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Serum Lipoprotein Biosynthesis and Orotic Acid

Theodore S. Musiala

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SERUM LIPOPROTEIN BIOSYNTHESIS
AND OROTIC ACID

by

THEODORE S. MUSIALA

A THESIS SUBMITTED TO THE FACULTY OF THE
GRADUATE SCHOOL OF LOYOLA UNIVERSITY IN
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1967
Theodore S. Musiala was born in Chicago, Illinois, on February 14, 1943.

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He is co-author of a publication entitled: "The Effects of Phospholipids on Serum Lipoprotein Biosynthesis", Federation Proceedings, 26, 849 (1967).
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CHAPTER I
INTRODUCTION AND REVIEW OF SERUM LIPOPROTEIN BIOSYNTHESIS

That serum lipoprotein biosynthesis takes place in the liver was demonstrated for low-density (d. < 1.063) lipoproteins in rat liver slices by Marsh (113) and in perfused rat liver by Marsh and Whereat (114) using the immunochronal technique of Peter and Anfinsen (140). Incorporation of carbon-14-labeled amino acids into low and high density serum lipoproteins was shown by Radding et al. (146) in rat liver slices. A chemical identity between the newly synthesized high-density lipoproteins and those circulating in rat plasma was established by Radding and Steinberg (147) by peptide mapping according to the procedure of Ingram (83). In the studies of Radding et al. (146) and Haft et al. (64), the incorporation of the amino acids was greater in the low-density (d.<1.063) than in the high density (den.1.063-1.210) lipoprotein fraction. Studies by Marsh (115) on a cell-free system from rat liver have shown that low and high density lipoproteins are synthesized in microsomes, although the possibility of mitochondrial participation was not ruled out.

The addition of excess carrier lipoprotein during the isolation of the small amount of labeled lipoprotein formed by microsomes in vitro may mask the lipid requirement for lipoprotein biosynthesis. Scanu and Hughes (168) found that the protein moiety of human high-density lipoproteins readily exchanged its lipid moiety. On this basis, when an ex-
cess of intact lipoprotein is added to a small amount of its labeled protein moiety, the lipid required for the conversion of the labeled protein moiety to lipoprotein is largely supplied by the excess of lipoprotein carrier. Under these circumstances, any additional lipid synthesized at the same time as the protein moiety has a negligible effect on the observed incorporation of amino acids into lipoprotein. Thus, Marsh (415) reported that rat liver microsomes were just as active in lipoprotein biosynthesis as the whole homogenate from which they were derived. However, under conditions where the lipid moiety as well as the protein moiety must be synthesized, mitochondria may contribute both lipid (Comatzer and Watser, (34)) and the adenosine triphosphate needed by the microsomal lipid synthesizing enzymes (Hokin and Hokin, (78)). It is apparent that any interference in the synthesis of the lipid moiety of lipoproteins will result in the failure to recover their labeled peptide moieties in the lipoprotein fractions.

According to known mechanisms of protein synthesis, the formation of lipoproteins can be viewed as a multi-step process. After translation of the peptide chains of each lipoprotein species on membrane-bound polyribosomes, chain release, accompanied by the development of secondary and tertiary structure, occurs. Next, the linkage of covalently bound carbohydrates takes place. The function of these carbohydrates and their contribution to lipoprotein structure has not been assessed,
however, by analogy to other serum glycoproteins and to some hormones, the function may be to protect the molecule against proteases. In the final step of synthesis characteristic amounts of phospholipid and neutral lipid complete the structure.

Amino acid analysis of human beta-lipoprotein (Margolis and Langson, 109) has shown it to be no richer in hydrophobic amino acids than lipid-free proteins. This finding suggests that it is the juxtaposition of the hydrophobic side chains of the molecule that confers the affinity for lipids. Attempts to remove the bound lipid in the absence of detergent stabilizers have resulted in the formation of insoluble inter-molecular aggregates (Banaszak and McDonald, 14).

Although the fatty acid composition of serum lipids varies with diet (Olson and Vester, 134; Best et al 18), the distribution of fatty acids between neutral and phospho-lipids is remarkably constant. This constancy has led to the postulate that phospholipids form an integral part of lipoproteins (Fredrickson and Gordon, 55). In choline-deficiency, failure to form lecithin and sphingomyelin in required amounts results in marked depression of serum lipoproteins and the accumulation of fat in the liver (Cornatzer and Watser, 34; Wilgram et al 206; Rosenfield and Lang, 156). Since the triglycerides which form the bulk of the accumulated lipid are unable to substitute for these phospho-lipids, it follows that the charged structure of phospholipids contributes
strongly to lipoprotein stability. It is not surprising, then, that phosphatidyl choline is the major phospholipid in each lipoprotein class (Philips, (144)).

A diagram showing known pathways for the synthesis of choline-containing and other phospholipids is shown below. Lecithin has more than one immediate precursor. The most active path begins with the intact choline molecule. In the alternate, de novo pathway phosphatidyl N, N-dimethylethanolamine is the immediate precursor of phosphatidyl choline. Artrom (11) has evaluated the relative contributions of each pathway. He has found that four to eight times as much lecithin is synthesized through cytidine intermediates as through methylation with S-adenosyl methionine, even though the methylation pathway is six to twelve times as active in liver as in other tissues.

Wittenberg and Kornberg (211) have shown that phosphorylcholine can be formed in the liver at the expense of adenosine triphosphate by the enzyme choline phosphokinase. In the next reaction catalyzed by cytidine diphosphate choline pyrophosphorylase, cytidine diphosphate choline is formed. Although the reaction is freely reversible as written, the ubiquitous presence of pyrophosphatase favors the synthetic forward reaction. The following reaction utilizes the cytidine diphosphate choline intermediate and also a D-1,2-diglyceride unsaturated in the beta fatty acid moiety (Kennedy, (93)). The particular enzyme which catalyzes
the above reaction, cytidine diphosphate choline: 1,2-diglyceride cholinephosphotransferase (2,7,8,2), has been shown by Wilgram et al. (207) to be markedly reduced in carbon tetrachloride poisoning. The enzyme is also strongly inhibited by calcium ions (Weiss et al. (201)). In order to demonstrate stimulation of the reaction by added diglyceride, Kennedy and Weiss found it necessary to emulsify the lipid with Tween-20, a non-ionic detergent.

The phosphatidic acid phosphatase which furnishes the diglyceride for lecithin synthesis has been shown to be inhibited by magnesium ions (Rossiter and Strickland, (157a)). The behavior of this enzyme is opposite to that of cytidine triphosphate: cholinephosphate cytidyl-transferase (2,7,7,15) which requires magnesium for optimal activity. Since only small amounts of phosphatidic acid are present in tissues (Dawson, (38); Marinetti and Stotz, (112); Marinetti et al. (110)), it would appear to be rate limiting. However, Cornatzer and Watser (34) have found that phosphatidic acid is the only phospholipid that accumulates in the fatty liver resulting from choline deficiency.

In addition to the pathway in which choline participates, an alternate, de novo pathway exists for the formation of phosphatidyl choline. Because of this pathway, rats will not develop fatty livers on a choline-deficient diet when it contains adequate methionine. The methyl groups of methionine do not appear as limiting as the homocystine which is re-
BIOSYNTHESIS OF LIVER LIPIDS

L-ALPHA-PHOSPHATIDIC ACID

CDP-DIGLYCERIDE

CTP

CDP-DIGLYCERIDE

INOSITOL

PHOSPHATIDYL INOSITOL

PHOSPHATIDYL SERINE

PHOSPHATIDYL ETHANOLAMINE

INOSITOL

ACYL CO-A

SERINE

ETHANOLAMINE

ATP

PHOSPHORYL ETHANOLAMINE

CTP

CDP-ETHANOLAMINE

THFA

VITAMIN B12

N-METHYL ETHANOLAMINE

ATP

PHOSPHORYL N-METHYL ETHANOLAMINE

CTP

CDP-N-METHYL ETHANOLAMINE

N,N-DIMETHYL ETHANOLAMINE

ATP

PHOSPHORYL N,N-DIMETHYL ETHANOLAMINE

CTP

CDP-N,N-DIMETHYL ETHANOLAMINE

GLYCINE

CHOLINE

ATP

PHOSPHORYL CHOLINE

CTP

CDP-CHOLINE

SARCOSINE/BETAINE

DIMETHYL GLYCINE

HOMOCYSTINE

CERAMIDE

SPHINGOMYELIN

METHIONINE

S-ADENOSYL METHIONINE
methylated by methyl-vitamin B₁₂ with methyl groups derived directly from 5-methyl tetrahydrofolic acid which in turn are derived from the one-carbon pool. Furthermore, betaine is capable of remethylating homocystine directly. Finally, the formation of 5-methyl tetrahydrofolic acid from methionine complicates the question of the origin of the methyl groups of lecithin. Clearly, more attention must be given to precursor-product relationships as defined by Zilversmit et al (214). Still, support for the limiting role of homocystine has been obtained by du Vigneaud et al (42), who found that rats failed to develop fatty liver on a low protein, choline-deficient diet supplemented with sarcosine and homocystine. In view of this finding, surprisingly little attention has been given to the sparing effect of cysteine in the conservation of homocystine when fatty livers have been produced by feeding proteins low in methionine content.

