Genesis of Hyperlipemia in Experimental Nephrosis

by

Harbhajan Singh Sodhi, B.A., M.B., B.S., M.Sc.

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Investigative Medicine,
McGill University,
Montreal

May, 1962

Acknowledgements

To Dr. J. S. L. Browne, Chairman, Department of Investigative Medicine, I owe a debt of profound gratitude and respect. I am thankful for having an occasion to associate with and learn from the teacher, the scientist and the gentleman in him.

It gives me a great pleasure to place on record my heartfelt appreciation and thankfulness to Dr. N. Kalant, Assistant Director, Research Laboratories of the Jewish General Hospital, for helping me along the way of scientific inquiry. I owe this work to his continuous supervision, stimulating criticism and patient guidance.

Studies on the effects of delipidized proteins of plasma lipoproteins on cholesterol synthesis by liver homogenates, were done in collaboration with Dr. K. H. Lee, whose help in measuring cholesterol synthesis by liver homogenates is gratefully acknowledged. My thanks are also due to Dr. I. Guttman of McGill University for his help in the statistical analysis of turnover rates of cholesterol.

Invaluable technical assistance rendered by Miss E. Brunner and the help of Mrs. M. Aikman and Mrs. N. Philips are acknowledged with thanks. I would like to express my appreciation for the cheerful cooperation of Mr. George Berci and Mr. William Gally in studies on animals; Mrs. H. Gally in preparation of charts and Mr. N. Feifer

in photographing all the figures presented in this work.

My special thanks go to Miss M. Wheeler for typing this manuscript.

It pleases me to mention my friends for their help in the preparation of this thesis: Dr. R. Schucher, for many helpful discussions throughout the period of this work: Mrs. Lolly Galt and Dr. H. Bergman for checking and correcting the manuscript.

I would like to convey my appreciation for the help so kindly extended by Dr. C. Giroud and the Staff of the Endocrinology Research Laboratories of the Montreal Children's Hospital, in the use of the Liquid Scintillation Counter.

Lastly, I acknowledge the material support and facilities given by the Medical Research Council of Canada and the Jewish General Hospital, Montreal, for the execution of the present work.

Table of Contents

Introduction

The nephrotic syndrome has been defined as "renal loss of albumin resulting in diminished serum concentration, variably associated with hyperlipoidemia and edema" (1). It has been clear since the time of Richard Bright (2) that some patients with renal disease are edematous and have lipemic serum and marked proteinuria. Association of the nephrotic syndrome with other systemic diseases such as lupus erythematosus (3), diabetes mellitus (4, 5) and amyloidosis (6), and with infections such as syphilis (7, 8) and malaria (9) has been observed. It has also been described as the result of renal vein thrombosis (10, 11), drug toxicity, especially from mercurials (12), bee sting (13), and poison oak (14) reactions. Finally, the development of this syndrome has been noted during the course of chronic glomerulonephritis (15-17) and so called "lipoid nephrosis" of children may or may not fall into this group (18).

A similar type of disease can be produced experimentally in rats either by intravenous injections of antiserum against rat kidney produced in the rabbit (19) or by daily subcutaneous injections of 6-dimethyl-amino-9-3'-amino-3'-deoxy-b-D-ribofuranosyl purine, the amino nucleoside derivative of the antibiotic Puromycin (20).

Examination of kidney sections with the light microscope

reveals in the "lipoid nephrosis" of children and in both varieties of experimental nephrosis, a thickening of the basement membrane, an increase in the number of Periodic Acid Schiff positive droplets in the proximal convoluted tubules, and a decrease in alkaline phosphatase in the tubules (21-23). Electron microscopic studies of kidney sections also show identical changes in the lipid nephrosis of children and in the aminonucleside nephrosis in rats. These changes consist of swelling and coalescence of the foot processes of the epithelial cells covering the glomerular basement membrane, and an increase in the number and size of the cytoplasmic vacuoles within these cells (24-26).

The data obtained (27) in various experiments support the idea that aminonucleoside acts as an antimetabolite during the production of the nephrotic syndrome in rats. Aminonucleoside inhibits the formation of adenine-triphosphate from inorganic phosphate and adenosine by brewer's yeast (28) but its effects on organs other than kidney have not been studied adequately so far.

When antikidney serum (AKS) is injected into the rat its nephrotoxic effects result from an antigen-antibody reaction, as evidenced by a fall in complement (29) and a localization of antibody in the renal glomerulus (30). The specific

site of antibody localization and therefore by inference the localization of the specific antigen(s) is in, or on, the glomerular basement membrane (31, 32). There are multiple glomerular antigens (33) which are presumed to be associated with basement membrane of pre-capillary, capillary and post-capillary vessels (34, 35). After injection of AKS antibodies are detected in various organs of the body (36) but the clinical picture of hyperlipemia, hypoproteinemia, proteinuria, and anasarca resulting from the antibody localization is referable to the kidney; it may therefore be inferred that the basement membrane of the glomerulus is the site of the most important lesion of AKS disease.

After a detailed study of AKS nephrosis in rats, Heymann and Lund (22) came to the conclusion that "In symptoms and course, in the deviation of the blood chemistry and in the morphology of the renal lesion, this disease in rats simulates the nephrotic syndrome as observed in infants and children".

In order to elucidate the genesis of hyperlipemia of nephrosis, the experimental disease produced in rats by the injection of AKS was used as a model for the studies herein reported.

Survey Of The Literature

Body Lipids

Since the levels of the several lipids in the blood mirror the conditions which obtain in the tissues, any abnormality which results in variations in the blood picture is usually to be ascribed to an alteration of lipid metabolism in the tissues themselves.

According to Reed et al. (37), normal rats, weighing 250 g., after having completed their period of most active growth, had the following approximate distribution in per cent of total fat in the several fat depots: intramuscular, 9; genital, 13; subcutaneous, 53; peritoneal, 16.4; mesenteric, 6; and omental 2.6. Female rats had somewhat larger proportions of subcutaneous and genital fat.

When animals are allowed to starve to death, their carcasses still contain an appreciable amount of fat, which appears to be fairly constant for each species (38). The component of fat used as a source of calories is called the variable component and consists almost exclusively of neutral fat, whereas the constant components consist predominantly of phospholipids and cholesterol (39). It is believed that constant components serve as essential elements of the protoplasm, they are thus called essential lipids (39).

Adipose Tissue

Adipose tissue constitutes an important link in the metabolic processes of the animal (40). It has been shown to develop from specific primitive cells which are distinct from fibroblasts of the connective tissue (41), and it has its own supply of blood vessels and nerves (42).

Adipose tissue in rats is usually found in the subcutaneous and retroperitoneal areas; it is also found in the mesentery, in the mesometrium of females, and in the epidydimal fat pads of males (43). Adipose tissue is not limited to these regions but may develop in almost any areolar connective tissue. The amount present varies greatly with the nutritional state of the animals (43).

Of the lipids extracted from normal human adipose tissue, more than 99% is triglyceride, only 0.3% is cholesterol, and less than 0.1% is phospholipids (44). Major fatty acids present in rat adipose tissue contain 10 to 18 carbons; C16 and C18 fatty acids, saturated as well as unsaturated, form by far the greatest fraction of total fatty acids (45) present in adipose tissue.

The metabolic activities of adipose tissue reside primarily in the synthesis of lipids from different substrates, their storage, mainly as triglycerides, and the release of lipids as unesterified fatty acids (46).

It is now well established that adipose tissue is able to synthesize glycogen (47-49) and glycerol (50), to oxidize glucose, pyruvate and acetate (49, 51-54), and to synthesize long-chain fatty acids from acetate (51, 55), glucose (52, 53) and propionate (56). Adipose tissue can also synthesize phospholipids (46).

In addition to its ability to synthesize lipids, adipose tissue is capable of removing fat from serum and other media (57-59). This uptake has been observed with neutral fat and fatty acids but not with phospholipids or cholesterol (57). The uptake is markedly influenced by the nutritional state of the animal. It is small in tissues obtained from fasted animals and greatly increased in tissues obtained from animals re-fed following a starvation period (57).

Regulation of Mobilization and Deposition of Fats in Adipose Tissue:

The deposition of fat in adipose tissue and its subsequent mobilization is one of the major processes in the energy metabolism of the animal organism. The bulk of the food fat (60, 61), as well as a considerable portion of the carbohydrates ingested (62) is converted into adipose tissue fat. The rapid mobilization of fat under various conditions brings about an accumulation of fat in the liver (63, 64).

The autonomic nervous system plays an important role in regulating fat mobilization and deposition. Unilateral denervation of symmetric fat bodies results in diminished rate of lipid depletion from the denervated site during fasting (40). Spinal cord section produces a significant inhibition of fat mobilization from the epidydimal fat pad in fasted restrained rats (65).

The sympathetic nervous system plays a particularly important role in the regulation of the mobilization of fatty acids from adipose tissue (66 , 67) , probably via the release of norepinephrine from nerve endings (46). Norepinephrine and epinephrine both have been shown to enhance lipolysis in vitro (68 , 69). They also enhance lipolysis in vivo , as indicated by an increase in unesterified fatty acid concentration in blood (70 , 71). Norepinephrine has a greater lipolytic activity than epinephrine in vivo. The addition of epinephrine to the incubation medium produces a significant increase in the lipolytic activity extractable from adipose tissue (72). After total adrenalectomy , both epinephrine and cortisone are required to bring fat mobilization back to normal (73). Neither is effective alone. In the normal animal , injection of epinephrine leads to a decrease in carcass fat (74) and increase in liver lipids (75).

Adrenocortical hormones seem to have another effect on fat deposition. The depletion of fat stores of adrenalectomized animals with restricted food intake is much more rapid than that of normal animals (76 , 77). This can be prevented by treatment with cortisone.

The effect of the anterior pituitary on fat mobilization is well established. (78 - 80). The hypophysectomized animal has a higher proportion of body fat than normal animals on the same caloric intake. At the same time liver fat levels are low (81) and are not increased by fasting (82) as they are in the normal animal , due to a relative inability to mobilize fat from peripheral depots.

It is probable that the fat mobilizing activities of anterior pituitary extract reside in growth hormone as well as in a distinct pituitary fraction (83 ,84). The effects of treatment with anterior pituitary extract or purified growth hormone on fat metabolism are essentially the reverse of those subsequent to hypophysectomy. The total fat content of the body decreases concomitantly with a net increase in protein. Fat is rapidly mobilized to the liver producing a fatty liver (79 , 85-87) and ketosis (88 ,89). ACTH has also been shown to have fat mobilizing effects in both <u>in vitro</u> and <u>in vivo</u> experiments (90-93). A new hormone , prepared from the anterior pituitary , which

causes a marked increase of the plasma unesterified fatty acid concentration in the rabbit has been reported. This preparation has been assayed for the activity of all the other known pituitary hormones, and no other activity could be demonstrated (94, 95). There are other reports which provide evidence of the existence of a factor elaborated by the posterior pituitary gland and possessing marked mobilizing effects on the depot fat (96-98).

Liver Lipids.

The liver plays an important and unique role in the metabolism of the various lipid components. Hepatic cells have the ability to synthesize fatty acids (99), cholesterol (99, 100) and phospholipids (101), and to incorporate these lipids into lipoproteins (102).

The liver of normal rats on a stock diet contains lipids amounting to 3.5 to 4% of the weight of the liver (103). The data in Table 1 show the distribution of the different lipid components in the cell fractions of rat liver (104).

It is clear that while the fractions vary with respect to amount of lipid present, the phospholipid as per cent of total lipid, is quite similar from one fraction to another except for the supernatant. The relatively small amount of lipid, other than neutral fat, in the supernatant indicates that the

Table 1

Lipid Composition of Rat Cell Fractions (104)

Fraction	Total Lipid % Dry Weight	% Total Lipid		
		Phospholipid	Cholesterol	Neutral Fat
Nuclei	16	93	4.5	2.5
Mitochondria	21	93	5.5	1.4
Microsomes	32	94	5.8	0
Supernatant	7	28	3.9	68

intracellular lipids of the liver cell are predominantly associated with particulate fractions.

In another study the composition of liver lipids in rats 7 weeks after weaning and on a diet containing 10% cotton seed oil was as follows: total lipid, 519 ± 20 mg.; total cholesterol, 31 ± 3 mg.; unesterified cholesterol, 22 ± 0.4 mg.; phospholipids, 357 ± 6 mg. (105). Linoleic acid seemed to be selectively retained in the liver and used in esterification of cholesterol, and the proportion of fatty acids of cholesterol esters consisting of linoleic acid was higher than in plasma, whereas the proportion consisting of arachidonic acid was much lower than in the plasma (105). The fatty acid components of the liver triglycerides resembled those of the dietary fat to a greater extent than did the fatty acids of cholesterol esters. The composition of the phospholipids was more nearly independent of diet than was that of other lipids (105).

The adult levels of liver cholesterol in rats are lower than those of fetal or newborn, though the highest levels are observed around the age of 15 days (106). The liver of the female rat contains less total sterol and the amount esterified is significantly lower than in males (107). The weight of the liver of an animal starved to death is decreased considerably, but there is no decrease in the absolute amount of sterol present (38).

Lipids in Erythrocytes.

Brun (108) reported that the total cholesterol content of erythrocytes from 58 normal subjects was 139 mg./100 ml. of packed red blood cells, with a standard deviation of 5.2 mg. He also found that all the cholesterol was unesterified. This has been recently confirmed (109). Erickson et al. (110) reported that the cholesterol in the red blood cells of children averaged 129 mg.%, as much as 32% being in ester fraction. The same workers later reported (111) that the amount of esterified cholesterol varied from 0 to 1.1% between animal species. In a more recent study, using more refined methods (112), cholesterol esters were shown to be present to the extent of 5% of the total cholesterol in the pooled human red blood cells. The fatty acids of the red cell cholesterol esters were saturated in contrast to those of serum cholesterol esters. In rabbit erythrocytes no cholesterol esters were detected (113).

Sixty five per cent of total lipids in the erythrocytes of the normal adults are made up of phospholipids (109). Triglycerides could not be detected in the red blood cells, according to Abderhalden (114). Recently however, triglycerides were detected in the lipids of pooled human erythrocytes, though they constituted less than 0.3% of the total lipids (112); this amount could not have been detected by the techniques available

to Abderhalden. Unesterified fatty acids were reported to make up to 2.5% of total lipids of the red cells (112).

Brun (108) reported that repeated saline washings of the red blood cells before extraction caused no loss of cholesterol; this was confirmed by Reed et al. (109) who showed that repeated washings up to 6 times caused no loss of lipid from cells.

Lipids of Leucocytes.

The average values for white cells of women expressed in mg. per 100 g. were as follows: total lipid, 1710 ± 734; neutral fat, 536 ± 536; total fatty acids,, 73 ± 65; total cholesterol, 300 ± 60; unesterified cholesterol, 194 ± 110 and phospholipids, 802 ± 255 (115).

Plasma Lipids.

Introduction:

All types of lipids which are present in the tissues of animals occur in greater or lesser amounts in the plasma. Although the lymphatics provide the chief initial pathway for the transport of fats from the intestines, the blood is the ultimate avenue for the further distribution of these substances to the liver and to the fat depots. In addition, plasma serves as the medium of transfer of the lipid components from one organ to another.

The Nature of Plasma Lipids:

The plasma lipids consist of fatty acids, neutral fats, phospholipids and unsaponifiable components including cholesterol, carotenoids, and vitamins A, D, E and K. All the lipids are present in association with proteins in the plasma (116-119). Unesterified fatty acids exist in loose association with albumin (120, 121), and practically all the remaining lipids form structural complexes called "lipoprotein molecules", with proteins. The blood lipids, which are carried from the intestinal tract in the chyle, are mixed with the blood when the thoracic duct empties its contents into the blood stream. The fine droplets, which are composed largely of neutral fat,

originally present in the chyle, are retained in suspension in the blood as chylomicrons. Chylomicron lipids also have small amounts of protein associated with them (122), and, thus, they can also be classified as lipoproteins.

Plasma Lipoproteins.

Introduction:

Even though association of some lipids with proteins had been established (116, 117) it was believed that most of the blood lipids were present in solution in the blood until the classical studies of the Cohn group at Harvard, which involved a large-scale fractionation of human plasma at low ionic strength, by the use of ethanol-water mixtures at low temperatures (123). It was therefore expected that the lipids would remain in the residual ethanol-water mixture from which the proteins had been precipitated. However, such was not found to be the case. The blood lipids were identified in two distinctly different types of lipoproteins, which were present in readily separable fractions.

Nomenclature of Lipoprotein Fractions:

Fractionation of lipoproteins can be obtained by a variety of methods, e.g., floation in solutions of various densities under centrifugal forces, migration on electrophoresis or

precipitation by ethanol-water mixtures at low ionic strength. The Committee on Lipid and Lipoprotein Nomenclature of the American Society for the Study of Atherosclerosis (124) recommended that each fraction be described in terms of its method of separation, e.g., Sf classification is to be confined to flotation rates of lipoproteins on ultracentrifixation at density 1.063; α- and β-lipoproteins are to be confined to electrophoretic analysis; lipoprotein separated by the Cohn fractionation are to be designated by the appropriate fraction number. Fortunately, it is possible to relate these different methods (Table 2) (125).

The low density lipoproteins (LDL_p) have a density less than 1.063 and correspond to the Sf classes greater than 0 because they undergo flotation and not sedimentation at that density. All these lipoproteins have the electrophoretic mobility of β-globulins, except the chylomicrons, which have a density less than 1.006 and the mobility of α_2-globulins on starch (126). The lipids of low density lipoproteins correspond to those of Cohn fractions I and III.

The high density lipoproteins (HDL_p) undergo sedimentation at density 1.063 on ultracentrifixation, have the electrophoretic mobility of α_1-globulins and are usually found in the Cohn fractions IV and V.

Table 2

Interrelationship of Various Methods of Lipoproteins' Determination (125)

Density	S_f	Starch Electrophoresis	Cohn Fraction	
1.006 (Chylomicrons)	20	α_2	1 + 111	
1.006–1.019	12–20	β_1	1 + 111	(LDL_p)
1.019–1.063	0–12	β_1	1 + 111	
1.063–1.125	HDL_2	α_1	IV + V	
1.125–1.21	HDL_3	α_1	IV + V	(HDL_p)

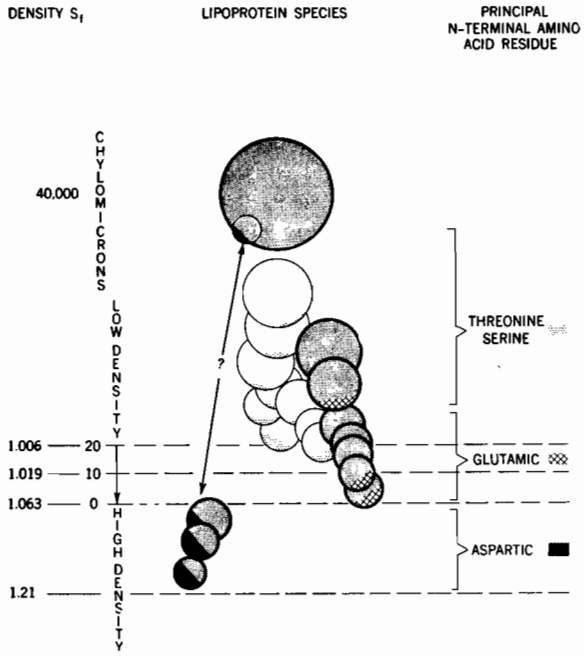
Lipid Composition of Lipoproteins:

The lipids of LDL_p and chylomicrons are composed chiefly of triglycerides and small amounts of cholesterol and phospholipids. There is a progressive increase in the triglyceride content of LDL_p as the density decreases. In these low density fractions the ratios of unesterfied to total cholesterol and of cholesterol to phospholipids are constant for each fraction but vary from one fraction to another.

High density lipoproteins are particularly rich in phospholipids and have a cholesterol-phospholipid ratio of 0.5, as compared to ratios of 1.0 or over in the low density group (127).

Protein Composition of Lipoproteins:

Avigan, Redfield and Steinberg (128) determined the amino acid composition of the N-terminal residues of lipoproteins by preparing dinitrophenyl derivatives. They showed that in the D 1.019-1.063 fraction (LD) glutamic acid appears to be N-terminal, whereas in the D 1.063-1.2. fraction (HD) aspartic acid is N-terminal. Studies by Shore (129) have also shown glutamic acid to be N-terminal in the D 1.029 lipoprotein and aspartic acid to be N-terminal in the D 1.093 and 1.149 lipoproteins. In the D 1.006 fraction serine, glutamic acid and threonine were present in the N-terminal position (Fig. 1).



Schematic conception of human plasma lipoprotein groupings according to present information. Polypeptide portions of the molecules are represented by the cross-hatching or stippling shown at the extreme right of the figure for the predominant N-terminal amino acid residue(s) present. No attempt has been made to conform to scale. Taken from D. S. Fredrickson and R. S. Gordon, *Physiol. Rev.*, **38**, 585 (1958).

Figure 1

Threonine was found to be the principal C-terminal amino acid of the HDL_p, while serine is probably C-terminal in the D 1.029 fraction (121).

Immunochemical Studies on Serum Lipoproteins:

Different classes of LDL_p were found to be antigenically similar to each other in various immunochemical reactions, but they did not react with antibodies to HDL_p (130). Middleton (131) showed that when the chylomicrons were pretreated with lipase, their proteins would react with antibody to HDL_p.

Physical Characteristics of Plasma Lipoproteins:

The HDL_p have molecular weights ranging from 165,000 to 435,000 (129). The LDL_p have much higher molecular weight, the lowest being about 2 million and this may increase to as high as 250 million for the chylomicrons (129).

Oncley et al. (132) concluded from their measurements that the β-lipoprotein molecule was a sphere with a diameter of 185 Å, and that the α-lipoprotein was ellipsoid in shape with a long axis of 300 Å and a short axis of 50 Å.

Nature of Lipid-Protein Combination of Lipoproteins:

There is little evidence that lipids and proteins ordinarily combine with each other through primary covalent linkages, such as ester bonds. "It is quite possible that strong

binding of lipids to proteins may depend upon closeness of fit, multiple attachment and matching of polarity that are recognized to determine the combination of substrates, inhibitors, and coenzymes with enzymatically active proteins" (133).

The hypothesis that varying types of union occur between lipids and proteins in the lipoprotein is largely based on extraction studies (134), and on use of cationic soaps (135). In the presence of ether these soaps assist in solubilization of almost all cholesterol, but not of phospholipids, while in the absence of ether, these soaps precipitate most of the lipids, including phospholipids. Avigan (136) found that practically all of the cholesterol in the D 1.019 - 1.063 lipoproteins could be removed without producing any apparent change in the protein residue. The phospholipids, however, could not be removed without denaturing the protein. On the other hand, the cholesterol in the HDL_p could not be removed without denaturing the protein, while the phospholipid could be more easily removed.

Specific lipid molecules in the lipoproteins appear to leave and enter the lipoprotein macromolecule with relative ease under physiological conditions. Cholesterol has been shown to transfer readily from plasma to red cells (137), from chylomicrons to HDL_p both <u>in vitro</u> and <u>in vivo</u> and between high and low density lipoproteins (138).

Unesterified cholesterol appears to transfer or exchange much more readily than esterified cholesterol (138). Phospholipids also undergo exchange between lipoproteins (125) and Lindgren (139) has observed the apparent transfer *in vitro* of labeled triglycerides between various low density classes. James *et al.* (140) have recently shown that triglycerides from the formed elements of blood are transferred preferentially to the HDL_p and the phospholipids to the low density molecules.

Stability of Plasma Lipoproteins:

Little specific information is available on the general nature of the chemical binding and of the metabolic relationship of lipid to protein in the lipoproteins. From the chemical and physical properties of lipoproteins it has been assumed (132) that the peptide residues are on the surface of the molecule, where their charge will be most available. Calculations based on protein content, estimated size of protein molecules and thickness of protein films suggest that too little protein exists to cover the entire surface of soluble lipoproteins or chylomicrons (141, 142). Hence, phospholipid may also promote surface stability, since it contains a charged group (121).

Lipoprotein Interconversions:

Our present knowledge of the chemical nature of the protein portions of the major lipoprotein classes in the human has been discussed previously. It is apparent that the N-aspartic acid-C-threonine (Fig. 1) proteins of HDL_p molecules are quite different from the N-glutamic acid-C-serine peptide chains predominating in the class of Sf 0-20 of LDL_p. It is also clear from the work of Shore (143) and Rodbell (144) that polypeptides with N-terminal serine and threonine begin to predominate as the low density molecules assume higher flotation rates. Although the largest fraction of the chylomicron protein has N-terminal amino acid identical to that of the protein in HDL_p (144, 145), it is not yet known whether this protein is present in or on the surface of chylomicrons, merely as a polypeptide, or as part of the full high density lipoprotein complex. The cholesterol of chylomicrons transfers very rapidly to the HDL_p in plasma or lymph (121).

Pierce (146) injected purified LDL_p of various Sf classes intravenously in rabbits and observed increases in the successively lower Sf classes of LDL_p of the serum in a stepwise fashion until Sf 5-15 class was elevated and eventually returned to normal levels. Graham et al. (147) found in the intact rabbit that heparin induced a fall in the Sf 20-400 lipoproteins and a concomittant rise in the Sf 12-20 class of LDL_p followed

by decline of the latter class to normal concentrations within a few hours. The increase in Sf 12-20 lipoproteins did not account for all the fall in Sf 20-400 lipoproteins. Pierce and Bloom (148) and Hewitt et al. (149) observed during the disappearance of cortisone-induced and radiation-induced lipemia in rabbits that first there is a fall in the lightest classes of LDL_p (Sf 100-400) with serial rise and then decline in the concentrations of the more dense classes of LDL_p, in a stepwise fashion.

It is safe to assume, that conversion of low density lipoproteins of high flotation rates to others of lower flotation rates occurs, and that this conversion depends largely upon a loss of triglyceride, catalyzed by lipoprotein lipase.

Sources of Plasma Lipids and Lipoproteins:

Neutral Fat

It is virtually certain that practically no synthesis of lipids occurs in the plasma itself as shown by Lovelock et al. (150) among others. It is generally believed that the neutral fat present in the plasma may originate from the gastro-intestinal tract, the liver, or from the fat depots depending upon the state of alimentation (39).

Phospholipids

The experimental data leave little doubt that plasma phospholipids are largely, if not entirely, synthesized in the liver (151, 152). Recent investigations (153) have shown that leucocytes are capable of incorporating acetate into phospholipids and there is a limited exchange of some phospholipids between the cells and plasma.

Cholesterol

Biosynthesis

Acetate is the principal and probably the only common simple metabolite from which cholesterol is biosynthesized. Every carbon in cholesterol may be derived from acetate (154). Block and his collaborators (155) suggested that cholesterol might be formed from acetate by (a) condensation to an isoprenoid unit, (b) polymerization to squalene, and (c) cyclization of squalene to give cholesterol. Much experimental evidence has since provided the factual backing to the suggestion (156 - 165).

A recently discovered compound β-hydroxy-βmethyl-ω-valerolactone was shown by Tavorinina et al. (166) to be converted to cholesterol with high efficiency. The name "mevalonic acid" or MVA has been proposed for this compound. The lactone is the form customarily employed in experiments, since the lactone

ring opens rapidly in the physiological pH range and does not close readily; it is probably the acid form which is reactive (154).

The administration of mevalonic acid-2-C^{14} to intact rats and mice has been reported to result in urinary excretion of almost exactly half the labeled material, and the conversion of up to 37% to cholesterol-C^{14} and about 10% to $C^{14}O_2$ (167). Theoretical yields on the assumption that one enantiomorph is quantitatively utilized are 41.7% cholesterol, 8.3% CO_2, and 50% in urine. Conversion of mevalonic acid into squalene and cholesterol is apparently the only metabolic reaction it undergoes in mammalian tissues. The biosynthesis of cholesterol from acetate (in contrast to that of fatty acids) is irreversible (154).

The development of a method of homogenization of liver tissue without destruction of the capacity to synthesize cholesterol (168) has made it possible to study cholesterol biosynthesis from an enzymological standpoint. Neither the nucleus nor the mitochondria are required (169). The system requires Mg. ions, nicotinamide adenine diphosphate (NAD), niacinamide (Presumably to inhibit the breakdown of NAD), an energy source, and oxygen to convert acetate into cholesterol. Coenzyme A and adenosine triphosphate are necessary but need not be added to this system (154).

Sources:

A Unesterified Cholesterol

All exogenous cholesterol enters the blood by way of the thoracic lymph. When cholesterol-C^{14} was given to dogs by mouth, all radioactivity appeared in the lymph and none in the portal vein blood (170); the radioactive cholesterol was associated with chylomicrons in greater amounts than with other lipoproteins in the lymph (171). After entering the blood, the major portion of the cholesterol enters the liver (172-174). Once it has entered the liver, exogenous cholesterol is metabolized in the same way as endogenous cholesterol (175). Absorbed cholesterol adds quantitatively to plasma cholesterol only during the persistence of alimentary lipemia and even then its quantitative effect is slight. (172).

Almost all tissues synthesize cholesterol to some degree (176), and some of the newly synthesized cholesterol quickly appears in plasma (177). The removal of various organs except the liver however, does not significantly alter the plasma cholesterol concentration (177-179), and it seems fairly well established that practically all the plasma cholesterol of endogenous origin is derived from the liver (180). In liver cells microsomes appear to be the site of synthesis of cholesterol (166, 181, 182). Full enzymatic activity was retained in the supernatant after

sedimenting mitochondria and heavy microsomes (167, 181). In fact, an extract made from liver cell mitochondria after breaking them with ultrasonic vibration was shown to inhibit the hepatic synthesis of cholesterol both *in vitro* and *in vivo* (182, 183).

B Esterified Cholesterol

Sperry (184) reported that incubation of human serum for several days produces an increase in the esterified fraction. It is, however, still not clear whether this is the physiological method of formation of these esters.

Esterification of cholesterol can occur in liver *in vivo*, as may be inferred from the rapid appearance of cholesteryl-C^{14} esters in liver after the administration of acetate-C^{14} (177). However, evidence exists suggesting that in normal rats a large proportion of plasma cholesterol esters arise from extrahepatic sources (185, 186). Of the cholesterol absorbed from the intestines, 60-80% is esterified before reaching the thoracic duct and being delivered to the blood (171); since the exogenous cholesterol mixes in the pool of cholesterol in the gut before esterification and absorption (187), and since after absorption the dietary cholesterol is assumed to be metabolized in a manner identical to that of the endogenous plasma cholesterol (154), the relative contributions to plasma cholesterol esters of normal rat by the liver and extrahepatic tissues are difficult to estimate at present.

Lipoproteins:

Serum lipoproteins are known to exist in a spectrum of varying density, from very low density particulate chylomicrons composed chiefly of triglycerides through various classes of LDLp relatively rich in cholesterol to HDLp in which phospholipids are prominent.

As has been discussed earlier, the liver seems to be the principal source of endogenous lipids present in the serum, and it has been assumed, but not proven, that the liver is also the chief source of the protein moiety of the serum lipoproteins. Incorporation of labeled amino acids by perfused rat liver and rat liver slices into proteins with densities similar to those of serum lipoproteins has been reported (188, 189).

Net synthesis, by isolated perfused liver and liver slices *in vitro*, of low density (190) and high density (191) lipoproteins, immunochemically and in amino acid composition identical to the plasma low and high density lipoproteins, has been reported. It thus seems quite probable that practically all the plasma lipoproteins are synthesized by the liver.

Fate of Plasma Lipids.

Chylomicrons:

Once chylomicrons enter the blood, they are normally

rapidly removed, although a transient rise in total blood fatty acids is a normal accompaniment of moderate fat ingestion (192). The metabolism of chylomicrons containing isotopically labeled fats or protein, and obtained from lymph of donor animals fed appropriate precursors have been recently studied in many laboratories. Havel and Fredrickson (193) observed that over 90% of the radioactivity injected in the form of palmitate-1-C^{14}-containing chylomicrons disappeared from the blood at an exponential rate in anesthetized dogs, and the early rates of disappearance were inversely proportional to the load of lipids injected (138). This has also been observed in rats by French and Morris (194, 195). During the removal of the labeled chylomicron triglycerides, it was observed that the labeled fatty acids rapidly appeared as unesterified fatty acid (193). Over half of the chylomicron triglyceride fatty acids are directly oxidized and the transport of triglyceride fatty acids in the unesterified form is not a necessary step for their oxidation (195). The rapid oxidation of the fatty acids in chylomicron triglyceride also has been demonstrated in the rat by Morris (196) and by Bragdon (197). French, Morris and Robinson (195) have noted that hepatectomy caused a delay in removal of chylomicrons, and after injection of labeled chylomicrons most of the radioactivity was noted in the liver and small amounts in spleen,

kidneys, lungs and depot fat, in descending order.

Although it seems well established that intravascular hydrolysis of chylomicrons does occur, the evidence obtained from animal experiments is against the necessity of intravascular hydrolysis operating in the normal removal of chylomicrons. (121). However the removal of chylomicron lipid from blood is accelerated by lipoprotein lipase (195).

Lipoprotein Lipase:

The enzyme lipoprotein lipase isolated from plasma, adipose tissue and myocardium (198-200) appears to be identical to clearing factor first observed after injection of heparin into lipemic animals (201-203).

Following the observations *in vivo* and *in vitro* that post-heparin plasma contained a factor which could decrease visible lipemia in blood (201, 204, 205) it was demonstrated that such clearing was the result of hydrolysis of triglycerides in the large, light scattering, triglyceride-rich lipoprotein (198, 206) with the acceptance by the serum albumin of the fatty acids released by hydrolysis (206-208). Beyond the fact that heparin appears to form an essential part of the enzyme complex, nothing further is known about the manner in which heparin causes lipoprotein lipase activity to appear in the blood, presumably

'spilling out' from the tissues. Lipoprotein lipase acts specifically upon the ester linkages in triglycerides (143), not attacking the cholesterol esters or phospholipids. The enzyme is absorbed tightly to the surface of chylomicrons (209) and it is possible that a lipoprotein, specifically high density lipoprotein, may facilitate this bond and thus serve to 'activate' particles of triglyceride for enzymatic hydrolysis (198, 199).

Spontaneous activity of lipoprotein lipase has been observed in certain strains of rats after fat feeding (210), such activity being increased by removal of liver from the circulation (211). The importance of its role, under physiological conditions, in clearing the chylomicron lipid from circulation is not clearly established.

Cholesterol:

The term "turnover" may be defined as the rate of replacement of the molecules of a body constituent or the rate of disappearance due to excretion, and metabolic conversion into other substances. In the steady state these two rates will be equal.

Turnover in plasma, strictly speaking, reflects the rates of entry into and exit from the plasma but has been more loosely applied to include synthesis and degradation in plasma-tissue pools. When a labeled substance is injected intravenously,

the early part of the time course of disappearance from plasma has been thought to represent "mixing" within the vascular compartment, and, "exchange" between it and the extravascular compartment in which the injected material rapidly equilibrates. Mixing within the vascular compartment for most purposes can be considered to occur instantaneously (less than 2 minutes). Since metabolic degradation takes place only intracellularly, exchange is the only kind of turnover that goes on in the plasma proper. The part of the curve which has been considered to represent mixing of the injected material into the plasma-tissue pool, in fact reflects the process of "equilibration" of the specific activities of the injected material in the plasma and tissue compartments of that pool. This will depend largely upon the rate of entry into the plasma from the tissue and rate of exit from plasma to the extravascular compartment of the plasma-tissue pool.

