration and an inverse relationship was again indicated (Figure 3).

C. Rate of disappearance of unesterified fatty acid from serum

An albumin-sodium palmitate-1-C¹⁴ complex was obtained by incubation of the labeled acid (1 mc. per mMole) with normal rat serum at room temperature for 30 minutes. Four normal and four nephrotic rats were given only 50 per cent glucose in water to drink overnight, and 3 ml. of 50 per cent glucose by stomach tube the next morning. One hour later they were given intravenously 1 ml. of the labeled serum containing 1 μ c. of C¹⁴. Aliquots of blood (0.05 ml.) were obtained from the tail tip at accurately timed intervals thereafter and plated directly for measurement of radioactivity. UFA concentrations were measured in other groups of eight normal and eight nephrotic animals similarly prepared with 50 per cent glucose.

In both normal and nephrotic rats the concentration of radioactivity fell rapidly in the first five minutes, reached a minimum value at 10 to 15

minutes and then began to rise (Figure 4). There was no gross difference in the initial rates of fall between the two groups. However, the level of activity reached in the secondary rise was considerably higher in the nephrotic than in the control animals.

Serum unesterified fatty acid concentrations (mean \pm S.D.) were 0.11 ± 0.02 mEq. per L. for normal rats and 0.15 ± 0.07 mEq. per L. for nephrotics. The difference was not significant at the 5 per cent level.

In order to determine the nature of the radioactive material re-entering the circulation, normal and nephrotic animals were exsanguinated one hour after an injection of albumin-sodium palmitate-1-C¹⁴. A negligible amount of radioactivity was associated with erythrocytes. The plasma activity was nondialyzable and entirely soluble in Bloor's reagent. It could not be extracted from carbon tetrachloride-ether (18:1) by 1 N KOH (preliminary experiments established that 95 per cent of unesterified fatty acids and less than 1 per cent of esterified fatty acids were extractable by 1 N KOH). On paper electrophoresis it was distributed in the regions of α and β globulins (Figure 5), while on starch electrophoresis it moved ahead of the albumin.

D. Rate of removal of labeled chylomicrons from serum

Normal and nephrotic rats were prepared with 50 per cent glucose feedings as above, and then injected with 1 ml. of labeled chyle containing 46

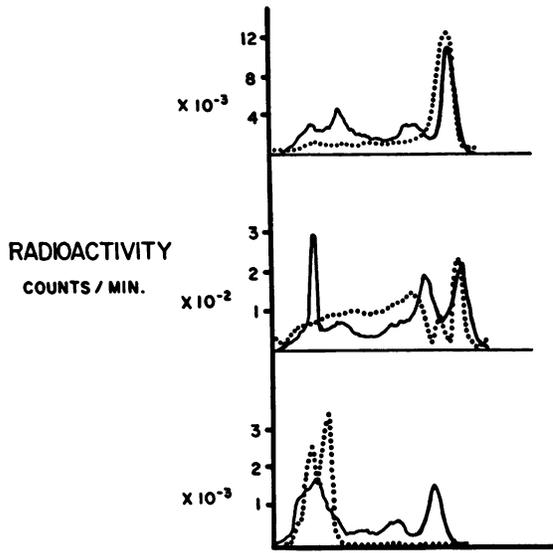


FIG. 5. ELECTROPHORETIC DISTRIBUTION OF RADIOACTIVITY

Top: palmitic acid-1-C¹⁴ bound to serum albumin, as injected into normal and nephrotic rats. Middle: plasma from nephrotic rat 60 minutes after receiving albumin-palmitate-1-C¹⁴ complex. Bottom: chyle containing palmitic acid-1-C¹⁴ as injected into normal and nephrotic rats. In all cases the solid line represents protein concentration, the dotted line, radioactivity.

mg. lipid. Blood samples of 0.05 ml. each were taken at 5 to 10 minute intervals and plated directly for radioactivity measurement. In normal rats the time curve of C¹⁴ concentration had at least two components (Figure 6), similar to that found by French and Morris (10). The mean half-life (by inspection) during the interval 15

to 40 minutes was eight minutes, compared to a value of 11.5 minutes found by these workers after injection of somewhat more lipid into smaller animals. In the present experiment, sampling was not continued long enough to get a measure of the half-life for the later part of the curve. In nephrotic rats the time curve was quite different, since there appeared to be only one component with a half-life varying from 28 to 160 minutes. The chylomicron count on blood from both normal and nephrotic animals prepared with 50 per cent glucose feedings as described was lower than five.