Early evidence for the conversion of ethanolamine into lecithin was derived from the feeding experiments carried out by Stetten (188). He found that more than half of all choline in phosphatides arises from dietary ethanolamine. More importantly, he also found (Stetten, 189) that the conversion of ethanolamine to choline phosphatides was not significantly reduced by diets poor in labile methyl groups. His results are consistent with the origin of the methyl carbons of choline from serine via 5-methyl tetrahydrofolic acid. The fact that the synthesis of choline phosphatides by this pathway was not affected by an absence of
dietary methyl groups sufficient to cause fatty liver indicates that the pathway does not possess an absolute requirement for free S-adenosyl-methionine.

The finding of Weissback et al. (2014) that S-adenosyl-methionine is required for the transfer of the methyl group of methyl-vitamin B₄₂ to homocysteine but that the methyl group of S-adenosyl-methionine is itself not transferred, indicates that enzyme-bound S-adenosyl-homocysteine may be important in the overall reaction which may be written as:

\[
\begin{align*}
5-\text{CH}_3 \text{THFA} + \text{Enz-S-adenosyl-homocystine} & \rightarrow \text{THFA} + \text{Enz-S-adenosyl methionine} \\
\text{Enz-S-adenosyl-methionine} + \text{homocysteine} & \rightarrow \text{Enz-S-adenosyl-homocystine} + \text{methionine} \\
\text{Enz-S-adenosyl-methionine} + \text{ethanolamine} & \rightarrow \text{Enz-S-adenosyl homocysteine} + \text{N-methylethanolamine}
\end{align*}
\]

When adequate homocysteine is present in the diet, this intermediate reacts with it as shown below yielding methionine:

When homocysteine is absent, however, the enzyme may react with ethanolamine or its monomethyl or dimethyl derivatives according to the equation:

\[
\begin{align*}
\text{Enz-S-adenosyl-methionine} + \text{ethanolamine} & \rightarrow \text{Enz-S-adenosyl homocysteine} + \text{N-methylethanolamine}
\end{align*}
\]
The failure of the methyl group of free S-adenosyl-methionine to be transferred to ethanolamine in many systems may simply be due to the exclusion of 5-methyl tetrahydrofolic acid and vitamin B₁₂. Although Stekol et al (186) working with folic-acid-deficient rats, found that only N,N-dimethylaminoethanol improved the utilization of methionine for phosphatidyl choline synthesis, this does not mean that N,N-dimethylaminoethanol cannot be converted to choline by the above mechanism. It only signifies that the enzyme methylating phosphatides and utilizing S-adenosyl-methionine is relatively specific for phosphatidyl-N,N-dimethylaminoethanol. This result has been confirmed by Bremer and Greenberg (23). Therefore, the results previously obtained by Stetten do not conflict with those of Stekol. Furthermore, experiments by Ansell and Chojnacki (6) and Artrom (10) showing the conversion of N,N-dimethylaminoethanol into phosphatidyl N,N-dimethylaminoethanol via cytidine intermediates supports the basic importance of these cytidine intermediates for lecithin synthesis even in the pathway utilizing S-adenosyl methionine.

Finally, exchange pathways by which choline may exchange with phosphatidyl serine forming phosphatidyl choline in enzyme systems activated by calcium ions such as that elucidated by Hubscher (81) do not detract from the basic role of cytidine nucleotides. The only known pathway for the formation of phosphatidyl serine is via cytidine diphosphate diglyceride which in turn is synthesized from cytidine-5'-triphosphate and
phosphatidic acid.

In summary, measurable incorporation of a suspected radioactive precursor into the desired product does not furnish evidence of a significant metabolic pathway. Indeed, tracer techniques have been used to measure the reversal of the hexokinase reaction for which the thermodynamic equilibrium constant is $6.5 \times 10^3$. One would hardly conclude that the measured reversal is important in gluconeogenesis. Although all the methyl groups of lecithin can be donated by S-adenosyl methionine (Wilson et al. (209)), the introduction of the first methyl group into phosphatidyl ethanolamine has been shown to be rate-limiting (Bremer et al. (24)). Because of this limitation, it is unlikely that any significant percentage of phosphatidyl choline is synthesized in which all three methyl groups are derived directly from S-adenosyl methionine.

Whatever functions N-methylphosphatidyl ethanolamine and N,N-dimethylphosphatidyl ethanolamine have apart from the synthesis of phosphatidyl choline has not been thoroughly investigated. However, Jacobi and Bauman (84) have suggested that these compounds may be effective in preventing renal lesions in rats on a diet devoid of labile methyl groups. The full significance of these compounds which account for approximately one-third (Artrom, (11)) of the total methylated phospholipids is yet to be determined.

The above discussion has shown that cytidine triphosphate is essen-
tial in all known significant pathways for the synthesis of choline-containing phospholipids. Any compound, then, that interferes with either the formation or utilization of cytidine triphosphate would be expected to cause the development of fatty liver.

The Relationship of Orotic Acid-Fatty Liver to the Problem of Serum Lipoprotein Biosynthesis

The applicability to man of results obtained in any study of lipoprotein biosynthesis in rats depends upon the species similarities in their lipoprotein patterns. In an early comparative ultracentrifugal study of lipoproteins, Lewis et al (100) found that the rat had only two major lipoprotein classes compared to the four classes found in human sera. In addition, the study showed that rat serum had a larger proportion of high density, phospholipid-rich lipoproteins than was found in human sera. This difference in the number of components may reflect differences in the stability of rat and human lipoproteins during isolation. In particular, the two classes of lipoproteins which were to be found missing in the rat are those which are now believed to be subunits of their respective low and high density counterparts by analogy with the human system.

Electrophoretic techniques, especially disk electrophoresis in polyacrylamide gel, have helped to characterize the lipoproteins of rat sera. Utilizing the technique for prestaining lipoproteins with Sudan
Black B originally developed by McDonald and Bermes (117), Narayan et al (124, 125) have demonstrated the presence of about nine to twelve components in ultracentrifugally isolated rat serum lipoproteins. This distribution of lipoprotein species is similar to that determined for human sera by Ewing et al (49) using computer methods. Thus, the lipoproteins of the rat are essentially similar to those of man with the exception that the relative amounts of the individual lipoprotein species present may be different.

Some of the discrepancies reported in lipoprotein composition are undoubtedly due to chemical alterations. Thus, Ray et al (150) reported that when salt solutions used for the ultracentrifugal preparation are made up with laboratory distilled water, approximately 0.05 p.p.m. of copper ion is present to catalyze the peroxidation of the unsaturated fatty acids present in lipids. To overcome this difficulty, it has become customary to prepare solutions with the addition of 0.1 gram per liter of the complexing agent disodium ethylenediaminetetraacetic acid. Even with this precaution, however, other changes may occur. Glomset et al (58) reported the presence of a fatty acid transferase in serum which catalyzed the transesterification of a fatty acid from lecithin to cholesterol. That the enzyme activity involved may be associated with high density lipoproteins is indicated by the work of Lossow et al (104). A free sulfhydryl group is necessary for transferase activity and mercurial inhibitors,
such as p-chloromercurialbenzoate have proved effective in preserving serum. In obtaining serum from rats fed ad libitum there is the danger of contamination by lipoprotein lipase (Engelberg, (45)). However, both sodium fluoride and diethyl p-nitrophenylphosphate (Zemplenayi and Grafnetter, (213)), as well as concentrated sodium chloride solutions, such as used in floatation, inhibit the enzyme (Korn, (96)). There is less danger of degradation of isolated lipoproteins by the lipase since it requires the presence of serum albumin as lipid acceptor.

Although there are many substances which will cause disturbances in lipid metabolism, most are not normal metabolites. Examples of such compounds include carbon tetrachloride (Smuckler and Benditt, (180)), ethionine (Harris and Robinson, (70)), chloropromazine (Ansell and Marshall, (7)), aureomycin (Bateman et al (15)), dimethylchlorotetracycline (Searcy et al (174)), and phenobarbital (Holtzman and Sillette, (80)). In contrast, orotic acid (6-carboxy-uracil) is an intermediate in pyrimidine metabolism and is present in milk. Furthermore, while most of the aforementioned compounds cause irreversible liver damage, the effects of orotic acid can be prevented or reversed if adenine is included in the diet (Jatlow, (84a)). The relative amounts of orotic acid and adenine needed to produce these effects are, respectively, 1.00 and 0.25 per cent of total diet.

The reversal or prevention of orotic acid fatty liver by adenine is
due to its effect on the synthesis of two key enzymes necessary for orotic acid metabolism. According to Krooth (97) the structural information for both orotidylic acid decarboxylase and orotidylic pyrophosphorylase is encoded in a single operon. The mutant gene causing orotic aciduria is at the corresponding regulator locus. The mutant regulator gene produces a primary chemical change in the repressor molecule, so that the repressor now has an increased avidity for both inducer and operator gene. Relative to normal, the avidity of the repressor molecule for the inducer is the most affected. An inducer for the operon is biosynthesized by all cultured (diploid) human cells and tends to leak out of the cells into the medium. The intracellular concentration of inducer in cells of all genotypes is diminished when the culture is grown in the presence of adenosine.