In the case of cholesterol, exchange between liver and plasma is rapid compared to the rate of its synthesis by the liver. The turnover rate of the plasma cholesterol can be calculated from the rapid phase of the disappearance curve after intravenous injection of C^{14}-cholesterol labeled lipoproteins and the turnover rate in the plasma-liver cholesterol pool is calculated from the slower phase of the same disappearance curve. These assumptions have been validated by Zilversmidt (212) while working on plasma

lecithin, which like cholesterol is derived mainly from the liver and has a rapid exchange rate between liver and plasma.

In the fasting state the liver is the principal source of plasma cholesterol (177-179), and in the steady state the rate of production must be the same as the rate at which cholesterol disappears from the plasma-tissue pool.

Cholesterol enters the plasma either as chylomicrons or as a component of lipoproteins from the liver. The cholesterol which is absorbed from the intestines becomes indistinguishable from that synthesized in the liver (175). Unesterified cholesterol from the liver equilbrates with that of the plasma which in its turn equilibrates rapidly with that of erythrocytes (177).

The transformation of unesterified to esterified cholesterol is, as compared to its exchange with liver and erythrocytes, a relatively slow process. Absorbed cholesterol becomes partially esterified during absorption, presumably in the intestine, whereas endogenous cholesterol becomes esterified in the liver and possibly in the plasma and other tissues (154).

Cholesterol-C^{14} introduced into the body by feeding, intravenous or intraperitoneal injection, mixes with that in liver and erythrocytes rapidly, and a little more slowly with that in other viscera. The specific activity of cholesterol in kidney, lung, heart, intestine and diaphragm reached half that of plasma

in 1-2 days (in dogs) and in adrenal gland , aorta and skin considerably more slowly (213). Overall metabolism of cholesterol in rats consists to a very large extent in oxidative removal of the terminal 3 carbon atoms of the side chain and eventual excretion of the remaining 24 carbons (214 , 215) , as fecal sterols (10%) , as fecal bile acids , (85%) and in the urine, presumably as steroid hormone metabolites (1%) , (154).

Some Factors Influencing Cholesterol Biosynthesis , Turnover and Distribution in Tissues:

Fasting:

Only a slight decrease was noted in the rate of hepatic synthesis of cholesterol in the intact rat after 24 hours of fasting , but after 48 hours of fasting the rate of synthesis was half of the control value (216).

Cholesterol biosynthesis appears to be sensitive to the nutritional state of the animal , particularly when estimated by in vitro methods. Presence of glycogen stores in the liver are essential for hepatic synthesis of cholesterol (181).

Phospholipid and Triglyceride Injection:

It has been shown that a change in the plasma levels of any one of the three classes of lipids , triglycerides , phospholipids and cholesterol , usually is associated with changes in

the others. Friedman and Byers (217) reported that sustained, elevated levels in plasma of triglycerides and phospholipids increased the plasma cholesterol level in rats from 50 - 60 to 80 -128 mg.%. In a second study similar effects were produced by injection of soya bean lecithin emulsion (218).

Nephrosis:

In human nephrosis and antikidney serum disease in rats, hyperlipemia and hypercholesterolemia are marked features. Literature relevant to the genesis of hyperlipemia in this condition is reviewed in detail in the following pages.

Hyperlipemia in Nephrosis.

Introduction:

The most striking manifestation of the disease in man or experimental animal is loss of albumin through the diseased kidney in amounts sufficient to lead to a marked decrease in its concentration in blood. Thus most of the experimental work in the genesis of hyperlipemia has been done in relation to changes in metabolism of albumin in the disease.

Serum Lipids and Lipoprotein Alterations in Nephrosis:

When nephrosis was recognized as a clinical entity, an increased concentration of serum cholesterol was observed as a consistent feature of the syndrome (219). Later it was found that phospholipid and triglyceride also are increased in nephrosis (220).

A relative and absolute reduction in "α" lipoprotein and a marked increase in "β" lipoprotein cholesterol on alcohol fractionation of serum proteins, were found by Barr, Russ and Eder (221). Kunkel and Slater (222) observed that the major lipoprotein had a mobility intermediate between α and β components of normal sera, though in chemical composition it resembled β-lipoprotein. The abnormal pattern of serum lipoproteins by zone

electrophoresis was, in part, a result of increased quantities of unesterified fatty acids bound to the lipoproteins in nephrosis (223). Gofman, Rubin, McGinley and Jones (224) noted in nephrotic children that all classes of LDL_p studied by them in the analytical ultracentrifuge (S_f 0-400) were increased.

In 44 hospitalized patients the concentrations of total cholesterol, phospholipid, triglyceride and total lipid were related inversely and non-linearly to serum albumin levels. Either or both of the D < 1.019 and the D 1.019-1.063 fractions of LDL_p were abnormally large; however, a marked increase occured in only one or the other of the two fractions in any one case. In cases where decrease in albumin levels was not great the increase was principally in D 1.019-1.063 lipoprotein fraction, whereas severe nephrosis was characterized by high levels of the D < 1.019 lipoprotein fraction and subnormal D 1.063-1.21 (High Density) lipoproteins (225).

Hepatic and Extra-hepatic Depot Lipids in Experimental Nephrosis:

Hepatic and extra-hepatic fat depots were compared in nephrotic and control rats by Heymann and Hackel (226). When the control animals were housed, like nephrotic rats, one to a small cage, the carcass fat values did not differ between the two groups, whereas liver fat content was significantly lower in the

nephrotic animals. These results differ from those obtained by Marsh and Drabkin (227) and by Rosenman, Friedman and Byers (228). Marsh and Drabkin found liver cholesterol to be increased whereas extra-hepatic fat depots were markedly decreased; however these studies were done on a small group of moribund animals, and thus the animals probably had diminished intake of food. Rosenman, Friedman and Byers found both the hepatic and extra-hepatic cholesterol to be normal in well established nephrotic syndrome of moderate severity.

Saffran and Kalant (229) found a progressive increase in hepatic content of cholesterol during the induction stages of nephrosis, while carcass lipids remained normal. Hypertrophy of the liver accompanied by a rise in both ribonucleic and deoxyribonucleic acids has been observed in nephrotic rats (230).

Urinary Lipoproteins, Proteins and Lipids in Nephrosis:

Plasma albumin is the main, but not the only protein in the urine of nephrotic human subjects (231). Lipoproteins are also lost through damaged glomeruli in patients with proteinuria of any etiology (232). Part of this filtered lipoprotein is then reabsorbed by tubular cells. In experiments with amphibians, damage to glomeruli was found to be a necessary antecedent to appearance of cholesterol in the urine (233). Some of the cholesterol

ester which enters the tubular cells is altered to an anisotropic state and re-enters the urine either as the material extruded from cells or as sloughing tubular cells. The anisotropic material, which is composed largely of cholesterol ester with a smaller component of unesterified cholesterol, appears in the urine sediment as the "Maltese Cross" bodies (234).

The nephrotic urine also contains some cholesterol and other lipids which remain in the supernatant presumably as lipoproteins, on spinning down the sediment (234).

Electrophoretic patterns (Fig.3) demonstrate that in experimentally nephrotic rats, in addition to albumin, the low molecular weight globulins such as the mucoproteins and siderophilin also can traverse the damaged glomerular filtering membrane (102). Low density lipoproteins, with molecular weights of the order of 1,000,000 were absent from the pooled nephrotic urine, but HDL_p, with an average molecular weight of 200,000 were present as determined by ultracentrifugation analysis (102).

The daily loss of albumin in urine of nephrotic rats may amount to twice the total circulating albumin of normal rats. After intravenous injections of C^{14} glycine into rats the specific activity of plasma and urinary albumin were found to be similar, thus it was concluded that urinary albumin is derived

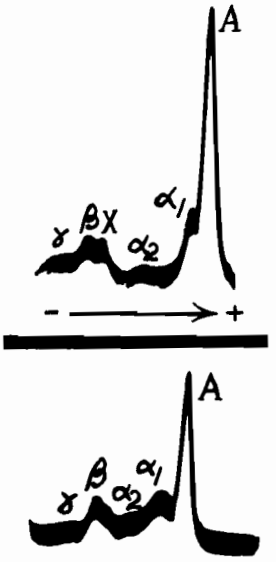


Fig. 1.—Electrophoretic patterns of normal rat plasma after 120 minutes of migragration *(top)* and nephrotic rat urine after 90 minutes of migration *(bottom)*.

The arrow indicates direction of migration. The symbol A is for albumin, α_1,α_2, X, β and γ for the globulins. X, probably a beta globulin, is usually not indentified in human plasma (cf. ref. 2). It should be pointed out that the electrophoretogram of the urine was made on the fourth day of the experimental disease, when the concentration of albumin in the plasma was very low. These electrophoretic determinations were made by G. A. Braun in our laboratory.

Figure 2

Taken from Marsh, J.B., and Drabkin, David L., Metabolism 9:946 (1960)

from plasma (227).

Metabolism of Cholesterol and Other Lipids in Experimental Nephrosis:

Gastro-intestinal tract:

Exogenous cholesterol, absorbed from the intestinal tract, is transported into blood by the lymph of the thoracic duct. By means of thoracic duct cannulation and subsequent lymph collection following stomach feeding of 100 mg. of cholesterol dissolved in olive oil, it was found that the nephrotic rat has a diminished rate of cholesterol absorption. This could not be ascribed to a deficiency of lymph flow or of bile in the intestinal tract (235). However, feeding excess cholesterol to the nephrotic rat intensified its hypercholesterolemia (236) and increased the hepatic cholesterol (237). A similar phenomenon was also seen in normal rats though the increase in plasma cholesterol was not as great as in nephrotics on the same diet (237). When rats on a fat and cholesterol free diet were injected with AKS, the increase in the concentration of plasma lipids was greater than that observed in rats fed a stock ration before the injection of AKS (236). The fecal excretion of cholesterol and total lipids in nephrotic rats was no greater than that of normal rats (238).

Liver - Byers et al. (239) studied the "hepatic rate of synthesis"

of cholesterol at 2, 5 and 14 days after the last injection of AKS, in order to determine the cholesterol synthesis during the early and "chronic" stages of nephrosis. The results demonstrated either an unchanged or a diminished hepatic synthesis of cholesterol by the nephrotic rat. The assessment of hepatic rate of synthesis of cholesterol was made in these studies very indirectly from the amount of cholesterol excreted in the bile. The role of liver in nephrotic hypercholesterolemia was further investigated by this group (240) and they observed that hypercholesterolemia could not be correlated with any accumulation of liver cholesterol and also could not be ascribed to any failure of hepatic conversion of cholesterol to cholate or to any abnormality of Kupffer cell function. An increase in the plasma level of bile acid (cholate) in experimental and human nephrosis (241) may suggest an abnormality in metabolism of cholesterol in the liver, but may also be secondary to an increase in the cholesterol levels of plasma.

The hepatic origin of plasma cholesterol and plasma lipoproteins is well established for the normal animal (242) and it is quite reasonable to find a similar metabolic pattern in the nephrotic rats as suggested by a decrease in plasma lipids after partial hepatectomy before or after induction of nephrosis (243, 244).

Studies done on liver slices *in vitro* give very conflicting results. Heymann *et al.* (245) studied the incorporation of C^{14} labeled acetate into lipid by liver slices from normal rats and from rats during the induction phase of nephrosis, i.e., 8, 24 and 48 hours after the injection of AKS. The values in nephrotic rats were within the normal range. Marsh and Drabkin (227), using liver slices from gravely ill nephrotic rats with depleted glycogen stores, found a decrease in the rate of incorporation of acetate into lipids by the liver. However, studies done on moderately severe nephrotic rats, 48 hours after the induction of nephrosis, demonstrated a definite increase in hepatic rate of synthesis of lipids (246). These results were corroborated by studies *in vivo* of incorporation of labeled acetate into cholesterol; nephrotic rats studied up to 48 hours after the injection of AKS demonstrated a marked increase in hepatic synthesis of cholesterol. This is in agreement with the findings of Saffran and Kalant (229).

The hepatic lymph is a diffusion product. When a load of lipids in the form of hyperlipemic rat serum is injected into a normal animal, the concentrations of cholesterol and other lipids in the hepatic lymph are rapidly increased but in nephrosis despite marked hyperlipemia and hypercholesterolemia the hepatic lymph levels of lipids, including cholesterol, remain

unchanged (247).

Kidneys:

Bilateral nephrectomy prevented the development of hyperlipemia in rats injected with AKS (244). A correlation between the severity of the hyperlipemia and the quantity of diseased renal tissue was indicated by the finding that hyperlipemia was decreased progressively by removing one and then the other kidney. The level of hyperlipemia was less in rats with unilateral renal disease than those with bilateral renal disease.

When AKS was injected into bilaterally nephrectomized rats, the rise in cholesterol was only the same as that which occurred in control nephrectomized animals (243). Ligation of the renal pedicles or ureters produced identical results in both normal and nephrotic animals. That the rise of lipid and cholesterol in the plasma of the AKS injected animal was not due to the acute renal injury per se, was shown by the lack of rise in plasma lipids when a ureterovenacaval anastomosis was established prior to injection of AKS (243).

In nephrotic rats the intraperitoneal administration of non-protein containing rat urine decreased the hyperlipemia almost as regularly and markedly as when protein-containing nephrotic urines were used (248).

Blood:

Rosenman *et al.* (243) inferred from their data that the excess of cholesterol and lipids in the nephrotic state is due to an accumulation which is confined to the plasma and cannot be ascribed to any change in the animals' usual modes of synthesizing, absorbing, excreting, or, metabolising cholesterol. The clearance of C^{14}-labeled trilaurin, administered intravenously in the form of a synthetic emulsion was normal in five nephrotic rats studied (248); however, when triglycerides were injected in physiologic state as chylomicrons, a marked decrease in the rate of their clearance was observed in two independant studies (229, 249). The delay in clearance of chylomicrons in nephrotic animals could not be corrected by administeration of bovine albumin (249). In another study, clearance of intravenously injected sodium palmitate-1-C^{14} bound to albumin was found to be normal in four nephrotic rats (229) though reappearance of injected C^{14}-palmitate in the circulation, after esterification was markedly increased in nephrosis.

Rosenman and Byers (250) studied the metabolism of heparin and lipoprotein lipase in AKS nephrotic rats. There was no abnormality in plasma disappearance rate of exogenous heparin, nor was there any difference in lipoprotein lipase response between normal and nephrotic rats. The clearing ability of post-

heparin plasma from nephrotic animals was found to be normal, whereas Seifter and Baeder (251) had presented data suggesting the presence of an inhibitor in the nephrotic serum to the lipoprotein lipase clearing. Lastly, no abnormality was detected in the ability of nephrotic plasma to support lipase-induced release of unesterified fatty acids from a triglyceride substrate suspended in such plasma (249, 250).

Metabolism of Proteins in Nephrosis:

The daily loss of albumin in the urine of nephrotic rats may amount to more than twice the total circulating albumin of control rats (227). Evidence that the urinary albumin represents newly synthesized protein was obtained from the observation that after the injection of glycine-2-C^{14} (227) the specific radioactivity of urinary protein in nephrotic rats was several times higher than that of the plasma albumin of the normal rat. These results also indicated that the synthesis of plasma albumin in the nephrotic rat was accelerated. This was later confirmed by the demonstration of an increase in the synthesis of albumin in liver slices from rats with experimental nephrosis (252).

Gitlin et al. (253) reported that in several children with nephrosis the major metabolic alteration encountered was an increase in the rate of albumin catabolism, with little alteration in the rate of synthesis. Since these authors employed I^{131}-

labeled albumin, the interpretation of their results is made difficult by the possibility that I^{131}-labeled albumin may not be catabolized in the same manner as normal albumin (254). Gitlin and Cornwall (255) also deduced an increased synthesis of β lipoproteins in nephrotic children, again using lipoprotein labeled with I^{131}.

An increase in the synthesis of protein moieties of low density (190) as well as high density (191) lipoproteins by nephrotic liver slices or isolated liver has also been demonstrated recently.

Endocrine Glands:

Cortisone and ACTH are reported to have a beneficial effect on the nephrotic syndrome in children (256). The mechanism of these actions is not known, but studies have been done to determine the effect of various endocrine alterations in the nephrotic rats.

Hypophysectomy or adrenalectomy of animals before injection of AKS resulted in decreased proteinuria, but hyperlipemia was similar in degree to that of the control animals (257). Adrenalectomy after the injection of AKS resulted in a slight decrease in proteinuria, but had no effect on the course of the disease. The nephrotic hyperlipemia was not affected by hypophysectomy or adrenalectomy in established nephrosis. However,

in another study, hypophysectomized rats developed severe hypoalbuminemia after injection of AKS but only mild hyperlipemia (258). Injection of ACTH into nephrotic rats resulted in a decrease in the blood concentration of triglycerides (259).

Rosenman and Smith (260) studied the effects of altered thyroid function on plasma lipids of experimentally nephrotic rats. Acute administration of excess thyroid to rats with established nephrosis did not produce any effects; however, if nephrosis was induced on a pre-existing hyperthyroid state it resulted in a greater increase in blood lipids than normal rats. Pre-existing hypothyroidism depressed the production of hyperlipemia on induction of nephrosis. On the other hand, the effects of thyroidectomy and thyroxine replacement therapy on serum cholesterol were qualitatively similar in normal and nephrotic rats. (261).

Theories of Hyperlipemia.

Introduction:

An increase in the rate of entry or a decrease in the rate of removal of lipids from plasma can lead to progressive hyperlipemia until there is an equal change in the rate of exit or entry respectively, so as to result in a new steady state lipemia at a higher concentration. The lipids entering the plasma may be directly derived from exogenous sources or synthesized de novo endogenously.

Increase in the rate of entry as well as a decrease in the rate of exit of lipids from plasma have been invoked to explain the hyperlipemia in experimental nephrosis.

Increased Synthesis of Lipids and Their Entry into Plasma:

An increase in hepatic synthesis of cholesterol and fatty acids has been reported in in vivo (246, 229) as well as in in vitro (246) studies during early stages of AKS nephrosis, though earlier studies (227, 229, 245) had indicated either diminished or normal rates. As has already been pointed out the conclusions drawn from these early studies (except those of Heymann et al.) (245) are questionable. Recently, increases in the synthesis of lipids as well as of the protein moiety of lipoproteins have been

demonstrated by studies on isolated liver and liver slices from nephrotic rats (102, 191).

Marsh and Drabkin (102) implicate the proteinurea as the causative factor in the development of hyperlipemia in nephrosis. They suggest (102) that there is a generalized increase in hepatic synthesis of a whole spectrum of plasma proteins in response to the protein loss in the urine of nephrotic animals. This increase in synthesis of proteins by the liver is just able to maintain plasma levels of most of the plasma globulins which are less readily lost in the urine, but cannot compensate adequately for the massive losses of plasma albumin. Plasma lipoproteins, particularly LDL_p cannot filter through the damaged glomeruli on account of their molecular size, so their concentration in the plasma rises.

Increase in Mobilization of Lipids from Depots and Their Entry Into Plasma:

Drabkin and Marsh (227) noted a marked decrease in carcass lipid in nephrotic animals, and they postulated that mobilization of lipids from extra-hepatic fat depots was responsible for lipemia of nephrosis. However, the diminution of carcass fat

was later attributed to the moribund state of animals, resulting in lack of food intake (226). Saffran and Kalant (229), from their studies, also concluded that mobilization of carcass lipids did not account for the hyperlipemia of nephrosis.

Decrease in the Rate of Exit of Lipids from Plasma:

It has been proposed (262) that hypoalbuminemia initiates and maintains the process both of hyperlipemia and hypercholesterolemia. Albumin is an essential component of the lipemia clearing process (207) and the hydrolysis of plasma fats is markedly inhibited by the absence of adequate amounts of plasma albumin with available binding sites for fatty acids (207), leading to accumulation of neutral fats in the plasma. The increase in concentration of plasma neutral fats secondarily produces hypercholesterolemia. Considerable data has been marshalled in support of this hypothesis, as follows:

1. When sufficient albumin was given to AKS-injected rats to overcome the plasma deficit, hyperlipemia and hypercholesterolemia were either prevented or markedly inhibited during the induction of nephrosis, as well as ameliorated in the previously established nephrotic state (262).

2. A close inverse relationship between the lowered albumin and elevated lipid levels has been noticed in children with

nephrosis (263). A rise of plasma albumin, when induced by ACTH in nephrotic patients, is associated with a fall of plasma cholesterol, regardless of the pattern of diuresis (264). During clinical remissions in nephrotic patients it has been observed that shifts of plasma cholesterol and albumin occur together, both reaching normal levels nearly simultaneously (265).

3. In most of the conditons associated with albumin losses from the body, hyperlipemia also occurs; examples are repeated plasma loss from animals (266), patients with severe proteinuria due to constrictive pericarditis and renal thrombosis (267), dogs fed low protein diet (268), and animals made proteinuric by a nephrotoxin such as uranium (269).

In a subsequent study (270), the group advocating hypoalbuminemia as the sole cause for increased lipid concentrations in blood, modified their views in view of the following observations. They noted that heparin was found to inhibit the rise of plasma lipids and cholesterol in experimental nephrosis. Such inhibition was marked when heparin was administered prior to or immediately after the injection of AKS, but of much lesser degree when administered to the chronic nephrotic rat with established hyperlipemia (235). Infusion of bovine serum albumin (262) was far more effective than heparin in preventing and correcting the hyperlipemia of nephrosis in rats. However, the infusion of

albumin was not found capable of completely normalizing the plasma lipids of the nephrotic animals unless they were concomittantly nephrectomized.

It was found (270) that heparin administration induced only a minimal lipemia clearing response in severely hypoalbuminemic rats, unless albumin was administered simultaneously. The concurrent administration of heparin markedly augmented the ability of infused albumin to correct the hyperlipemia and hypercholesterolemia of the nephrotic rat. Thus it was suggested that in addition to deficiency of plasma albumin, a causal factor underlying nephrotic hyperlipemia may be the renal loss of "clearing factor" or some co-factor essential to the lipolytic process.

In spite of the loss of "clearing factor" and deficiency of albumin in plasma, the appearance of lipoprotein lipase in response to heparin and its clearing effect in nephrotic animals were found to be normal (250). Injection of heparin in nephrotic animals increased the rate of clearance of chylomicron lipid from the blood, suggesting that in spite of the low levels of albumin, all the receptor sites for unesterified fatty acids were not saturated (249). Injection of bovine serum albumin in nephrotic animals did not normalize the delay in the rate of clearance of chylomicrons indicating that hypoalbuminemia was not responsible for this delay (249).

Lipoprotein Lipase Inhibition:

Seifter and Baeder (251) demonstrated the presence of an inhibitor to post-heparin clearing factor in the plasma of nephrotic rats. Since there is a delay in clearance of chylomicrons from the blood in nephrosis (229, 249) the possibility that this inhibitor was responsible for the retention of lipids in plasma of nephrotic rats exists. However, after injection of heparin, the action of lipoprotein lipase has been shown to be normal in nephrotic rats (250).

Summary:

1. It has been well established that there is delay in clearance of chylomicrons from the blood of nephrotic rats (229, 249) and there is suggestive but unconfirmed evidence of an inhibitor to post-heparin clearing factor in nephrotic plasma (251).

2. Studies on isolated liver and liver slices in vitro suggest an increase in the rate of synthesis of lipids and lipoproteins in nephrosis (102, 191), but previous studies on nephrotic liver slices had shown no increase in synthesis of lipids (227, 245).

3. Direct evidence (246) for increased rate of synthesis of lipids by the liver during the induction stages of nephrosis is supported by some indirect evidence. (229).

4. The plasma concentration of albumin is in some way related to the hyperlipemia of nephrosis, though the exact relationship is not yet clear.

Methods

Animals.

Animals used for experiments were rats of the Sprague-Dawley strain. Most of the rats were male, about 250 g. in weight, unless otherwise stated. They were kept in groups of about 6 to a cage, in a room with constant temperature (80°F.) and humidity (42%). They were fed standard commercial laboratory chow (Purina) and given tap water to drink, unless otherwise noted. For collection of urine they were kept in single metabolic cages and the cages were rinsed with distilled water for quantitative collections.

Before collection of their blood from the cut end of the tails and before giving intravenous injections, animals were placed under an infrared lamp for a few minutes to warm them in order to facilitate the procedure. For collection of blood from the abdominal aorta, the animals were anesthetized with pentobarbital sodium, 0.1 grain (gr.) per 100 grams (g.) body weight dissolved in alcohol, propylene glycol and water mixture at a concentration of 1 gr. per mililitre (ml.) injected intraperitoneally; the abdomen was then opened, and, the abdominal aorta exposed and cleaned. A needle attached to a catheter or a syringe was inserted into the aorta at its bifurcation, and the blood was allowed to flow out.

The livers were excised when required and immediately

frozen in liquid nitrogen or in well crushed solid carbon dioxide. When perfusion of the liver was required, a #16 needle was inserted into the portal vein, the hepatic and superior mesenteric arteries were immediately ligated and the liver was flushed with normal saline. The hepatic vein as it came out of the liver was cut to provide an easy outlet for the perfusing fluid. The entire liver became bloodless, as judged by the change in color, within 30 to 90 seconds. The clearing at the surface could be expedited by gently rubbing with the flat of the fingers.

Liver homogenates were prepared by the method of Bucher and McGarrahan (181). The livers were perfused in situ for a few seconds with ice-cold medium containing 0.1 M potassium phosphate buffer, pH7.4, 0.03 M nicotinamide, 0.004 M magnesium chloride, and 0.125 M sucrose, and then homogenized in a glass homogenizer at 300 r.p.m. for 30 seconds. The homogenates were centrifuged for 10 minutes at 700 g. to eliminate tissue fragments, cells and nuclei, and the supernatants were used for study of cholesterol synthesis.

Nephrosis was produced in rats by administration of anti-rat serum (AKS) prepared in rabbits (22). Most of the studies were done in the chronic stage of the experimental disease when the rats were free of edema and were assumed to be in the steady

state. Unless otherwise stated, the animals were used about two weeks after the last injection of AKS.

The animals were tested for the development of the disease by examining the degree of hypercholesterolemia. All animals used as nephrotics had cholesterol levels above 100 mg. per 100 ml. of serum and most of them were above 250 mg. per 100 ml. The values for total cholesterol in normal rat serum rarely exceeded 50 mg. per 100 ml.

For collection of normal rat chyle, the thoracic duct was cannulated (271) and chyle was collected overnight after feeding the animals 6 ml. of corn oil through a gastric tube. Labeled rat chyle was collected by the method of Morris and French (249) using tripalmitin-1-C^{14} to label the chylomicrons.

Methods for Studies on Chylomicron Lipid.

Chylomicron counts were made under dark ground illumination. In a pilot experiment, different amounts of chyle were added to identical aliquots of the same serum, and mixed well. Fifty microlitres (µl) samples from each were transferred to a glass slide and chylomicrons counted in a standard field. The standard field was a circle about one cm. in diameter drawn with grease pencil, which was divided into quadrants by two lines etched on the cover slip. The sample volume of 50 µl. was spread

evenly within the circle marked off by the pencil. The amounts of chyle added to the serum had a linear relationship to the number of chylomicrons in a standard field, so long as the number did not exceed 50. Beyond this figure the chylomicron counts were not accurate. Thus all samples of sera having a number of chylomicrons greater than 50 were diluted with a serum of known and low chylomicron count.

For studies on lipoprotein lipase the substrate was prepared by adding normal rat chyle to normal rat serum. The optical density of serum at 550 mμ was observed to have a linear relationship to the concentration of chylomicrons in the serum. Plasma obtained from normal rats 15 minutes after intravenous injection of 4 mg. per kg. of body weight of heparin served as a source of lipoprotein lipase.

Fractionation of Sera.

Paper Electrophoresis:

Paper electrophoresis was done on Whatman paper #3MM in barbital buffer, pH 8.6, at ionic strength of 0.075, either for 16 hours at 110 volts or for 4-6 hours at 240 volts. For detection of the proteins, the strips were stained with bromphenol blue; for detection of lipids, the serum was prestained with acetylated sudan black B (272) before application to the paper

strip for electrophoresis at a voltage of 210, or the strip was stained with sudan black after the electrophoresis. The distribution of radioactivity on the dried paper strips was determined by scanning in an automatic scanning device, or by radioautographs made on X-ray plate, as explained later.

Ultra-Centrifugation:

The specimens of plasma or serum for fractionation into low and high density lipoproteins were obtained from animals which had been fasted overnight and given 50% dextrose to drink. The number of chylomicrons in such sera was negligible. Low density lipoproteins were separated after adjusting the density of the solution to 1.063, either by the addition of sodium chloride solution (273) or by the addition of sodium chloride and potassium bromide solution (274). The solution was centrifuged in a rotor model 30.2 for 16 hours at 30,000 r.p.m. in a Spinco model "L" preparative ultracentrifuge. The LDL_p was concentrated as an opalescent layer at the top. It was carefully pipetted out or separated by cutting the tube in a test tube slicer and collecting the solution quantitatively.

The lipoproteins remaining in the solution after removal of LDL_p were HDL_p. The entire solution was extracted to obtain lipids of HDL_p, or the HDL_p themselves were floated after adjusting the density of the solution to 1.21 with sodium chloride

and potassium bromide mixture (274), and centrifuging it at 30,000 r.p.m. for 20 hours.

Polyanion Precipitation of Low Density Lipoproteins:

In one study LDL_p were separated from plasma by polyanion precipitation method described by Florsheim and Gonzales (275). This procedure uses "mepsulfate", the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside (Hoffman-La Roche) as the polyanion.

The amounts of cholesterol in LDL_p separated from equal amounts of plasma whether by precipitation or by ultracentrifugation were similar in various experiments (Table 3). The cholesterol in HDL_p remaining after removal of LDL_p by the two methods were also within experimental error (Table 4). The completeness of precipitation of LDL_p was further established by adjusting the density of plasma after removal of precipitates to 1.063 and separation of LDL_p in a preparative ultracentrifuge. No cholesterol was detected in the top ml. of the solution, in any of the experiments. When LDL_p precipitated by "Mepsulfate" were redissolved in a solution of density 1.063, all the cholesterol was recovered in the top ml. fraction after ultracentrifugation. The chylomicrons were separated by centrifuging the serum at 30,000 r.p.m. for 10 minutes.

Table 3

Mean amounts (µg) of cholesterol in LDL_p separated by "Mepsulfate" or by ultracentrifugation from identical amounts of plasma in various experiments.

Experiment	"Mepsulfate"	Ultracentrifuge
1	72.5	75
2	170.5	161.5
3	56.0	45.0
4	220.0	231.0

Table 4

Mean amounts of cholesterol in HDL_p separated from identical amounts of plasma after removal of LDL_p either by "Mepsulfate" or by ultracentrifugation.

Experiment	"Mepsulfate"	Ultracentrifuge
1	3.84 mg.	3.90 mg.
2	197 µg	176 µg

The proteins of high and low density lipoproteins were delipidized, according to the method of Scanu et al. (276).

Extraction of Lipids.

Extraction of Lipids from Serum, Serum Lipoproteins and Red Blood Cells:

For each milliliter sample of serum, solution of lipoproteins or cell suspension, 25 ml. of alcohol:acetone (1:1) were used. The sample was pipetted into a volumetric flask and alcohol: acetone added to fill the flask to two thirds its volume. The mixture was brought to a boil, allowed to cool to room temperature, and made up to volume.

In one of the earlier experiments, the solution containing HDL_p was lyophilized, taken up in 0.5 ml. of distilled water and boiled for 60 minutes with 200 ml. of alcohol:acetone; it was allowed to cool to room temperature and then transferred quantitatively into a volumetric flask.

Extraction of Lipids from the Liver:

Each liver was homogenized in approximately 30 ml. of chloroform:methanol (2:1) for five minutes in a "virtis" homogenizer. The homogenate was filtered into a 50 or 100 ml. volumetric flask. The vessel in which the liver was homogenized, the filter

paper, the precipitates and the funnel were all thoroughly washed into the volumetric flask with chloroform:methanol to bring the filtrate up to volume. The lipid extracts were then washed in a large volume of distilled water by the method of Folch et al. (277), and remade up to volume. Aliquots of this were evaporated to dryness and the lipids were redissolved in petroleum ether, leaving behind some of the proteins extracted by chloroform: methanol.

Chemical Determinations.

Determination of Cholesterol:

For screening of nephrotic animals for hypercholesterolemia, total serum cholesterol was determined by the procedure of Zak et al. (278). For all other purposes extraction, hydrolysis and separation of cholesterol by precipitation with digitonin were done according to the method of Sperry and Webb (279). The precipitates of cholesterol digitonide were washed with distilled water in addition to the washings with acetone-ether and ether as recommended by Sperry and Webb. The color development for the determination of the amounts of cholesterol was done according to the method of Zak et al. (278).

The following studies were undertaken to establish the reliability of the latter method.

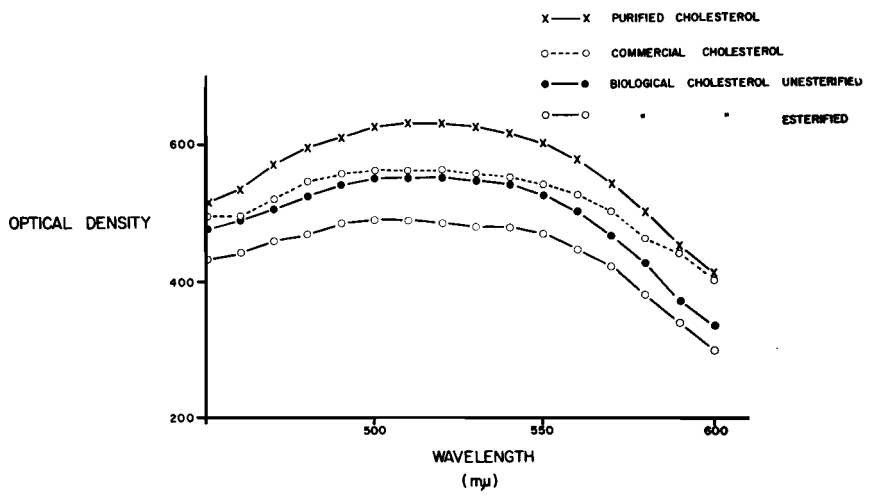


Figure 3

Absorption spectra of cholesterol.

Absorption Spectra:

Absorption spectra were obtained for commercial cholesterol, cholesterol purified by dibromination (280) and biological cholesterol both unesterified and esterified (Fig. 3), and the identity of biological cholesterol with the purified cholesterol was established. The commercial cholesterol had somewhat greater absorption at higher wave lengths than the other two. The purified cholesterol was therefore used as a standard in all the experiments.

Standard Curves:

Color was developed with different amounts of purified cholesterol and its optical density was plotted against the cholesterol concentration in the mixture (Fig. 4). A straight-line relationship from 5 µg. up to 300 µg. was observed. For each set of determinations on unknown samples, a standard curve with at least three different known amounts of purified cholesterol was made; from this the amounts in the unknowns were assessed.