DISCUSSION

The first experiment described above differs in two respects from similar work previously reported: body lipids were labeled prior to development of nephrosis, and dilution of labeled fats by exogenous lipids was controlled by tube feeding. Total fatty acids are heterogenous, being derived from neutral fat, phospholipid and cholesterol esters; cholesterol may be free or esterified. The results, as in earlier work, therefore reflect the averages of the changes within these subclasses. In addition, the specific activities determined experimentally were means for each of the three anatomical pools, since each such pool may consist of more than one "functional" pool, each with its own initial specific activity and turnover rate. Within the framework of these facts certain qualitative conclusions may be drawn from the relationships among the curves of specific activity.

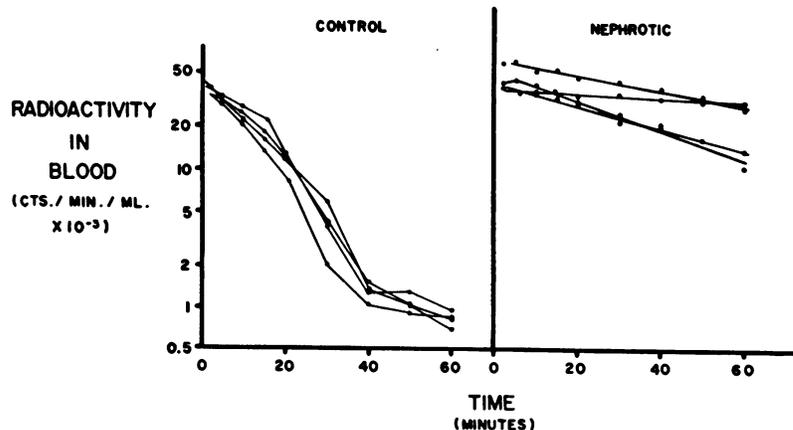


FIG. 6. RADIOACTIVITY IN WHOLE BLOOD FOLLOWING INJECTION OF C¹⁴-LABELED CHYLE, CONTAINING 36 MG. OF TOTAL LIPID

With regard to fatty acids, the rapid fall in liver specific activity during the first 15 hours suggests that this organ contained at least two separate pools, one having a very high turnover rate, the other(s) a lower rate (Figure 2). A similar situation may also have obtained in the carcass but the data are not as definite. It can reasonably be assumed that an insignificant amount of lipid synthesis or degradation occurred in the serum, and that the decrease in serum specific activity was entirely due to replacement by lipid from lower specific activity sources. The shape of the specific activity curve in serum may be explained by several hypotheses, one of which is that serum lipid was derived in part from the rapidly metabolizing pool in the liver and in part from a carcass pool whose specific activity was close to that of the whole carcass and whose turnover rate was low.

In the nephrotics there occurred: (1) increases in the lipid content of serum and liver, (2) an increase in the rate of fall of liver specific activity, (3) a fall in serum specific activity below that of the whole carcass. These changes may have been due to (a) retention of lipid in the serum, (b) increased mobilization of lipid to the plasma, and subsequent movement of part of this "excess" lipid to the liver, (c) increased synthesis and discharge of lipid by the liver, or (d) a combination of these.

Retention of lipid in the serum would not of course account for the increase in lipid content of the liver; if hyperlipemia were due to retention alone, there must also have occurred an increase in synthesis or a decrease in degradation of lipid in the liver.

Increased mobilization from the carcass pool with which serum normally appeared to equilibrate, in the absence of any other change, would have caused a more rapid fall than normal in serum specific activity to the equilibrium level. In addition, mobilization of lipid from the carcass to liver, via the serum, in an amount sufficient to produce the observed increase in liver content (even if entirely derived from a constituent pool of zero specific activity) would have altered the rate constant for liver specific activity by a maximum of 20 to 25 per cent rather than by the observed value of approximately 90 per cent. However, mobilization together with an increase in the rate of interchange between serum and a hepatic

pool of higher specific activity could have produced all changes noted.

Alternatively all observed changes could have been produced by increased hepatic synthesis and discharge of lipid alone or with either of the other two mechanisms. The fall in the plasma specific activity below that of the whole carcass indicates that the average specific activity of the pools with which the plasma equilibrated was lower in nephrotic than in normal animals. (The experimental finding was confirmed in a second group of animals in which the last feeding was omitted in order to obviate the possibility of isotope dilution by dietary lipids.) One explanation for such a phenomenon is that one or more of these pools underwent an increase in size, turnover rate (by pathways other than exchange with serum) or both. In the case of the liver this could explain the increases in lipid content and in the rate of fall of specific activity of the whole organ, as well as the inverse relationship between serum lipid concentration and the ratio: $\frac{\text{serum specific activity}}{\text{liver specific activity}}$.