These results confirm the finding that no build-up of orotidylic acid is found in orotic aciduria due to the absence of orotidylic acid pyrophosphorylase. In addition, there are no signs of fatty liver in this disease. These results suggest that orotidylic acid itself may have a prime role in the development of the fatty liver.

A biochemical analysis of the changes which accompany orotic acid-fatty liver has been made by Creasey et al (36). In addition to an accumulation of triglycerides and uridine nucleotides, decreases in the incorporation of inorganic phosphate into liver phospholipids and in hepatic
adenosine-5′-triphosphate levels were observed. A specific depression of amino acid incorporation into serum lipoproteins with no decrease in the formation of other liver proteins was also reported. These investigators found that the administration of 6-azauridine in conjunction with the orotic acid diet had no effect on the development of orotic acid-fatty liver. Previous work by Handschumacher and Pasternak (69) had indicated that this compound was converted to 6-azauridylic acid in vivo and that this latter compound was a potent competitive inhibitor of orotidylic acid decarboxylase. The ineffectiveness of 6-azauridine in preventing orotic acid-fatty liver proved that the elevated levels of uridine nucleotides were not responsible for the fatty liver. The investigators surmized that the specific depression of amino acid uptake into serum lipoproteins was due to the shortage of adenosine-5′-triphosphate which is found in orotic acid-fatty liver. They postulated a competition between orotic acid and adenosine for a limited supply of 5-phosphoribosyl-1-pyrophosphate. When orotidylic acid was formed, adenylic acid synthesis suffered. Their hypothesis is consistent with the reversal of orotic acid-fatty liver by adenine in that, when no excess of orotidylic acid is formed, no fatty liver is produced. However, the specific effect of low adenosine-5′-triphosphate levels on amino acid incorporation into lipoproteins has been sharply criticized (Roheim et al (155)). In ethionine fatty liver, for example, much larger decreases in adenosine-5′-triphosphate occur, and the doses of
ethionine which will lower the adenine nucleotide level to the degree to which one finds it in orotic acid fatty liver will do very little in the way of inhibiting protein synthesis or inducing a fatty liver. Furthermore, besides the general decrease in protein synthesis observed in ethionine fatty liver due to the formation of abnormal ethylated transfer ribonucleic acid (Natori, (126)), there is extensive trapping of adenosine-5'-triphosphate as S-adenosyl-ethionine which cannot be utilized by all methyl transferases. For example, the ethyl group cannot be transferred to nicotinamide (Stekol et al (185)). Surprisingly, the ethyl group can be transferred to phospholipids (Kaneshuo and Law, (86)) and in ethionine fatty liver there is actually stimulated synthesis of phosphatidyl "choline" (Ulsamer and Glenn, (197)).

The decreased uptake of amino acids into lipoprotein has been confirmed by Rubin and Pendleton (160) and deserves comment. While it is obvious that any hepatic condition interfering with protein synthesis will also interfere with lipoprotein formation, because of the additional steps involved in lipoprotein formation, it does not follow that decreased specific activity in isolated lipoproteins reflects inhibition of apolipoprotein (Oncley, (135)) formation. For instance, under conditions in which the synthesis of some essential phospholipid necessary for the conversion of apolipoprotein to stable lipoprotein is inhibited, the pool of this apolipoprotein will increase. If labeled amino acids are then administered, any
synthesized apolipoprotein will have its specific activity reduced by the enlarged pool. Because the bound lipids of lipoproteins determine their characteristic density properties, the specific activity of isolated lipoproteins will appear to have been decreased.

Further confirmation of reduced serum lipoprotein specific activities in orotic acid-fatty liver has been given by Roheim *et al* (155). The most marked depression occurred in the very low density lipoproteins (density less than 1.006) in which the total incorporation was only 3.5 per cent of that in control animals. Incorporation into low density lipoproteins (density greater than 1.006 and less than 1.063) was 30 per cent of normal, and that into high density lipoproteins (density greater than 1.063 and less than 1.210) 42 per cent. It is important to note that the above experiments were done *in vivo* and that the incorporation of amino acids into total plasma proteins was only slightly depressed. Furthermore, this experiment showed that orotic acid feeding specifically affected lipoproteins, but it did not distinguish between an interference in the coupling of lipid to protein and a block in the release of lipoproteins into the circulation.

In order to distinguish between these possibilities, Roheim *et al* (154) carried out additional experiments. They had previously found that very low density lipoproteins were formed when the livers from cholesterol-fed rats were perfused with a biosynthetically labeled normal
plasma protein preparation. When these same livers were perfused with a similar lipoprotein-free plasma protein preparation from orotic acid-fed rats, similar amounts of very low density lipoproteins were formed. From these studies, it was apparent that the orotic acid-fed rats did synthesize the low density apolipoprotein and release it into the circulation. When orotic acid-fed rat livers were perfused with a similar plasma protein preparation from normal rats, there was more than a 90 per cent decrease in the total incorporation of apolipoprotein into very low density lipoproteins. The possibility that the liver of the orotic acid-fed rat could have removed or destroyed the apolipoprotein in the perfusing fluid was eliminated by first perfusing a normal plasma protein preparation through the liver from an orotic acid-fed rat and then through the liver from a normal rat fed cholesterol. The rate of lipoprotein synthesis in the normal liver was not affected by the prior perfusion. The results of these investigations indicate that the apolipoprotein of very low density lipoprotein can be released into the circulation without its normal lipid load. This release also occurs in the normal liver so that there seems to be no necessity for apolipoproteins of very low density lipoproteins to combine with lipids before being released into the blood. The possibility that high density lipoproteins may have an important role in the mobilization of essential fatty acids from the liver is suggested by the marked rise in the content of unsaturated esters in high density lipo-
proteins that occurs when rats are fed unsaturated fats such as olive oil (Gidiz et al (57)).

That a defect in phospholipid synthesis could be responsible for the decreased release of lipid-mobilizing high-density lipoproteins from the liver is indicated by the work of Creasey et al (36). After ten days on a one per cent orotic acid diet, rat liver cephalin dropped from 26.3 per cent of total liver lipids to 3.0 per cent. Lecithin fell from 46.9 per cent to 7.3 per cent. Sphingomyelin likewise decreased from 3.4 per cent to 0.4 per cent and was accompanied by a drop in lysolecithin from 1.7 to 0.3 per cent. Free sterols declined from 4.8 to 0.7 per cent and sterol esters diminished to 0.4 per cent from 3.4 per cent. Only triglycerides increased, from 11.1 per cent to 86.1 per cent. Liver diglycerides showed higher amounts of linoleic, palmitic, palmitoleic, and oleic acids and decreased amounts of stearic acid. The specific activity of acetate-2-14C incorporated into liver phospholipids decreased 64 per cent. However, the incorporation of inorganic phosphate into liver phospholipids decreased by only 12 per cent. The discrepancy between the incorporation of acetate and inorganic phosphate into phospholipid is consistent with the key role of phosphatidic acid in both triglyceride and phospholipid synthesis. The greater decrease in the incorporation of acetate into phospholipid means that more of the phosphatidic acid is being diverted to triglyceride synthesis. It is this additional triglyceride
which produces the fatty liver. The exact reversal of this change, the
diversion of phosphatidic acid from triglyceride to lecithin synthesis, has
been accomplished with rat liver mitochondria by the in vitro addition of
cytidine diphosphate choline (Strickland and Rossiter, 192).

Changes of vitamin content of orotic acid-fatty livers seems to be
restricted to niacin. Both thiamine and riboflavin remain unchanged. The
drop in total liver niacin from 825 micrograms to 594 micrograms may
be caused by its increased conversion to $^1$-methylnicotinamide during
which it discharges the S-adenosyl methionine not utilized in the methyla-
tion of N,N-dimethylaminoethanol. The ability of nicotinamide to com-
pete for these methyl groups is clearly demonstrated by its ability to pro-
duce fatty livers when administered in large amounts.

The pronounced decrease in the synthesis of liver phospholipid might
be expected to result in a rapid loss of structural integrity in the liver.
This does not occur and orotic acid-fed rats survive over a year. A
possible explanation of this phenomenon is suggested by the experiments
of Thompson and Balou (195). Using tritium labeled water, they found
that a rather large proportion of the total phosphatide of the rat is found
in an inert or "long-lived" component. The biological half-life of this
long-lived phosphatide component was estimated at 220 days compared to
20 days for a"shorter-lived" component. In contrast, the rapid turnover
of serum lipids is indicated by the fact that the specific activity of serum
phospholipids becomes equal to the specific activity of liver phospholipids 24 hours after injection of inorganic phosphorus-32 (Sakagami et al (161a) ). Therefore, it is the rapidly turning-over phospholipid that is most affected when overall phospholipid synthesis is impaired (Zilversmit and Duleszic, [214] ).

Histologically, the most pronounced structural changes in the orotic-acid fed rats are seen after one week on the diet. Among these changes are the following:

1. Loss of cytoplasmic basophilic aggregates.
2. Intracellular accumulation of numerous small discrete lipid droplets within scattered hepatocytes with an abrupt variation between normal and abnormal cells.
3. Focal silation of and loss of the parallel orientation of the perinuclear profiles of the rough endoplasmic reticulum. There is dilation of the cisternae and the population density of the ribosomes attached to the membranes appears to be lowered.
5. No alteration of structure in either the mitochondria nor in lysosomes was observed.
6. A characteristic accumulation of triglycerides in the endo-
plasmic reticulum develops.