Optical Densities of Free Cholesterol Versus Cholesterol Digitonide:

Most of the quantitative determinations of cholesterol were done on the digitonide precipitates of cholesterol; however, the standards used for all the determinations were made up with unesterified cholesterol. It was thus relevant to determine the

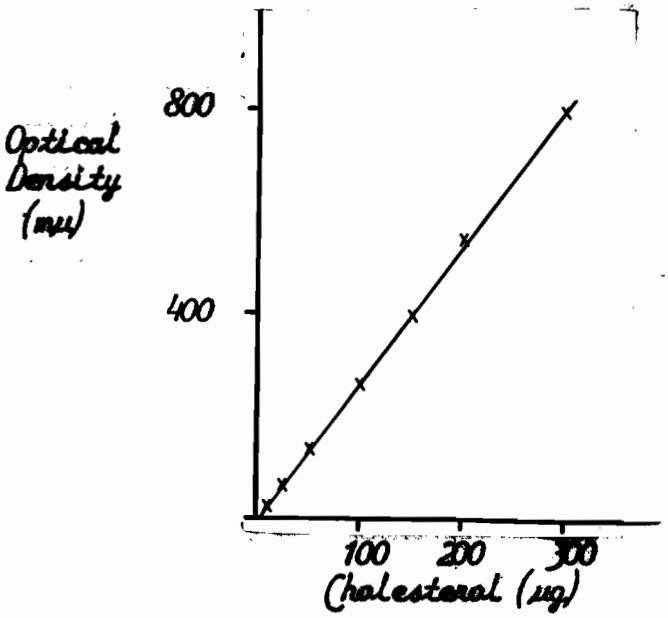


Figure 4

Standard curve of optical density versus cholesterol concentration.

Table 5

Optical Densities of Unesterified Cholesterol and Cholesterol Digitonide.

Amount (µg)	Cholesterol Unesterified	Cholesterol Digitonide
10	25	30
25	70	76
50	151	149
100	298	301
150	452	445
200	597	585
300	880	905

optical densitis of identical amounts of cholesterol in both unesterified and digitonide forms.

Different amounts of the standard cholesterol were taken in duplicate and one aliquot was precipitated with digitonin and washed. The optical densities and the absorption spectra (Fig. 5) of the two were compared. The results are given in Table 5 and do not show any systematic difference.

Reproducibility of Estimations:

Replicate aliquots from samples of serum, plasma or their lipid extracts were taken for the determination of the amounts of unesterified and total cholesterol. The results are listed in Tables 6 and 7. The coefficients of variation were between 2 to 4% for unesterified and 2 to 3% for total cholesterol.

Recoveries:

A fixed amount of purified cholesterol was added to aliquots of several sera and determinations of the cholesterol in sera with and without the added cholesterol were made. The results (Table 8) indicate a recovery of 96.8% with a standard deviation of 1.5

Changes in Optical Density of the Color with Time:

The optical densities of two aliquots of standard

Table 6

Unesterified Cholesterol (µg) in Replicate Aliquots of Various Samples

Aliquot No.						
1	43.0	22.5	25.0	67.0	115.0	99.0
2	41.5	22.0	25.0	67.0	115.0	104.0
3	43.0	21.5	25.0	67.0	220.0	100.0
4	40.5	21.5	27.0	63.0	108.0	104.0
5			27.0			
6			25.0			
7			25.0			
8			27.0			
9			27.0			
Mean	42	21.9	25.9	66	112	101.7
Standard Deviation	1.2	0.47	1.02	2.0	3.5	2.6
Coef. Variation %	2.8	2.1	3.9	3.0	3.1	2.4

Table 7

Total Cholesterol (ug) in Replicate Aliquots of Various Samples

Aliquot No.				
1	62.0	122.0	177	152
2	60.5	130.0	180	152
3	64.0	123.5	175	154
4	61.5	128.0	172	158
5	61.5			153
6	63.0			151
Mean	62.1	12.5	176	153.3
Standard Deviation	1.24	3.7	3.32	2.5
Coef. of Variation	2.0	2.9	1.9	1.6

Table 8

Recoveries of Unesterified Cholesterol Added to Serum

Aliquot No.	Amt. in Serum (μg.)	Amt. Added (μg.)	Amt. Recovered (μg.)	Recovery %
1	67.0	100.0	158.0	94.6
2	65.5	100.0	160.0	96.7
3	66.5	100.0	157.0	94.3
4	68.0	200.0	258.0	96.3
5	70.0	200.0	263.0	97.4
6	66.5	200.0	256.0	96.1
			Mean	96.8
			Standard Deviation	1.5

cholesterol solution were noted at various time intervals after color development. No changes in optical density were noted between 15 minutes and 8 hours.

Effect of Washing Cholesterol Digitonide Precipitates with Water:

Early in our studies it was noticed that occasionally on adding ferric chloride reagent to the solution of cholesterol digitonide, a brownish color developed in place of the pink-violet given by unesterified cholesterol. Digitonin alone when dissolved in acetic acid gives a brown color on addition of ferric chloride reagent. This suggested that the brownish tinge in the samples of cholesterol may be due to digitonin.

Digitonin is very sparingly soluble in acetone or acetone-ether, so that washing the cholesterol digitonide precipitates with these solvents would not remove any excess digitonin which might coprecipitate with cholesterol digitonide. In view of the fact that digitonin was always used in excess, the possibility of coprecipitation of digitonin could not always be guarded against. Since digitonin is soluble in water and cholesterol digitonide is not, the precipitates of cholesterol digitonide were washed with distilled water before washing with organic solvents.

The effect on its absorption spectrum, of washing the cholesterol digitonide with distilled water was tested by comparison with the spectrum of unesterified cholesterol (Fig. 5).

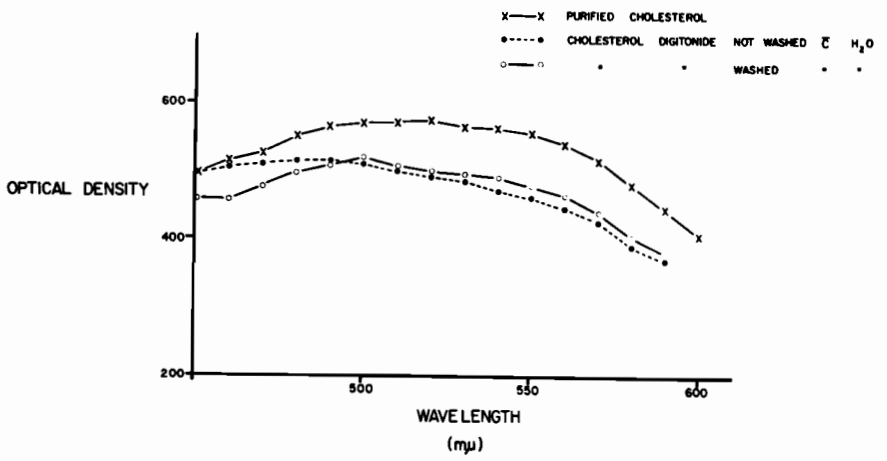


Figure 5

Effect of washing cholesterol digitonide precipitates with distilled water on its absorption spectrum.

The spectra of unesterified cholesterol and cholesterol digitonide washed with water were identical, whereas the absorption spectrum of the cholesterol digitonide washed only with organic solvents showed a greater absorption at lower wave lengths as compared with the other two.

Chemical Reaction of Cholesterol and Digitonin:

It was suggested by Windaus (281) that one molecule of cholesterol combines with one molecule of digitonin, and, since then separation of cholesterol by precipitating it with digitonin has become a standard procedure (279). However, it is now well known that substances other than cholesterol are also precipitated by digitonin (280).

To confirm the mono-molecular nature of the reaction between cholesterol (M.W. 387) and digitonin (M.W. 1,229) and to determine the effects of concentrations of cholesterol, purified by dibromination (280) and of digitonin on their precipitation as cholesterol digitonide, the following experiment was performed.

To various amounts of purified cholesterol in 2 ml. of alcohol-acetone, different amounts of digitonin dissolved in 2 ml. of 50% alcohol were added as in Table 9. The cholesterol in the precipitates was determined and the estimated values as well as expected values of cholesterol on an equimolecular basis are given in Table 10. The results indicate that:

Table 9

Reaction between Cholesterol and Digitonine when Present in Different Concentrations

NO.	Cholesterol (mg.)	Digitonine (mg.)	Cholesterol (mg.)	
			Determined	Expected
1	2.0	8.0	2.224	2.00
2	2.0	4.0	1.404	1.250
3	2.0	2.0	0.700	0.625
4	2.0	0.8	0.261	0.250
5	1.0	8.0	1.060	1.000
6	1.0	4.0	1.110	1.000
7	1.0	2.0	0.656	0.625
8	1.0	0.8	0.261	0.250
9	0.5	8.0	0.500	0.500
10	0.5	4.0	0.528	0.500
11	0.5	2.0	0.500	0.250
12	0.5	0.8	0.234	0.200
13	0.2	8.0	0.210	0.200
14	0.2	4.0	0.210	0.200
15	0.2	2.0	0.186	0.200
16	0.2	0.8	0.160	0.200

1. The reaction between purified cholesterol and digitonin is on an equimolecular basis.

2. The optical density of color given by precipitates of cholesterol digitonide was slightly greater in some instances than that which could have been given by all the cholesterol present in the system, or by amounts of cholesterol in the precipitates, calculated theoretically on monomolecular basis. Since the solution of purified cholesterol for making the standards was the same as used for the studies, error in the weights of cholesterol could not account for the observed values. Thus it seems that digitonin in cholesterol digitonide precipitates contributes a little to the optical density of the color developed.

3. When the concentrations of cholesterol as well as digitonin in the solution were low, complete precipitation of cholesterol did not occur.

Determination of Fatty Acids:

Total fatty acids in the serum lipoproteins and their specific activity were determined by the method used by Saffran and Kalant (229). The lipids were extracted in ethanolic KOH and cholesterol was separated by extraction with ether. The fatty acids were separated from the aqueous phase after acidification and were measured gravimetrically.

Albumin:

Albumin in the urine was determined by the method of Gornall *et al.* (283) after precipitation of protein with 20% tricholoracetic acid.

Chromatography.

Chromatographic separation of lipids was done in several instances on silicic acid columns, using the method of Hirsch and Ahrens (282).

Labeling of Serum Lipoproteins with C^{14}-Cholesterol.

Most of the studies involved the use of serum lipoproteins labeled with C^{14}-cholesterol. Serum lipoproteins can be labeled with C^{14}-cholesterol by injecting into donor animals C^{14}-labeled precursors such as acetate or mevalonic acid. The specific activities obtained by these *in vivo* techniques are not very high. To obtain high specific activities, *in vitro* methods have been reported (284, 285). Both types of methods were used in the present work.

Incubation of Serum with C^{14}-Cholesterol Dispersed on an Inert Powder (284):

C^{14}-cholesterol is dissolved in pentane and mixed with

"Celite". The solvent is evaporated under nitrogen, leaving cholesterol dispersed on the inert powder, which is then incubated with serum (or serum lipoproteins) at 37° C. for 20 hours. The serum is separated and has been shown to contain C^{14}-cholesterol associated with the lipoproteins.

Several other sterols and steroids were also taken up by the serum lipoproteins by this method of labeling. The cholesterol content of the serum was increased by such an incubation. It was therefore concluded that the labeling was obtained by non-specific "solubilization" of the cholesterol and other sterols in the lipid phase of serum lipoproteins. It was claimed however, that the labeled cholesterol incorporated in vitro behaved like the C^{14}-cholesterol incorporated into the serum lipoproteins in vivo after intravenous injection into rats and also in its ability to exchange with red blood cell cholesterol.

The following preliminary studies were done on serum labeled by this method (284).

To Determine if All the Radioactivity was Associated with Lipoproteins:

The proteins in 0.5 ml. of labeled serum were precipitated with sodium tungstate and sulphuric acid (286) and filtered. The precipitates were washed, dried, weighed and then extracted with alcohol acetone.

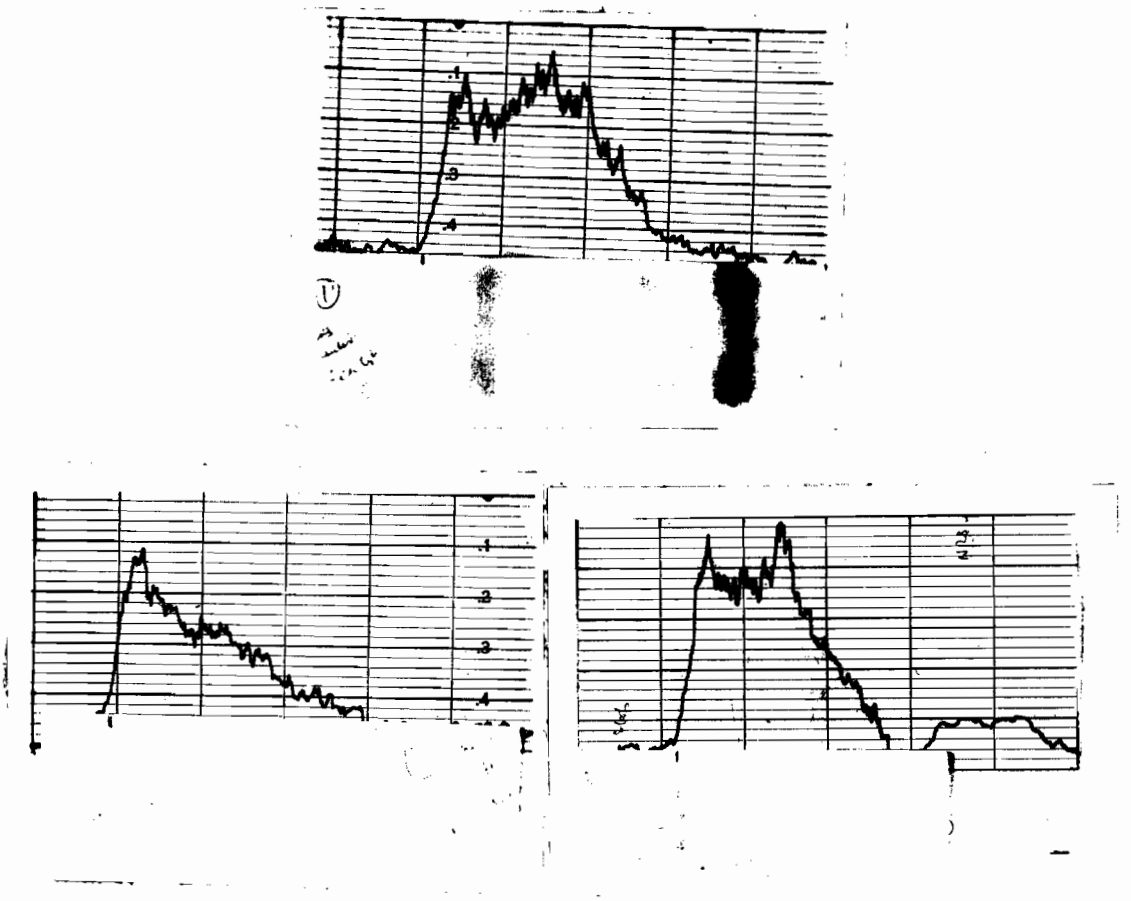


Figure 6

Distribution of radioactivity on separation of labeled sera by paper electrophoresis.
(Uppermost is stained for proteins and the lower strips for lipids)

The counting rates of the lipid extract of the precipitates and the filtrate were determined (Table 10). Almost no radioactivity was found in the filtrate indicating that the labeled cholesterol was in combination with the serum proteins.

Fractionation of Labeled Sera by Paper Electrophoresis:

Different samples of labeled sera were electrophoresed either for 16 hours at 110 volts and stained with bromphenol blue, or for 6.5 hours at 200 volts after prestaining the serum for lipids (272) (Fig. 6). The radioactivity present in the serum showed itself at the site of application and associated with α- and β- globulins.

Disappearance of Radioactivity from Blood:

Aliquots of normal rat serum labeled with C^{14}-cholesterol were injected into two normal rats and the disappearance of radioactivity from the serum of animals was observed. Another aliquot was electrophoresed and the paper strip was radioautographed as well as scanned for the distribution of radioactivity. The results are given in Figs. 7 and 8.

Conclusions:

It is concluded from these experiments that the radioactivity in the labeled serum is associated with the plasma lipoproteins and that the rate of disappearance of the label from the

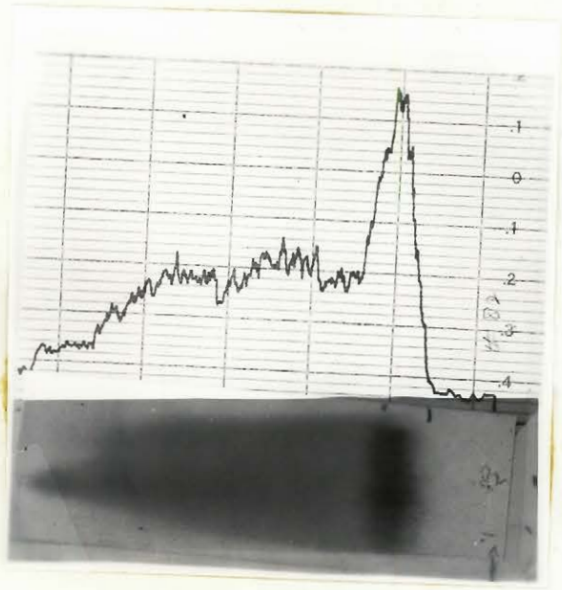


Figure 7

Radioautograph and scan of labeled serum after its fractionation by paper electrophoresis.

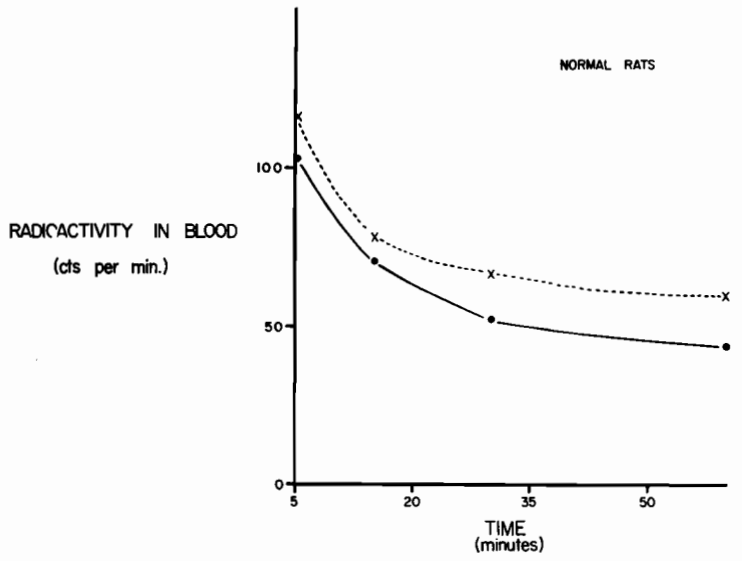


Figure 8

Time course of disappearance of radioactivity from blood of two normal rats after injection of normal rat serum labeled with C^{14}-cholesterol.

Table 10

Association of Radioactivity with Proteins in the Serum Labeled by Incubating Serum with C^{14}-Cholesterol on Celite.

Total Radioactivity in the precipitated proteins	21763 cts/min
Total Radioactivity in the filtrate	280 cts/min
Percentage of total radioactivity with proteins	98.73

Table 11

Association of Radioactivity with Proteins in the Serum Labeled with Tween-20 Cholesterol-C^{14} Suspension.

Total radioactivity in the lipid extract of the precipitates	256,000 cts/min
Total radioactivity in the filtrate	1,000 cts/min

Table 12

Relative Amounts of Radioactivity in Unesterified and Esterified Fractions of Cholesterol in Serum

Total radioactivity in the precipitates of unesterified cholesterol	16,860
Total radioactivity in the supernatant (esterified cholesterol)	2,120 cts/min

sera of injected rats were comparable to that observed (284) in rats after injection of the in vivo labeled serum.

Addition of Tween 20 C^{14}-Cholesterol Suspension to the Serum (285) :

This method is based on the assumption that labeled cholesterol which is "suspended" in aqueous solution of Tween 20 when added to the serum exchanges with the cholesterol molecules of the serum lipoproteins. It was shown that Tween 20 did not adsorb to globulins non-specifically and that the distribution of labeled cholesterol in high and low density lipoproteins corresponded to their cholesterol content. The process of labeling of lipoproteins was not temperature dependent.

The following studies were done to determine the disposition of the C^{14}-cholesterol in the serum labeled by this method.

To Determine if all the Radioactivity was Associated with Lipoproteins:

The proteins in 0.5 ml. of labeled serum were precipitated with sodium tungstate and sulphuric acid, filtered, washed and dried. The precipitates were extracted with chloroform-methanol. Counting rates of the lipid extract and of the protein free filtrate of the serum were determined (Table 11). The lipid extract of the precipitates had about 250 times the activity left in the filtrate, indicating that the label was associated with the proteins in the serum.

To Determine the Relative Amounts of Radioactivity in Unesterified and Esterified Fractions of Cholesterol in Serum:

The cholesterol was precipitated from the extract of 0.4 ml. of labeled serum, and the supernatant and washings of the cholesterol digitonide were pooled. The radioactivity was determined in the precipitates and in the supernatant (Table 12).

It was presumed that supernatants and the washings contained all the cholesterol esters and that all the unesterified cholesterol was precipitated by digitonin. The unesterified cholesterol had eight times more radioactivity than in its esters.

To Determine the Effect of Increasing the Amount of Label in Serum on the Disposition of the Radioactivity:

Six aliquots of 1 ml. each were taken from a pool of normal rat serum and different amounts of labeled cholesterol in Tween 20 suspension were added. Proteins in 0.5 ml. of serum in each tube were precipitated by sodium tungstate and sulfuric acid, centrifuged, and washed with distilled water. Supernatant and washings of the precipitates were pooled and their counting rates determined. The precipitates were extracted with chloroform-methanol and radioactivity determined (Table 13).

The results indicated that for amounts of C^{14}-cholesterol used to label the sera in subsequent work, almost all the radioactivity was associated with lipoproteins.

Table 13

Radioactivity Free and in Association with Proteins in Sera Labeled with Different Amounts of Tween-20 C^{14}-Cholesterol

Specimens	cts/min/ml	
	Proteins	Free
1	90,820	1,280
2	128,540	240
3	160,780	520
4	249,500	640
5	307,200	320
6	464,620	1,240

Labeling of Serum Lipoproteins by Incubation with Labeled Red Blood Cells:

Red blood cells were labeled by the addition of C^{14}-cholesterol suspension in Tween 20 to heparinized blood and incubating the mixture overnight in the cold. The cells were separated, washed twice with normal saline and then incubated with fresh serum overnight in the cold. The serum, after incubation, had C^{14}-cholesterol presumably incorporated into lipoproteins by a process of exchange with cells.

The necessity of adding Tween 20 into the serum was circumvented by labeling the lipoproteins indirectly by a process of exchange from red cells though the possibility of the Tween molecules remaining linked with C^{14}-cholesterol during all the processes of exchange could not be ruled out., The concentration of Tween in the labeled serum was probably much smaller, if not insignificant, as compared to the method of labeling when Tween is added to the serum.

Determination of Radioactivity.

Liquid Samples:

Counted as Solids:

Liquid samples, such as serum lipid extracts in organic

solvents, cholesterol digitonide solution in glacial acetic acid, fatty acids dissolved in alcohol and aqueous solutions of serum lipoproteins were counted by plating aliquots on aluminum planchets of known weights. The samples were evaporated to dryness, weighed, and the weight of the sample determined by difference, and counted in a gas flow Geiger counter with a thin window (Nuclear Chicago D 47) or a gas flow proportional counter (Measurement Engineering) up to a total of 10,000 counts in order to obtain a standard deviation of 1% of the counts. In most of the studies the amounts of material plated were small enough to give "infinitely thin" samples; however, when the amount of the material plated was enough to cause self absorption, adequate corrections were made from self absorption curves made with the same material and on the same counter.

Background radioactivity was determined daily. The stability of the counting equipment was checked every few hours by the use of standards.

Samples of blood were plated on lens paper discs put in the aluminum planchets to obtain even spread.

The reliability of plating was checked in the following experiment: Replicate aliquots of different amounts of C^{14}-cholesterol dissolved in glacial acetic acid were plated and the counting rates were determined.

Results (Table 14) indicate that the coefficients of

Table 14

Counting rate of replicate aliquots of C^{14}-Cholesterol dissolved in glacial acetic acid.

Counts per minute

	Aliquot size (µL)					
	10	25	50	100	200	300
1	69	165	330	640	1355	1990
2	65	158	312	690	1310	1970
3	68	167	340	650	1300	1940
4	70	150	320	670	1325	2000
5	66	165	335	664	1334	1980
6	68	160	325	681	1326	2010
Mean	68	161	327	666	1325	1982
Standard Deviation	1.86	6.3	10.2	18.7	19.1	24.8
Coefficient of variation as percent of mean	2.7	3.9	3.1	2.8	1.4	1.2

variation of the counts were between 1.2 and 3.9%. Counting rates of aliquots of volumes between 10 to 300 µL., all infinitely thin samples, were proportional to the volumes plated.

The variation in the efficiency of the counting equipment from moment to moment was tested by counting a number of planchets repeatedly (Table 15). The coefficient of variations of the counts were between 0.44 and 1.8% of the means.

Liquid Scintillation Counting:

In some of the studies a liquid scintillation counter with automatic sample changer and two photomultiplier tubes (TriCarb) was used to determine the counting rates of aliquots of lipid and cholesterol. The aliquots to be counted were evaporated to dryness in the sample holders and then dissolved in 10ml. of the scintillation mixture (287).

The lipid samples gave a yellowish color to their solution and the absorption of scintillations by the color in each sample was assessed by addition of a fixed volume (100 µL) of toluene containing C^{14}-cholesterol into all samples. The radioactivity added into samples was estimated by determining its counting rate in colorless blank scintillation mixture. From the counting rates of the additional radioactivity in the colored samples, absorption by the color was determined and the original counts corrected.

Table 15

Repeated Determinations of Counting Rates of Different Samples.

Time (minutes) to count 10,000 counts

	Sample					
	I	II	III	IV	V	VI
1	6.19	6.34	3.42	3.42	1.49	1.47
2	6.23	6.31	3.45	3.43	1.49	1.46
3	6.22	6.28	3.48	3.39	1.51	1.46
4	6.16	6.29	3.43	3.46	1.49	1.46
5	6.17	6.28	3.38	3.32	1.50	1.45
6	6.21	6.26	3.30	3.45	1.51	1.47
Mean	6.19	6.29	3.41	3.41	1.49	1.46
Standard Deviation	0.029	0.028	0.062	0.051	0.013	0.007
Coefficient of variation as percent of mean	0.47	0.44	1.8	1.5	0.87	0.47

For Paper Strips:

Radioautographs of paper strips with radioactive serum fractions were made on no-screen X-Ray plate by leaving the two in contact for periods of a few weeks. When developed, the site of radioactivity was represented by blackening of the corresponding area on the X-ray film.

The scanning for radioactivity was done on an automatic scanner, having a gas flow detector with a thin window. The strip of paper was drawn past the detector and the radioactivity in the area of paper over the window was recorded on a strip chart recorder. The drive motor of the recorder was used to drive the paper strip so that the count rate meter was synchronized with the recorder.

Experiments and Results

Introduction

Lipoprotein lipase accelerates the clearing of alimentary lipemia (198, 199) and an alteration in its activity may cause the delay observed (229, 249) in the clearance of injected chylomicrons from the blood of nephrotic rats. Lack of adequate amounts of albumin and the presence of an inhibitor in the plasma of nephrotic animals have also been considered (262, 251) as possible explanations for the retention of lipids in their plasma. Studies were done to elucidate the role of albumin and lipoprotein lipase inhibitor in nephrotic hyperlipemia.

There is an increase in all classes of plasma lipids in nephrosis (220) but most of the studies reported herein were done to evaluate factors contributing primarily to hypercholesterolemia. Liver is the principle endogenous source of plasma cholesterol. Thus rates of hepatic synthesis of cholesterol from acetate and mevalonic acid and also the rates of its incorporation into plasma lipoproteins were compared in normal and nephrotic rats. Turnover rates of plasma cholesterol were also determined in normal and nephrotic rats.

The possibility of a qualitative or quantitative alteration in the plasma lipoproteins contributing to hyperlipemia was considered. To evaluate this, rates of exchange of cholesterol from normal and nephrotic plasma and plasma lipoproteins

with that of red blood cells and liver were compared both *in vitro* and *in vivo*.

It has been shown (102) that there is an increase in synthesis of plasma albumin and lipoproteins by the liver. It is possible that increased synthesis of lipoproteins results in an increased concentration of the protein moities of the lipoproteins in the liver and that this in turn stimulates synthesis of lipids. To test this hypothesis, liver homogenates were prepared (181) and the effects of protein moities of high and low density lipoproteins were studied on the rates of synthesis of cholesterol by the homogenates.

Removal of Chylomicron Lipid from Plasma

In order to elucidate the role of various factors in causing delay in the removal of chylomicrons from the blood of nephrotic animals, the following studies were carried out.

Experiment 1:
Effect of Albumin Administration on Rate of Disappearance of Chylomicrons from Blood of Nephrotic Rats.

It has been postulated (262) that the retention of lipids in the blood of nephrotic rats is due to lack of albumin in amounts adequate for clearing of lipids at a normal rate. The concentration of circulating albumin was therefore raised to normal levels and its effect on the rate of clearance of chylomicrons determined.

Six nephrotic rates were used, each serving as its own control. Labeled chyle was injected into each rat intravenously twice at an interval of 2-3 days, and the disappearance rate of radioactivity from the blood was measured. Ten minutes before the second injection, each rat received 1 ml of rat serum albumin (9 g./100 ml), sufficient to increase serum albumin by approximately 1.5 g/100 ml.

The fractional rate constants before and after albumin administration were determined and are given in Table 16. The

Table 16

Fractional Rate Constant Per Minute of Chylomicron Disappearance in Nephrotic Rats.

	Before injection of albumin	After injection of albumin
1	0.0014	0.0037
2	0.0084	0.0113
3	0.0022	0.0027
4	0.003	0.0011
5	0.0018	0.0056
6	0.0070	0.0556
Mean	0.0035	0.0133
SD	0.0031	0.0210

P value for the difference before and after the injection of albumin < 0.05

results indicate that, except in one rat (#16), raising the levels of albumin raised the fractional rate constant of removal of chylomicrons to a degree which though statistically significant, was not marked enough to have any physiological importance. Even in rat #6 the fractional rate constant remained much lower than normal (229).

This shows that hypoalbuminemia per se is not an important factor for retention of chylomicron lipid in the blood of nephrotic animals.

Experiment 2:

Inhibitor to Lipoprotein Lipase Present in Plasma of Nephrotic Animals.

It was suggested (251) that nephrotic plasma contained an inhibitor to post-heparin clearing factor. Clearance of chylomicron lipid was studied in in vitro in order to confirm the presence of the inhibitor in the nephrotic plasma.

Normal rat serum was made lipemic by addition of normal rat chyle and was used as substrate for the clearing action of post-heparin plasma (lipoprotein lipase). To observe the effect of the inhibitor present in nephrotic serum, 0.1 ml or 0.3 ml aliquots of nephrotic serum were added to 1 ml of lipemic rat serum before addition of 0.1 ml of post-heparin plasma. The same amounts of normal serum were added to the clearing system as control. The optical density at 550 mμ was read at 0, 30 and 60

Table 17.

Changes in the Optical Density of Lipemic Serum Caused by Post-Heparin Lipoprotein Lipase in the Presence of Normal and Nephrotic Sera.

	Concentration of Test Serum % of Total	Mean Change in O.D.			
		30'	% difference	60'	% difference
Normal (6)	10	.112	27	.145	30
Nephrotic (6)	10	.082		.111	
Normal (6)	25	.090	29	.134	28
Nephrotic (5)	25	.059		.096	
Normal (6)	10	.059	44	.100	32
Nephrotic (8)	10	.033		.068	
Normal (6)	25	.029	62	.059	56
Nephrotic (8)	25	.011		.026	

minutes as a measure of clearing of plasma.

The results (Table 17 and Figure 9) indicate that nephrotic plasma in a concentration of 10% by volume caused a 27-44% inhibition of lipemia clearing. An increase in the concentration of nephrotic plasma to 25% by volume caused an inhibition of somewhat greater magnitude.

The results confirm the presence of some factor(s) in the nephrotic serum which inhibits the clearing of lipemic plasma *in vitro* by post-heparin lipoprotein lipase.

Experiment 3:

Effect of Nephrotic Plasma on Disappearance Rate of Chylomicrons from Blood of Normal Rats.

In order to evaluate the significance of the inhibitor in *in vivo* clearing of lipids, the following study was done:

Three normal rats were injected with 0.5 ml labeled chyle and the disappearance rate of radioactivity in blood was measured. Three days later, 1 ml of nephrotic serum was given to the same animals, followed in 10 minutes by a second injection of labeled chyle; radioactivity in blood was again measured.

The results (Table 18) show that no inhibition of removal occurred in any of the animals as a result of administration of nephrotic serum in volumes to give concentration equivalent to

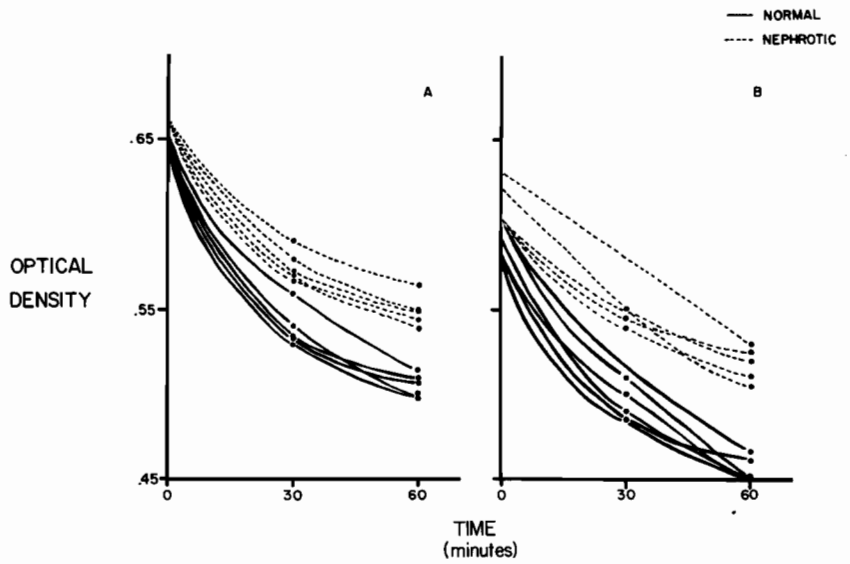


Figure 9

Clearing of lipemic rat serum by post-heparin lipoprotein lipase in presence of normal or nephrotic sera in concentrations of 10% (A) and 25%(B).

Table 18

Fractional Rate Constant Per Minute of Chylomicron Removal from Blood of Normal Rats before and after Injection of Nephrotic Plasma

No.	Before injection of nephrotic plasma	After injection of nephrotic plasma
1	0.158	0.163
2	0.193	0.177
3	0.190	0.188

what was obtained in *in vitro* experiments.

This indicates that nephrotic rats have an inhibitor to lipoprotein lipase in their plasma though this inhibitor does not appear to play a significant role in retention of chylomicrons in the plasma of intact animals. The possibility exists that the inhibitor did not remain in the circulation long enough to be effective.

It was suggested that the delay in the removal of chylomicrons in nephrotic animals is due to isotopic dilution in increased amounts of plasma lipids (249). The following two experiments were done to determine if there is any dilution of injected chylomicrons, and to determine the effect of isotopic dilution, if present, on the rate of removal of injected chylomicrons.

Experiment 4:

Effect of Glucose Feeding on Chylomicronemia.