As anticipated from the fact that the liver is the major site of cholesterol synthesis and that there is normally a constant interchange of cholesterol between liver and serum, there was in normal rats a close similarity between specific activities of cholesterol in these two pools. In principle, the considerations applied to the hypotheses of retention and hepatic synthesis of fatty acids are equally pertinent in the case of cholesterol. However, one need not here postulate an increase in the rate of interchange between serum and liver as an accompaniment of mobilization, since the normal rate of interchange was apparently sufficiently high to maintain a close relationship between the specific activities in these pools.

The observed changes then may be adequately explained on the basis of increased hepatic synthesis and release of lipid, increased hepatic synthesis together with retention of lipid in the serum, or mobilization from carcass to serum and liver, with an increased (fatty acids) rate of exchange between these two pools. It is apparent that the hypotheses of mobilization and of decreased movement of serum lipid to the liver (1) are mutually exclusive. Since later experiments did indicate the occurrence of "retention," we believe that mobilization did not play a role in producing hyper-

lipemia. This view is compatible with the findings of Marsh and Drabkin (5) and of Heymann and Hackel (13), indicating that the development of hyperlipemia resulted largely from increased lipid synthesis and release by the liver. They did not exclude the possibility that decreased removal of lipids from serum by liver was also an etiological factor in the hyperlipemia (1). In order to test this concept, the rate of disappearance of radioactivity from the circulation was measured after administration of labeled UFA-albumin complex and of labeled chylomicrons. In the former case, because of its initial extreme rapidity, the rate of removal of radioactivity could not be measured accurately; there was, however, no gross difference between the normal and nephrotic animals in this regard. That this was not an artifact due to differences in isotope dilution is indicated by the similarity in UFA concentrations.

There was a distinct decrease, however, in the rate of removal of chylomicron lipid from the serum of nephrotic rats. Isotope dilution was not a factor in this result since the dietary preparation of the animals reduced endogenous chylomicron levels to a minimum value. The results of the chyle experiment are compatible with those of early work in human nephrotic subjects (14, 15) and with those of Friedman, Rosenman and Byers on the metabolism of ingested cholesterol (16). They are in contrast to those of Heymann and associates (3), who injected intravenously labeled trilaurin together with synthetic emulsifiers and observed no difference between normal and nephrotic rats in the rate at which radioactivity left the blood stream. However, this was not a physiological form of serum lipid and its behavior may not reflect that of endogenously synthesized lipoproteins. Furthermore, the fate of intravenously administered emulsions is dependent on the particular emulsifying agent used (17). This does not argue against the recent observation (18) that urine contains an agent which reduces hyperlipemia; this agent may be lipoprotein lipase itself or a cofactor (1, 19).

It has been shown that triglycerides, including those of chylomicrons, may be converted to UFA by the action of lipoprotein lipase (20-23), which is released into the plasma following ingestion of lipid (24). It has been suggested (25) that a major factor in producing hyperlipemia in nephro-

sis is an inhibition of lipoprotein lipase activity secondary to the decrease in the amount of serum albumin available to act as an acceptor of UFA released by the enzyme. The present results are compatible with this view. However, since most chylomicron lipid is removed without prior conversion to UFA (26), it is likely that the defect is one of tissue rather than plasma lipoprotein lipase. These possibilities are presently being investigated.

An interesting incidental finding after administration of UFA-albumin complex was the reappearance of radioactivity in increased amount in the circulation of nephrotic rats. Positive identification has not been made of the form of this radioactivity, but in agreement with recent work in man (27), the evidence available suggests that it is associated with esterified fatty acids. This is further presumptive evidence of increased hepatic synthesis and turnover of lipid in nephrosis.

The present experiments indicate that there are at least two defects in lipid metabolism to account for the development of hyperlipemia in experimental nephrosis: (a) increased entry of lipid into the serum, and (b) decreased ability to remove chylomicron lipid from the circulation.

SUMMARY AND CONCLUSIONS

1. Normal rats were given injections of acetate- $1-C^{14}$ to permit endogenous labeling of fatty acids and cholesterol. Some of the animals were then injected with anti-kidney serum to produce nephrosis, and the distribution of radioactivity among body lipid compartments measured at intervals during the development of hyperlipemia. The changes in content and specific activity can best be explained on the basis of increased hepatic synthesis and discharge of lipid together with retention of lipid in the serum.

2. After intravenous injection of palmitic acid- $1-C^{14}$ bound to albumin, nephrotic rats disposed of the radioactivity from the serum at a rate similar to normal. The radioactivity later reappeared in the circulation, probably as esterified fatty acid, to a greater degree in nephrotic than in normal animals.

3. After intravenous injection of chyle containing tripalmitin- $1-C^{14}$ radioactivity disappeared from the circulation of nephrotic rats at a much lower rate than normal.

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