(7). There is a significant increase in liver glycogen.

A gross anatomical difference exists between the fatty liver caused by orotic acid and that caused by choline deficiency (Sidransky, (176) ). In orotic acid fatty liver most of the lipid accumulates in the right lobes of the liver while in choline-deficiency, most of the lipid appears on the left side. This distribution indicates that there is no deficiency of choline in the left lobes of orotic acid fed rats. Since the right lobes of the liver receive blood predominantly from the superior mesenteric veins which drain the small intestine while the left lobes receive blood from the splenic veins which drain, among other structures, the large intestine, it is conceivable that a greater concentration of orotic acid reaches the right than the left lobes. Since orotic acid does not accumulate in the liver, only those cells coming into direct contact with orotic acid are effected.

The histological evidence indicates that there is some decrease in ribonucleic acid synthesis (Palade and Siekevitz, (137) ). This decrease of microsomal ribonucleoprotein could be caused by a deficiency of cytidine-5'-triphosphate, but since microsomes contain equal quantities of phospholipid and ribonucleic acid, a defect in phospholipid synthesis could also be responsible for the loss of cytoplasmic basophilic aggregates.
Furthermore, the loss in parallel structure of the perinuclear profiles may also be due to the lack of the essential phospholipids in the structural model proposed for these components by Sjostrand (179).

The dilatation of the cisternae due to the accumulation of triglyceride is in accord with the function of the cisternae as outlined by Hirsch (76). The secretory products of cells, such as zymogen particles, aggregate in this region before entering the Golgi vacuoles. Presumably, it is in this region that lipoproteins are formed; it is here that orotic acid-fed rats fail to combine lipid triglyceride with the protein moiety of very low density lipoproteins. This combination entails the transfer of a quantity of lipid sufficient to drastically alter the density properties of the apoprotein and micellar structures or cellular lipoproteins may be involved in the transition. In contrast to the marked reduction of very low density lipoproteins in orotic acid-fed rats, the levels and specific activities of the high density lipoproteins are only slightly decreased. The higher affinity of high density apolipoprotein for lipid offers a possible explanation for this pattern (Scanu and Page, (170)). Furthermore, the longer half-life of high density lipoproteins and the ready exchange of protein moieties within this lipoprotein class contribute to the observed distribution (Scanu and Page, (170a)). To summarize, the pronounced reduction in the specific activity of low density lipoproteins following the administration of labeled amino acids to orotic acid fed rats does not
necessarily reflect an inhibition of protein synthesis. Rather, it could
represent the result of the differential affinities of the apolipoproteins
synthesized in normal amounts for a limited supply of lipid. When the
conversion of very low density apolipoprotein to lipoprotein is blocked as
it is in orotic acid fatty liver, the pool of this circulating apolipoprotein
is increased. When labeled apolipoprotein is formed after the admin-
istration of labeled amino acids, its specific activity is consequently re-
duced because of the enlarged pool. Since only the fraction of this pool
combining with lipid and possessing characteristic density properties is
isolated as lipoprotein, the specific activity of the low density lipoproteins
will appear to have been preferentially reduced.

The increased thiamine pyrophosphatase activity in the livers of
orotic acid-fed rats appears to be one of the many compensatory mech-
anisms that are operating in these systems. Thiamine pyrophosphate is
required for the conversion of pyruvate to acetyl CoA by the complex
pyruvic acid dehydrogenase. The acetyl CoA so produced is subsequently
converted to fatty acyl CoA which competes with cytidine intermediates
for available diglyceride. Thiamine pyrophosphatase decreases the for-
ination of fatty acyl CoA and thereby enhances the competition of the cy-
tidine intermediates for the diglyceride which leads to phospholipid for-
mation.

In the fatty liver of choline deficiency, there is actually an in-
creased incorporation of inorganic phosphorus into total phospholipids (probably phosphatidic acid) which lasts from the 36th to the 64th day of the deficiency (Cornatzer and Watser, (34)). Additional compensatory mechanisms in orotic acid-fatty liver will be discussed later when pyrimidine metabolism is reviewed. In addition, the explanation for the increase in liver glycogen in orotic acid-fatty liver will become apparent.

The lack of mitochondrial involvement and the characteristic accumulation of triglyceride in the endoplasmic reticulum is in accord with the localization of the cytidine pathway of phospholipid synthesis in the microsomes (de Duve et al (40); Wilgram and Kennedy, (205)).

The effects of hormones on the development of orotic acid-fatty liver concur with the previous conclusions directly implicating high density lipoproteins. Sidransky (176) found that female rats were more susceptible to orotic acid-fatty liver than were males. He also found that testosterone lessened the severity of orotic acid-fatty liver. Alaupovic et al (2) found that estrogen administration in rats resulted specifically in the stimulation of alpha-apolipoprotein synthesis. In a study of human serum lipoprotein distribution carried out by Ewing et al (49), women were found to have over three times as much of a particular high density lipoprotein. This lipoprotein is designated HDL$_2$ and it undergoes flotation at density 1.125 but not at density 1.063. The high levels of this lipoprotein found in women probably play an important
role in the mobilization of lipid from the maternal liver during pregnancy. In the non-pregnant state the full lipid-mobilizing capacity of these lipoproteins is probably not being utilized so that most of them return to the liver unchanged. However, because the female liver is synthesizing more high-density apolipoprotein, it also has a greater need for the essential phospholipids required to convert the apolipoprotein to lipoprotein. This extra phospholipid is needed only because each increment of phospholipid must be distributed over an enlarged pool of high density apolipoprotein and because only completed high density lipoprotein can enter the circulation. Testosterone, by counteracting the excess production of high density apolipoprotein, enables a given amount of phospholipid to more completely convert the decreased amount of apolipoprotein to lipoprotein which can then leave the liver.

As mentioned earlier, some difficulties are encountered when attempts are made to study hepatic phospholipid synthesis as it relates to lipoprotein formation. Total liver phosphatide is a poor gauge of phospholipid available for lipoprotein formation since many structural phospholipids are long-lived. A seemingly better approach would be to concentrate on just those phospholipids which leave the liver in the form of lipoproteins. However, the study of lipoprotein-bound phosphatides is itself a problem. Kunkel and Bearn (98) and Eder et al (43a) observed that alpha and beta lipoproteins readily exchanged phospholipids with each
other as well as within their own lipoprotein classes. Thus, any increased phospholipid synthesis leading to the formation of a single lipoprotein species (HDL) will be masked by equilibration with the entire pool of circulating lipoprotein phospholipid. These exchanges make it difficult to assign exact functional roles to individual lipoproteins in fat transport.

Another approach to the same problem is available when the same apolipoprotein forms two lipoprotein species of varying density. This reaction may be represented by the equation:

\[ \text{VHDL}_4 + \text{Lipid}_3 \rightarrow \text{HDL}_3 + \text{Lipid}_4 \]

The net result of this reaction is the addition of lipid to the apolipoprotein and a decrease in its density. That such a transformation can occur is indicated by data compiled by Aloupovic et al. (3) and by Scanu and Granada (167). Both VHDL\(_4\), Very High Density Lipoprotein floating between densities 1.210 and 1.250 and HDL\(_3\), High Density Lipoprotein floating between densities 1.125 and 1.210, have similar antigenic determinants. Both lipoproteins from human serum have identical N-terminal aspartic and C-terminal threonine. Both contain identical peptide backbones with an approximate molecular weight of 75,000. The molecular weight of the lipoprotein VHDL\(_4\) is 153,000 while that of lipoprotein HDL\(_3\) is 175,000. Since HDL\(_3\) differs from VHDL\(_4\) in containing more free cholesterol, cholesterol ester, and unesterified fatty acids, the con-
version of $^{131}I$-VHDL$_1$ to $^{131}I$-HDL$_3$ as a function of the addition of cholesterol, cholesterol ester or free fatty acids is not useful as far as phospholipid contributions to lipoprotein formation are concerned. However, by equilibrating $^{131}I$-VHDL$_1$ with a saturated lipid emulsion at various temperatures and observing the extent of its conversion to $^{131}I$-HDL$_3$, some idea of the changes in the free energy, enthalpy, and entropy involved in the intermolecular conversion of lipoproteins can be obtained. Such an experiment will be described later.