In one of the studies (229) demonstrating the delay in removal of chylomicrons in nephrosis, animals were given only 50% glucose to drink for 16 hours to the exclusion of all other food. Effect of this treatment on endogenous chylomicronemia was observed on normal and nephrotic rats.

Chylomicron counts were made on the serum samples of four normal and four nephrotic rats prepared by giving 50% glucose overnight. One normal rat so prepared was given 1 ml of chyle intravenously and serum was taken for chylomicron count 2 minutes

after the injection. The counts in one standard field were: normal, 3-5; nephrotic, 5-10; normal rat injected with chyle, 550.

Thus the levels of endogenous chylomicronemia were insignificant in the presence of the level produced by injection of chyle, so that isotopic dilution of injected chylomicrons could not account for the delay in their clearance in nephrotic animals.

Experiment 5:

Effect of Endogenous Hyperlipemia on Rate of Disappearance of Chylomicrons from Blood.

Two rats were fed a diet containing 60% butter fat and 5% cholesterol; this produced fasting serum lipid levels of 5.4 and 4.9 g./100 ml. The rate of removal of chylomicrons from the circulation was then measured in these animals and in two control animals given the standard diet.

The fractional rate constants of removal were essentially the same for the two pairs of rats; 0.038 and 0.053 per minute for control animals, and 0.042 and 0.043 per minute for hyperlipemic animals, suggesting that increased concentrations of serum lipids do not decrease the rate of removal of injected chylomicrons.

Conclusions.

1. These studies confirm the presence of an inhibitor

to post-heparin lipoprotein lipase in the plasma of nephrotic rats, however the inhibitor does not cause any delay in removal of chylomicron lipid from the blood of normal rats.

2. It was reported earlier (249) that administration of bovine albumin did not correct the delay in clearance of chylomicrons from the blood of nephrotic rats. Our studies done with rat serum albumin also show that correction of hypoalbuminemia did not significantly alter the rate of clearance of chylomicron lipid.

3. The hypothesis that the "apparent" delay in chylomicron clearance in nephrosis was due to isotope dilution could not be substantiated.

Plasma Volumes of Normal and Nephrotic Rats.

In subsequent studies knowledge of sizes of plasma pools of cholesterol and other lipids was necessary. In order to obtain this plasma volumes were determined in normal and nephrotic rats.

Most of the studies reported hereafter were done on rats 2-3 weeks after injection of AKS when they had no manifest edema. Therefore similar animals were used for the determinations of plasma volume in this experiment. Normal rats of equivalent weights were also studied.

Experiment 6

In a preliminary experiment to determine the rate of disappearance of I^{131}-albumin from the blood of animals two normal rats weighing 250 g. were each injected with 1 µc of I^{131}-albumin dissolved in 0.5 ml of normal saline. Blood (0.05 ml) from the cut end of their tails were plated at 1, 2, 3, 4, 5, 6, 8, and 10 minutes after injection. The time course of radioactivity (counts/minute) in the samples was determined and showed a plateau from 1 to 6 minutes (Fig. 10). A 2-minute period was selected as the interval of choice after the injection for calculating the pool size in which the I^{131}-albumin was mixed.

Nine nephrotic and seven normal rats were weighed and

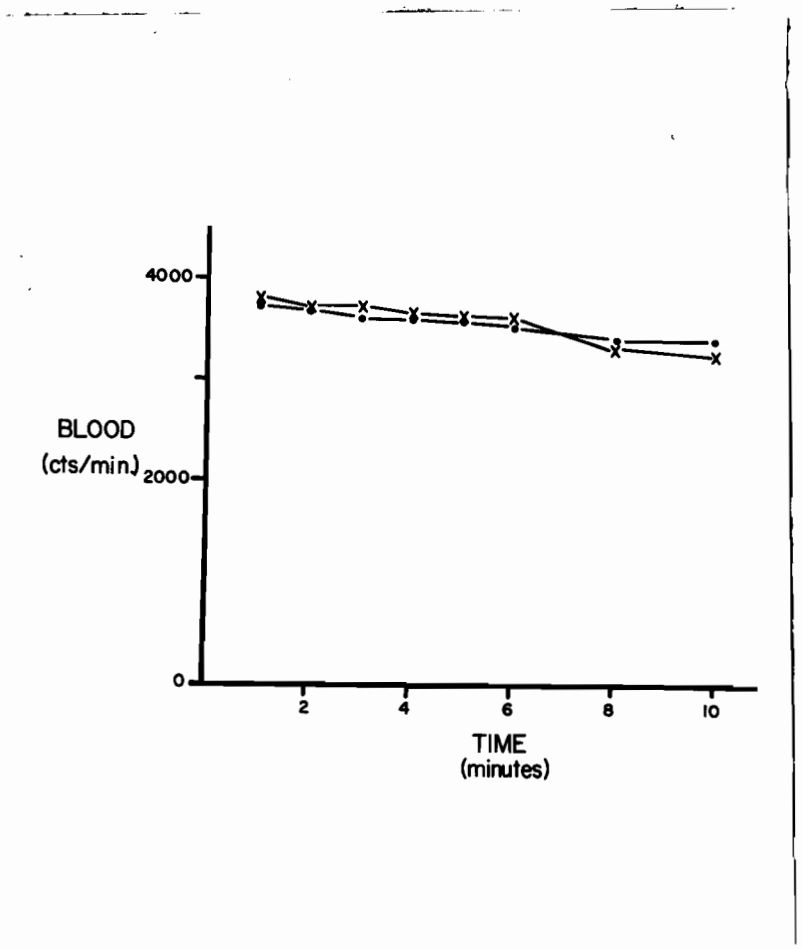


Figure 10

Time course of radioactivity in blood (0.05 ml) after injection of I^{131}-albumin.

Table 19

Plasma Volumes and Hematocrits

Body Weight (g)	Plasma cholesterol (mg/100ml)	Plasma volume (ml) per animal	Plasma volume (ml) per 100g body weight	Hematocrit
		Normal		
190	-	6.656	2.977	42.8
178	-	5.841	3.281	45.4
193	-	6.301	3.265	43.9
185	-	6.223	3.640	42.9
190	-	5.665	2.981	46.5
187	-	7.065	3.767	50.0
177	-	5.275	2.980	45.1
		Mean	3.2	45.2
		S.D	0.33	2.4
		Nephrotic		
200	403	7.208	3.604	44.5
208	408	7.035	3.382	40.3
202	310	6.596	3.265	43.8
240	505	8.049	3.265	40.5
196	476	6.835	3.407	40.4
170	325	5.896	3.468	34.4
280	153	7.994	2.855	47.7
190	315	6.354	3.344	30.7
190	257	7.054	3.713	47.3
		Mean	3.4	41.9
		S.D.	0.24	4.2

P value for the difference in plasmas volumes of normal and nephrotic rats > 0.05

injected with 0.4 ml of I^{131}-albumin solution intravenously. Their blood was collected 2 minutes after injection for determination of hematocrit and plasma radioactivity.

Plasma volumes were calculated from the dilution of injected dose (288).

The plasma volumes and hematocrits are given in Table 19. The blood volumes were calculated from the mean values of plasma volume and hematocrit; they were 5.9 ml and 5.8 ml per 100 g. body weight in normal and nephrotic rats respectively.

Conclusions.

The plasma volumes (as well as blood volumes) of nephrotic animals 2-3 weeks after the injection of AKS were not different from those of normal rats of similar weights, even in the presence of severe nephrosis as manifested by marked hypercholesterolemia.

Studies on Synthesis of Plasma Cholesterol in Normal and Nephrotic Animals

It is well established (180) that the most important endogenous source of plasma cholesterol is the liver. Recently synthesized cholesterol is incorporated into plasma lipoproteins but no data in the literature is available comparing the rates of incorporation separately into high and low density lipoproteins. Several experiments were carried out to determine in normal and nephrotic rats the rates of synthesis of cholesterol from acetate and mevalonic acids and the rates of incorporation of the newly synthesized cholesterol into plasma lipoproteins.

Experiment 7

Purpose:

To compare the rates of hepatic synthesis of fatty acids and cholesterol from acetate in normal and nephrotic rats and the rates of incorporation of recently synthesized fatty acids and cholesterol into high and low density lipoproteins of plasma.

Methods:

Fourteen normal (average body weight 220 g.) and 14 nephrotic (2 weeks after the last injection of AKS) rats (average body weight 300 g.) were fasted overnight and given 50% glucose to drink. The following morning 25 μc of

Table 20

Concentration of Unesterified Cholesterol in Plasma Lipoproteins
(mg./ml. of Plasma)

Group No.	Low Density Lipoprotein		High Density Lipoprotein	
	Normal	Nephrotic	Normal	Nephrotic
1	35	620	70	135
2	50	1060	40	150
3	70	610	60	100
4	50	910	50	285
5	35	150	45	350
6	45	1250	-	200
Mean	47.5	766.7	53.0	203.3
S.D.	12.9	387.5	4.2	69.6
Ratio	1	16	1	4

acetate-1-C^{14} (Merck, Sharp and Dohme) approximately 0.4 mg. in weight were injected intraperitoneally into all rats. At intervals of 12, 25, 40, 60, 90 and 180 minutes thereafter two rats from each group were anesthetized with nembutal and their livers and blood collected. The blood from two rats in each group was pooled and plasma separated immediately. Low density lipoproteins were separated from the specimens of plasma in a preparative ultracentrifuge at density 1.063. The mixture of plasma and salt solution remaining after separation of LDL_p was treated as a solution of HDL_p. Concentrations and specific activities of unesterified and total cholesterol and of total fatty acids in the plasma lipoprotein fractions were estimated. The amount and specific activity of unesterified cholesterol in all livers were also determined.

Results and Discussion:

All lipid moieties studied were increased in concentration in nephrotic sera as compared to controls, the increase being more marked in the constituents of LDL_p (Tables 20-22). The increase in various lipids in LDL_p was 3-4 times the increase noted in the lipid content of HDL_p.

The time course of specific activity in high and low density lipoproteins of normal and nephrotic plasma are given in Figures 11-13. Early peaks in the curves of specific

Table 21

Concentrations of Total Cholesterol in Plasma Lipoproteins
(µg/ml. of Plasma)

Group No.	Low density lipoprotein		High density lipoprotein	
	Normal	Nephrotic	Normal	Nephrotic
1	600	10700	625	800
2	375	18800	1050	3100
3	700	8500	650	6600
4	650	12300	500	8000
5	550	17400	-	6200
6	425	15100	-	4500
7	-	11500		
Mean	550	13471	706	4867
S.D.	127	3741	238	2622
Ratio	1	24	1	7

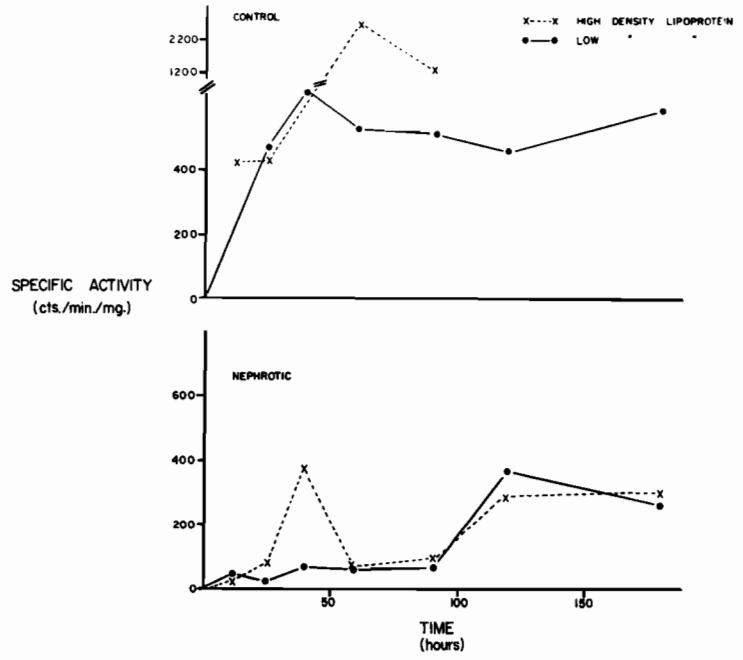


Figure 11

Specific activities of total cholesterol of plasma high and low density lipoproteins after injection of acetate-1-C^{14} into normal and nephrotic rats.

activities of cholesterol are probably due to contaminants of cholesterol which are precipitated by digitonin and have high specific activities. They have been shown to be present in the liver and plasma for about one-half hour after injection of labeled acetate (280, 302, 303). The contaminants may be intermediaries in the synthesis of C^{14} cholesterol from labeled sodium acetate (280), though they have also been thought to be alcoholic intermediaries in the synthesis of fatty acids (302). The second peaks which corresponded in time to the peak incorporation of acetate into cholesterol (246) were therefore used to calculate the incorporation of acetate into cholesterol of plasma lipoproteins.

Total Cholesterol:

The concentrations of total cholesterol in nephrotic low and high density lipoproteins were 24 and 7 times normal (Table 21).

The peak specific activity of total cholesterol in LDL_p of normal animals was about 1.6 times that of nephrotic rats. The peak values for cholesterol in HDL_p could not be compared because some samples of normal high density lipoproteins were lost. Thus considering the LDL_p cholesterol alone, the nephrotic rats had about 15 times more label incorporated into cholesterol

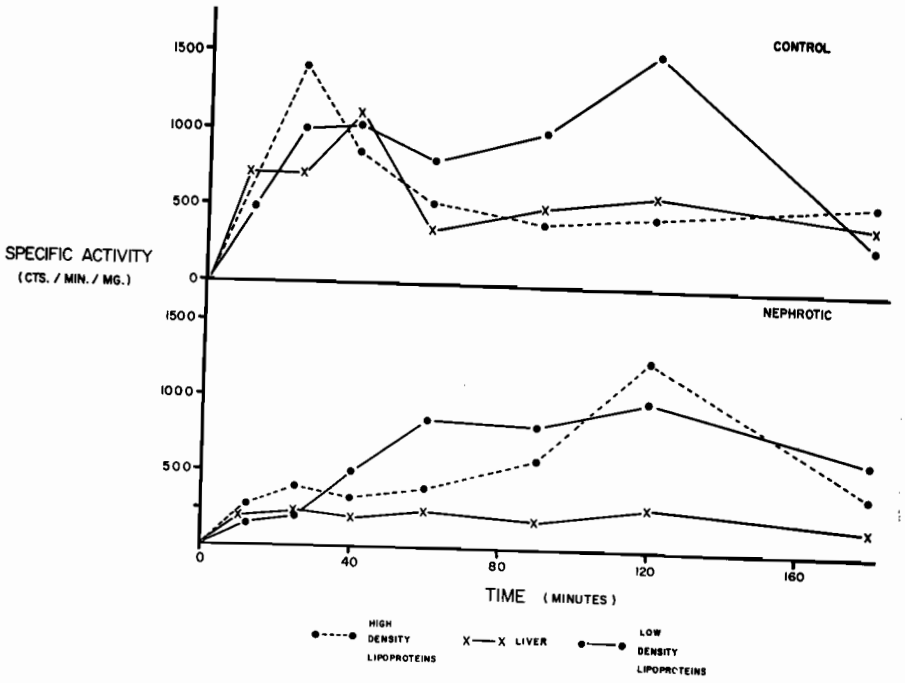


Figure 12

Specific activities of unesterified cholesterol in plasma lipoproteins and liver after injection of acetate-1-C^{14} into normal and nephrotic rats.

per ml of plasma than did normal rats. Since plasma volumes in normal and nephrotic rats are the same (see Experiment 6), the ratio of incorporation of acetate into total plasma LDL_p cholesterol is also the same.

Unesterified Cholesterol:

The concentrations of unesterified cholesterol in nephrotic low and high density lipoproteins were 16 and 4 times normal (Table 20). The peak specific activity of cholesterol in normal LDL_p was 1.5 times greater than that in nephrotic animals, whereas specific activity of HDL_p unesterified cholesterol in nephrotic plasma was about two times normal. Assuming that the plasma volumes are the same in normal and nephrotic animals the incorporation of acetate in nephrotic LDL_p cholesterol was about 10 times normal and in plasma HDL_p about 8 times greater than normal.

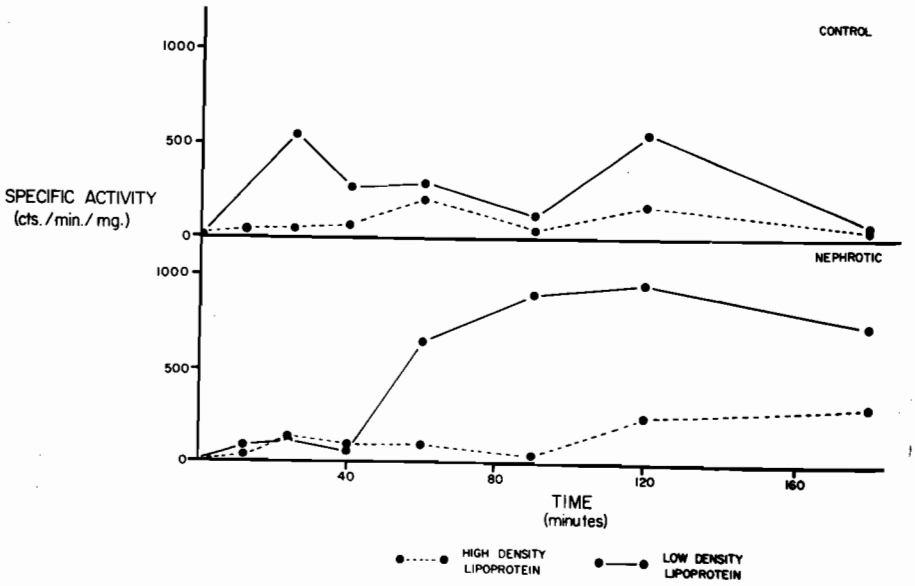


Figure 13

Specific activities of total fatty acids of plasma high (broken lines) and low (solid lines) density lipoproteins after injection of acetate-1-C^{14} into normal and nephrotic rats.

Fatty Acids:

The specific activity of total fatty acids was greater in nephrotic than in normal lipoproteins and the amounts of fatty acids in nephrosis were approximately 7 and 2 times greater than normal in low and high density lipoproteins respectively (Table 22). Since the peak values of specific activities of fatty acids for low and high density lipoproteins in nephrotic animals were 1.5 to 2 times normal, the total incorporation of acetate into plasma fatty acids in nephrosis was about 14 and 4 times greater than normal in low and high density lipoproteins respectively. The curves of specific activity of fatty acids also show two peaks, and the early peaks are more marked in normal animals. Possibly some of the activity in early peaks could be due to esterification of the contaminants of cholesterol with radioactive fatty acid.

In normal animals the specific activities of unesterified cholesterol in the two lipoprotein classes were markedly different from each other, whereas in nephrotic animals the values of specific activities for high and low density lipoproteins were close to each other. The specific activities of fatty acids in the two lipoproteins were different both in normal and nephrotic rats. This would indicate that the exchange of cholesterol and fatty acids (138, 139) between high and low density lipoproteins is not rapid enough for them to be considered as one pool.

Table 22

Concentration of Total Fatty Acids in Plasma Lipoproteins
(mg./ml. of Plasma)

Group No.	Low Density Lipoproteins		High Density Lipoproteins	
	Normal	Nephrotic	Normal	Nephrotic
1	1.3	4.3	1.7	3.4
2	1.4	8.4	1.1	2.7
3	2.1	7.7	1.3	3.3
4	1.2	7.7	1.1	3.3
5	1.0	12.1	2.1	5.3
6	1.0	17.2	1.4	2.9
Mean	1.3	9.6	1.5	3.5
S.D.	0.4	4.5	0.4	0.9
Ratio	1	7.4	1	2.3

The amounts of unesterified and total cholesterol in the livers of all animals are listed in Table 23. The specific activities of liver cholesterol at various times after the injection of acetate are shown in Fig. 12. The liver unesterified cholesterol appears to have two peaks, as was the case with plasma cholesterol. As pointed out earlier, early peaks are probably due to high specific activity contaminants of cholesterol which are precipitable with digitonin (280, 303, 304). The specific activities of these contaminants are similar in liver and plasma, suggesting that they are readily incorporated into plasma after their synthesis by the liver.

The values of specific activity of unesterified cholesterol in high and low density lipoproteins were different from each other, suggesting that plasma lipoproteins are either not mixing with the same pool in the liver or have different rates of exchange with the same pool. Since the specific activity of liver total unesterified cholesterol is lower than that of LDL_p cholesterol in both normal and nephrotic rats and of HDL_p in nephrotic animals, the former could not be a precursor of higher specific activity cholesterol of plasma lipoproteins, so that liver probably has at least two pools of cholesterol one of which has at least the same specific activity as the plasma LDL_p cholesterol.

The peak specific activity of unesterified cholesterol in

Table 23

Liver Cholesterol (mg.)

	Normal		Nephrotic	
	Total	Unesterified	Total	Unesterified
	36.4	30.2	59.3	50.4
	28.0	26.0	59.6	59.6
	23.3	20.8	59.3	54.4
	23.3	23.6	82.6	75.0
	23.6	18.4	64.0	43.6
	18.4	17.6	66.6	58.4
	17.6	15.6	68.4	68.4
	19.3	15.6	58.0	56.4
	18.0	16.4	18.0	17.6
	19.3	17.2	115.3	65.6
	20.3	18.8	59.3	56.0
	22.0		65.6	65.6
			56.4	56.4
			43.0	42.8
Mean	22.2	20.1	62.5	55.0
S.D.	5.4	1.5	21.3	5.0
Amount 100g. body weight		9.16		18.34

normal livers was about twice that of nephrotic livers (Fig. 12) and correspondingly the amount of cholesterol was greater in the latter (Table 23).

Hepatic Synthesis of Cholesterol:

From the amount (Table 23) and specific activity of hepatic unesterified cholesterol (Fig. 12) total radioactivity in unesterified cholesterol of livers was calculated (Table 24). Plasma volumes of the animals were calculated from their body weights (Experiment 6) and from concentrations of cholesterol in lipoproteins the amounts of unesterified cholesterol in plasma high and low density lipoproteins were deduced. The total radioactivity in plasma unesterified cholesterol was also calculated and is shown in Table 24.

Not all the radioactivity incorporated into unesterified cholesterol by the liver was accounted for in the above calculations. Some of the radioactive (that is recently synthesized) cholesterol undoubtedly became esterified and some was transferred to red blood cells by the time peak specific activity of unesterified cholesterol was obtained. In order to determine the total amounts of radioactivity incorporated into cholesterol, the amounts esterified and transferred into cells should be included but the data is not available. However, unesterified cholesterol in liver and plasma makes up the major fraction

Table 24

Radioactivity in Unesterified Cholesterol of Liver and Plasma Lipoproteins (cts./min.)

Time after injection	Normal				Nephrotic			
	Liver	LDL_p	HDL_p	Total	Liver	LDL_p	HDL_p	Total
60	7245	278	100	7,632	7700	4596	812	13,108
90	9873	345	154	10,392	11,000	6358	1238	18,596
120	11687	524	177	12,388	15,950	7813	2517	26,280

of the total cholesterol pool in equilibrium with plasma (Tables 20, 23), and in the first two hours cholesterol radioactivity is present predominently in unesterified cholesterol of the liver-plasma pool (177). Moreover, if the rates of esterification of newly synthesized cholesterol are assumed to be normal in nephrosis (Experiment 9), the total amount of radioactivity present in esterified cholesterol in nephrotic plasma will be more than normal, since there is more esterified cholesterol than normal. It can thus be safely concluded that the hepatic rate of incorporation of labeled acetate into cholesterol is about twice normal in nephrotic animals. Since it is generally assumed that the rate of incorporation of labeled acetate reflects the true rate of synthesis, it may be further concluded that the actual rate of synthesis of cholesterol from all sources is increased. Table 24 indicates that rates of incorporation of labeled acetate into plasma lipoprotein cholesterol is markedly increased in nephrosis.

Conclusions.

1. There is probably an increase in synthesis of cholesterol by the liver and in the rate of incorporation of newly synthesized cholesterol into plasma lipoproteins in nephrotic rats 2 weeks after the injection of AKS.

2. The liver has at least two pools of unesterified cholesterol one of which has higher specific activity than the other.

3. The cholesterol in the two lipoproteins of plasma either equilibrate with different pools in the liver or have different rates of exchange with the same pool.

4. In normal rats there is a greater incorporation of recently synthesized cholesterol in LDL_p than in HDL_p and a similar but more marked increase is noticed in the nephrotic animals.

Experiment 8

Purpose.

The nephrotic animals (2 weeks after injection of AKS) used in the last experiment had body weights somewhat greater than the control animals, and the cholesterol in the livers and plasma lipoproteins was not freed of the high specific activity contaminants which are known to be present after injection of labeled acetate. The following experiment was undertaken to overcome these objections. However two changes in experimental design were made. 1. The nephrotic rats were injected with AKS 6 weeks prior to the experiment. 2. Since mevalonic acid lies on the pathway of cholesterol synthesis from acetate, an increase in synthesis of cholesterol from acetate necessarily implies an increase in synthesis from mevalonic acid. It may therefore be reasonably assumed that mevalonic acid would have given results identical to those with acetate in the previous experiment. Cholesterol of higher specific activity can be obtained from mevalonic acid than from acetate; for this reason, therefore, the former substance was used as a labeled precursor of cholesterol in this experiment.

Methods:

Fourteen normal and 14 nephrotic (6 weeks after AKS injection) rats of similar weights were given 50% dextrose in water to drink as the only food for 20 hours. DL-Mevalonic-5^{H3} DBED Salt (New England Nuclear Corp.) was injected intraperitoneally into each. Two animals from each group were sacrificed at 15,

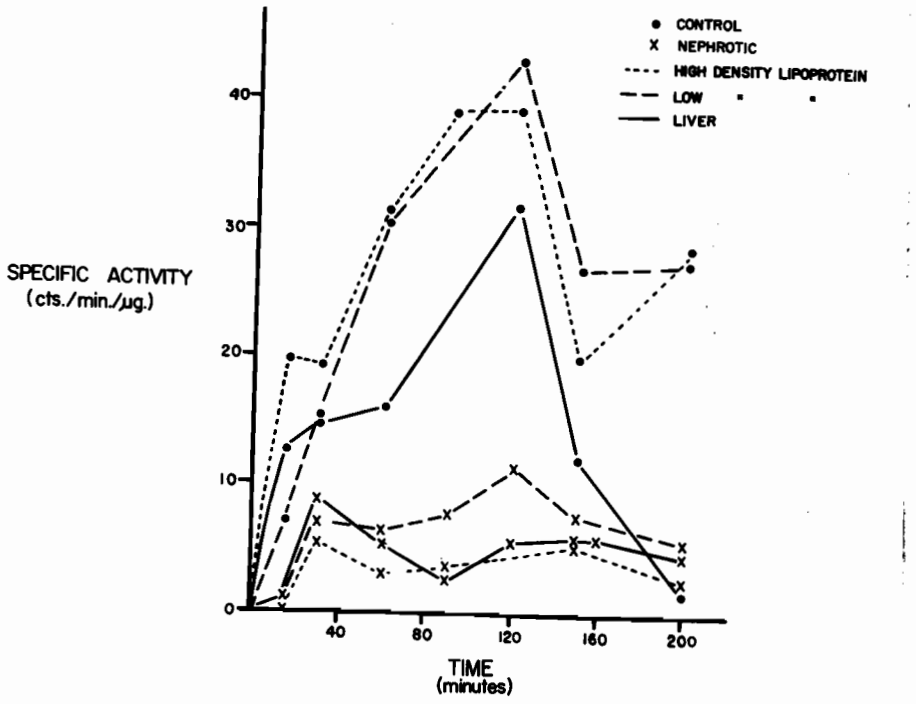


Figure 14

Time course of specific activity of unesterified cholesterol in liver and plasma lipoproteins of rats after injection of radioactive mevalonic acid.
(Ninety minute specimens of normal liver and LDL_p and 120 minute specimen of nephrotic HDL_p were lost.)

30, 60, 90, 120, 150 and 200 minutes after the injection; blood and livers were collected. The plasma was separated in the cold, equal volumes from the two rats in the group were pooled and LDL_p were precipitated with "mepesulfate". The amounts of unesterified cholesterol in the lipid extracts of plasma lipoproteins and the livers were determined. In order to obtain specific activities of cholesterol in lipid extracts of liver lipoproteins it was purified by dibromination (280). Since the amounts of unesterified cholesterol in plasma lipoproteins were not enough for purification procedure, previously purified unlabeled cholesterol was added to the extracts in known ratios as carrier before precipitation of cholesterol by digitonin. From the specific activity of purified cholesterol (including carrier) and from the relative amounts of endogenous and carrier cholesterol in the mixture subjected to purification, the specific activity of lipoprotein cholesterol was calculated.

Results:

The time course of specific activity for liver and plasma lipoproteins is shown in Figure 14 and the amounts of cholesterol in the livers and plasma lipoproteins in Tables 25, 26. The amount of radioactivity present in unesterified cholesterol of liver and plasma lipoproteins is given in Table 27.

Table 25

Unesterified Cholesterol (mg.) in Livers of Normal and Nephrotic Animals.

Livers of two animals in group.			Liver per 100 g. body weight	
	Normal	Nephrotic	Normal	Nephrotic
	33.60	21.00	6.20	4.79
	25.44	30.24	5.04	6.54
	26.16	35.76	4.84	7.39
	25.44	32.04	4.88	6.51
	31.92	24.84	5.95	4.50
	26.04	25.20	5.40	4.65
	24.24	24.52	4.86	4.93
Mean	27.55	27.65	5.3	5.6
Standard Deviation	5.3	5.0	0.56	1.16

P Between 0.3 and 0.4

Discussion:

The curves of specific activities demonstrate a progressive rise with time reaching their peaks at 120 minutes and then they decline with time. Deviations from this pattern appear to be random and therefore probably due to biologic variations in the groups of rats sacrificed at different times.

In normal rats, the specific activities of the unesterified cholesterol in the two lipoproteins appear to be similar and they are higher than those of the liver. Since the product cannot have higher specific activity than the precursor and plasma cholesterol is derived from liver the total unesterified cholesterol in the liver cannot be a precursor of plasma cholesterol, but that liver has two pools one of which has a specific activity higher than that of cholesterol in plasma lipoproteins. The two lipoproteins in the normal plasma appear to be part of the same pool.

In nephrotic animals the specific activities of unesterified cholesterol were lower than the corresponding values in controls. The specific activities of cholesterol in high and low density lipoproteins were different from each other, that in LDL_p being higher than those of HDL_p and of liver cholesterol. This suggests that in nephrosis the two plasma lipoproteins mix with liver pools of different specific activities or that the two lipoporteins have different rates of exchange with the same pool in the

Table 26

Unesterified Cholesterol (mg.) in Plasma High and Low Density Lipoproteins.

Normal		Nephrotic	
LDL_p	HDL_p	LDL_p	HDL_p
.0748	.0096	0.385	0.1250
.0768	.0106	0.269	0.1325
.0658	.0269	0.284	0.1138
.0986	.0075	0.694	0.1325
.0648	.0180	0.408	0.0625
.0750	.0265	0.494	0.1159
.088	.0217	0.612	0.1555
Mean .0777	.0173	0.449	0.1197
S.D. .0118	.0077	0.160	0.0286

liver.

The peak amount of radioactivity present in unesterified cholesterol of liver and plasma lipoproteins of normal rats appears to be 5 times the peak amount present in nephrotic rats. If the assumption is made that the esterification of recently synthesized cholesterol in rats 6 weeks after the injection of AKS is normal, as it was in rats 3 weeks after the injection of AKS then, as has been pointed out in the last experiment, peak amounts give a fairly close approximation of relative rates of synthesis. Even if all the esterified cholesterol in liver and plasma had the same specific activity as unesterified cholesterol, the total cholesterol radioactivity in nephtonic rats would still be less than 50% of that present in only the unesterified cholesterol of normal rats. Because of the relationship of acetate and mevalonic acid in cholesterol synthesis, a decreased synthesis of cholesterol from mevalonic acid implies a similar decrease in synthesis from acetate. It is therefore clear in comparing this experiment with the preceeding one that the rate of synthesis of cholesterol was increased 2 weeks after the injection of AKS but was decreased 6 weeks after the injection. That this decrease in synthesis is not associated with recovery from nephrosis is demonstrated by the fact that the degree of hypercholesterolemia in nephrotic animals was as great in this experiment as in the preceeding one.

The incorporation of newly synthesized cholesterol into

Table 27

Radioactivity in Unesterified Cholesterol of Liver and Plasma Lipoprotein per 100 g. Body Weight (cts/min)

Time after injection (minutes)	Normal				Nephrotic			
	Liver	LDL_p	HDL_p	Total	Liver	LDL_p	HDL_p	Total
60	77,440	6,535	2,779	86,754	39,758	5,998	1201	46,957
90	117,120	9,132	970	127,222	17,447	17,863	1662	36,972
120	189,269	9,193	2,334	200,796	24,795	15,349	1444	41,588
150	62,192	6658	1748	70,598	27,335	12,390	2027	41,752
200	8,797	7957	2048	18,802	22,875	11,713	1386	35,974

plasma lipoproteins was about 1.5 times greater in nephrotic animals than in controls, in spite of the decrease in the rate of synthesis of cholesterol by the liver. The incorporation of radioactivity into HDL_p cholesterol was not much different in the two but that in LDL_p was greater in nephrotic animals as compared to normal rats.

The amounts of cholesterol in livers of normal and nephrotic rats were not significantly different.

Conclusions:

1. The rate of hepatic synthesis of cholesterol in nephrotic rats (6 weeks after injection of AKS) is markedly decreased while the concentration of plasma cholesterol is still markedly elevated.

2. The incorporation of recently synthesized cholesterol into plasma LDL_p is greater in nephrotic rats than in normals.

3. The liver has at least two pools of unesterified cholesterol, one or both of which are precursors of plasma cholesterol.

Experiment 9

Introduction and Purpose:

In experiments 7 and 8 specific activities of cholesterol in plasma and liver at different times after the injection of labeled precursors were determined on samples taken from different animals. In order to overcome the possibility of variations in the rates of hepatic synthesis of cholesterol in different animals, studies on incorporation of mevalonic -2-C^{14} lactone into plasma and red blood cell cholesterol were made on samples taken from the same animals. The purpose of the study was to compare the rates of incorporation of recently synthesized cholesterol into plasma lipoproteins of normal and nephrotic animals and to compare the rates of equilibration of plasma unesterified cholesterol with that in the red blood cells.

Methods:

Five microcuries (1.19 mc./mM.) of mevalonic-2-C^{14} lactone were injected intraperitoneally into each of 4 normal and 4 nephrotic* rats, paired according to their weights. Blood (0.2-0.3 ml) was collected in heparinized capillary tubes at different times after the injection and plasma was separated from cells immediately after collection. Animals in groups 2-4 were exsanguinated 25 hours after the injection and those in group 1, 8 hours after the injection, and the blood collected.

* #1, 3 weeks and #2-4, 4 weeks after the induction of nephrosis.