Another more suitable high density lipoprotein interconversion is represented by the equation:

$$\text{HDL}_3 + \text{Phospholipid} + (\text{VHDL})_2 \rightarrow \text{HDL}_2$$

This reaction is more sensitive to phospholipid variations since HDL$_2$ contains 36.2 per cent phospholipid while HDL$_3$ contains only 26.2 per cent. As in the previous reaction, HDL$_3$ has a molecular weight of 175,000; HDL$_2$ has a molecular weight of 360,000. The difference or molecular weight change is contributed by a peptide of 40,000 molecular weight and a phospholipid-cholesterol complex of approximately 145,000 molecular weight. The small peptide designated as $(\text{VHDL})_2$ is composed of two of the basic subunits found in all high density lipoproteins. Both VHDL$_1$ and HDL$_3$ contain five of these subunits while HDL$_2$ contains seven. The possibility of calculating the thermodynamic data for the
above reaction is limited due to the difficulty of obtaining the protein subunit (VHDL)_2 in native form. Because of its small size, it is not readily isolatable by ultracentrifugal techniques. This protein fragment, though, is potentially contained in the other high density lipoprotein species and should be obtainable from them through degradation. Such a degradation procedure based upon extraction of high density lipoproteins with ether-ethanol mixtures at -4°C Centigrade has yielded a fragment of 36,500 molecular weight (Shore and Shore, (174a)). Other very similar techniques have yielded fragments about twice this size (Scanu et al (169a)). The reaction itself is still of value in studying the conversion of ^131^I-HDL_3 to ^131^I-HDL_2 under conditions where phospholipids are limiting. Such a situation exists in orotic acid fatty liver. Instead of studying the effects of individual phospholipids on the conversion, the effects of cytidine triphosphate in the presence of the appropriate enzymes of the endoplasmic reticulum was studied. According to the review of phospholipid synthesis previously presented, cytidine triphosphate would be expected to accelerate the synthesis of all the essential, limiting phospholipids and thereby enhance the conversion of ^131^I-HDL_3 into ^131^I-HDL_2. The study of these lipoprotein intermediates and their interconversions is free from the complications encountered in studying the biosynthesis of lipoproteins from free amino acids.

The previous paragraphs put forth the idea that a basic deficiency
of cytidine triphosphate is responsible for orotic acid-fatty liver. The difficulty involved in directly determining the levels of high-energy phosphates in the liver is illustrated in the variety of results obtained by experts in this field. Thus, Pappenberg (138), Mandel et al (107), and Chance et al (32) reported the hepatic levels of cytidine-5'-triphosphate in the normal rat as 180, 144, and 80 μmoles per gram respectively. These same workers reported their respective evaluations of uridine-5'-triphosphate levels as 300, 234, and 350 μmoles per gram of tissue. Although these workers disagree quantitatively, they are in agreement that the hepatic levels of cytidine triphosphate are generally lower than the levels of the other nucleoside triphosphates. In these assay procedures the concentration of the nucleotide is estimated from its enhancement of a specific enzyme reaction. This same approach has been taken when comparing the cytidine triphosphate levels of normal and orotic acid fed rats. The measure of cytidine triphosphate concentration in the livers has been judged by its unique ability to accelerate phospholipid synthesis.

Since orotic acid is a precursor of cytidine triphosphate, orotic acid-fatty liver seems to be the result of substrate inhibition of the enzymatic formation of cytidine-5'-triphosphate. The purpose of the following paragraphs is to explain how such a situation is possible.

The initial reaction in the utilization of dietary orotic acid is its condensation with 5-phosphoribosyl-1-pyrophosphate to form orotidylic
acid. This reaction is shown below:

\[
\begin{align*}
\text{HCO}_2\text{N} & + \text{CH}_2\text{O} \text{H} \text{H} \text{H} \text{P}_2\text{O}_6 & \text{H}_2\text{O} \text{H} \text{H} \text{N} \text{O}_2 \text{C} \\
\text{H}_2\text{O} \text{H} \text{H} & + \text{CH}_2\text{O} \text{H} \text{H} \text{H} \text{P}_2\text{O}_6 & + \text{H}^+ \\
\end{align*}
\]

This reaction is catalyzed by orotidine 5'-phosphate pyrophosphorylase and it confers solubility on the otherwise slightly soluble orotate. The efficiency of the above reaction is illustrated by the apparent lack of urinary blocks in rats maintained on orotic acid diets for a long period of time. Combined with the active secretion of orotic acid by the renal tubules this efficiency explains why orotic acid itself does not accumulate in the liver.

In contrast to the above behavior of orotic acid in orotic acid fed rats, is the fate of orotic acid in individuals suffering from the hereditary disorder known as orotic aciduria. These individuals are unable to produce the enzymes, orotidylic pyrophosphorylase and orotidylic decarboxylase in amounts necessary for growth (Smith Jr. et al (178)).
These two enzymes are normally present in mature erythrocytes even though the enzymes required for the formation of orotic acid are absent. They are also present in saliva. Determination of the levels of these enzymes in a parent and two siblings of a person suspected to have died of orotic aciduria have revealed that the hereditary disorder is transmitted as an autosomal recessive trait. Decreased levels of both enzymes, especially orotidylic acid pyrophosphorylase, were found in the siblings and in other individuals heterozygous for the recessive gene. This genetic defect is almost always lethal in the homozygous individual. Because of this lethality, the above investigators were not able to make direct enzyme determinations on the erythrocytes of the homozygote. However, an autopsy showed signs of urinary complications but surprisingly enough, no signs of fatty liver. The failure to find fatty liver in cases of orotic aciduria indicates that orotic acid itself is not responsible for the fatty liver, but that its prior conversion to orotidylic acid is essential.

The next reaction involved in the metabolism of orotic acid is the decarboxylation of orotidylic acid forming uridylic acid. This reaction is catalyzed by orotidylic acid decarboxylase and the purified enzyme from rat liver has been studied in detail by Creasey and Handschumacher (35). The enzyme possesses a sulfhydryl group at the active site and requires no cofactors to catalyze the following reaction:
Because of the activity of this enzyme, there is a large increase in uridylic acid in the livers of orotic acid fed rats (Von Euler et al. (199)). There is also a rise in the concentration of uridine diphosphate. Other changes in nucleotide levels found in orotic acid fatty liver have been determined by Marchetti et al. (108a) and are summarized in Table I. The histological finding of increased liver glycogen is in accord with the finding of increased concentrations of uridine diphosphate glucose. Preference for glycogen formation seems to occur in the synthesis of lipid and carbohydrate from common intermediates. This adaptation acts to lessen the effect of lipid infiltration.

At this point, a digression is needed in which to discuss the feedback inhibition of pyrimidine synthesis as it occurs in the rat. Yates and Pardee (242) demonstrated that the enzyme aspartate carbamyl trans-
<table>
<thead>
<tr>
<th>Diet</th>
<th>CMP (μmoles)</th>
<th>AMP (μmoles)</th>
<th>GMP (μmoles)</th>
<th>IMP (μmoles)</th>
<th>UMP (μmoles)</th>
<th>ADP (μmoles)</th>
<th>UDP-acetyl-glucosamine (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>59 ± 4.5</td>
<td>153 ± 11.3</td>
<td>21 ± 2.8</td>
<td>66 ± 5.9</td>
<td>36 ± 4.2</td>
<td>105 ± 8.8</td>
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<tr>
<td>Orotic Acid (0.001%)</td>
<td>91 ± 8.7</td>
<td>183 ± 13.9</td>
<td>40 ± 4.5</td>
<td>69 ± 7.1</td>
<td>49 ± 6.1</td>
<td>107 ± 6.1</td>
<td>52 ± 5.1</td>
</tr>
<tr>
<td>1% Orotic Acid</td>
<td>47 ± 2.4</td>
<td>124 ± 6.5</td>
<td>33 ± 4.2</td>
<td>41 ± 7.8</td>
<td>115 ± 9.9</td>
<td>38 ± 2.1</td>
<td>119 ± 7.4</td>
</tr>
<tr>
<td>1% Orotic Acid and 1/4% Adenine</td>
<td>81 ± 9.3</td>
<td>152 ± 17.2</td>
<td>23 ± 3.1</td>
<td>38 ± 8.6</td>
<td>83 ± 9.2</td>
<td>54 ± 4.3</td>
<td>104 ± 13.2</td>
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</tbody>
</table>

<table>
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<tr>
<th></th>
<th>UDP-glucose</th>
<th>CTP (μmoles)</th>
<th>UDP (μmoles)</th>
<th>ATP (μmoles)</th>
<th>GTP (μmoles)</th>
<th>UTP (μmoles)</th>
<th>GDP and Adenosine Polyphosphates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>73 ± 3.6</td>
<td>5 ± 0.2</td>
<td>49 ± 6.7</td>
<td>59 ± 7.1</td>
<td>9 ± 1.1</td>
<td>19 ± 1.5</td>
<td>55 ± 8.2</td>
</tr>
<tr>
<td>Orotic Acid (0.001%)</td>
<td>54 ± 4.3</td>
<td>20 ± 3.1</td>
<td>33 ± 5.2</td>
<td>50 ± 7.9</td>
<td>11 ± 1.4</td>
<td>8 ± 1.1</td>
<td>59 ± 7.6</td>
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<tr>
<td>1% Orotic Acid</td>
<td>124 ± 11.5</td>
<td>Trace</td>
<td>59 ± 7.3</td>
<td>24 ± 1.7</td>
<td>Trace</td>
<td>9 ± 1.6</td>
<td>48 ± 3.9</td>
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<tr>
<td>1% Orotic Acid and 1/4% Adenine</td>
<td>92 ± 8.7</td>
<td>Trace</td>
<td>54 ± 8.9</td>
<td>30 ± 4.9</td>
<td>3 ± 0.5</td>
<td>4 ± 0.9</td>
<td>47 ± 6.2</td>
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</table>

Marchetti et al (108a), (1964)
Ferase was subject to feedback inhibition by cytidine triphosphate in *Escherichia coli*. Feedback inhibition of de novo pyrimidine biosynthesis in mammalian systems has been reviewed by Bresnik (25). In addition to its sensitivity to cytidine nucleotides, the rat liver system was also inhibited by uridine nucleotides. Thus, the feedback inhibition of orotic acid synthesis produced by the accumulation of uridine nucleotides in orotic acid fatty liver is in part responsible for its lack of severity compared to other forms of fatty liver. Further explanation for the relative mildness is derived from the enzymatic studies of Creasey and Handschumacher (35). They found that uridylic acid was a potent competitive inhibitor of the enzyme orotidylic acid decarboxylase. The $K_i$ of the inhibitor was $1.5 \times 10^{-4}$ M. They also found cytidylic acid to be somewhat less inhibitory to the enzyme. As mentioned above, Creasey et al. (35) found that 6-azauridine had no effect on the development of orotic acid-fatty liver though it is converted to the corresponding ribonucleotide which had a $K_i$ of $1.0 \times 10^{-7}$ for the enzyme. The failure of 6-azauridine to affect orotic acid-fatty liver pinpoints excess orotidylic acid as the prime cause of the abnormality; that is, the fatty liver develops even though the formation of excess uridine nucleotides is inhibited. Therefore, neither uridine nor cytidine derivatives are primarily responsible for the pathology.