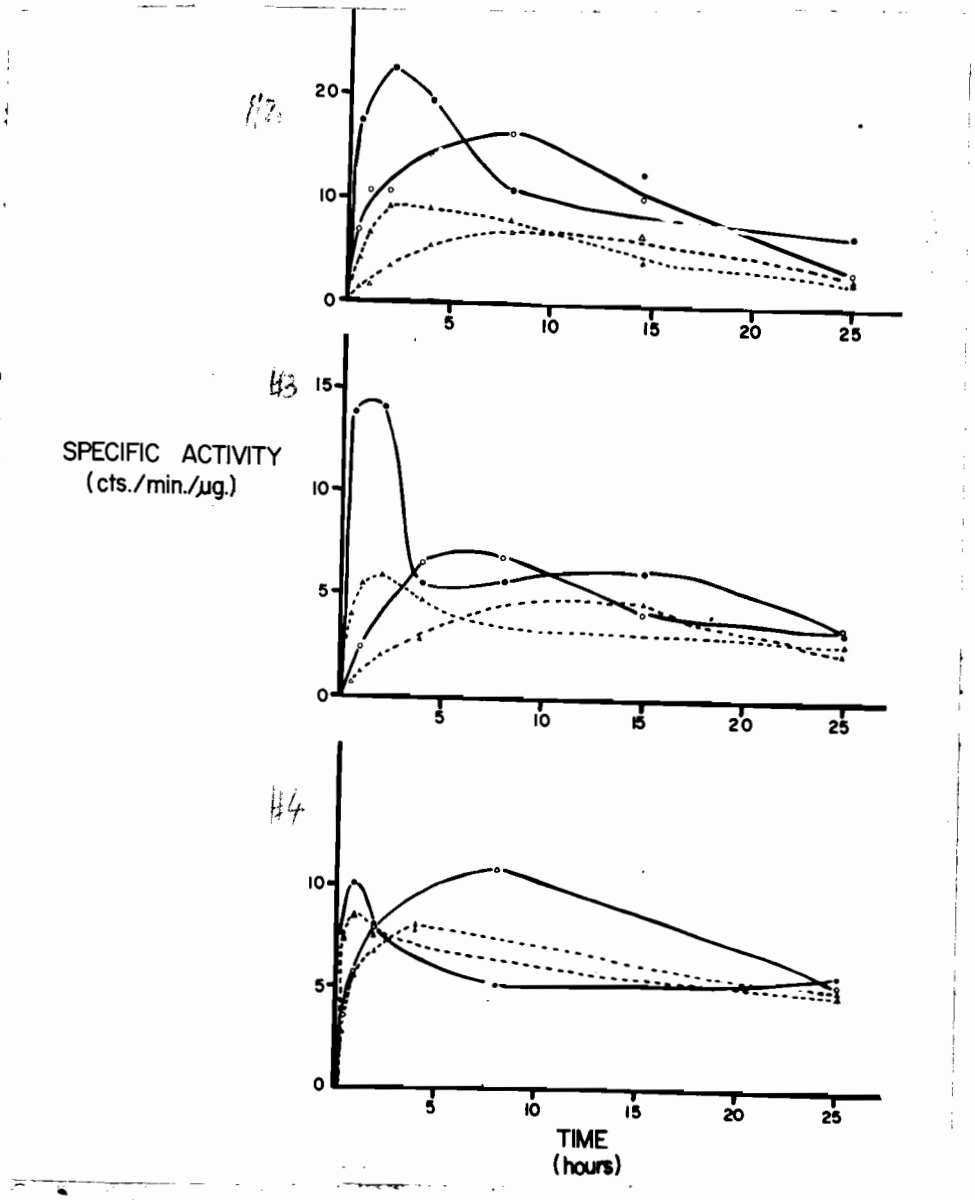


Figure 15

Specific activities of unesterified (closed symbols) and esterified (open symbols) cholesterol after injection of mevalonic-2-C^{14} lactone in normal (solid lines) and nephrotic (broken lines) rats, paired by weight.

The concentrations and specific activities of unesterified and total cholesterol in the plasma of samples collected at 25 hours (group 2-4) were determined, and concentration and specific activity of only unesterified cholesterol were determined in the plasma of the sample collected at 8 hours (group 1) after the injection. The cells obtained in the earlier samples were washed twice with normal saline, extracted in alcohol:acetone, and the specific activity of the cell cholesterol was determined.

Known aliquots of plasma samples collected in capillary tubes were extracted and their cholesterol precipitated after the addition of a fixed amount of cholesterol as carrier to ensure complete precipitation of the labeled cholesterol. The total radioactivity in the unesterified and total cholesterol of all plasma samples was determined. Assuming that there was no change in the plasma cholesterol concentration during the experimental period, the specific activities of unesterified and esterified cholesterol were calculated from their concentration in the last samples of plasma.

Results and Discussion:

Plasma concentrations of cholesterol of rats in all groups are given in Table 28. Since the two rats in each group had similar weights, their plasma volumes were assumed to be identical (see Experiment 6); thus the ratio of total cholesterol in plasma

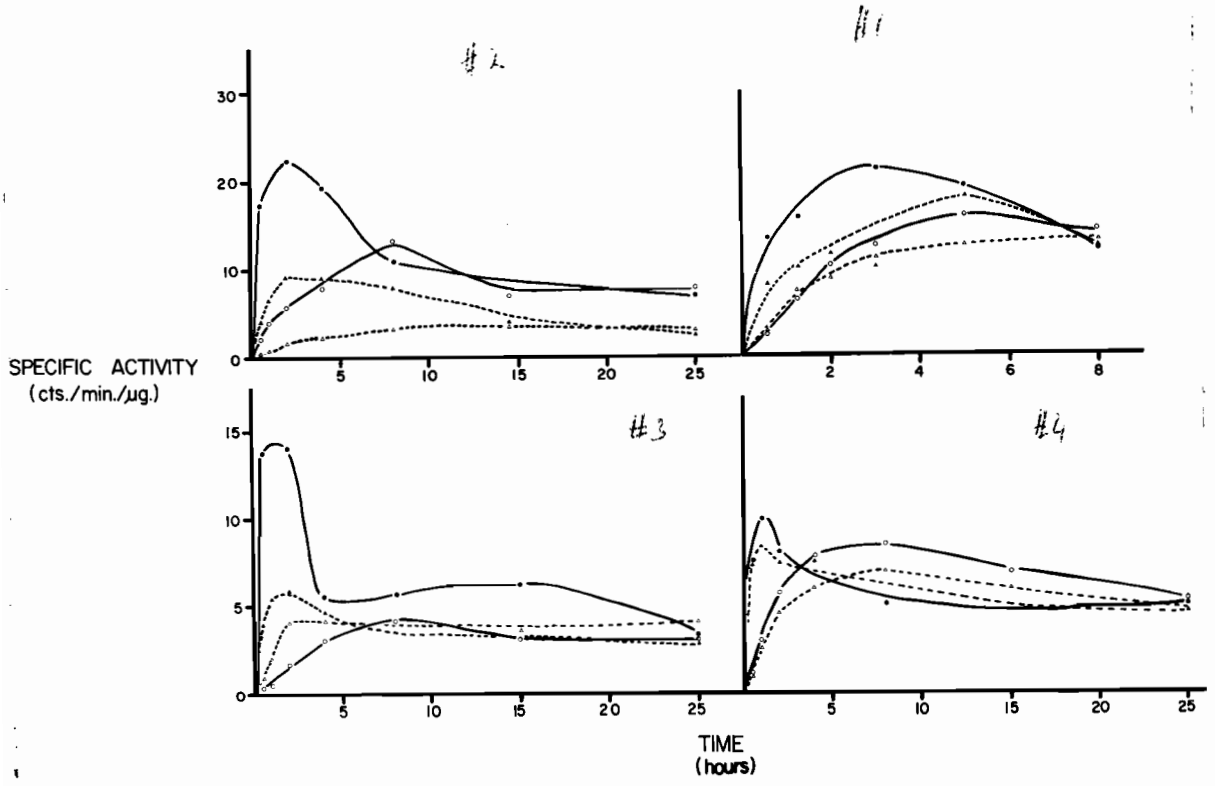


Figure 16

Specific activities of unesterified cholesterol in plasma (closed symbols) and red blood cells (open symbols) after injection of mevalonic-2 C^{14} lactone in normal (solid lines) and nephrotic (broken lines) rats, paired by weight.

pools was the same as the ratio of the cholesterol concentrations in plasma (Table 29).

The time course of specific activities of unesterified and esterified cholesterol are given in Fig. 15. The peaks of specific activity of plasma unesterified cholesterol for normal as well as nephrotic rats were reached about 2 hours after the administration of mevalonic acid. The peak of control plasmas were always higher than in nephrotics. The ratios of their peak specific activities are given in Table 29, and taken with the amounts of cholesterol present in plasma, the ratios of peak incorporation of mevalonic acid into plasma unesterified cholesterol were calculated and are shown in the same table.

If the deviation from normal concentration of unesterified cholesterol in plasma is taken as an index of the severity of the AKS disease, the animals with most severe nephrosis had greatest incorporation of labeled mevalonic acid into cholesterol (Table 29) and the animals with the mildest degree of nephrosis demonstrated mevalonic acid incorporation into cholesterol only slightly greater than normal. This indicates that increased plasma concentrations of cholesterol are associated with increased rate of entry of unesterified cholesterol from liver into plasma, provided the pools of mevalonic acid (or its products, which act as precursors of cholesterol) are of the same size in normal and nephrotic rats.

Table 28

Concentrations of Plasma Cholesterol in Normal and Nephrotic Rats

(mg./100 mL.)

Group No.	Normal		Nephrotic	
	Total	Unesterified	Total	Unesterified
1	-	11.2	-	23.5
2	23.0	6.0	258.4	125.1
3	33.5	9.0	119.8	33.7
4	44.5	13.9	72.1	22.6

The appearance of radioactivity in esterified cholesterol was much slower than its appearance in unesterified cholesterol. The specific activity appeared to achieve its peak around 8 hours after the injection in all animals. The patterns of the time course of specific activity of unesterified and esterified cholesterol did not show any consistent or significant difference between normal and nephrotic rats of each group. Since the ratio of unesterified to esterified cholesterol does not change in nephrosis (18), the specific activity curves suggest that fractional rate of esterification of newly synthesized cholesterol is normal in nephrosis.

Radioactive cholesterol appeared also in red blood cells. In normal animals the specific activity of erythrocyte cholesterol reached its peak at 8 hours except in one case in which it reached a peak at 5 hours; in nephrotic animals the peak specific activity of cell cholesterol was reached about the same time as in normals. The peak specific activity of plasma cholesterol was always greater than that of cell cholesterol. The time for equilibration of specific activities of plasma and cellular unesterified cholesterol did not appear to be different in normal and nephrotic animals.

Conclusions:

1. In AKS nephrotic rats there was an increase in the entry of newly synthesized cholesterol from liver into plasma and this increase was greater in rats with greater degrees of hypercholes-

Table 29

Ratios (Nephrotic/Normal) of Concentrations, Peak Specific Activities and Total Radioactivity of Plasma Unesterified Cholesterol.

Group No.	Concentration	Peak Specific Activity	Total Radioactivity
1	2.1	0.8	1.7
2	20.8	0.4	8.3
3	3.7	0.4	1.5
4	1.6	0.9	1.4

terolemia.

2. The fractional rate of esterification of newly synthesized cholesterol appeared to be normal in nephrotic rats.

3. The fractional rate of entry of newly synthesized cholesterol from plasma into red blood cells appeared to be normal in nephrosis.

Turnover Rates of Plasma Cholesterol

To determine the turnover rates of plasma cholesterol, 4-C^{14}-cholesterol was introduced into the plasma as a tracer and after the period of its equilibration in the readily exchangeable pool, the rate of its disappearance was observed.

Since cholesterol is only sparingly soluble in water and disappears from the blood very rapidly when injected in aqueous suspension (284), 4-C^{14}-cholesterol was "solubilized" in normal rat serum lipoproteins (284) before injection. The rates of disappearance of the label from the blood of normal and nephrotic rats were determined in the following two studies to see if the disappearance of the tracer from the blood was comparable to that of cholesterol present in a physiological state.

Experiment 10.

One normal and one nephrotic rat of equal weight were injected with equal amounts of serum labeled with 4-C^{14}-cholesterol by the method of Avigan (284). Aliquots of blood were collected at hourly intervals and radioactivity in equal amounts of plasma was determined in all samples.

The time courses of disappearance of radioactivity from normal and nephrotic plasma from 1 to 6 hours are shown in Fig. 47.

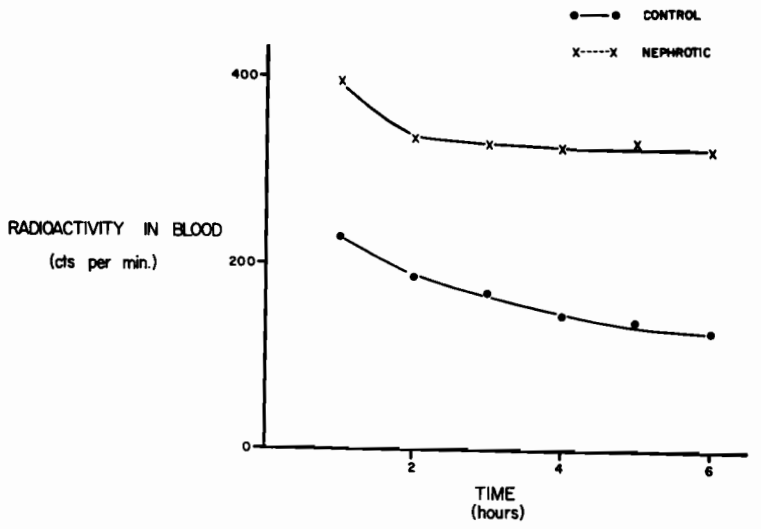


Figure 17

Time course of disappearance of radioactivity from blood (0.05ml) of normal and nephrotic rats after injection of normal rat serum labeled with C^{14}-cholesterol.

Experiment 11

Three normal and two nephrotic rats were injected with the same amount of serum labeled with 4-C^{14}-cholesterol by the method of Avigan and aliquots of blood collected at 1, 3, 7, 13, 23.5 and 47.5 hours after the injection. The amount of radioactivity in aliquots of plasma was determined in all samples.

The concentration of radioactivity in blood at various times after injection are shown in Fig. 18.

Discussion:

1. Cholesterol in unphysiological form, when injected into rats disappears very rapidly from the blood, only to reappear after being incorporated into lipoproteins (284). The label administered in the last two experiments did not disappear from blood like cholesterol in suspension, and the half-life of disappearance of radioactivity from blood was about 17 hours which is comparable to 15 hours calculated from the data obtained from rats injected with *in vivo* labeled serum (Fig. 4, Line 1, Ref. 284).

2. The curves of disappearance of radioactivity with time show two components; the initial slope, presumably due to equilibration of label in the readily exchangeable pool, lasted up to 7 hours both in normal and nephrotic rats, and the second slope, which was smaller than the first, reflected the rate of disappearance of radioactivity from the pool.

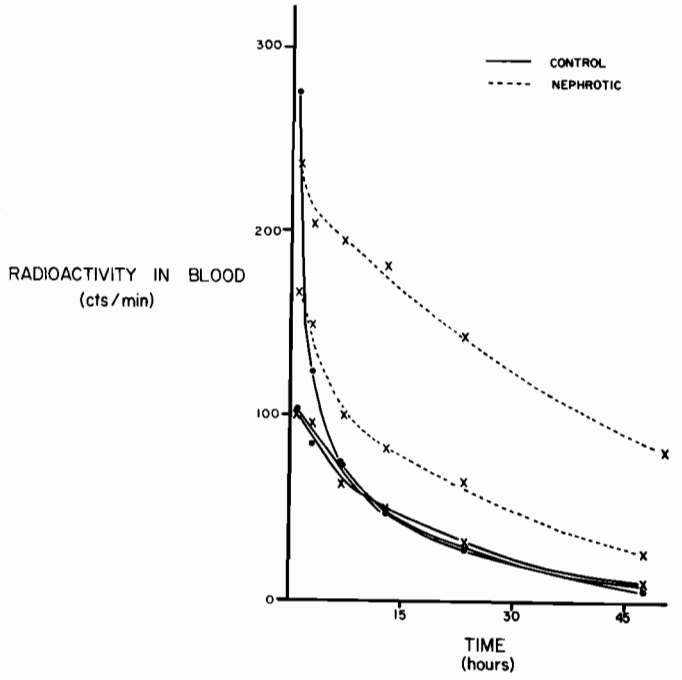


Figure 18

Time course of disappearance of radioactivity from blood (0.05ml) of normal and nephrotic rats after injection of normal rat serum labeled with C^{14}-cholesterol.

3. The blood had greater amounts of radioactivity in nephrotic animals than controls indicating either a decrease in size of the pool in which the isotope was diluted or that the fraction of the readily exchangeable pool in the vascular compartment was greater in nephrotic animals than in normal rats.

Experiment 12

Purpose:

To determine the turnover rates of cholesterol in plasma, low and high density lipoproteins in normal and nephrotic rats (3 weeks after the induction of nephrosis) and to study the rate of transfer of cholesterol from low density to high density lipoproteins.

Methods:

Serum lipoproteins were labeled with 4-C^{14}-cholesterol by the method of Avigan and the LDL_p were isolated from the labeled serum in a preparative ultracentrifuge. Aliquots of labeled LDL_p diluted in normal saline were injected intravenously into normal and nephrotic rats. Blood from three rats in each group was collected at 0.5, 1.25, 4, 8, 14, 24, 48 and 82 hours after injection and pooled. Plasma lipoproteins were separated into two classes and amounts and specific activity of unesterified cholesterol determined in all specimens.

Results and Discussion:

Unesterified cholesterol was increased in concentration in nephrotic plasma as compared to normal. The increase was found both in high and low density lipoproteins (Table 30). The variability of the increase in cholesterol in different groups of nephrotic animals seemed to be related to variability in increase in LDL_p; increases

Table 30

Amounts of Unesterified Cholesterol in Plasma Lipoproteins
(mg./ml. of Plasma)

	Normal		Nephrotic	
	LDL_p	HDL_p	LDL_p	HDL_p
1	.0237	.0529	1.1649	0.3621
2	.0387	.0527	1.0406	0.2000
3	.0262	.0329	0.3281	-
4	.0427	.0474	0.9984	0.2920
5	.0388	.0600	0.9257	-
6	.0380	.0469	0.2109	0.2506
7	.0485	-	0.4734	0.1934
8	.0410	.0921	1.1344	0.3266
9	.0263	.0653	-	-
Mean	0.0360	0.0569	0.7845	0.2708
S.D.	0.0083	0.0173	0.3870	0.0666

in cholesterol in HDL_p were more uniform but less than the increases in cholesterol of LDL_p. The specific activities of unesterified cholesterol in plasma high and low density lipoproteins are given in Table 31. The values of total plasma unesterified cholesterol specific activity were obtained from the total amounts of cholesterol and radioactivity in the two lipoproteins and are also given in Table 31.

The time curves of specific activity in all cases showed two components: a steep fall in early period up to 4 hours after injection, which probably represented predominantly the mixing of injected material in the pool with which plasma cholesterol is in equilibrium, and a slower decline representing the net loss of radioactive cholesterol from that pool.

The second component was used to calculate the pool size and the fractional turnover rates (288). Logs of specific activity values were plotted against time and the lines of best fit were calculated by the method of least squares (Fig. 19). Fractional turnover rates for the two lipoproteins as well as total plasma unesterified cholesterol were calculated from the coefficients of regression of these lines and are given in Table 32. By extrapolation the values of specific activity at '0' time were estimated. Half an hour after injection of the labeled LDL_p

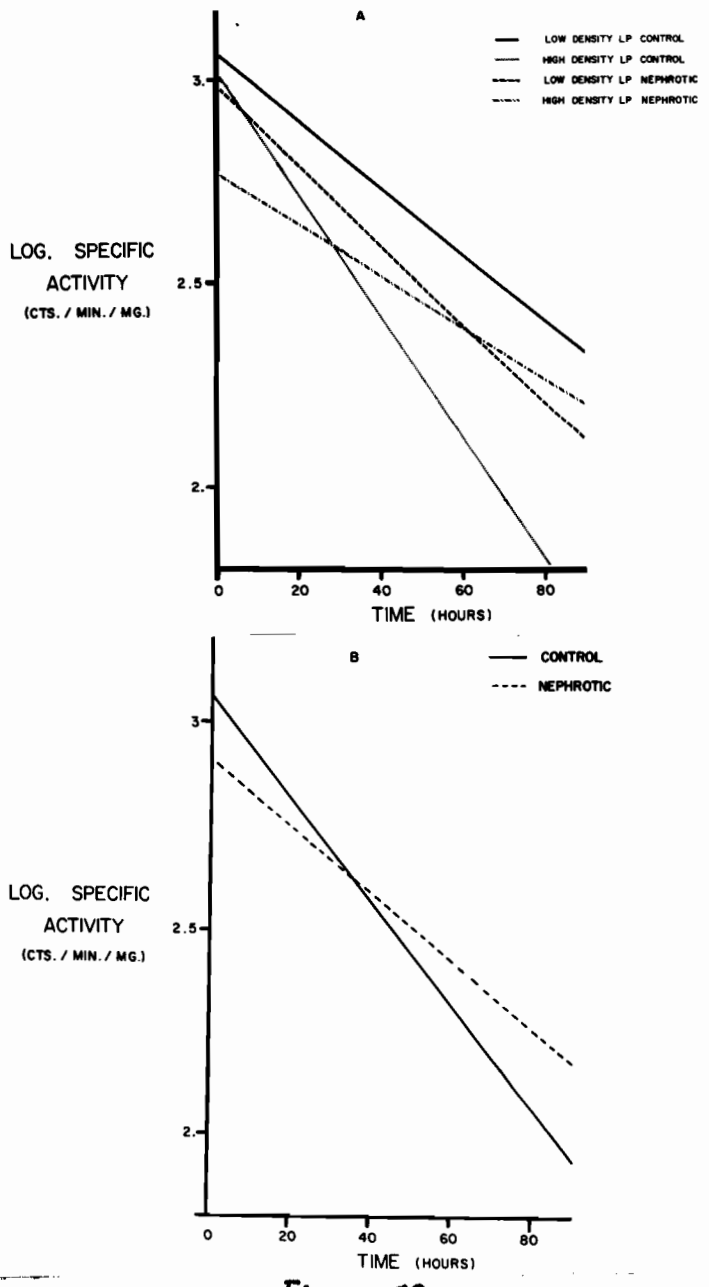


Figure 19

Time course of specific activity of unesterified cholesterol in plasma lipoproteins (A) and of total unesterified cholesterol in plasma (B)

solution the specific activities of HDL_p cholesterol in both normal and nephrotic rats were half that of the LDL_p cholesterol (Table 31), suggesting a more rapid incorporation of label from the injected LDL_p into native HDL_p than would be expected from the estimated turnover rates of native lipoproteins, even if all the HDL_p unesterified cholesterol were derived from LDL_p. One will have to assume that the initial fate of the injected LDL_p and its radioactive cholesterol was not like that of native LDL_p molecules. This observation was confirmed by injecting another batch of labeled LDL_p into a small group of animals.

Nevertheless the radioactive cholesterol present after 30 minutes in high and low density lipoproteins, appeared to be in physiological state for the following three reasons:

1. During the process of normal exchange of cholesterol between different molecules of lipoproteins, progressively greater amounts of labeled cholesterol would become associated with native lipoproteins.

2. The fractional turnover rate of plasma unesterified cholesterol in normal rats was in agreement with published reports

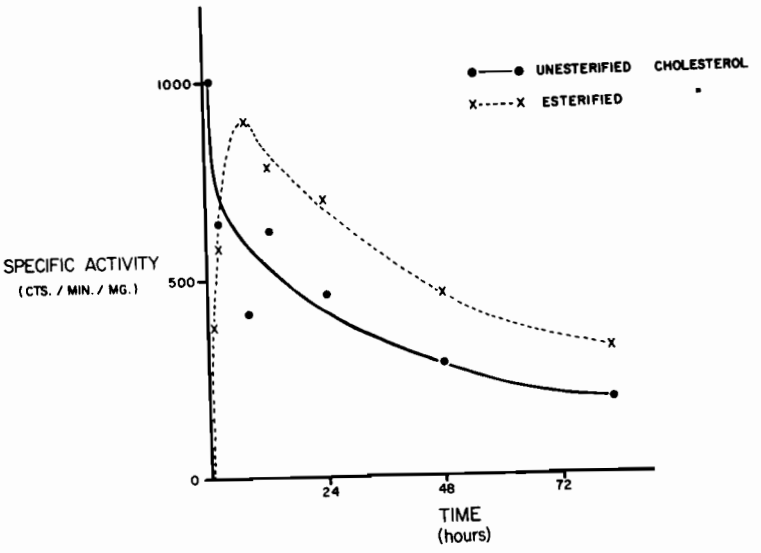


Figure 20

Time course of specific activities of unesterified and esterified cholesterol of HDL_p after injection of normal LDL_p labeled with C^{14}-cholesterol into nephrotic rats.

(289, 290).

3. The esterification of labeled cholesterol in nephrotic HDL_p was normal in so far as slopes of specific activity are concerned (Fig. 20).

The difference in fractional turnover rates of LDL_p unesterified cholesterol in normal and nephrotic animals was statistically not significant, though the fractional turnover rate of HDL_p unesterified cholesterol in nephrotics was lower (significant at 5% level) than that of control animals (291).

In normal animals the fractional turnover rate of unesterified cholesterol in HDL_p was greater than that of LDL_p cholesterol. Since HDL_p carries more cholesterol than LDL_p in plasma of normal rats, the actual turnovar rate of plasma HDL_p cholesterol in mg./hour is greater than that of LDL_p cholesterol. Hence all the HDL_p unesterified cholesterol could not have been derived from LDL_p plasma. In nephrotic rats the fractional turnover of HDL_p unesterified cholesterol was less than that of LDL_p cholesterol in plasma. Since in nephrosis plasma LDL_p have greater amounts of cholesterol than HDL_p (Table 30), the actual turnover of LDL_p unesterified cholesterol is greater than that of HDL_p unesterified cholesterol.

The fractional turnover rates of plasma lipoproteins reflect also the fractional turnover rates of pools in the liver with which plasma lipoproteins equilibrate rapidly, and in liver no

Table 31

Specific Activities of Unesterified Cholesterol in Plasma and Lipoproteins.

(cts/min./mg.)

Time after injection (hours)	Normal			Nephrotic		
	LDL_p	HDL_p	Total Plasma	LDL_p	HDL_p	Total Plasma
0.5	3500	1557	2121	1921	788	1652
1.25	2226	1022	1012	1289	992	999
2.5	1781	907	1294	1505	635	-
4.0	1015	992	1002	792	592	746
8.0	1124	918	999	848	406	-
14.0	885	658	759	1244	613	900
24.0	842	-	-	678	453	497
48.0	327	138	197	348	274	331
82.0	283	75	134	135	173	-

such differentiation into two classes of lipoproteins is known. Thus the total amounts (mg./hour) turned over in the combined plasma-liver pools cannot be calculated.

There is transfer of cholesterol from one lipoprotein to the other not only *in vivo* (138) but also during the time taken for their separation in the preparative ultracentrifuge (Experiment 14). The rates of transfer between the two lipoproteins were not known so that the true turnover rates for the lipoproteins could not be calculated. However, the observed difference in the turnover rates of cholesterol in HDL_p probably reflects a real difference, since the LDL_p cholesterol had similar turnover rates in normal and nephrotic rats.

When cholesterol in the two lipoproteins is considered as one pool, the determination of its fractional turnover rate is not affected by transfer between lipoproteins. In order to compare normal and nephrotic rats, the specific activities of plasma unesterified cholesterol were estimated from the amounts of cholesterol and radioactivity present in both lipoproteins at different times and fractional turnover rates calculated as for individual lipoproteins. They were 1.2%/hour for normal animals and 0.8%/hour for nephrotics, indicating that the normal rats had a fractional turnover rate 1.5 times that of nephrotic animals. When the slopes of specific activity of total plasma unesterified cholesterol

Table 32

Fractional Turnover Rates (%/hour) of Unesterified Cholesterol from the Pools of Plasma Lipoproteins in Normal and Nephrotic Rats.

Cholesterol in	Normal	Nephrotic	P values
LDL_p	1.9	2.3	> 0.05
HDL_p	3.5	1.5	< 0.05
Plasma	1.2	0.8	< 0.05

were extrapolated back to '0' time, the values of specific activities obtained were 1260 and 800 counts/minute/mg. for normal and nephrotic rats, respectively. This indicates that nephrotic rats have a liver-plasma pool of unesterified cholesterol about 1,5 times larger than normal rats. Thus the turnover rate in mg./hour of this pool is very similar in the two groups of rats.

Though the exchange of labeled cholesterol of plasma lipoproteins with unlabeled cholesterol of other tissues goes on to a minor degree (213), the turnover rates determined from specific activities of unesterified cholesterol from 4-82 hours after the injection of labeled lipoproteins reflect predominantly the rate of replacement of cholesterol of the plasma-liver pool by newly synthesized molecules. Thus any difference between normal and nephrotic rats in the rates of exchange of their plasma cholesterol with other tissues would not alter the significance of turnover rates as reflection of rates of synthesis.

Since plasma cholesterol mixes with the pool in liver very rapidly (177), the difference in the specific activities of unesterified cholesterol of high and low density lipoproteins of plasma suggest that liver either has different pools for the two lipoproteins or has one pool with different rates of mixing with the two lipoproteins.

Conclusions:

1. In normal rats the fractional turnover rate of unesterified cholesterol of HDL_p is greater than that of LDL_p. In nephrosis the fractional turnover rate of LDL_p cholesterol remains normal but that of HDL_p cholesterol is decreased to about half that of the normal HDL_p and is also less than the fractional turnover rate of LDL_p unesterified cholesterol in nephrosis.

2. The fractional turnover rate of plasma total unesterified cholesterol is decreased in nephrosis to about two-thirds normal, but the actual amounts turned over are probably the same as normal on account of the increase in the size of the pool in nephrosis.

3. The liver either has two separate pools for the lipoproteins of plasma or has one pool with different rates of equilibration.

Studies on Exchange of Labeled Cholesterol Between High and Low Density Lipoproteins

Experiment 13

Purpose:

In Experiment 12 the turnover rates of unesterified cholesterol in plasma lipoproteins were determined after injection of LDL_p separated from serum labeled with C^{14}-cholesterol *in vitro* (284). A significant transfer of radioactive cholesterol from the injected material to the native HDL_p was noted to have taken place within 30 minutes after the injection. An experiment was therefore done to assess the extent of transfer of labeled cholesterol from LDL_p prepared as in Experiment 12 and also from HDL_p to other plasma lipoproteins when incubated together *in vitro*.

Method:

Twenty-one ml. of normal rat serum were labeled with C^{14}-cholesterol *in vitro* (284), using 25 μc. of the radioactivity dispersed on 50 mg. of celite powder. Twenty ml. of labeled serum, 10 ml. of unlabeled normal rat serum and 5 ml. of nephrotic rat serum were each separated into high and low density lipoproteins in a preparative ultracentrifuge. The upper 1 ml. from each of the four tubes containing labeled LDL_p, and the subnatant containing labeled HDL_p were pooled. Similarly, LDL_p were separated from

the HDL_p from the samples of unlabeled normal and nephrotic sera. Labeled lipoprotein solutions were mixed with solutions of unlabeled lipoproteins and sera in the volumes as indicated in Table 33. The mixtures were incubated for 30 minutes at 37° and were again fractionated into high and low density lipoproteins. The specific activities of unesterified and total cholesterol were determined in all samples.

Results and Discussion:

The results are given in Table 33. The shift of radioactivity from the labeled lipoproteins to the unlabeled lipoproteins was very marked in all instances.

When labeled LDL_p were incubated with normal or nephrotic serum the specific activity of unesterified cholesterol in HDL_p was found to be more than that of LDL_p cholesterol. Even if the rate of exchange of cholesterol between the two lipoproteins was so rapid that the two lipoproteins had reached equilibrium, the specific activity of HDL_p cholesterol should not have become more than that of LDL_p cholesterol. Lipoproteins lose part of their native lipids on centrifugation in preparative ultracentrifuge (292) and the cholesterol which has been added in the plasma lipoproteins in excess of their physiologic amounts may be more likely to break loose from the lipoproteins and sediment. Since the HDL_p were not isolated by floating and the mixture remaining in the tube after the

Table 33

Specific Activities of Cholesterol in Lipoproteins on Separation in a Preparative Ultracentrifuge after Incubating their Mixtures at 37° for 30 Minutes.

(cts/min/mg)

Mixtures	Unesterified Cholesterol		Total Cholesterol	
	Low Density	High Density	Low Density	High Density
Labeled LDL_p (0.5ml) + control serum (5.0 ml)	13,397	16,157	13,013	8,249
Labeled LDL_p (0.5ml) + Nephrotic (5.0ml) Serum	3,219	5,566	5,326	2,032
Labeled LDL_p (1.0ml) + control HDL_p Solution (8.0ml)	154,955	67,939	65,583	15,044
Labeled LDL_p (1.0ml) + nephrotic (8.0ml) HDL_p Sol.	56,960	39,651	29,409	8,036
Labeled HDL_p solution (8.0ml) + control LDL_p (1.0ml)	56,577	225,045	51,100	85,172
Labeled HDL_p sol/(LDL_p) (8.0ml) + neph. (1.0ml)	14,587	76,162	14,984	29,003

removal of LDL_p was taken as the HDL_p solution, the labeled cholesterol which may have sedimented was counted along with that of HDL_p.

The specific activities of total cholesterol contained in HDL_p were only half that of the cholesterol in LDL_p, apparently because the HDL_p contained much higher percentages of esterified cholesterol than the LDL_p and the label was introduced in unesterified cholesterol.

When the labeled LDL_p was mixed with solutions containing unlabeled HDL_p, the transfer of label was much less than during incubation with the sera, perhaps because of different physical conditions in which the lipoproteins existed.

When labeled HDL_p solutions were mixed with unlabeled LDL_p, the HDL_p total cholesterol had a specific activity about twice and the unesterified cholesterol 4-5 times the specific activity of LDL_p cholesterol. The difference, in part at least, may also be due to the presence of loose labeled cholesterol in the solution containing HDL_p. However, the presence of labeled cholesterol in the LDL_p in this mixture indicated a significant exchange of cholesterol between the two lipoproteins in <u>in vitro</u> conditions.

There was no significant difference in the exchange of labeled cholesterol in mixtures containing normal or nephrotic lipoproteins. The label was introduced into all mixtures only with the

normal lipoprotein and thus not much significance can be attached to this observation.

Conclusions:

1. The labeled cholesterol from the lipoproteins of the serum labeled in vitro by Avigan's method (284) exchanges in significant amounts with other lipoproteins when incubated together at 37° C. for 30 minutes, and then separated in a preparative ultracentrifuge.

2. The labeled cholesterol, when introduced into the serum in vitro (284), may leave the lipoproteins more easily than the native cholesterol molecules of the lipoproteins.

3. The transfer of labeled cholesterol from the injected LDL_p to native HDL_p in experiment 12 may have been due either to physiologic exchange of cholesterol molecules between the two classes of lipoproteins in vivo, or to an artefact of preferential release of labeled versus non-labeled cholesterol molecules from the injected LDL_p.

Experiment 14

Introduction:

The transfer of labeled cholesterol from the injected LDL_p to native HDL_p in experiment 12 could have been due to the unphysiologic state of the labeled cholesterol which was "solubilized"

in the serum LDL_p. Thus the following experiment was done to determine if there is any transfer of cholesterol from *in vivo* labeled lipoproteins.

Purpose:

To compare the extent of transfer of labeled cholesterol from isolated lipoproteins to the lipoproteins of normal and nephrotic sera during the course of separation of lipoproteins in a preparative ultracentrifuge.