To recapitulate, a paradoxical change occurs in orotic acid-fatty
liver: the concentration of every intermediate in the de novo formation of cytidine-5'-'triphosphate, the essential coenzyme required for phospholipid synthesis, is increased, but phospholipid synthesis is still reduced. Evidence for the presence of increased concentrations of orotidylic acid in the livers of orotic acid-fed rats is given by the excretion of orotidine in the urine (Habermann, (63)). This orotidine is formed from orotidyllic acid through the action of a nucleotide phosphatase present in microsomes (Webster and Lingrill, (200)). This response is but another example of the mechanisms which are operating to counteract the full hazards of orotic acid-fatty liver.

The exact manner in which excess orotidylic acid exerts its effect on phospholipid synthesis is obscure. The work of Creasey and Handschumacher (35) showing cytidylic acid to be a mild inhibitor of orotidylic acid decarboxylase demonstrated similarities in these nucleotides as substrates. The possibility that orotidylic acid might also be an enzymatic antagonist of the phosphorylation of cytidine nucleotides was, therefore, investigated.

Herbert et al (74) reported that a cytoplasmic fraction of rat liver was capable of converting uridylic acid to uridine-5'-diphosphate and uridine-5'-diphosphate to uridine-5'-triphosphate. In 1956 Herbert and Potter (74) demonstrated that the cytoplasmic fraction could also convert cytidylic acid to cytidine-5'-diphosphate and cytidine-5'-diphosphate to
cytidine-5'-triphosphate. These phosphorylations occurred at the expense of adenosine-5'-triphosphate. In contrast to these cytoplasmic enzymes, isolated mitochondria could not convert uridylic acid to uridine-5'-diphosphate nor cytidylic acid to cytidine-5'-diphosphate, although they could convert both diphosphates to the corresponding triphosphates. The inability of mitochondria to phosphorylate cytidylic acid, together with the observations of Kammen and Hurlbert (85) that soluble enzymes are responsible for the conversion of uridine-5'-triphosphate to cytidine-5'-triphosphate in the presence of glutamine and guanosine nucleotides, suggests that the lack of mitochondrial involvement in orotic acid fatty-liver may be taken as an indication that the phosphorylation of cytidylic acid to cytidine-5'-diphosphate by cytoplasmic enzymes is unaltered. The possibility still exists, however, that orotidylic acid may interfere with the phosphorylation of cytidine-5'-diphosphate to cytidine-5'-triphosphate by the cytoplasmic enzymes and thereby deprive the cytidyl transferases of the endoplasmic reticulum of their cofactor.

Mention has already been made of the inhibition of orotidylic decarboxylase by 6-azauridylic acid. In 1961 Rychlik (158) found that 6-azauridine-5'-diphosphate inhibited the exchange of labeled pyrophosphate with cytidine-5'-triphosphate in the presence of transfer ribonucleic acid. This reaction represents the addition of the characteristic sequence pCpCpA to the amino acid binding terminal of transfer ribonucleic acid (Hecht et al...
If one alters an inhibitor of cytidine triphosphate by eliminating its terminal 5'-phosphate group, it is probable that the resulting compound will show some inhibitory activity towards cytidine-5'-diphosphate. If this reasoning is correct, one would expect 6-azauridylic acid to be an inhibitor of cytidine-5'-diphosphate. Also, since azauridylic acid is such a potent inhibitor of orotidylic acid decarboxylase, the possibility that orotidylic acid might be an inhibitor of cytidine-5'-diphosphate does not seem untenable. At this point, the lack of the commercial availability of orotidylic acid rendered the direct test of this hypothesis impractical.

Instead, an indirect experiment based on the following assumptions was done. If orotidylic acid does inhibit the phosphorylation of cytidine-5'-diphosphate, and if this phosphorylation is the rate limiting step in the conversion of phosphoryl choline into phospholipid lecithin, then one would expect to find higher levels of cytidine-5'-diphosphate in the cytoplasm of orotic acid fatty livers. Furthermore, if it is assumed that the rate limiting step follows Michaelis-Menten kinetics, then it can be shown that the only way that one can obtain a smaller proportional stimulation of phosphoryl choline incorporation in the case of orotic acid fatty livers with a constant addition of cytidine diphosphate is by having higher endogenous levels of this substrate initially. The mathematical expression for the rate of an enzymatic reaction is:
If an increment, $s$, of the limiting substrate is added, the new velocity will be:

$$V_s = \frac{V_{\text{max}} (S + s)}{K_m + S + s}$$

The acceleration of the reaction produced by $s$ is:

$$V'_s = \frac{V_{\text{max}} K_m}{(S + s + K_m)^2}$$

The proportional acceleration produced by $s$ is then given by:

$$\frac{V'_s}{V_s} = \frac{K_m}{(S + s) (S + s + K_m)}$$

The effect of competitive inhibition is not on the maximum velocity of the reaction, but on the apparent $K_m$. The apparent $K_m$ obtained is equal to the standard $K_m$ times the factor $(1 + I/K_i)$, where $I$ is the concentration of inhibitor and $K_i$ is the disassociation constant of the enzyme inhibitor complex.

Even if one allows for the presence of competitive inhibition of the reaction in the orotic acid livers, it can be shown that if:
From the last derived equation, this is equivalent to:

\[
\frac{K_m(1 + I/K_i)}{(S_i + s)(S_i + s + K_m(1 + I/K_i))} \leq \frac{K_m}{(S_c + s)(S_c + s + K_m)}
\]

\[
\frac{(S_c + s)^2I}{K_i} \leq \frac{(S_i - S_c)(S_i + S_c + 2s + K_m + \frac{K_m}{K_i})}{K_i (S_i + S_c + 2s + K_m) + K_m I} \leq \frac{S_i - S_c}{S_i}
\]

Since the deciding middle term has both numerator and denominator positive, the relation stands proven.

Experimentally, if the conversion of cytidine-5'-diphosphate to cytidine-5'-triphosphate is the rate limiting step in phospholipid synthesis in orotic acid fatty liver, then one would expect a build-up of the diphosphate so that the addition of a constant amount of diphosphate to cell-free preparations from orotic acid-fed and control rats would produce a higher percentage stimulation of phospholipid synthesis in the latter. As shown above, any inhibitory action of orotidylic acid enhances the effect, though
it need not be present for the effect to be observed. In calculation of the percentage stimulation in orotic acid-fed and control rats it is advantageous to express the result as the ratio of cytidine-5'-diphosphate stimulation to cytidine-5'-triphosphate stimulation. Since, according to the above hypothesis, there is no block in the utilization of cytidine-5'-triphosphate in either control or orotic acid fed rats, expression of the data in this manner correct for any changes in the levels of the cytidyl transferases involved in phospholipid synthesis. Utilizing the incorporation of phosphoryl choline as an assay, one would expect the result:

\[
\left( \frac{V'_{CDP}}{V'_{CTP}} \right)_{\text{orotic acid}} \ll \left( \frac{V'_{CDP}}{V'_{CTP}} \right)_{\text{control}}
\]
CHAPTER II
MATERIALS AND METHODS

Unless otherwise stated, all chemicals used were of analytical reagent grade and all solutions were made with doubly-distilled water. Nucleotides were obtained from the California Biochemical Corporation. Uniformly carbon-14-labeled yeast protein hydrolysate with a specific activity of 1500 microcuries per milligram was obtained from Schwarz Bio-research, Inc. Carrier-free sodium iodide-131 (40 mc./ml) was obtained from New England Nuclear Corporation.