Method:

One nephrotic and two normal rats were each given 5 μc. of mevalonic-2-C^{14} lactone and 4 hours later their blood was collected and plasma lipoproteins separated in a preparative ultracentrifuge.

Labeled lipoproteins from 5 ml. of normal and nephrotic sera were mixed with 5 ml. aliquots of fresh unlabeled sera from normal and nephrotic rats respectively and immediately transferred into preparative ultracentrifuge for separation into low and high density lipoproteins.

The amounts and specific activities of unesterified and total cholesterol were determined and the specific activities and amounts of esterified fractions calculated.

Results and Discussion: (Table 34)

When labeled LDL_p were mixed with normal serum, the specific

Table 34

Exchange of Radioactive Cholesterol between Lipoproteins Isolated from in-vivo Labeled Plasma of Normal or Nephrotic Rats.

		Cholesterol						
		Total		Unesterified		Esterified		
		mg.	cts/min/mg.	mg.	cts/min/mg.	mg.	cts/min/mg.	
				Normal				
LDLp *	LDLp **	0.494	3660	0.217	3050	0.277	4137	44 ***
	HDLp **	1.494	560	0.142	2730	1.354	332	9 ***
HDLp *	LDLp **	0.375	1470	0.143	1420	0.232	1500	38 ***
	HDLp **	1.450	1920	0.180	3650	1.270	1675	13 ***
				Nephrotic				
LDLp *	LDLp **	6.110	720	2.432	2760	3.678	-	40 ***
	HDLp **	5.885	580	1.301	690	4.584	549	22 ***
HDLp *	LDLp **	4.310	100	1.786	90	2.524	107	41 ***
	HDLp **	4.460	220	0.795	220	3.662	415	8 ***

* Labeled lipoprotein mixed with plasma before its separation.
** Lipoproteins separated from plasma after mixing isolated labeled lipoproteins with unlabeled plasma.
*** Unesterified cholesterol as per cent of total.

activity of unesterified cholesterol in the two lipoproteins was found to be similar. The transfer between esterified fractions was comparatively much less. When labeled HDL_p was mixed with normal serum, the transfer from the esterified fraction was more than that from unesterified fraction.

The labeled lipoproteins were obtained from sera of animals 4 hours after the injection of mevalonic-2-C^{14} lactone and at that time the specific activity of esterified cholesterol was observed to be fairly close to that of unesterified cholesterol (Experiment 9). Considering that HDL_p have more total cholesterol than LDL_p in normal animals, and the esterified cholesterol makes up a much greater fraction of this total, it is not surprising that absolute transfer of esterified fraction from the labeled HDL_p to LDL_p was so much that the specific activity of LDL_p esterified cholesterol became quite close to that of HDL_p cholesterol. Since the HDL_p was found to have only 9 and 13% of its cholesterol in unesterified form, as against 38 and 44% in LDL_p, the rise in specific activity of unesterified cholesterol in LDL_p was much less than in that of esterified cholesterol.

The transfer of radioactive cholesterol in mixtures of nephrotic lipoproteins and sera were found to be much less as compared to normals, indicating that under the same conditions, the fractional rates of exchange between nephrotic lipoproteins was much lower

than that between normal lipoproteins except in the case of unesterified cholesterol when labeled HDL_p were mixed with the serum.

The transfer of cholesterol from the isolated lipoproteins may not reflect the rate of transfer from lipoproteins *in vivo*. The specific activities of normal high and low density lipoproteins became similar to each other during the course of their separation in the preparative ultracentrifuge after isolated labeled LDL_p were mixed with unlabeled normal serum. On the other hand, the specific activities of plasma lipoproteins of animals after the injection of acetate-1-C^{14} were found to be very different from each other after their separation in the preparative ultracentrifuge. It is probable that the conditions for separating the lipoproteins in preparative ultracentrifuge weaken the bonds of lipids to lipoproteins. It has been observed that isolated lipoproteins lose 20-30% of their lipids each time they are recentrifuged under the same conditions (292). Since the specific activities of HDL_p never became more than those of LDL_p when labeled LDL_p were mixed with unlabeled sera, as happened in Experiment 13, the impression that the labeled cholesterol added to the serum *in vitro* (284) is released more easily than the native cholesterol of plasma lipoproteins is corroborated.

Conclusions:

1. There is exchange of cholesterol molecules between isolated lipoproteins *in vitro*. Such an exchange, however, may not reflect the rates of exchange between native lipoproteins *in vivo*.

2. There seems to be an exchange of esterified cholesterol as well as of unesterified cholesterol between the lipoproteins, though the fractional rate of exchange of unesterified cholesterol molecules may have been more than that of esterified cholesterol.

3. The percentage of unesterified cholesterol in HDL_p was much smaller than that in LDL_p of normal and nephrotic rats.

Studies on Exchange of Labeled Cholesterol Between Serum Lipoproteins, and the Liver.

Introduction:

On injection of AKS in rats, an increase in cholesterol concentration occurs predominantly in the vascular compartment (262). The rate of equilibration of cholesterol in plasma with that in liver is so rapid as compared to the rates of incorporation of newly synthesized cholesterol into plasma lipoproteins and the rates of exchange between plasma and other tissues, that for most purposes liver and plasma cholesterol can be considered as one pool (154). In disease states any increase in hepatic rate of synthesis or degradation of cholesterol should affect the plasma + liver components of the pool equally unless:

1. The rate of equilibration of cholesterol between plasma and liver is markedly decreased from normal so that changes in hepatic rates of synthesis or degradation are not reflected in plasma to the same extent as in liver. If this were so then an increase in hepatic synthesis would be most evident as an increase in concentration of cholesterol in the liver rather than in the plasma.,

2. In addition to the increase in amount of cholesterol in the pool, other changes also occur which alter the distribution of cholesterol in the pool.

To explain the increased concentration of plasma cholesterol and normal content of hepatic cholesterol it was suggested (262) that there was "trapping" of the lipid in the plasma. The turnover rate of plasma cholesterol reflects the rate of entry and exit of cholesterol, into and from, the entire liver-plasma pool and was found normal in rats 3 weeks after induction of nephrosis (Experiment 12). In order to study the rates of entry and exit of cholesterol, into and from plasma alone the following experiments were done.

Experiment 15

Purpose:

To compare the fractional rates of exchange of unesterified cholesterol between plasma LDL_p and liver.

Method:

Normal and nephrotic sera were labeled with 4-C^{14}-cholesterol suspended in Tween 20 and LDL_p were then separated from them. Aliquots of normal LDL_p containing 575,000 counts per minute in 22 mg. of unesterified cholesterol were injected into each of 5 normal rats and aliquots of nephrotic LDL_p containing 480,000 counts

Table 35

Radioactivity (percent of injected dose) in Livers, 10 minutes after the Administration of LDL_p Labeled with C^{14}-Cholesterol

	Normal	Nephrotic
1	47.8	21.9
2	62.6	63.2
3	42.8	42.2
4	46.3	32.9
5	30.6	48.0
6	–	65.0
Mean	46.0	48.3
S.D.	11.7	17.1
P	>0.005	

per minute in 44 mg. of unesterified cholesterol were injected into each of 6 nephrotic rats. The nephrotic rats had serum cholesterol concentrations between 110 and 248 mg./100 ml.

The blood and livers (after being perfused free of blood) were collected from all animals, 10 minutes after injection of labeled cholesterol. Amounts and specific activities of unesterified cholesterol of plasma and liver were determined.

Results:

The results of hepatic uptake of injected radioactivity are given in Tables 35 and 36.

Discussion and Conclusion:

The results suggest that labeled LDL_p cholesterol distributes itself in the plasma-liver pool rapidly after intravenous injection. The mean specific activities of liver total unesterified cholesterol 10 minutes after the injection of labeled lipoproteins were 9.5 and 15.4% of the specific activities of unesterified cholesterol of plasma in normal and nephrotic rats, respectively. The difference was not significant at 5% level.

Subsequent to completion of the above study a report (293) was published showing that cholesterol suspended in Tween 20 is selectively picked up by the liver. Hence the quantitative aspects of the above study are questionable.

Table 36

Specific Activity of Liver Unesterified Cholesterol as percent of Specific Activity of Plasma Unesterified Cholesterol 10 minutes after the Injection of LDL_p Labeled with C^{14}-Cholesterol

	Normal	Nephrotic
1	19.5	16.1
2	4.7	18.6
3	10.3	12.7
4	7.1	21.2
5	5.9	10.0
6	-	14.0
Mean	9.5	15.4
S. D.	$\pm$ 5.9	$\pm$ 4.2
P		>0.05

Experiment 16

Purpose:

The last experiment indicated that 10 minutes after injection of LDL_p labeled with 4-C^{14}-cholesterol suspended in Tween 20 up to 63% of the radioactivity injected was detected in liver. It is possible that the labeled cholesterol and Tween 20 remained linked in the injected LDL_p (294) and liver selectively picked up the cholesterol associated with it (293). The following experiment was therefore undertaken to overcome the presence of Tween in the injected material by comparing the hepatic uptakes in normal and nephrotic animals of radioactivity from the plasma lipoproteins labeled in vivo with C^{14}-cholesterol.

Method:

Red blood cells were collected from normal and nephrotic rats 2 hours after injection of C^{14}-mevalonic acid. The cells were washed and their aliquots were incubated with fresh normal and nephrotic sera in the cold overnight. The sera were separated and 0.5 ml. aliquots of normal and nephrotic sera were injected intravenously into 9 normal and 9 nephrotic (3 weeks after injection of AKSO) rats, all paired by weight.

Aliquots of normal serum had 23,895 counts per minute in 650 γ. of cholesterol and aliquots of nephrotic serum had 60,780

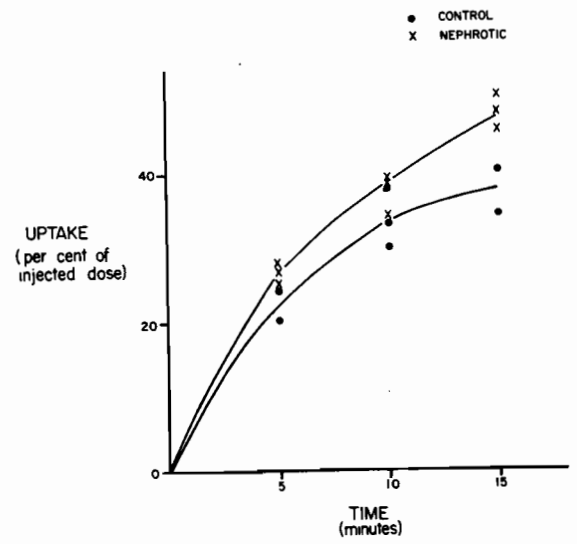


Figure 27

Transfer of radioactive cholesterol into liver after injection of sera labeled with C^{14}-cholesterol into normal and nephrotic rats.

counts per minute in 1,540 γg. of cholesterol. However, the lipids other than cholesterol in the aliquots had 1-1.5 times the radioactivity present in cholesterol. All the radioactivity was obtained ultimately from C^{14}-mevalonic acid.

The mevalonic acid has two isomers; only one is irreversibly incorporated into cholesterol and the other is excreted in the urine within 4 hours after injection in a mouse (167). It is possible that the metabolically inactive enantiomorph can pass from plasma to cells and back freely before being excreted in the urine, so that it may have been present in the red blood cells obtained 2 hours after the injection of mevalonic acid, and passed into the serum during the incubation. Thus the radioactivity present in lipids other than cholesterol, in the aliquots injected, may have been a biologically inactive isomer of C^{14}-mevalonic acid, and it was assumed that the radioactivity present in lipids other than cholesterol was not incorporated into liver cholesterol during the short time of this experiment.

After the injection of labeled sera, livers were perfused free of blood and removed from three control and three nephrotic rats at intervals of 5, 10 and 15 minutes. The amounts of total cholesterol and radioactivity were determined in livers of all animals (two samples from normal rats for the determination of radioactivity were lost).

Table 37

Amounts of Cholesterol (mg) in Livers.

	Normal	Nephrotic
	9.27	11.25
	8.34	12.20
	10.40	13.22
	10.74	14.62
	11.43	15.48
	11.53	15.66
	11.82	17.25
	12.55	17.92
	14.42	19.90
Mean	11.17	15.28
S.D.	1.79	2.9
P value	< 0.01	
Values per 100g body weight	6.03	8.9

Results:

The livers of the nephrotic animals had greater amounts of cholesterol than normal rats of identical weights (Table 37). The difference was significant at the level of 1%.

The hepatic uptake of labeled cholesterol increased with time both in normal and nephrotic rats (Fig. 27), but the nephrotic livers had a slightly greater fraction of injected radioactivity than normal livers.

Conclusions:

The fractional rate of exchange of cholesterol between plasma and liver in nephrotic rats was slightly greater than that in normal rats and in view of the increased concentration of cholesterol in nephrotic plasma (around 250 mg/100ml as against 45 mg./100ml in normal) the actual rate of transfer from plasma to liver was markedly increased in nephrotic animals.

Exchange of Labeled Cholesterol Between Plasma Lipoproteins and Red Blood Cells.

Introduction:

The exchange of cholesterol between plasma lipoproteins and liver (Experiments 15, 16) appears to be of an entirely different nature than that between plasma lipoproteins and red blood cells. Concentration of cholesterol in liver can be easily increased by increasing the concentrations in the plasma (237) whereas the amount of cholesterol in red blood cells cannot be altered even if the plasma concentration increases ten fold (133). The cholesterol in plasma lipoproteins exchanges isotopically with that of red blood cells, from and onto specific sites (133). Thus in order to detect any alteration in the avidity of nephrotic lipoproteins for cholesterol which may have a bearing on the hypercholesterolemia, the following studies on rates of exchange of cholesterol between plasma lipoproteins and the red blood cells were conducted.

Methods:

Serum lipoproteins were labeled with C^{14}-cholesterol either in vitro on by in vivo synthesis from acetate-C^{14} or mevalonic acid-C^{14}. Labelling of red blood cells by C^{14}-cholesterol was achieved either in vivo subsequent to injection of C^{14}-acetate or

(C^{14} mevalonic acid, or *in vitro* by incubation in the cold with labeled serum.

Aliquots of serum or plasma were taken in small erlenmeyer flasks to which a suspension of red blood cells in saline was added. The mixtures were incubated at 37° C. under an atmosphere of 95% oxygen-5% carbon dioxide. The mixtures were constantly but gently shaken and were thoroughly mixed before taking the samples.

The samples were transferred into chilled centrifuge tubes and were immediately separated in a refrigerated centrifuge. The supernatants (plasma plus normal saline in which red cells were suspended) were collected and the cells washed with 10-20 times their volume of normal saline and frozen.

The fractional rates of exchange were determined from the specific activities of unesterified cholesterol of cells and plasma according to the method of Solomon (295).

Experiment 17

Purpose:

To compare the rates of exchange of cholesterol between normal cells and normal or nephrotic plasma.

Method:

To normal heparinized rat blood, cholesterol-4-C^{14} suspension in Tween 20 was added and left overnight in the cold. Next morning the cells were separated and washed twice with normal saline.

Eight ml of fresh unlabeled normal and nephrotic sera were incubated in duplicate with 3 ml of labeled normal cells. Aliquots of 2 ml were taken at 0, 1, 2, 3 and 4 hours and immediately centrifuged to separate the sera and cells. One ml aliquots of the supernatants were taken to determine the concentration and specific activity of unesterified cholesterol. The content and specific activity of cholesterol in washed red cells were determined.

Results:

Results are given in Fig. 22. There was a progressive increase in the amount of radioactivity in serum cholesterol with time. The drop in specific activities of unesterified cholesterol in 4 hours was 32 and 58% of their value at '0' time for cells incubated with normal and nephrotic sera respectively, indicating

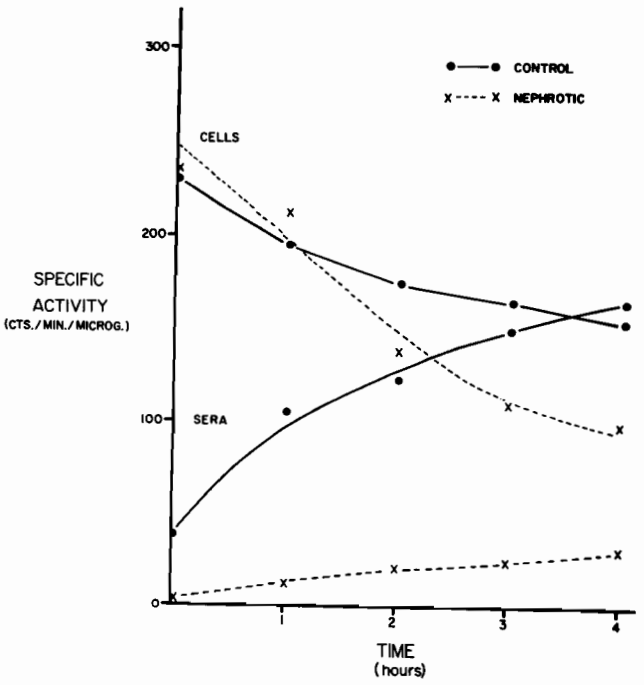


Figure 22

Changes in the specific activities of unesterified cholesterol in labeled normal cells and unlabeled normal and nephrotic sera when incubated together.

that the loss of radioactivity from cells incubated with nephrotic serum was 1.8 times greater than from cells incubated with normal serum.

The specific activity of unesterified cholesterol in normal serum was 5 times greater than nephrotic, indicating a greater fractional rate of exchange of cholesterol from normal than from nephrotic serum. At the end of 4 hours the specific activities of unesterified cholesterol of cells and control serum were similar whereas that of nephrotic serum was only one-third of the specific activity of cell cholesterol. This suggested that the time of equilibration of nephrotic serum cholesterol with that of red cells was markedly increased.

The actual rates of exchange of cholesterol between cells and sera could not be determined because the data on hematocrits were needed to calculate the amounts and the specific activity at equilibrium of cholesterol in the two compartments (sera and cells) of the system. The aliquots for hematocrits were not taken in this experiment.

Experiment 18

Purpose:

In experiment 17, labeled cells were incubated with unlabeled sera. During incubation over periods of hours some

hemolysis of red blood cells, however slight, does occur. Even small amounts of cell cholesterol would make a significant difference in the specific activity of cholesterol in the serum or plasma. However if plasma is labeled and cells are unlabeled, a very slight hemolysis will not markedly alter the rate of change of specific activity of cholesterol in plasma.

Thus plasmas were labeled by incubating with cells prepared as in Experiment 20 and the rates of exchange of their cholesterol with red blood cells were determined.

Method:

Normal red blood cells were labeled with C^{14}-cholesterol suspension in Tween 20 as in the last experiment and aliquots were incubated with normal or nephrotic plasma in cold overnight. The plasmas were separated in the morning and used for the experiment. Ten ml. of normal and 5 ml. of nephrotic labeled plasma were incubated in duplicate with 8 and 4 ml. aliquots of the same suspension of unlabeled normal cells. Aliquots of the incubation mixtures were taken at 0.5, 1, 1.5, 2, 3 and 4 hours. Specific activities of unesterified cholesterol in samples of cells and plasma, hematocrits of the mixture, and total cholesterol concentration in the plasmas (supernatants on spinning down of cells) were determined at different times.

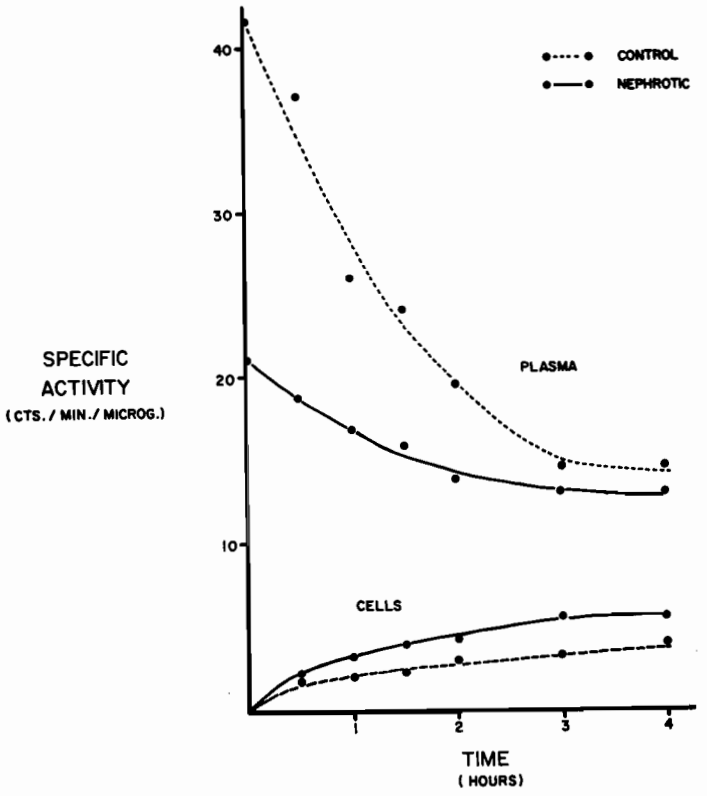


Figure 23

Changes in the specific activity of unesterified cholesterol in normal and nephrotic plasmas when incubated with normal cells.

Results:

The hematocrits and total cholesterol in the plasmas are given in Tables 38 and 39, and indicate that there was no change in the size of the two compartments (cells and plasma) due to hemolysis or any other cause. The amounts of radioactivity and specific activity of unesterified cholesterol in cells and plasma calculated per ml. of the mixture are given in Table 40. The curves of specific activities of unesterified cholesterol of cells and plasma samples are charted in Fig. 23.

Discussion:

The first aliquot taken was at one half hour because the true value for the specific activities at '0' time could not be determined. Ideally the specific activity of the supernatant plasma of the mixture at '0' time should be taken after the plasma and cell suspensions had been mixed but before any exchange of cholesterol molecules takes place. This was technically impossible because of the time it took to separate the cells and plasma. The reason against using the specific activity of the plasma before mixing with cells as the specific activity of the supernatant at '0' time is that any hemolysis in cells after reconstituting them in the suspension and mixing them with plasma may result in release of significant amounts of unesterified

Table 38

Hematocrits of Plasma Cell Mixtures at Various Times after the Start of Incubation.

	Time (hours)						
	0	0.5	1.0	1.5	2	3	4
Control 1	34.1	34.8	33.3	32.6	35.2	34.2	34.1
Control 2	37.5	32.0	33.9	33.0	-	34.6	32.1
Nephrotic 1	35.9	-	33.0	34.2	33.1	34.3	34.7
Nephrotic 2	32.7	-	33.8	33.3	35.6	36.4	36.4

Table 39

Total Cholesterol in Identical Aliquots of Supernatant at Various Times after the Start of Incubation (µg).

	Time (hours)					
	0.5	1.0	1.5	2	3	4
Control 1	162	200	200	192	186	190
Control 2	202	200	192	202	170	173
Nephrotic 1	610	585	550	560	550	530
Nephrotic 2	620	550	575	550	512	535

cholesterol in the supernatant, thus decreasing the '0' time specific activity of the plasma. Such a possibility would have significantly altered the results in this experiment, considering that the cells had 2 and 8 times the amount of cholesterol present in nephrotic and normal plasmas respectively.

However, calculating the gain of radioactivity by the cells and loss of radioactivity by the plasmas from the first to the last determination, it became apparent that practically all the radioactivity was accounted for (Table 40). Thus the values of the specific activity at '0' time could be calculated from the total radioactivity present in the system and amount of cholesterol in plasma. Similarly the specific activity values at equilibrium could also be calculated since at equilibrium both pools should have the same specific activity, assuming that all the unesterified cholesterol in the two compartments is exchangeable.

The fractional rates of exchange between red cells and plasma were calculated as per methods outlined earlier and were (% per hour):

	Normal	Nephrotic
From serum to cells	37.9	16.6
From cells to serum	5.1	13.5

Conclusions:

The results indicate that fractional rate of transfer of cholesterol from plasma lipoproteins of nephrotic animals

Table 40

Amounts of Unesterified Cholesterol and Radioactivity in Cells and Supernatants (Plasma + Saline) in One ml. Each of the Mixtures Incubated.

	Normal	Nephrotic
Amount of cholesterol in cells (μg)	462.1	386.8
Amount of cholesterol in supernatant (μg)	53.3	270.6
Radioactivity lost by plasma from 1st to last determination (cts/min)	1119	1623
Radioactivity gained by cells from 1st to last determination (cts./min)	1202	1392
Specific activity of cholesterol at equilibrium. Ant. of radioactivity/ ant. of cholesterol. (cts./min/μg) in both compartments of the system	4.8	8.6

is markedly reduced under the conditions of the above experiment.

Experiment 19

Purpose:

In the conditions of the last experiment the cholesterol in nephrotic lipoproteins had a fractional rate of exchange with that in red blood cells less than half that observed with normal plasma. The fractional rate of exchange of cells incubated with nephrotic plasma was about 2.5 times that of cells incubated with normal plasma. Though the fractional rate of transfer from nephrotic lipoproteins was decreased, the actual amount of cholesterol transferred was greater than normal. It is conceivable that the rate of transfer from cells could not exceed that obtained in the last experiment thereby having a rate-limiting effect on the exchange from nephrotic lipoproteins. In order to determine whether or not the decrease in fractional rate of transfer from nephrotic lipoproteins was due to rate-limiting effect of cells the following experiment was done.

Method:

Two aliquots of 5 ml. each of nephrotic plasma labeled with C^{14}-cholesterol as in the last experiment, were incubated with 4 ml. and 1 ml. of unlabeled normal cells. Aliquots of 1 ml

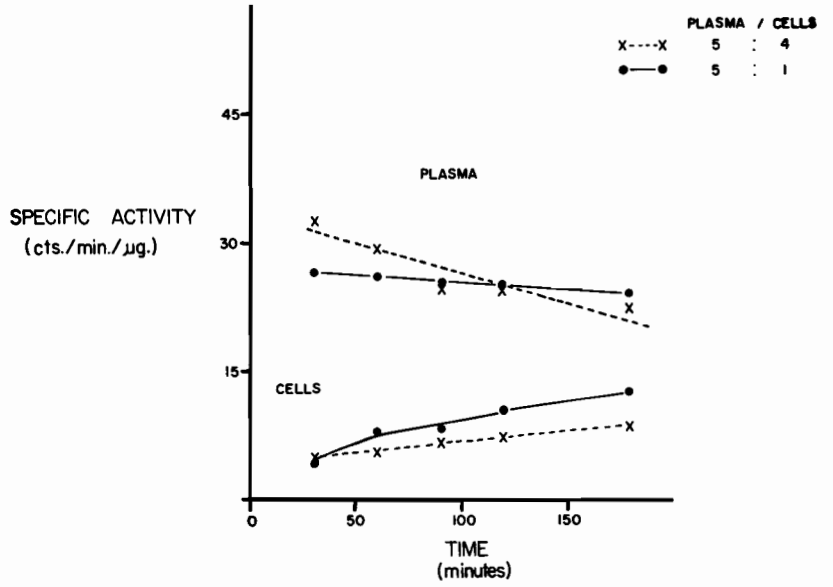


Figure 24

Change in specific activites of unesterified cholesterol in nephrotic plasma and unlabeled normal cells when incubated in different proportions.

were taken at 0.5, 1, 1.5, 2 and 3 hours, and hematocrits, amounts and radioactivity of the unesterified cholesterol of cells and the plasmas at different times were determined.

Results:

The hematocrit values are given in Table 41, the amounts and radioactivity of unesterified cholesterol in Table 42, and the specific activity are charted in Fig. 24.

The rates of exchange were calculated as per cent per hour, as outlined in the methods:

	Plasma/Cells	
	5/4	5/1
From cells to plasma	11.1	17.7
From plasma to cells	13.9	3.3

The results indicate clearly that normal red blood cells are capable of about 38% greater fractional rate of exchange than that obtained in the last experiment. Thus in Experiment 18 the rate-limiting factor for a decreased transfer to red blood cells from nephrotic plasma must reside in the latter and it is therefore probable that the decreased rate of exchange from nephrotic plasma probably represents a qualitative rather than a quantitative alteration in serum lipoproteins.

Table 41

Hematocrits of Plasma Cell Mixtures at Various Times after the Start of Incubation.

Plasma/Cells	Time (hours)				
	0.5	1	1.5	2	3
5/4	33.3	33.0	34.4	33.9	34.7
5/1	12.6	11.6	12.1	12.8	12.6

Table 42

Amounts and Specific Activity of Unesterified Cholesterol in Cells and Supernatant in One ml each of the Mixtures Incubated

	Plasma / Cells	
	5/4	5/1
Amount of cholesterol in cells (µg)	448	161
Amount of cholesterol in supernatant (µg)	247	591
Specific activity of cholesterol in cell/supernatant at equilibrium (cts/min/µg)	13.9	21.6

Experiment 20

Purpose:

Studies with *in vitro* labeled plasma indicated that nephrotic lipoproteins have decreased fractional rate of exchange when incubated with normal red blood cells. In order to corroborate this finding under more physiological systems, normal and nephrotic plasmas were labeled *in vivo* and they were incubated with normal and nephrotic cells respectively in more or less physiological proportions to determine their fractional rates of exchange of unesterified cholesterol.

Method:

Blood was collected from normal and nephrotic rats 2 hours after injection of labeled mevalonic acid, plasmas were separated and aliquots incubated with suspensions of unlabeled red blood cells obtained from other animals which had not been given labeled mevalonic acid. The incubation vessels contained the following:

(Normal) 4 ml. of normal cell suspension + 4 ml of labeled normal plasma. (Nephrotic) 2 ml of nephrotic cell suspension + 2 ml of labeled nephrotic plasma. Aliquots were taken from the mixtures at 0.5, 1, 1.5 and 2 hours after the start of incubation for determination of hematocrits and of cholesterol concentration and specific activity in plasma and cells.

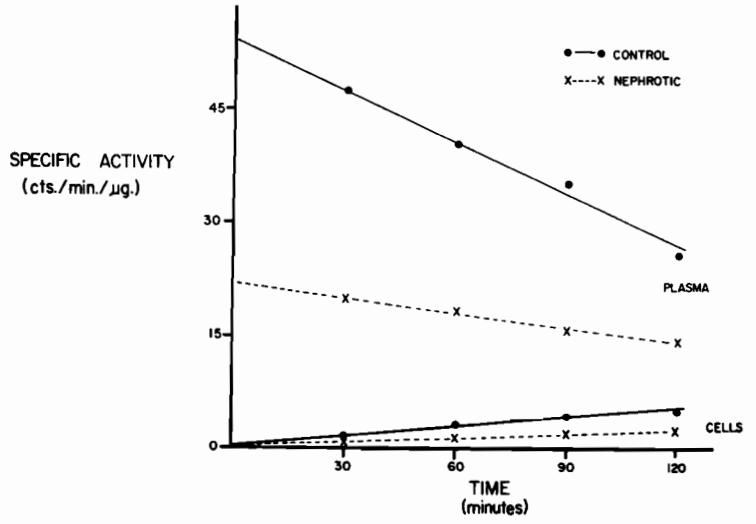


Figure 25

Change in specific activities of normal and nephrotic plasmas labeled in vivo when incubated with unlabeled normal and nephrotic cells.

Results:

The hematocrits are given in Table 43, the amounts and radioactivity of unesterified cholesterol in Table 44, and the specific activities in Fig. 25.

The rates of exchange in per cent per hour of unesterified cholesterol and plasmas were:

	Normal	Nephrotic
From plasma to cells	44.6	21.5
From cells to plasma	5.9	5.5

Discussion and Conclusions:

These results confirm the impressions gained from experiments using *in vitro* labeled plasma that the fractional rate of exchange of cholesterol between plasma and red blood cells is markedly reduced in nephrosis. Since plasma and cells were incubated in concentrations and conditions more or less physiological in this experiment, the comparison of the rates between plasma and cells probably reflects the situation obtained *in vivo*. The decreased transfer of cholesterol from lipoproteins to cells in nephrosis is probably due to changes in the lipoproteins and not the cells, as was suggested by the results of Experiment 19.

Table 43

Hematocrits of Plasma Cell Mixtures at Various Times after the start of Incubation.

	Time (hours)		
	1	1.5	2
Normal	25.9	25.4	26.0
Nephrotic	32.9	33.6	34.5

Table 44

Amounts and Specific Activities of Unesterified Cholesterol in Cells and Supernatant in One ml each of the Mixtures Incubated.

	Normal	Nephrotic
Amount of cholesterol in cells (µg)	565	359
Amount of cholesterol in supernatant (µg)	57.1	151.2
Specific activity of cholesterol in both compartments at equilibrium (cts/min./µg)	7.07	4.48

Experiment 24

Purpose:

The alterations(s), quantitative or qualitative, responsible for the decrease in the rate of transfer of cholesterol from nephrotic plasma may occur in either or both of the two classes (high and low density) of lipoproteins. In order to compare the purified lipoproteins of normal and nephrotic animals, the following experiment was done.

Methods:

Blood was collected from normal and nephrotic rats 4 hours after injection of mevalonic acid-C^{14} and the plasma was separated after centrifuging in the cold. Chylomicrons were removed from the plasma and then high and low density lipoproteins were isolated from the plasma in a preparative ultracentrifuge. The lipoprotein solutions were dialyzed against normal saline to rid them of excess salt and make them isotonic to red cells, and then were incubated with a suspension of normal cells at 37° C., according to the methods outlined earlier.

Aliquots were taken at 0.5, 1, 1.5 and 2 hours after the start of incubation for determinations of hematocrits, amounts and specific activities of unesterified cholesterol in cells and lipoprotein solutions.

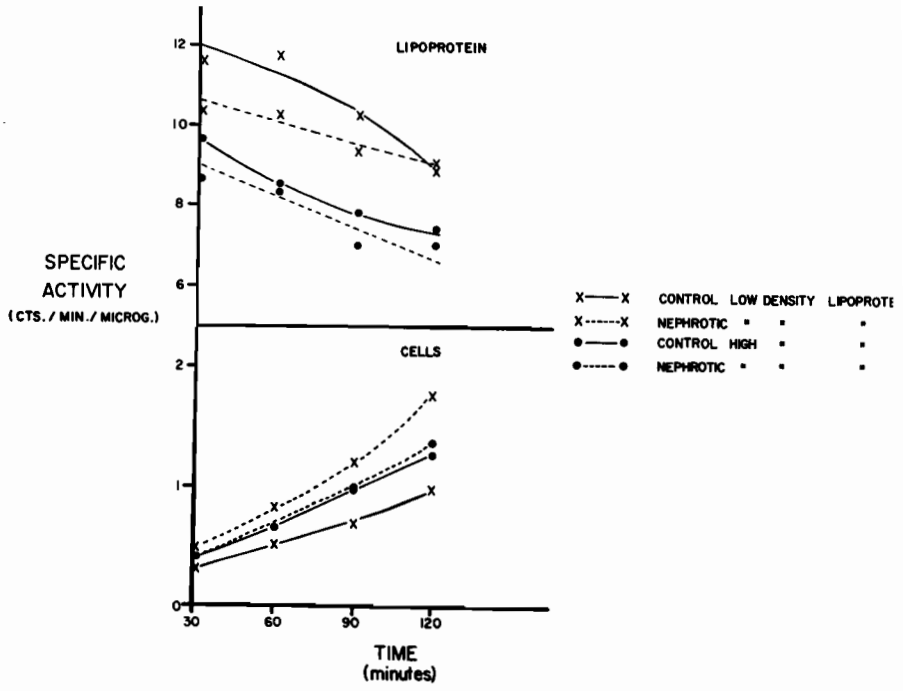


Figure 26

Changes in specific activities of unesterified cholesterol in labeled plasma lipoproteins of normal and nephrotic rats when incubated with unlabeled normal and nephrotic red blood cells respectively.