Fractionation of lipoproteins was carried out in the Spinco Model L preparative ultracentrifuge and the method of Bragdon et al. (22a) was followed with some modifications. All separations were carried out at a rotor temperature of 16°C. Potassium bromide solutions used in density adjustment were prepared from a stock solution nearly saturated at 10°C. This stock solution was prepared from U.S.P. grade potassium bromide by recrystallization once from distilled water and twice from doubly-distilled water. The solution was then extracted with diphenylthiocarbazone in carbon tetrachloride, filtered through a fine sinistered glass filter, and boiled to remove carbon tetrachloride before its density was determined. Density determinations were performed using a Westphal balance with a plummet that displaced five grams of water at 15°C.
The solution was placed in a twenty-five milliliter jacketed receiving flask and water at $15^\circ$ C from an Aminco thermostatic water bath was pumped through the flask during density determinations. In these determinations, the density of water at $15^\circ$ C. was taken as unity instead of 0.99913, the actual value. The error introduced, approximately one part per thousand, was within the experimental error of the density determination. The use of this assumption greatly facilitated the calculation of densities and the preparation of solutions. Protein solutions were adjusted to the desired density either by prolonged dialysis against several changes of the appropriate salt solution, or by mixing with more concentrated or dilute salt solutions according to the relation:

$$V_f D_f = V_i D_i + V_a D_a$$

where:

- $D_f$ = final solution density
- $V_f$ = final solution volume
- $V_i$ = initial solution volume
- $D_i$ = initial solution density
- $V_a$ = volume of added salt solution
- $D_a$ = density of added salt solution

The use of this equation does not take into account the change in partial specific volume of the salt solution with concentration. However, the
error involved is minimized by avoiding extreme dilutions during the adjustment. This method was employed principally because it involves less cross-contamination of solutions and equipment when many radioactive replicates must be processed. In the use of this method, the density of rat serum exclusive of protein was taken as 1.0060 gm./ml.

The time of ultracentrifugation varied with the density of separation. Chylomicrons were separated in one hour at 105,000 x g. Separations at density 1.063 were carried out at 36,000 r.p.m. for a period of 24 hours. High density lipoproteins, because of their lower molecular weights, required a longer period of time, 40 hours at 40,000 r.p.m. (105,000 x g.). The time required for these centrifugations somewhat limited the number of experiments which could be performed.

In a typical amino acid incorporation experiment, four male rats of the Sprague-Dawley strain, fed ad libitum, were used. The rats, weighing 150 - 200 grams apiece were stunned and decapitated. The livers were removed and washed and weighed in ice cold buffer. The livers were then blotted with tissue and minced in 2.5 times their weight of fresh cold buffer and homogenized by three passes with a motor driven teflon pestle. The buffer usually consisted of the following components when a medium approaching isotonic was used:

\[
0.05 \, M \, \text{Tris (hydroxymethyl) aminomethane-HCl} \quad \text{pH 7.8}
\]

\[
0.175 \, M \, \text{sucrose}
\]
0.008 M magnesium chloride

0.054 M potassium chloride

This buffer is similar to that employed by Webster and Lingril (200) in studies on the synthesis of albumin by rat liver microsomes. The concentrations of magnesium and potassium as well as the pH were determined to be optimal for the synthesis of albumin by microsomes. The cell fractionation procedure above served primarily as a means of concentrating the microsomes of the cell. No attempt was made to check the morphological integrity of the isolated fractions. When a better morphological preparation was desired, hypertonic medium was employed and a slight modification of the method of Moule et al (122) was followed. The buffer then consisted of 0.84 M sucrose, 0.05 M triethanolamine-HCl, pH 7.4, and 0.01 M magnesium chloride. The microsomal fraction was isolated by ultracentrifugation for one hour using the number 40 rotor at 35,000 r.p.m. instead of the number 30 rotor at 30,000 r.p.m. as in the original procedure.

Generally, 8 µcuries of carbon-14-labeled yeast protein hydrolysate (1500 µc/mg) was incubated with the microsomes from 2.0 grams of rat liver together with the necessary cofactors in a total volume of 2.0 ml. in 25 ml. Erlenmeyer flasks in a shaker bath maintained at 37°C. A solution containing all common cofactors as well as the radioactive
amino acids was made up in buffer prior to use. From this 10 ml. solution, 0.8 ml. aliquots were pipetted into the individual incubation flasks, usually twelve in number. Then 0.3 ml. of solution containing the variable factor was added and the incubation of successive flasks commenced with the addition of 0.9 ml. of microsomes resuspended in cell sap (100,000 x g. supernatant). Each flask contained the following cofactors:

1. 0 μmole guanosine-5'-triphosphate
2. 0 μmole nicotinamide adenine dinucleotide
3. 0 μmole adenosine-5'-triphosphate
4. 0 μmole phosphoenolpyruvate
5. 0 μmole pyruvate kinase

The adenosine triphosphate and the adenosine triphosphate generating system are required for the activation of the free amino acids by their specific activating enzymes. The resulting enzyme-bound amino-acyl adenylates are then attached to specific transfer ribonucleic acid molecules and are transported to the membrane-bound polyribosome consisting of a molecule of messenger ribonucleic acid and several attached ribosomes. Guanosine triphosphate is a specific requirement of an enzyme which activates bound transfer ribonucleic acid molecules prior to the formation of the peptide bond. The role of nicotinamide adenine dinu-
nucleotide in protein synthesis has not been completely elucidated, but, according to Webster and Lingrill (200) it is a peculiar requirement of the rat liver system. It is believed to be a cofactor required by enzymes responsible for the release of completed peptide chains from the polyribosome.

Although the membranous fragments of the endoplasmic reticulum are not essential for protein synthesis and are not found in bacterial cells, they serve an important function in the mammalian system (Noll, 130). They help to stabilize messenger ribonucleic acid from the destructive effects of ribonuclease. As a consequence, the template lifetimes in the mammalian system are longer and the system is less dependent on messenger ribonucleic acid synthesis.

Incubations were terminated by the addition of one ml. of cold 10 per cent unlabeled yeast protein hydrolysate and the contents of each flask was poured into 12.0 ml. stainless steel ultracentrifuge tubes. The flasks were then rinsed with 2.0 ml. of buffer containing the lipoproteins from 0.5 ml. of rat serum. These rinses were added to their respective tubes. The tubes were centrifuged at 40,000 r.p.m. for one hour to separate particulate material and the supernatant fractions containing soluble proteins was adjusted to density 1.063 or 1.210 with potassium bromide or sodium bromide respectively.

Following ultracentrifugation, the top ml. from each tube containing
lipoproteins or the bottom ml. containing lipid-free proteins was precipitated with 5.0 ml. of 6.0 per cent trichloroacetic acid. The precipitate was separated in a clinical centrifuge at 700 x g. and 0°C. After dissolution in 1.0 ml. of 0.1 N sodium hydroxide, the protein was reprecipitated with 5.0 ml. of 9:1 (v/v) methanol-ether containing sufficient trichloroacetic acid to bring its final concentration to 5.0 per cent. After reprecipitation, the residue was taken up in 1.0 ml. of hyamine hydroxide p-(diisobutyl-cresoxyethoxyethy)-dimethylbenzyl-ammonium hydroxide and heated in capped tubes for 24 hours at 50°C. to effect solution. The resulting solution was mixed with 15 ml. of scintillation fluor, which consisted of 85% dioxane-15% benzene (v/v) containing 120 grams of napthalene, 0.25 grams 1,4-bis-(5-phenyloxazolyl-benzene) and 7.0 grams of 2,5-diphenyloxazole per liter. The dioxane used was a spectral grade obtained from Matheson, Coleman, and Bell, and the secondary and primary scintillation solutes were products of Packard Instruments. The Tri-Carb model 314X liquid scintillation spectrometer was used to assay all samples. Because of the low activities of the samples, they were generally counted for a period of 100 minutes. For carbon-14 samples, a high-voltage of 1140 volts was employed while for iodine-131 samples, a voltage of 830 sufficed to produce the maximum counting rate. The results are usually expressed in terms of total activity rather than specific activity due to the fact that carrier lipopro-
teins were added during the isolation procedure.

The preparation of cesium chloride density gradients according to the data compiled by Ifft et al (82) was accomplished with the aid of concentration-density relationships published in the International Critical Tables. Purified cesium chloride was obtained from K and K Laboratories. These separations were also carried out at $15^\circ$ C. The practicality of density-gradient separations in the fixed-angle number 40 rotor has been verified by Fischer et al (52).

In several experiments radioactively phosphoryl choline was required and was not obtainable commercially. It was, therefore, prepared by the method of Plimmer and Bruck (144). The labeled choline chloride was refluxed for seven hours in 5.0 ml. of concentrated phosphoric acid containing 0.2 grams phosphorous pentoxide. A calcium chloride drying tube was used. Following precipitation of calcium phosphate the product was isolated as the calcium salt by precipitation with ethanol. Although this same method was followed by Kennedy (89), the yield of product was very low. Considerable losses of the product probably resulted from the co-precipitation of the calcium salt of phosphoryl choline with calcium phosphate. A yield of approximately 7 per cent was obtained.

An alternate procedure for synthesizing phosphoryl choline was tried. This procedure, based on the Michaelis-Arbusov rearrangement, was employed with labeled chlorocholine chloride as starting material.
This compound labeled in the one and two positions with carbon-14 was obtained from New England Nuclear Corporation. The type reaction is indicated below:

\[ \text{Phosphonium intermediate} \]

\[ P(OCH_3)_3 + RCl \rightarrow (CH_3O)_3PRCl \rightarrow (CH_3O)_2PR + CH_3Cl \]

R represents choline in the above reaction steps. The reaction is driven to the right due to the volatility of methyl chloride. The next step in the preparation requires the saponification of the two esterified methyl groups with the resultant production of the desired product. The data of Plimmer and Bruck (144) indicate that phosphoryl choline is very resistant to alkaline hydrolysis while data from the International Critical Tables show that phosphate methyl esters are the most rapidly hydrolyzed of organic phosphate esters. Therefore, it seemed possible to selectively remove the two methyl groups from dimethyl choline phosphonate by treatment with alkali.