Results and Discussion:

The hematocrits are given in Table 45 and indicate no progressive hemolysis of the red blood cells, so that the concentration of cholesterol in the two compartments (cells and lipoprotein solution) can be assumed to remain constant. The amounts of cholesterol present in the cells and lipoprotein solutions per ml of the mixture are given in Table 46 and the change in the values of their specific activity are plotted in Fig. 26. The fractional rates of exchange between lipoproteins and the cells were calculated as in the previous studies and are given in Table 47.

The normal LDL_p cholesterol had a higher rate of exchange with cells than the normal HDL_p. There was a similarity in the fractional rates of transfer from normal and nephrotic HDL_p, whereas the fractional rate of transfer from LDL_p was markedly lower than that from normal LDL_p. The fractional rate of transfer from cells incubated with nephrotic LDL_p was 20%/hour as against 5%/hour from cells incubated with normal LDL_p. This probably was due to the fact that concentration of cholesterol in nephrotic LDL_p cholesterol was much higher and the actual rate of exchange greater than with the normal LDL_p.

Since the red cells were capable of greater rates of exchange than those obtained on incubation with normal lipoproteins and with nephrotic HDL_p it is suggested that the rate limiting factor in these incubations resided in the lipoproteins.

Table 45

Hematocrits of Mixtures of Cells and Lipoproteins at Various Times (in hours) after the Start of Incubation

Mixture of cells and	0.5	1.0	1.5	2.0
Normal LDL_p	19.5	19.6	20.2	19.1
Normal HDL_p	19.8	19.5	20	20
Nephrotic LDL_p	20.2	21.3	19.8	20.9
Nephrotic HDL_p	20.1	20.0	19.6	20.2

Table 46

Amounts of Unesterified Cholesterol (per ml. of Mixture) of Cells and Lipoprotein Solutions. (μg)

	Normal		Nephrotic	
	Lipoprotein Sol.	Cells	Lipoprotein Sol.	Cells
Low Density	65.8	294.0	374.2	307.0
High Density	96.5	280.0	129.0	297.0

Table 47

Fractional Rates of Exchange between Normal Red Blood Cells and Normal or Nephrotic Lipoproteins.

(%/hour)

	Normal	Nephrotic
From LDL_p to cells	28.2	19.8
From cells to LDL_p	5.3	20.2
From HDL_p to cells	17.2	15.8
From cells to HDL_p	6.8	6.4

Previous experiments indicated that the decreased rate of exchange of cholesterol between red cells and nephrotic whole plasma was due to a qualitative abnormality in the plasma: the present result showing a decreased rate of exchange between cells and nephrotic LDL_p suggests that the abnormality in the whole plasma may be due to the LDL_p.

Conclusions:

1. Under physiological conditions the fractional rate of exchange of cholesterol between cells and plasma was decreased in nephrotic animals indicating a qualitative or quantitative change in nephrotic plasma lipoproteins.

2. Under identical conditions, normal LDL_p differs from normal HDL_p in being able to release its unesterified cholesterol more readily than HDL_p.

3. Nephrotic LDL_p probably differs from normal LDL_p in having greater avidity for cholesterol, provided that the smaller fractional rate of exchange of cholesterol was not due to the rate-limiting effect of red blood cells with which the lipoprotein was incubated.

Experiment 22

Purpose

The increase in plasma cholesterol concentration in nephrotic rats more than 2 weeks after the injection of AKS could not be ascribed either to "trapping" of the lipid within the vascular compartment or to an increase in synthesis of cholesterol by the liver. Protein moities of lipoproteins have an avidity for lipids (276) and evidence suggesting that nephrotic lipoproteins may have a greater avidity for cholesterol than normal was obtained (experiment 19). An increase in the concentration of protein moities of plasma lipoproteins may increase the capacity of nephrotic plasma to transport more lipids by providing greater number of binding sites. The following study was therefore undertaken to determine if there was an increase in the concentration of protein moieties of plasma lipoproteins in experimental nephrosis at a time when cholesterol synthesis is not increased.

Methods:

Normal (30ml) and nephrotic (8 weeks after injection of AKS) (27ml) sera were collected from rats in the morning without fasting or any other preparation of donor rats. Low density lipoproteins were separated at densities 1.006 and 1.003 and HDL_p were isolated at density 1.21, in a preparative ultrafuge. The two classes of LDL_p were washed once and HDL_p were washed twice by repeated floatation in the preparative ultracentrifuge.

The lipids from the lipoproteins were extracted with alcohol: acetone; the protein precipitates were washed twice with alcohol:

Table 48

Amounts of Cholesterol and Protein Moities of Lipoproteins in Normal and Nephrotic Sera

(μg/ml)

Lipoprotein	Cholesterol (μg)		Protein (μg)		Ratio: Normal / Nephrotic	
	Normal	Nephrotic	Normal	Nephrotic	Cholesterol	Protein
LDL_p D<1.006	52.1	1150.0	36.7	249.0	22.0	6.8
LDL_p D>1.006 <1.063	112.0	1390.0	24.0	216.0	12.4	9.0
HDLp D>1.063 <1.21	312.5	655.0	144.5	298.0	2.1	2.1
Total	476.6	3195.0	205.2	763.0	6.7	3.7

acetone and the washings were added to the original extracts. The lipid extracts were evaporated to dryness and redissolved in petroleum ether. Cholesterol was determined in aliquots of lipid extracts.

The proteins precipitated were dissolved in 2 ml of 1.0N NaOH and duplicate aliquots of the solution were taken out for the determination of amounts of proteins (296).

Results:

The concentrations of cholesterol and proteins of various lipoproteins are given in Table 48.

Discussion:

The concentrations of various lipoproteins in the present study represent the distribution in the phase of alimentary lipemia. In normal rats LDL_p of density <1.006 had only 11% of the total plasma cholesterol whereas in the nephrotic plasma they carried 36% of the total plasma cholesterol. In nephrosis, the increase in the lipid of lowest density lipoprotein was 22 times compared to 6.7 times for total plasma cholesterol.

The increase in cholesterol concentration was least marked in HDL_p which in normal rats carry more cholesterol than the LDL_p. However, the increase in the cholesterol concentration of HDL_p was of the same degree as in the protein moiety of HDL_p. The increase in the concentrations of protein moities of both classes of LDL_p was not as much as the increase in their cholesterol concentration.

The concentration of protein moities of plasma lipoproteins was greater in nephrotic rats than normal. This was particularly marked for the proteins of LDL_p. The presence of large amounts of lipids and proteins of lowest density lipoproteins was undoubtedly related to the presence of alimentary lipemia.

Conclusion:

The nephrotic plasma has an increased capacity to transport lipids due to an increase in concentration of proteins of lipoproteins and to an increased lipid binding ~~per~~ unit weight of proteins.

Effect of Proteins of Serum Lipoproteins on the Synthesis of Cholesterol by Rat Liver Homogenates.

Introduction and Purpose:

It was hypothesized (102) that there is an increase in the synthesis of all plasma proteins, including lipoproteins, by the liver in experimental nephrosis. The possibility existed that protein moieties of lipoproteins stimulate the liver to synthesize lipids which normally are associated with them. To test this hypothesis effects of proteins isolated from serum lipoproteins of human and rat blood on the synthesis of cholesterol by rat liver homogenates were studied. Proteins from the sera were prepared according to the method of Scanu et al (276) who had demonstrated that the proteins of HDL_p were completely delipidized by this method without being denatured. They observed that LDL_p could not be freed of all lipids; since for our work it was not essential that the proteins should be completely delipidized, LDL_p protein was also prepared by the same method. The proteins were dissolved in phosphate buffer used in preparation of homogenates.

Methods:

Liver homogenates were prepared from female rats according to the method of Bucher and McGarrahan (181) and aliquots of the homogenate were incubated according to Migicovsky (182). The proteins, when required, were dissolved in the solution containing ATP and DPN. Mevalonic lactone-2-C^{14} in amounts of 0.5 mg., containing 1-2 µc of radioactivity, was used as substrate. Incubation was

carried out at 37° C, under an atmosphere of 5% O_2 in oxygen. To isolate cholesterol, the contents of the incubation vessels were quantitatively extracted in chloroform:methanol, 2:1; the extracts were evaporated to dryness and the residues redissolved in alcohol: acetone. In order to obtain complete precipitation of cholesterol in the lipid extract, 1 mg. of cholesterol was added as carrier before precipitating it with digitonin. Total radioactivity in the cholesterol was determined after plating the cholesterol digitonide solution in pyridine.

Since homogenate prepared from livers of different animals differ in their rates of incorporation of mevalonic acid into cholesterol (181), all studies to determine the effects of various proteins were done on identical aliquots of the same homogenate preparation.

Experiments and Results:

A. In preliminary studies, to establish the rate of incorporation of mevalonic-2-C^{14} lactone into cholesterol by rat liver homogenates, aliquots of homogenates were incubated for periods of 0.5-2 hours and radioactivity in the cholesterol determined.

The incorporation of radioactive mevalonic acid into cholesterol increased with time as demonstrated by the results (Fig. 27) of one typical experiment. Thus in subsequent studies, to determine the effect of proteins on the incorporation of mevalonic acid into cholesterol, the homogenates were incubated for a period of 2 hours.

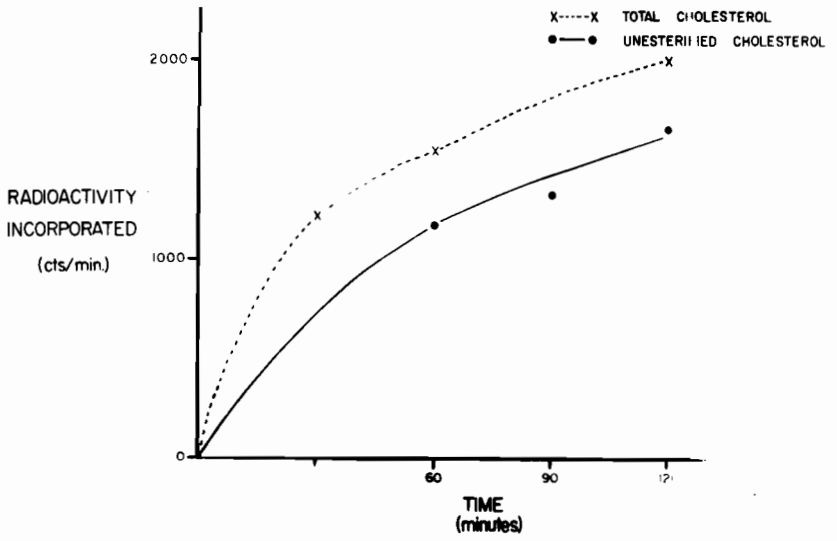


Figure 27

Time course of mevalonic-2C^{14} lactone incorporation into cholesterol by normal rat liver homogenates.

B. Four different homogenates of female rat livers were used to study the effects of protein moieties of lipoproteins of human sera and three different preparations were tested for the effects of rat serum lipoprotein proteins on their rates of synthesis of cholesterol. Included in the studies on the latter three homogenates was the effect of bovine albumin to serve as a control for non-specific effects of lipoprotein proteins.

(1) Studies with Human Serum Lipoprotein Proteins:

Six aliquots from each of the four homogenates were taken in six vessels and incubated as follows:

#1, 2 - Control

#3, 4 - Control + 0.5 mg. proteins of HDL_p

#5, 6 - Control + 0.3 mg. proteins of LDL_p

The vessels were incubated for 2 hours and the radioactivity incorporated in cholesterol was determined.

(2) Studies with Rat Serum Lipoprotein Proteins:

Identical aliquots from each of three homogenate preparations were incubated for 2 hours in eight vessels as indicated below:

#1, 2 - Control

#3, 4 - Control + 1.3 mg. HDL_p protein

#5, 6 - Control + 0.7 mg. LDL_p protein

#7, 8 - Control + 1.0 mg. bovine albumin

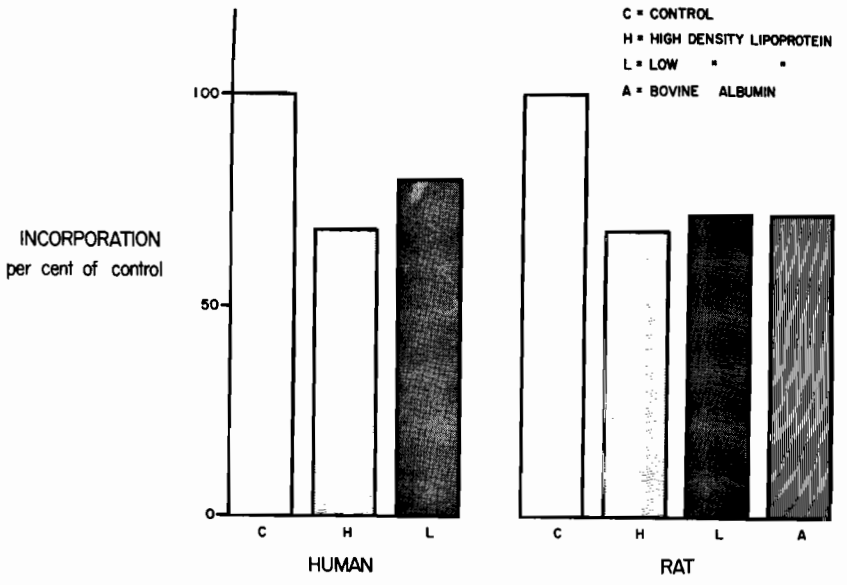


Figure 28

Effect of protein moities of plasma lipoproteins (human and rat) on the incorporation of mevalonic-2C^{14} lactone into cholesterol by rat liver homogenates.

The radioactivity incorporated in the cholesterol was determined.

Results:

The results of similar experiments were pooled and are given in Fig. 38. The mean of the radioactivity incorporated into cholesterol of control vessels was taken as 100 and the effect of lipoprotein is expressed as percentage of the control value.

High density lipoprotein proteins whether from human serum or from rat serum depressed the incorporation of mevalonic acid into cholesterol by rat liver homogenates to about 68% of the control values. Low density lipoproteins from human serum depressed the incorporation to 80% and those for rat serum to 72% of the control values.

Bovine albumin, when added to the homogenates, also depressed the synthesis of cholesterol to about 72% of the control value.

Discussion:

It is clear from the results of these studies that presence of lipoprotein proteins did not stimulate the synthesis of cholesterol from mevalonic acid by rat liver homogenates. The proteins added to the homogenates were assumed not to have been denatured during the process of delipidization as was shown by Scanu et al. (276). The fact that the lipoprotein proteins went into solution

readily corroborates this assumption.

High density lipoprotein protein caused a somewhat greater depression of synthesis than LDL_p proteins, however, the amounts of HDL_p protein used were also larger than those of LDL_p proteins. In order to test whether this depression of synthesis was non-specific action of any protein effect of bovine albumin in comparable concentrations was also sutdied. Like other two proteins, albumin also caused suppresion of synthesis by homogenates, however, the depression caused by proteins of HDL_p was greater than that caused by albumin.

Conclusions:

Serum lipoprotein proteins when added to rat liver homogenates caused depression of cholesterol synthesis by the homogenates.

Discussion

Methods

Cholesterol Determination.

Schonheimer and Sperry's method, as modified by Sperry and Webb (279), has been used as a standard method for the determination of cholesterol though it has been found to give values somewhat lower than other methods (278). When the concentration of cholesterol in the mixture containing cholesterol and digitonin was less tha 50 µg./ml., the precipitation of cholesterol by digitonin was not complete. Ferric chloride reagent (278) used to develop colour with cholesterol, also gave colour with digitonin. Digitonin is not soluble in acetone or ether, thus any co-precipitation of digitonin with the precipitates of cholesterol digitonide would interfere with the determinations of cholesterol by Ferric chloride reagent. Since digitonin is soluble in water and cholesterol digitonide is not, washing the cholesterol digitonide with distilled water was found to get rid of excess digitonin (Experiment, p. 74.

Labeling of Plasma Lipoproteins with C^{14}-Cholesterol.

From C^{14}-Cholesterol Adsorbed on Celite Powder (284):

It was claimed that C^{14}-cholesterol solubilized in the serum by this method, is associated with plasma lipoproteins in the physiological condition (284). Fate of labeled cholesterol in isolated

low density lipoproteins, after intravenous injection into rats was, however, different from the cholesterol incorporated into plasma lipoproteins *in vivo* (Experiment 12).

In vitro studies on exchange of cholesterol between lipoproteins isolated from sera labeled by this method, also suggested that all the radioactive cholesterol was probably not present in the same state as native cholesterol (Experiment 14). Though the process of isolation appears to alter the rates of exchange of cholesterol in plasma lipoproteins (Experiment 14), the results of *in vitro* studies on the lipoproteins suggest that at least part of the radioactive cholesterol dissociated from lipoproteins more readily than the non-radioactive physiological cholesterol.

However, the radioactive cholesterol remaining in association with plasma lipoproteins 30 minutes after its injection, appeared to be in the physiological state as judged from its turnover time and its esterification (Experiment 12).

C^{14}-Cholesterol Suspended in Tween 20 (285):

It was assumed that the labeled cholesterol in suspension exchanges with that in the molecules of lipoproteins, and it was also thought that a small amount of Tween present in the serum does not influence the fate of cholesterol (285). However, in studies on the exchange of cholesterol between plasma lipoproteins and red blood cells, the fractional rate of exchange of *in vivo* labeled

plasma lipoproteins was different from that of the plasma labeled *in vitro* by this method. Another report (293) demonstrated clearly that the presence of Tween markedly altered the uptake of C^{14}-cholesterol by the isolated perfused liver.

Labeling of Plasma Lipoproteins by a Process of Transfer from Cells:

Since presence of Tween in the labeled plasmas was noted to cause a change in the rate of transfer of cholesterol between plasma lipoproteins and red blood cells, and incubating labeled cholesterol with the serum at 37° C. failed to transfer all the label to serum lipoproteins in the physiological state, a method was devised which circumvented the necessity of having Tween 20 in the serum, and added the label(cholesterol)to lipoproteins in the physiological state.

Cholesterol Metabolism in Normal Rats.

In the plasma of normal rats, HDL_p carried more cholesterol than LDL_p (Experiment 12). This is in accord with the data in the literature (274). The ratios of unesterified, to total, cholesterol in the two lipoproteins differ. Of LDL_p, cholesterol, about 40% was found to be unesterified, whereas the latter made up only about 10% of the total cholesterol present in HDL_p.

The esterification of newly synthesized plasma cholesterol is a relatively slow process compared to the rate of synthesis of unesterified cholesterol. After the injection of a labeled precursor, it took only 2 hours for unesterified cholesterol to reach its peak of specific activity, whereas the esterified cholesterol took about 8 hours to reach its specific activity peak.

In normal rats, the unesterified cholesterol in the plasma HDL_p had a significantly greater fractional turnover rate than that in LDL_p. This suggests that the two lipoproteins cannot be regarded as one pool.

Unesterified cholesterol of the plasma freely mixes with that in the liver (177). In dogs half-time of equilibration between plasma and liver unesterified cholesterol is 20 minutes (177), whereas half-life of plasma cholesterol is 8-10 days (177). Thus it was suggested that for purposes of turnover the unesterified cholesterol in the plasma and liver can be considered as one pool (177). In rats,

about 40% of the radioactive cholesterol in the labeled serum was found in the liver 15 minutes after its injection (Experiment 16). Considering that about half of the radioactivity in the injected cholesterol was in the esterified fraction which has a much lower rate of equilibration with the liver than unesterified cholesterol (138), the actual uptake of unesterified cholesterol by the liver in that time would be much higher. The fractional turnover rate of plasma unesterified cholesterol, on the other hand, was only 1.2% per hour (Experiment 12), thus corroborating the evidence gained in dogs (177), that the liver and the plasma in rats also may be considered as one pool, for purposes of turnover of unesterified cholesterol.

It is the cholesterol in the microsomes of the liver cells that exchanges with that in the plasma (133), and such a rapid exchange probably does not involve intact lipoprotein molecules.

It has been assumed that all the liver unesterified cholesterol is part of the pool with which plasma is in rapid equilibrium (154). This was based upon the fact that after injection of labeled acetate into a dog, the specific activity of plasma cholesterol was found to be the same as that of the liver unesterified cholesterol, about 2 hours after injection. However, the cholesterol was not purified free of high specific activity contaminants in these studies, and it has been shown that soon after the injection of acetate,

precipitation of cholesterol with digitonin is not adequate, for its isolation in pure form(280).

In one experiment, isolated liver was perfused with blood containing C^{14}-cholesterol in Tween 20 suspension (293), and the specific activities of cholesterol in liver and blood were measured after various periods of equilibration. It was observed that within 2-3 hours the specific activity of liver cholesterol became 2-3 times greater than that of blood. However, if blood used for perfusing the liver was obtained from an animal 24 hours after the injection of C^{14}-cholesterol suspension in Tween 20, the liver and blood cholesterol unesterified cholesterol had similar specific activities after 2.5 hours, then the specific activity of liver cholesterol became much lower than that of the blood if perfusion was continued longer. The possibility of Tween influencing the liver uptake could not be ruled out.

The results of our studies on specific activities of liver and plasma lipoprotein cholesterol, purified by dibromination, clearly indicate that the liver has at least two pools, only one or both of which are in rapid equilibration with plasma and are the precursor of plasma cholesterol.

In normal rats, approximately 90% of the total cholesterol in the liver was unesterified (Table 23). The content of unesterified cholesterol varied from animal to animal. In three different

studies, the values obtained were 5.3, 6.8 and 9.1 mg/100 g. body weight. The total plasma unesterified cholesterol, on the other hand, is less than 0.5 mg./100 g. body weight.

For the same amount of protein, the LDL_p, normally carry more lipids than HDL_p, and the increase in floatation rates of LDL_p is, in fact, due to greater proportion of lipids (Table 48). Cholesterol, in association with normal plasma LDL_p, had a greater rate of transfer to red blood cells than that of normal HDL_p <u>in vitro</u> (Experiment 23), suggesting that the association between the cholesterol and the rest of the molecule may be weaker in LDL_p than in HDL_p molecules.

Proteins of the plasma lipoproteins have a great "avidity" for the lipids (276). When delipidized, HDL_p was allowed to equilibrate with plasma, the protein picked up lipids rapidly by a process which appeared non-enzymatic (276). It is conceivable that higher rates of transfer of cholesterol from LDL_p, than from HDL_p, to red blood cells, may be due to proportionally greater amounts of lipids associated with protein, thereby causing a relative decrease in the avidity of protein.

Hyperlipemia in Nephrosis

Since most of the experiments were related to the study of alterations in cholesterol metabolism in experimental nephrosis, the selection of nephrotic rats was based upon the degree of hypercholesterolemia. At the time of experiments, all the nephrotic rats had relatively severe nephrosis of duration of two weeks or longer.

The concentration of circulating lipids appears to be dependent upon three principal factors:

1. Changes in the degree of hydration of the plasma.
2. Changes in the rates of entry and exit of lipids into and from the vascular compartment.
3. The distribution of lipids between the vascular and extravascular compartment.

In so far as hypercholesterolemia of experimental nephrosis is concerned, in chronic stages, i.e., 2 weeks or more after the injection of AKS, the plasma and blood volumes were found to be normal. Thus, changes in the hydration of the vascular compartment do not account for any increase in the plasma concentrations of lipids in nephrosis.

Changes in the rates of entry and exit into and from the vascular compartment can increase the plasma concentrations of lipids only during the "unsteady states" of the disease. Once the steady state is established at any level of lipemia, any difference from normal in the rate of entry (and exit) will not explain the existing

concentrations.

The fractional rate of exchange of cholesterol between the vascular compartment and the liver in nephrotic animals, is at least equal to that observed in normal rats (Experiment 16). Since the actual amounts of cholesterol in plasma are markedly increased in nephrosis, the rate of entry and exit of cholesterol in mg. per hour is much greater than normal. Thus the hypothesis (262) of "trapping" of lipids in plasma (262) to explain hyperlipemia of nephrosis (if, by "trapping" is meant inability of lipid to leave the plasma) is not applicable to cholesterol. This also indicates that endothelium of hepatic vasculature offers no barrier in nephrosis, and that the liver and the plasma cholesterol mix as freely as in normal rats.

The exact sizes of the pools could not be determined from the data available from the present studies. The comparative estimates of the total pool in normal and nephrotic aminals were, however, made and the results indicated, that in nephrotic rats 3 weeks after the injection of AKS, the pool was about 1.5 times larger than in control animals.

It has been shown that soon after injection of AKS the hepatic content of cholesterol starts to rise and progressively increases. By 96 hours, the nephrotic liver had about 50% more cholesterol than normal (229). Nephrotic rats, 2 weeks after the injection of AKS, also had twice the normal amount of cholesterol

(Experiment 7) and by 3 weeks the nephrotic rats had about 30% more cholesterol than normal rats (Experiment 16). By six weeks after the injection of AKS, the difference in the hepatic content of cholesterol between normal and nephrotic aats had completely disappeared. The controversy in the literature (226-228) on the amounts of lipids in the livers of nephrotic rats is probably due to the difference in intervals of time after the injection of AKS.

The plasma concentration of cholesterol rises rapidly after the injection of AKS (246) and remains elevated even after the amount of liver cholesterol has returned to normal. The percent increase in plasma cholesterol is much greater than that in liver cholesterol at all times after the induction of nephrosis.

The increase in hepatic andplasma cholesterol during induction stages of the AKS disease may be due either to an increase in the rate of synthesis or to a decrease in the rate of degradation. Studies with labeled acetate (229, 246) clearly implicated the former mechanism.

The increase in synthesis persists until about 2 weeks after the induction of nephrosis (Experiment 7). By three weeks the rate of synthesis of cholesterol in nephrotic animals returns to normal (Experiment 12) and then becomes even less than normal (Experiment 8).

On the other hand, the plasma cholesterol concentaations as well as incorporation of recently synthesized cholesterol into

plasma lipoproteins remain elevated in nephrotic rats even when there is a very marked decrease in the rate of hepatic synthesis of cholesterol.

Therefore it may be concluded that hypercholesterolemia in the later stages of experimental nephrosis is not caused by an increase in the rate of hepatic synthesis of cholesterol.

The disproportionate increase in the liver and plasma cholesterol can occur only if, in addition to increased synthesis, some other factors influence the distribution of cholesterol. If there were no disproportion, the increase in plasma concentration (and the amounts in liver) would only be 1.5-2 times normal. That it is this redistribution of the pool which is predominantly responsible for the characteristic marked hypercholesterolemia in nephrosis, is shown conclusively (Experiment 10, 11, 12) by the fact that even though the cholesterol pool was only slightly increased, the intravascular fraction of the pool was much greater in nephrotic than in normal rats (Experiments 10, 11, 12).

In order to determine the possible factor(s) responsible for this redistribution, a comparison of nephrotic hyperlipemia with hyperlipemia due to other causes may help. Hyperlipemia in rats induced by feeding cholesterol, or by administering Triton WR-1339, has been extensively studied and will be compared with nephrotic hyperlipemia.

In rats fed a high cholesterol diet, the increase in concentration of cholesterol occurs mostly in LDL_p (297). In the

lipoproteins synthesized by the isolated liver from such animals, there is 3 times more cholesterol present with the same amount of protein as the normal (297). The increase in liver cholesterol in such rats is also about 3 times the normal (297) indicating that partitioning of the pool between the plasma and the liver is unlike that in nephrosis, and is more proportionate. In spite of marked increase in plasma cholesterol concentration in rats fed high cholesterol, there is no increase in the protein moiety of plasma lipoproteins (297). Rates of incorporation of amino acids into lipoproteins by perfused rat liver (298), or by rat liver slices (191) were normal.

It is known that Triton WR-1339, when administrated to dogs, combines with lipids and weakens the association of lipids and proteins (294). Lipid and protein moieties of normal dog lipoproteins move together on electrophoresis, but the lipids and proteins of dog plasma after the dog has been treated with Triton move in opposite directions (299). The increase in plasma lipids in animals treated with Triton is only in the LDL_p (299) as in rats fed high cholesterol diet, and it is not associated with any increase in the protein moieties of lipoproteins. Livers from rats which had been administered Triton demonstrated a marked increase in the synthesis of cholesterol (300), but showed no increase in the rate of synthesis of proteins of plasma lipoproteins (191).

Hepatic lymph is a product of diffusion of plasma (247).

In normal rats, the concentration of cholesterol in hepatic lymph is slightly lower than in plasma (247), indicating that the lipid diffuses out of the vascular compartment easily. When hyperlipemia is induced in a normal rat, either by injection of hyperlipemic serum from a cholesterol-fed donor rat or by the administration of Triton WR-1339, the cholesterol concentration in the hepatic lymph immediately goes up (247). In nephrotic rats, on the other hand, the concentration of cholesterol in hepatic lymph does not increase above normal, despite marked hyperlipemia (247). This suggestes that plasma lipids of nephrotic rats differ from those in other hyperlipemias in their ability to diffuse out of the vascular system.

Capillary endothelium in the liver has been shown to offer no barrier to the egress of cholesterol in nephrotic rats (Experiment 15). Thus, the lipids are probably held by the lipoprotein molecules more firmly in nephrosis than in Triton, or cholesterol-fed hyperlipemias. This difference in nephrotic lipoproteins, from those in other conditions, may be related to the nature of circulating lipoproteins. In contrast to other conditions, in nephrosis, there is increase in the rate of synthesis of protein moieties of all lipoproteins (102, 191) and in the concentration of protein (along with lipid) moieties of plasma lipoprotein (274) (Experiment 23).

When normal lipoproteins were added to the medium in which liver slices were incubated, a shift of lipids from tissue to medium

was noticed (301). That nephrotic plasma has a capacity to hold more lipids and cause this shift in the distribution of cholesterol in the liver-plasma pool, is also demonstrated by the observation that the increase in the amount of liver cholesterol in nephrotic rats fed a high cholesterol diet, is only one-fifth of the increase in plasma, whereas in normal rats on the same diet the relative increase in liver and plasm was similar, and the absolute increase of cholesterol in plasma was only one-tenth of that produced in nephrotic plasma (237). When AKS is injected into hyperthyroid rats (which have decreased plasma cholesterol concentration and an increased hepatic rate of cholesterol synthesis), the concentration of plasma cholesterol was observed to be even higher than in normal rats made nephrotic (260). In vitro studies on exchange of cholesterol between plasma lipoproteins and red blood cells, showed a marked decrease in the fractional rate of exchange from nephrotic plasma (Experiment 20). This was probably due to the decrease in the fractional rate of exchange from LDL_p of nephrotic animals (Experiment 24).

Thus, it is suggested, that nephrotic plasma offers not only more acceptor sites for lipids, but also a stronger bond between the protein and lipid moieties of lipoproteins, which results in the redistribution of liver-plasma pool of cholesterol.

The increase in the concentration of cholesterol within the vascular compartment is also not uniformly distributed between high and low density lipoproteins in nephrosis. In man the increase is

noted only in LDL_p (235). In rats, the increase is observed in all classes of lipoproteins, though the increase in LDL_p is more marked than that in HDL_p cholesterol (Experiment 7)

A delay in the clearance of radioactivity subsequent to intravenous injection of chylomicrons labeled with C^{14}-tripalmitate was observed in nephrotic rats (229), and also in animals treated with Triton (299). Rats fed high cholesterol and high fat diet had normal rate of clearance (Experiment 5), thus ruling out the hypothesis of isotope dilution as an explanation for the observed delay in nephrotic rats. In nephrotic animals the injection of rat serum albumin did not correct the delay (Experiment 1), thus suggesting that the delay in clearance of chylomicrons in nephrosis is not due to albumin deficiency.

The presence of an inhibitor to post-heparin lipoprotein lipase in nephrotic plasma was confirmed (Experiment 2), but it did not appear to have any role in the delay in the clearance of injected chylomicrons (Experiment 3).

A marked increase in the rate of incorporation of recently synthesized fatty acids into plasma lipoproteins, occurs in nephrosis (Experiment 7). A similar increase in the incorporation of fatty acids administered intravenously was also observed (229). The increased incorporation of fatty acids into plasma lipoproteins in nephrosis was the result of increased synthesis, and probably, the shift of the

pool into the vascular compartment similar to that observed for cholesterol. Thus the rate of reappearance into blood of radioactive triglycerides would be much higher in nephrotics than in normal rats, and the total radioactivity in blood of nephrotics would be progressively greater than normal with time. This may at least be partially responsible for the impression of delay in clearance of chylomicron lipid in nephrotic animals.

To test the hypothesis that lipoprotein proteins stimulate the synthesis of lipids in the liver, studies on liver homogenates were done. Human, as well as, rat lipoprotein proteins failed to show any stimulatory effect on the rate of synthesis of cholesterol by the homogenates. On the contrary, a definite depression, more marked with the proteins of HDL_p was noted. The depression was caused by amounts of proteins which amounted to less than 5% of the total proteins of the homogenates. However, bovine albumin also depressed the synthesis of cholesterol by the homogenates.

It has been shown that the synthesis of proteins and lipid do not necessarily go hand in hand, i.e., the rates of their synthesis are not equally increased or decreased in various conditions (297). Thus the depression of cholesterol synthesis by liver homogenates, on adding lipoprotein proteins, is ulikely to be due to a depression in the protein synthesis. Thus, the results suggest, that the proteins of lipoproteins do not stimulate the synthesis of cholesterol by the liver.

Summary

1. The delay in clearance of chylomicrons from the blood of nephrotic rats could not be attributed to hypoalbuminemia, lipoprotein lipase inhibitor or hyperlipemia.

2. The rate of hepatic synthesis of cholesterol in nephrotic rats was increased above normal 2 weeks after injection of anti-kidney serum, was normal one week later and was decreased below normal 6 weeks after the injection. The incorporation of recently synthesized cholesterol into plasma lipoproteins of nephrotic rats however, was greater than normal in all instances.

3. The fractional rate of transfer of cholesterol from plasma low density lipoproteins to red blood cells was smaller than normal in nephrosis.

4. The liver has at least two pools of unesterified cholesterol; one or both of which are in rapid equilibrium with the unesterified cholesterol in plasma. The rate of exchange of cholesterol between liver and plasma is increased in nephrotic rats.

5. In nephrotic rats the intravascular fraction of the pool in rapid equilibration with plasma, was larger than in normal animals. This may have been brought about by an alteration in the nature of plasma lipoproteins.

Bibliography

1. Baldwin, D.S., and McCluskey, R.T., Post. Grad. Med. 26: 603 (1959)
2. Bright, R., Reports of Medical Cases Selected with a view of Illustrating the Symptoms and Cure of Diseases by a Reference with Morbid Anatomy. London, Longman, Rees, Orme, Brown and Greene, 1827.
3. Muehrcke, R.C., Kark, R.M., Pirani, C.L., and Pollak, V.E., Medicine 36: 1 (1957)
4. Kimmelstel, D., and Wilson, C., Am. J. Path. 12: 83 (1936)
5. Farquhar, M.G., Hopper, J. Jr., and Moon, N.D., Am. J. Path. 35: 721 (1959)
6. Muehrcke, R.C., Pirani, C.L., Pouak, V.E., and Kark, R.M., Guy's Hosp. Rep. 104: 312 (1955)
7. Bauer, B.M., Bull. John Hopkins Hosp. 65: 196 (1939)
8. Thomas, E.W., and Schur, M., A.M.A. Arch. Int. Med. 78:679 (1946)
9. Keitel, H.G., Goodman, H.C., Havel, R.J., Gordon, R.S., and Baxter, J.H., J.A.M.A. 161: 520 (1956)
10. Pollak, V.E., Kark, R.M., Pirani, C.L., Shafter, H.E., and Muehrcke, R.C., Am. J. Med. 21: 496 (1956)
11. Derow, H.A., Schlesinger, M.J., and Savitz, H.A., A.M.A. Arch. Int. Med. 63: 620 (1939)
12. Jockes, A.M., Heptinstall, R.H., and Porter, K.A., Quart. J. Med. 27: 495 (1958)
13. Rytand, D.A., Stanford M. Bull. 13: 224 (1955)
14. Rytand, D.A., Am. J. Med. 5: 548 (1948)
15. Bloom, W.L., and Seegal, D., Ann. Int. Med. 25: 15 (1946)
16. Longcope, W.T., Bull. Johns Hopkins Hosp. 45: 335 (1929)
17. Ellis, A., Lancet. [illegible]: 1: [illegible], 72 (1942)
18. Blainey, J.D., Brewer, D.B., Hardwicke, J., and Scoothill, J.F., Quart. J. Med. (N.S.) 29: 235 (1959)

Bibliography

19. Masugi, M., Beitr. Path. Anat. u Allg. Path. 91: 82 (1933)
20. Frenk, S., Antonowicz, I., Craig, J.M., and Mettoff, J., Proc. Soc. Exper. Biol. and Med. 89: 424 (1955)
21. Spater, H.W., Proc. 9th Ann. Conf. on the Nephrotic Syndrome, October, 1957, New York, 1958. National Nephrosis Foundation, p. 220
22. Heymann, W., and Lund, H.Z., Pediatrics, 7: 691 (1951)
23. Ehrich, W.E., Forman, C.W., and Seifer, J., Am. A. Arch. Path. 54: 463 (1952)
24. Vernier, R.L., Padermaster, B.W., and Good, R.A., J. Exper. Med. 109: 115 (1959)
25. Farquhar, M., Vernier, R.L., and Good, R.A., J. Exper. Med. 106: 649 (1957) Am. J. Path. 33:791 (1957)
26. Farquhar, M., Vernier, R.L., and Good, R.A., J. Exper. Med. 106: 649 (1957) Am. J. Path. 33: 791 (1957)
27. Hartman, M.E., Hartman, J.D., and Baldridge, R.C., Proc. Soc. Exper. Biol. and Med. 100: 159 (1959)
28. Kesner, D.M., Bonamskiy, B.A., and Recant, L., Proc. Soc. Exper. Biol. and Med. 98: 766 (1958)
29. Stavitsky, A.B., Hacker, D.B., and Heymann, W., Proc. Soc. Exper. Biol. and Med. 85: 593 (1954)
30. Pressman, D., Hill, R.F., and Foote, F.W., Science, 109: 65 (1949)
31. Krakowek, C.A., Greenspon, S.A., A.M.A. Arch. Path. 66:364 (1958)
32. Mellors, R.C., Siegel, M., Pressman, D., Serle Invest. 4: 69 (1955)
33. Yagi, Y., and Pressman, D., J. Immunol. 81: 7 (1958)
34. Goodman, H.C., and Baxter, J.H., J. Exp. Med. 104:467 (1956)
35. Krakower, C.A., and Greenspon, S.A., Arch. Path. 52:629 (1951)
36. Hill, A.G.S., and Cruickshank, B., Brit. J. Exp. Path. 34:27 (1953)

Bibliography

37. Reed, L.L., Yamaguchi, F., Anderson, W.E., and Mendel, L.B., J. Biol. Chem., 87: 147 (1931)
38. Perrain, E.F., J. Physiol. et Path. gen. 16: 408 (1914-1915)
39. Deuel, Harry J., Interscience Publishers, New York (1955) The Lipids and their Chemistry and Biochemistry.
40. Wertheimer, E., and Shapiro, B., Physiol. Revs. 28:451 (1948)
41. Maximow, A.A., and Bloom, W., Histology 6th Ed. Saunders, Philadelphia, 1952
42. Hausberger, F.X., Z. Mikroskp Anat. Forsch. 36:231 (1934)
43. Faucett, D.W., J. Morphol. 90: 363 (1952)
44. Hirsh, J., Farquhar, J.W., Ahrens, E.H., Peterson, M.L., and Stoffel, W., Am. J. Clin. Nutrition 8: 499 (1960)
45. Benjamin, William, Gellhorn, Alfred, Wagner, Mary, Kunder, Harold, Am. J. Physiol. 201: 540 (1961)
46. Jeanrenaud, Bernard, Metabolism 10: 535 (1961)
47. Faucett, D.W., Endocrinology 42: 454 (1948)
48. Tverkischer, E., and Wertheimer, E., J. Physiol. 100:385 (1942)
49. Rose, E., and Shapiro, B., Biochem. et Biophys. Acta 18:504 (1955)
50. Cahill, G.F., Leboeuf, B., Renold, A.E., J. Biol. Chem. 214:483 (1955)
51. Feuer, D.D., J. Biol. Chem. 206: 171 (1954)
52. Hausberger, F.X., Milstein, S.W., and Rutman, R.J., J. Biol. Chem. 208:431 (1954)
53. Hausberger, F.X., and Milstein, S.W., J. Biol. Chem. 214:483 (1955)
54. Perry, F.W., Canad. J. Biochem. Physiol. 36:237 (1955)
55. Miller, J.P., and Cooper, J.A.D., Biochim. et Biophys. Acta 24:430 (1957)

Bibliography

56. Feller, D.D., and Feist, E., J. Biol. Chem. 228:275 (1957)
57. Shapiro, B., Chowers, I., and Rose, G., Biochim. et Biophys. Acta 23:115 (1957)
58. Shapiro, B., Weissmann, D., Benton, V., and Wertheimer, E., Metabolism 1:396 (1952)
59. Stern, I., and Shapiro, B., Metabolism 3:539 (1954)
60. Schoenheimer, R., and Rittenberg, D., J. Biol. Chem. 111:163 (1935)
61. Bernhard, K., and Steinhauser, H., Helvet. Chim. Acta. 27:12 (1943)
62. Stetten, D., Jr., and Boxer, G.E., J. Biol. Chem. 155:237 (1944)
63. Barret, H.M., Best, C.H., and Ridout, J.H., J. Physiol. 93:367 (1938)
64. Stetten, D., Jr., and Salcedo, J., J. Biol. Chem. 156:27 (1945)
65. Levy, A.C., and Raney, E.R., Proc. Soc. Exper. Biol. and Med. 104:125 (1960)
66. Beznak, A.B.L., and Hasche, Z., J. Exper. Physiol. 27:1 (1937)
67. Clement, G., Compt. Rend. Soc. de Biol. 141:255 (1947)
68. Schotz, M.C., and Page, I.H., Fed. Proc. 18:139 (1959)
69. White, S.E., and Engel, F.L., Proc. Soc. Exper. Biol. and Med. 99:375 (1958)
70. Schotz, M.C., and Page, I.H., Proc. Soc. Exper. Biol. and Med. 101: 624 (1959)
71. Goldfien, A., and Havel, R.J., J. Clin. Invest. 38:1007 (1959)
72. Rizack, M.A., (Thesis) Rockefeller Institute, New York, N.Y., (1960) as quoted by Jeanaud, 1961

Bibliography

73. Shapiro, B., and Wertheimer, E., Metabolism 5:79 (1956)
74. Cullumbine, H., Am. J. Physiol. 169:525 (1952)
75. MacKay, E. M., Am. J. Physiol. 120:361 (1937)
76. Schiffer, F., and Wertheimer, E., Endocrinology, 5:147 (1947)
77. Stoerk, H. C. and Porter, C. C.,
Proc. Soc. Exper. Biol. and Med. 74:65 (1958)
78. Best, C. N., and Cambell, J., J. Physio. 99:91 (1938)
79. Payne, R. W., Endocrinology, 45:305 (1949)
80. Levin, L., and Farber, R. K.,
Recent Progress in Hormone Research, 7:399 (1952)
81. Entenmann, C., Chaikoff, I.L., and Reichert, F. L.,
Endocrinology, 42:210 (1948)
82. Levin, L., and Farber, R. K.,
Proc. Soc. Exper. Biol. and Med. 74:758 (1950)
83. Weil, R., and Stetten, D., J. Biol. Chem. 168:129 (1943)
84. Rosenberg, J. W., Exper. Biol. and Med. 82:301 (1953)
85. Campbell, J. and Davidson, I. W. F.,
J. Biol. Chem. 189:35 (1951)
86. Ennor, A. H., Brit. J. Exper. Path. 30:395 (1949)
87. Weil, R., and Ross, S., Endocrinology 45:207 (1949)
88. Bondy, P. K., and Wilhelmi, A. E., J. Biol. Chem. 186:245 (1950)
89. Bennett, L., Kress, R. E., Li, C. H., and Evans, H.,
Am. J. Physio. 152:210 (1948)
90. Engel, F. L., Engel, M. G., and McPherson, W. T.,
Endocrinology, 61:713 (1957)
91. Engel, F. L., Yale J. Biol. and Med. 35:201 (1957)
92. Lynn, W. S., MacLeod, R. M., and Brown, R. H.,
J. Biol. Chem. 235:1904 (1960
93. Schotz, M. C., Masson, G. M. C., and Page, I. H.,
Proc. Soc. Exper. Biol. and Med. 101:159 (1959)

Bibliography

94. Rudman, D., and Seidman, F., Proc. Soc. Exper. Biol. and Med., 99:146 (1958)
95. Rudman, D., Seidman, G., and Reid, M.D., Proc. Soc. Exper. Biol. and Med. 103:315 (1960)
96. Seifter, J., and Baeder, D.H., Proc. Soc. Exper. Biol. Med., 95:318 (1957)
97. Seifter, J., and Baeder, D.H., Proc. Soc. Exper. Biol. Med., 95:489 (1957)
98. Seifter, J., and Baeder, D.H., Proc. Soc. Exper. Biol. Med., 95:747 (1957)
99. Bloch, K., and Kramer, W., J. Biol. Chem., 173:811 (1948)
100. Little, H.N., and Bloch, K., J. Biol. Chem. 183:33 (1950)
101. Perlman, I., Ruben, S., and Chaikoff, I.L., J. Biol. Chem., 122:169 (1937)
102. Marsh, J.B., and Drabkin, David L., Metabolism, 9:946 (1960)
103. Deuel, H.J., Jr., Hallman, L.F., and Murray, S., J. Biol. Chem., 119:257 (1937)
104. Spiro, M.J., and McKibbin, J.H., J. Biol Chem., 219:643 (1956)
105. Okey, Ruth., Shannon, Angela., Tinoco, Joan., Ostwald, Rosemarie, and Miljanick, Peter., J. Nutrition, 75:51 (1961)
106. Lang, A., Z. Physiol. Chem., 246:219 (1937)
107. Okey, R., Gillum, H.L., and Yokela, E., J. Biol. Chem., 107:207 (1934)
108. Brun, G.C., Acta Med. Scandinav. Supp. 99:1 (1939)
109. Reed, Claude F., Swisher, Scott N., Marinetti, Guido, V., and Eden, Eva G., J. Lab. Clin. Med. 56:281 (1960)
110. Erickson, B.N., Williams, H.H., Bernstein, S.S., Avrin, I., Jones, R.L., and Macy, I.G., J. Biol. Chem., 122:515 (1938)

Bibliography

111. Erickson, B.N., Williams, H.H., Hummer, H.C., and Macie, I.G., J. Biol. Chem. 118:15 (1937)
112. Irie, Reiko,, Iwanaga, Michiko,, and Yamakawa, Tamid., J. Biochem. (Tokyo) 50:122 (1961)
113. Burt, N.S., and Rossiter, R.J., Biochem. J. 46:569 (1950)
114. Abderhalden, E., Physiol. Chem., Wiley, New York (1908)
115. Boyd, E.M., J. Biol. Chem., 101:623 (1933)
116. Macheboeuf, M.A., Bull. Soc. Chim. Biol., 11:268 (1929)
117. Blix, G., Tiselius, A., and Svensson, H., J. Biol. Chem., 137:485 (1941)
118. Pedersen, K.O., J. Phys. Chem., 51:156 (1947)
119. Gofman, J.W., Glazier, F., Tamplin, A., Strisower, B., and DeLalla, O., Physiol. Revs., 34:589 (1954)
120. Dole, V.P., J. Clin. Investigation, 35:150 (1956)
121. Fredrickson, I.S., and Gordon, R.S., Jr., Physiol. Rev. 38:585 (1958)
122. Bragdon, J.H., J. Lab. Clin. Med., 52:564 (1958)
123. Edsall, J.T., Advances in Protein Chem., 3:383 (1947)
124. Committee on Lipid and Lipoprotein Nomenclature of the American Society for the Study of Atherosclerosis. Circulation Res., 4:129 (1956)
125. Eder, Howard A., Am. J. Med. 23:269 (1957)
126. Kunkel, H.G., and Trautman, R., J. Clin. Invest. 35:642 (1956)
127. Bragdon, J.H., Havel, R.J., and Boyle, E., J. Lab. and Clin. Med. 48:36 (1956)
128. Avigan, J., Redfield, R., and Steinberg, D., Biochem. et Biophys. Acta, 20:557 (1956)
129. Shore, B., University of California Radiation Laboratory, No. 3489 (1956)

130. Levine, L., Kauffman, D.L., and Brown, R.K., J. Exper. Med., 102:105 (1955)
131. Middleton, E., Jr., Am. J. Physiol., 185:309 (1956)
132. Oncley, J.L., Scatchard, G., and Brown, A., J. Phys. Chem., 51:184 (1947)
133. Gurd, Frank R.N., Lipide Chemistry, John Wiley and Sons, New York (1960)
134. Delsal, J.L., Bull. Soc. Chim. Biol., 31:122 (1949)
135. Macheboeuf, M., and Polonovski, J., Bull. Soc. Chim. Biol., 31:125 (1949)
136. Avigan, J., J. Biol. Chem. 226:957 (1957)
137. Hagerman, JoAnne S., and Gould, Gordon R., Proc. Soc. Exper. Biol. and Med., 78:329 (1951)
138. Fredrickson, D.S., McCollester, D.L., Havel, R.J., and Ono, K., Chemistry of Lipides as Related to Atherosclerosis, Thomas, Springfield (Ill.) 1958, p. 205
139. Lindgren, F.T., Nichols, A.V., and Freeman, N.K., J. Phys. Chem., 59:930 (1955)
140. James, A.T., Lovelock, J.C., and Webb, Joan P.W., Biochem. J., 73:106 (1959)
141. McFarlane, A.S., Discussions Faraday Soc., 6:74 (1949)
142. Oncley, J.L., Gurd, F.R.N., and Melin, M., J. Am. Chem. Soc. 12:458 (1950)
143. Shore, B., Arch. Biochem. 71:1 (1957)
144. Rodbell, M., Science, 127:701 (1958)
145. Rodbell, M., and Fredrickson, D.S., Fed. Proc. 17:298 (1958)
146. Pierce, Frank T., Metabolism, 3:141 (1954)
147. Graham, D.M., Lyon, T.P., Gofman, J.W., Jones, H.B., Yankley, A., Simonton, J., and White, S., Circulation, 4:666 (1951)

Bibliography

148. Pierce, F.T., and Bloom, B., Metabolism, 1:163 (1952)
149. Hewitt, J.E., Hayes, T.L., Gofman, J.W., Jones, H.B., and Pierce, F.T., Cardiology, 21:353 (1952)
150. Lovelock, J.E., James, A.T., and Rowe, C.E., Biochem. J. 74:137 (1960)
151. Fishler, M.C., Enterman, C., Montgomery, M.L., and Chaikoff, I.L., J. Biol. Chem. 150:47 (1943)
152. Zilversmit, D.B., Enterman, C., and Chaikoff, I.L., J. Biol. Chem., 176:209 (1948)
153. Rowe, C.E., Biochem. J. 76:471 (1960)
154. Gould, Gordon R., Cholesterol, Chemistry, Biochemistry and Pathology. Academic Press, New York (1950) p. 211
155. Bloch, K., Harvey Lectures Ser. 48:68 (1954)
156. Wuersch, J., Huang, R.L., and Bloch, K., J. Biol. Chem., 195:439 (1952)
157. Cornforth, J. W., Revs. Pure and Appl. Chem. (Australia), 4:275 (1955)
158. Cornforth, J.W., Hunter, G.D., and Popjak, G., Biochem. J. 54:590 (1953). 54:597 (1953)
159. Cornforth, J.W., Hunter, G.D., and Popjak, G., Biochem. J. 54:590 (1953). 54:597 (1953)
160. Cornforth, J.W., and Popjak, G., Biochem. J. 58:403 (1954)
161. Cornforth, J.W., Yovhitsky-Gore, I., and Popjak, G., Biochem. J. 64:388 (1956). ~~65:94 (1957a)~~
162. Cornforth, J.W., Yovhitsky-Gore, I., and Popjak, G., Biochem. J. ~~64:388 (1956).~~ 65:94 (1957a)
163. Popjak, G., Chemistry, Biochemistry and Isotopic Tracer Technique. Roy. Inst. Chem. (London) Lectures, Monographs, Repts, No. 2. 23 (1955)

Bibliography

164. Langdon, R.G., and Bloch, K., J.Biol.Chem. 200:129 (1953)
~~J.Biol.Chem. 200:135 (1953)~~
165. Langdon, R.G., and Bloch, K., ~~J.Biol.Chem. 200:129 (1953)~~
J.Biol.Chem. 200:135 (1953)
166. Tavormina, P.A., Gibbs, N.H., and Hoff, J.W.,
J.Am.Chem.Soc., 78:4498 (1956)
167. Gould, R.G., and Popjak, G., Biochem.J. 66:518 (1953)
168. Bucher, N.L.R., J.Am.Chem.Soc. 75:498 (1953)
169. Dituri, F., and Gurin, S., New England J.Med. 248:965 (1953)
170. Biggs, M., Friedman, M., and Byers, S.O.,
Proc.Soc.Exper.Biol. and Med. 78:641 (1951)
171. Chaikoff, I.L., Bloom, B., Siperstein, M.D., Kiyasu, J.Y.,
Reinhardt, W.O., Dauben, W.G., and Eastham, J.F.,
J.Biol.Chem. 194:407 (1952)
172. Friedman, M., Byers, S.O., and Gonning, B.,
Am.J.Physiol., 172:389 (1953)
173. Gould, R.G., Am.J.Med., 11:209 (1951)
174. Hollman, L., Rosenfeld, R.S., and Gallegher, T.F.,
J.Clin.Investigation 33:142 (1954)
175. Hellman, L., Rosenfeld, R.S., Eidinoff, M.L., Fukoshima,
D.K., and Gallegher, T.F., J.Clin.Investigation, 34:48 (1955)
176. Friedman, M., Byers, S.O., and St.George, S.,
Ann.Rev.Biochem., 25:613 (1956)
177. Eckles, N.E., Taylor, C.Bruce., Campbell, Donald J., and
Gould, R.Gordon., J.Lab. Clin.Med. 46:359 (1955)
178. Tennent, D.M., Zanetti, M.E., Atkinson, D.J., Kuron, G.W.,
and Opdyke, O.F., J.Biol.Chem. 228:241 (1957)
179. Friedman, M., Byers, S.O., and Michaelis, F.,
Am.J.Physiol. 164:789 (1951)

180. Gould, R.G., Campbell, D.J., Taylor, C.B., Kelly, F.B., Jr., Warner, I., and Davis, C.B., Jr., Fed. Proc. 10:191 (1951)
181. Bucher, N.L.R., and McGarrahan, K., J. Biol. Chem. 222:1 (1956)
182. Migikofsky, B.B., and Wood, J.D., Canad. J. Biochem. and Physiol., 33:858 (1955)
183. Migicovsky, B.B., Canad. J. Biochem. and Physiol. 30:339 (1960)
184. Sperry, W.M., J. Biol Chem. 111:467 (1935)
185. Swell, L., Trout, E.C., Jr., Field, H., Jr., and Treadwell, C.R., J. Biol. Chem. 230:631 (1958)
186. Swell, L., Law, M.D., Field, Henry, Jr., Treadwell, C.R., J. Biol. Chem., 235:1960 (1960)
187. Swell, L., Trout, E.C., Jr., Field, H., Jr., et al. Proc. Soc. Exper. Biol. 101:519 (1959)
188. Radding, C., and Steinberg, D., Circulation, 18:484 (1958)
189. Radding, C.M., Bragdon, J.H., and Steinberg, D., Biochim. Biophys. Acta., 30:443 (1958)
190. Marsh, Julian B., and Whereat, Arthur F., J. Biol. Chem., 234:3196 (1959)
191. Radding, C.M., and Steinberg, D., J. Clin. Invest. 39:1560 (1960)
192. Man, E.B., and Gildea, E.F., J. Biol. Chem. 99:61 (1932)
193. Havel, R.J., and Fredrickson, D.S., J. Clin. Invest., 35:1025 (1956)
194. French, J.E. and Morris, B., J. Physiol. 138:326 (1957)
195. French, J.E., Morris, B., and Robinson, D.S., (3rd Internat. Conf. on Biochemical Problems of Lipids, July, 1956). Brussels: p. 323 (1958)
196. Morris, B., Quart. J. Exper. Physiol., 43:65 (1958)
197. Bragdon, J.H., Arch. Biochem., 75:528 (1958)

Bibliography

198. Korn, E.D., J. Biol. Chem. 215:1 (1955), ~~215:15 (1955)~~
199. Korn, E.D., J. Biol. Chem. ~~215:1 (1955)~~, 215:15 (1955)
200. Korn, E.D., and Quigley, T. W., Biochem. et Biophys. Acta 18:143 (1955)
201. Anderson, N.G., and Fawcett, B., Proc. Soc. Exper. Biol. and Med., 74:768 (1950)
202. Hahn, P.F., Science, 98:19 (1943)
203. Weld, C.B., Canad. M.A.J., 51:578 (1944)
204. Anfinsen, C.B., Boyle, E., and Brown, R.K., Science, 115:583 (1952)
205. French, J.E., Robinson, D.S., and Florey, H.W., Quart. J. Exper. Physiol. 38:101 (1955)
206. Robinson, D.S., and French, J.E., Quart. J. Exper. Physiol. 38:233 (1953)
207. Anfinsen, C.B., A Symposium of Fat Metabolism. Johns Hopkins Press (1954) p. 93
208. Gordon, R.S., Jr., Boyle, E., Brown, R.K., Cherkes, A., and Anfinsen, C.B., Proc. Soc. Exper. Biol. and Med. 84:168 (1953)
209. Robinson, D.S., Jeffries, G.H., and Poole, J.C.F., Quart. J. Exper. Physiol., 40:297 (1955)
210. Jeffries, G.H., Quart. J. Exper. Physiol., 39:77 (1954) ~~Quart. J. Exper. Physiol. 39:261 (1954)~~
211. Jeffries, G.H., ~~Quart. J. Exper. Physiol., 39:77 (1954)~~ Quart. J. Exper. Physiol., 39:261 (1954)
212. Zilversmit, D.B., Chemistry of Lipids as Related to Athrosclerosis. Page 156.

213. Gould, R.G., "Proc. Ann. Meeting Council for High Blood Pressure Research." Page 3, Am. Heart Assoc. New York, (1952)
214. Bergstrom, S., Kgl. Fysiograf. Sall Skap. Lund. Forh 22:1 (1952)
215. Siperstein, M.D., and Chaikoff, I.L., J. Biol. Chem., 198:93 (1952)
216. Van Broggen, J.T., Hutchens, T.T., Claycomb, C.K., Cathey, W.J., and West, E.S., J. Biol. Chem., 196:389 (1952)
217. Friedman, M., and Byers, S.O., Am. J. Physio., 186:13 (1956)
218. Byers, S.O., and Friedman, M., Proc. Soc. Exper. Biol. Med., 92:459 (1956)
219. Epstein, P.A., J. Amer. Med. Ass., 69:444 (1917)
220. Peters, J.P., and Man, E.B., J. Clin. Invest., 22:721 (1943)
221. Barr, D.P., Ross, E.M., and Eder, H.A., Amer. J. Med., 111:480 (1951)
222. Kunkel, H.G., and Slater, R.J., J. Clin. Invest., 31:677 (1952)
223. Shafrir, E., J. Clinic. Invest., 37:1775 (1958)
224. Gofman, J.W., McGinley, Rubin L., and Jones, H.B., Am. J. Med., 17:514 (1954)
225. Baxter, James H., Goodman, Howard C., and Havel, Richard J., J. Clin. Invest., 39:445 (1960)
226. Heymann, W., and Hackel, D.B., Metabolism, 6:169 (1957)
227. Drabkin, D.L., and Marsh, J.B., J. Biol. Chem., 212:633 (1955)
228. Rosenman, R.H., Friedman, M., and Byers, S.O., J. Clin. Invest., 34:700 (1955)
229. Saffran, Judith., and Kalant, Norman., J. Clin. Invest., 38:1717 (1959)

230. Marsh, Julian B., and Drabkin, David L., J. Biol. Chem., 230:1063 (1958)
231. Lewis, L., Med. Clin. North America, 39:1015 (1955)
232. Schradl, W., Bohle, E., and Becker, G., Dent. Arch. Klin. Med. 202:415 (1955-56)
233. Smetana, H., and Jonson, F.R., Amer. J. Path. 18:1029 (1942)
234. Zimmer, James G., Dewey, Richard, and Waterhouse, Christine, Annal. Intern. Med. 54:205 (1961)
235. Rosenman, R.N., Friedman, M., and Byers, S.O., Circulation Res. 2:256 (1954)
236. Friedman, M., Rosenman, R.H., and Byers, S.O., J. Clin. Invest. 33:1103 (1954)
237. Dubach, V., Recant, L., Hatch, E., and Koch, M.B., Proc. Soc. Exp. Biol. and Med., 106:136 (1961)
238. Byers, S.O., Rosenman, R.N., and Friedman, M., Am. J. Physiol., 182:73 (1955)
239. Byers, S.O., Friedman, M., and Rosenman, R.N., Am. J. Physiol., 178:327 (1954)
240. Rosenman, R.H., Breall, William, Byers, S.O., and Rabin, David D., J. Clin. Invest., 38:1434 (1959)
241. Roseman, R.H., Friedman, M., and Byers, S.O., J. Clin. Invest., 32:121 (1953)
242. Hotta, S., and Chaikoff, I.L., Arch. Biochem. and Biophys. 56: 28 (1955)
243. Rosenman, R.H., Proc. of the Annual Conference on the Nephrotic Syndrome, New York, 38 (1956)
244. Heyman, Walter, and Hackell, Donald B., Proc. Soc. Exper. Biol. and Med., 89:329 (1955)

245. Heymann, Walter, Nash, Gerald, Gilkey, Caroline, and Lewis, Mildred, J. Clin. Invest., 37:208 (1958)
246. Marsh, Julian, and Drabkin, David L., J. of Biol. Chem. 230:1083 (1958)
247. Friedman, Myer., Rosenman, R.N., Byers, S.O., Am. J. Physiol. 190:180 (1957)
248. Heymann, Walter., Matthews, LeRoy W., Olynk, Paul., Salehar, M., and Gilkey, Caroline., Metabolism, 3:27 (1954)
249. Morris, B., and French, J.E., Quart. J. of Exper. Physiol., 44:190 (1959)
250. Rosenman, R.H., and Byers, S.O., Proc. Soc. Exper. Biol. and Med., 103:31 (1960)
251. Seifter, J., and Baeder, D.H., Proc. Soc. Exp. Biol. and Med., 86:709 (1954)
252. Marsh, Julian B., and Drabkin, David L., J. Biol. Chem., 230:1073 (1958)
253. Gitlin, D., Janeway, C.A., and Farr, L.E., J. Clin. Invest., 35:44 (1956)
254. Gordon, A.H., Biochem. J., 66:255 (1953)
255. Gitlin, D., and Cornwall, D., J. Clin. Invest., 35:706 (1956)
256. Metcoff, J., Rance, C.P., Kelsey, W.M., Nakasine, N., and Janeway, C.N., Pediatrics, 10:593 (1952)
257. Heymann, Walter., Hacker, Donald B., Gilkey, Caroline, and Salehar, Milena., Lab. Invest. 2:423 (1953)
258. Bally, Peter., and Numa, Shosaku., J. Clin. Invest. 36:872 (1957)
259. Soshea, J.W., and Farnsworth, E.B., J. Lab. and Clin. Med. 38:414 (1951)
260. Rosenman, R.H., and Smith, M.K., Proc. Soc. Exp. Biol., 98:444 (1958)

261. Kalant, N., McIntyre, W.C., and Wilansky, D.L., Endocrinology, 64:333 (1959)
262. Rosenman, R.H., Friedman, M., and Byers, S.O., J.Clin.Invest., 35:522 (1956)
263. Thomas, E.M., Rosenblum, A.H., Langder, H.B., and Fisher, R., Am.J.Dis.Child., 81:207 (1951)
264. Luetscher, J.A., Jr., Piel, C.F., and Curtis, R.N., J.Chronic Dis., 1:442 (1955)
265. Gurin, S., Fat Metabolism Report of the 11th M. and R. Ped. Conf., Columbus, Ohio. M. and R. Lab., 1954, p.42
266. Sellers, A.L., Hubbard, R., Marmoston, J., Proc.Soc.Exper.Biol.Med., 95:67 (1957)
267. Koldl, H., and Peer, E., Klin.Med., 9:527 (1954)
268. Zeldis, L.J., Alling, E.L., McCoord, A.B., and Kulka, J.P., J.Exper.Med., 82:157 (1945)
269. Heymann, W., and Clark, E.C., Am.J.Dis.Child., 70:74 (1945)
270. Rosenman, R.H., and Friedman, M., J.Clin.Invest., 36:700 (1957)
271. Bollman, J.L., Cain, J.C., and Grindlay, J.H., J.Lab. and Clin.Med., 33:1349 (1948)
272. McDonald, Hugh J., Paper Electrophoresis, A Ciba Foundation Symposium. J. and A. Churchill Ltd., London (1956) p.183
273. DeLalla, O.F., and Golfman, J.W., Methods of Biochemical Analysis, Vol.1, Interscience Publishers Inc., New York, 1954
274. Havel, R.J., Eder, H.A., and Bragdon, J.H., J.Clin.Invest., 34:1345 (1955)
275. Florsheim, W.H., and Gonzales, C., Proc.Soc.Exp.Biol.Med., 104:618 (1960)

Bibliography

276. Scanu, A., Lewis, L.A., and Bumpus, F.M., Arch. Biochem. and Biophysics, 74:390 (1958)
277. Folch, J., Ascoli, I., Lees, M., Meath, J.A., and Lebaron, F.N., J. Biol. Chem., 191:833 (1951)
278. Zak, B., Am. J. Clin. Path., 24:1307 (1954)
279. Sperry, W.M., and Webb, M., J. Biol. Chem., 187:97 (1950)
280. Schwenk, Erwin., and Werthessen, Nicholas T., Arch. Biochem. Biophys., 40:334 (1952)
281. Windaus, A., Ber. Dent. Chem. Ges., 42:238 (1909)
282. Hirsch, J., and Ahrens, E.H., J. Biol. Chem., 233:311 (1958)
283. Gornall, A.G., Bardanill, C.J., and David, M.M., J. Biol. Chem., 177:760 (1949)
284. Avigan, Joel., J. Biol. Chem., 234:787 (1959)
285. Whereat, Arthur F., Staple, Ezra., Arch. Biochem. and Biophysics, 9:224 (1960)
286. Hawk, P.B., Oser, B.L., Summerson, W.H., Practical Physiological Chemistry. McGraw Hill (N.Y.) 1954. p. 493
287. Hayes, F.N., Packard Technical Bulletin, page 4, November 1960.
288. Zilversmit, D.B., Am. J. Med. 29:832 (1960
289. Hotta, S., and Chaikoff, I.L., Arch. Biochem. Biophys. 56:28 (1955)
290. Wong, K.L., Rose, Van Bruggen, J.T., J. Biol. Chem. 235:30 (1960)
291. Bennett, C., and Franklin, N., Statistical Analysis in Chemistry and Chemical Industry. John Wiley Incorp. (N.Y.)
292. Scanu, A., and Hughes, W.L., J. Biol. Chem., 235:2876 (1960)

293. Danielsson, H., Insull, W., Jordan, P., and Strand, O. Am. J. Physiol., 202:699 (1962)
294. Scanu, A., and Oriente, P., J. Exp. Med., 113:735 (1961)
295. Solomon, A.K., Advances in Biological and Medical Physics Vol. 3, Academic Press, New York
296. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem. 123:265 (1951)
297. Marsh, J.B., and Sherry, F., Proc. Soc. Exper. Biol. and Med., 109:14 (1962)
298. Haft, D.E., White, A., Roheim, P.S., Walcott, G., Maresca, L., and Eder, H.A., Fed. Proc., 18:240 (1959)
299. Scanu, A., and Page, I.H., J. Clin. Invest., 41:495 (1962)
300. Frantz, I.D. Jr., and Hinkelman, B.T., J. Exp. Med., 101:225 (1955)
301. Hamosh, Margit., and Shapiro, B., Am. J. Physiol. 201:1030 (1961)
302. Waelsh, H., Sperry, Wm., J. Biol. Chem. 132:788 (1940)
303. Bloch, K., and Rittenberg, D., J. Biol. Chem. 145:625 (1942)
304. Bloch, K., and Rittenberg, D., J. Biol. Chem. 178:971 (1949)

Contribution to Knowledge by the Author

1. A new *in vitro* method is suggested for labeling serum lipoproteins with radioactive cholesterol which does not have the drawbacks of methods previously published.

2. It is observed that in nephrotic rats there is increase in all classes of plasma lipoproteins and the increase is seen in lipid as well as protein moities of lipoproteins.

3. The delay in the clearance of chylomicrons from the blood of nephrotic rats is not attributed to hypoalbiminemia, lipoprotein lipase inhibitor or hyperlipemia.

4. It is indicated that total unesterified cholesterol in the liver is present in at least two pools, one or both of which are in equilibrium with plasma cholesterol. No difference in the fractional rates of exchange of cholesterol between liver and plasma were noted in normal and nephrotic rats.

5. It is shown that hepatic content and rate of synthesis of unesterified cholesterol in nephrotic rats are related to the length of interval after induction of nephrosis. The rate of synthesis is above normal 2 weeks after the injection of antikidney serum, normal one week later and below normal 6 weeks after the injection. The amounts of cholesterol is twice normal 2 weeks after injection, only slightly above normal 3 weeks after and normal 6

weeks after the injection of antikidney serum.

6. From these facts it is concluded that in the late stages of experimental nephrosis, hypercholesterolemia is the result of redistribution of cholesterol between the hepatic and plasma components of the cholesterol pool.

7. The fractional rate of transfer of cholesterol to red blood cells was smaller from low density lipoproteins of nephrotic animals than of normal rats. In addition there is an increase in the capacity of plasma to transport cholesterol due to increases in the plasma concentration of lipoprotein protein and in the cholesterol: protein ratio in the lipoproteins (especially in the case of the low density components). It is tentatively suggested that these changes are manifestations of an abnormality in the lipoproteins which may in turn be responsible for the altered plasma-liver distribution of cholesterol.