Assays of phosphoryl choline-cytidyl transferase activity in microsomes and in the supernatant were conducted according to the technique of Borkenhagen and Kennedy (22). Cytidine diphosphate choline was quantitatively absorbed on Norit A charcoal and the label was released from the charcoal by hydrolysis with 2N hydrochloric acid. Following
reprecipitation of the charcoal, an aliquot of the supernatant was assayed for radioactivity by liquid scintillation. Constant quenching was assumed for all samples except those which were grossly discolored or those in which the ratio of counts in the two windows employed differed significantly from the average. Difficulties in the assay of this enzyme such as those reported by Ansell and Marshall (7) were sometimes encountered.

Iodination of lipoproteins with radioactive sodium iodide-131 was carried out by the following technique. Isolated lipoprotein fractions were dialyzed against several changes of 0.1 N sodium N, N-dihydroxyethylglycine buffer, pH 9.0 before use. A minute amount of thymol blue indicator was then added to approximately 0.5 mc. of the carrier-free isotope in a conical centrifuge tube. Several drops of concentrated nitric acid were then added sufficient to bring the indicator to its red acidic color. After several minutes at room temperature, 1 N sodium hydroxide was added till the color of the indicator changed to blue at pH 9. Then the lipoprotein in buffer was added and the mixture was allowed to stand at room temperature (25° C.) for one hour. After the addition of two drops of 0.1 N sodium thiosulfate the reaction mixture was passed through an Amberlite ion exchange resin column in the chloride form. The eluant was dialyzed against several changes of incubation buffer before use. The exclusion of halide ions from the labeling mixture served to minimize the labeling of the lipid components.
of the lipoproteins.

In the course of this investigation, a promising density gradient technique for the fractionation of serum was tested. Solid cesium bromide was added to chylomicron-free serum to increase its density to 1.250. After placing 3.0 ml. of this high-density serum in 13 ml. ultracentrifuge tubes and layering 10.0 ml. of density 1.210 cesium bromide above, the tube is centrifuged for 24 hours. At 40,000 r.p.m. and an average force of 105,000 x g. serum was separated fairly sharply into four density bands. Undoubtedly, the application of the method with twice the above force as is currently available should lead to much sharper separation. The method is especially tailored to the lipoproteins because the lipoproteins banded in low density regions and low forces in the top region of the tube have higher molecular weights which contributes to the sharpness of the resulting bands.
CHAPTER III

EXPERIMENTAL RESULTS

EXPERIMENT 1: The Effects of Ultrasonication on the Release of Lipoproteins from Microsomes.

One of the first experiments in this series was concerned with the improvement of the incorporation of amino acids into lipoprotein in normal rats. One of the most promising techniques for increasing the yield of labeled lipoproteins seemed to be that of ultrasonication. The rational behind this procedure was that it might release completed lipoproteins bound loosely to the lipid components of the microsome.

The sonicating forces should break any weak hydrophobic bonds and free additional labeled lipoproteins as reported by Marsh (145). He doubled incorporation into low density lipoproteins and increased high density incorporation by a factor of ten by using the Raytheon sonic oscillator for 20 minutes at 0°C. The results of an attempt, shown in Table II, to reproduce Marsh's work do not substantiate this effect. Several explanations are possible for the observed differences.

Marsh's sonication mixture contained chylomicrons which may have been labeled during his incubation. Upon sonication, these chylomicrons may have been disrupted into low and high density lipoproteins (Scanu and Page, (170)). If this did in fact occur, it represents an unphysio-
TABLE II

The Effects of Ultrasonication on the Release of Lipoproteins from Microsomes. (Experiment 1)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time (min.)</th>
<th>den. 1.006-1.063 (c.p.m.)</th>
<th>den. 1.063-1.125 (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0° C.</td>
<td>0</td>
<td>2.2</td>
<td>9.5</td>
</tr>
<tr>
<td>0° C.</td>
<td>0</td>
<td>3.0</td>
<td>7.3</td>
</tr>
<tr>
<td>0° C.</td>
<td>15</td>
<td>3.3</td>
<td>2.8</td>
</tr>
<tr>
<td>0° C.</td>
<td>30</td>
<td>0.1</td>
<td>3.3</td>
</tr>
<tr>
<td>0° C.</td>
<td>45</td>
<td>6.0</td>
<td>16.7</td>
</tr>
<tr>
<td>0° C.</td>
<td>60</td>
<td>0.2</td>
<td>3.5</td>
</tr>
<tr>
<td>33° C.</td>
<td>60</td>
<td>15.2</td>
<td>2.4</td>
</tr>
</tbody>
</table>
logical disruption of the normal lipoprotein pattern. On the other hand, the observed increases may have been due to the disruption of microsomal complexes in which a soluble low density lipoprotein is bound to a semi-completed high density lipoprotein (Scanu and Hughes, (168)). Due to the possibility that growing peptide chains may be bound to the ribosome through the hydrogen bonding of a single soluble ribonucleic acid triplet, partially completed apolipoproteins able to combine with lipid may have been released during the ultrasonication process. This solution is especially plausible in view of the fact that polysomes are engaged in the synthesis of several peptide chains simultaneously. If this was indeed the case, then the failure of this release to occur in the above experiment may have been due to the less powerful sonication method employed.

Sonifications were carried out under milder conditions in a Branson sonogen D series ultrasonic cleaner at one-fifth the power output employed by Marsh. Nevertheless, ultrasonic disruption is unphysiological and the actual release of completed peptides is likely to involve the action of a specific esterase splitting the bond between the C-terminal amino acid of the peptide and adenosine hydroxyl group of its corresponding transfer ribonucleic acid molecule.

A check of the above explanation is possible through the use of puromycin, which also releases partially completed peptide chains (Williamson and Schweet, (208)).
EXPERIMENT 2: The Effects of Cytidine-5'-Triphosphate on the Incorporation of Amino Acids into High Density Lipoproteins by Microsomes.

A preliminary glance at the outcome of this experiment in Table III brings to light several facets of orotic acid-fatty liver. Without doubt, orotic acid fed rats are seen to be converting less of their apolipoproteins to lipoproteins. Although this conversion is stimulated in both normal and orotic acid fed rats by the addition of cytidine-5'-triphosphate, not all the converted apolipoprotein is high density apolipoprotein. In the control rats a relatively small proportion of the converted apolipoprotein is high density apolipoprotein. In the orotic acid fed rats, however, this proportion is much larger. The conclusion drawn from this experiment is that high density apolipoproteins take up the additional synthesized phospholipids preferentially. Only when they are relatively saturated with phospholipid does phospholipid become available for the conversion of low density apolipoprotein to low density lipoprotein. This conclusion accords with the greater severity of orotic acid fatty liver in female rats because of the larger amount of high density apolipoprotein synthesized. The preferential synthesis of high density lipoproteins is puzzling in view of role of low density lipoproteins as the major carriers of triglycerides (Rubin and Aladjem, (159); Olson and Vester, (134) ). Possibly, the main function of high density lipoproteins may be in the stabilization of chylomicrons (Rodbell and Frederickson, (152) ), but
TABLE III

The Effects of Cytidine-5'-Triphosphate on the Incorporation of Amino Acids into High Density Lipoproteins by Microsomes. (Experiment 2)

<table>
<thead>
<tr>
<th>System</th>
<th>den. 1.063-1.210 (c.p.m.)</th>
<th>den. 1.210 (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.2</td>
<td>84.2</td>
</tr>
<tr>
<td>Control and CTP</td>
<td>16.2</td>
<td>89.2</td>
</tr>
<tr>
<td>Orotic Acid</td>
<td>9.6</td>
<td>95.6</td>
</tr>
<tr>
<td>Orotic Acid and CTP</td>
<td>18.8</td>
<td>63.0</td>
</tr>
</tbody>
</table>
other functions for this density class are indicated by the symptoms present in Tangier's disease in which there is a congenital absence of this lipoprotein class. The predominate changes which occur include lipid engulfment of the liver and other tissues consisting primarily of cholesterol esters of oleic acid and the accumulation of linoleic acid in plasma (Fredrickson and Altrocchi, (54)).

The discovery of Glomset et al (58) of a fatty acid transferase in plasma catalyzing the transesterification of a fatty acid from lecithin to cholesterol is of greatest interest with regard to the true role of high density lipoproteins. The further findings of Lossow et al (104) of cholesterol esterifying activity in the high density lipoproteins themselves heightens the interest. The presence of linoleic acid as the main fatty acid on the central carbon of both lecithin and triglycerides (Hanahan, (66)) together with the specificity of phosphorylcholine cytidyl transferase for an unsaturated fatty acid in this position (Kennedy, (93)) implicate high density lipoprotein lecithin in the transport of unsaturated fatty acids. Complications in studies of this sort arise because both low and high density lipoproteins exchange lecithin, lysolecithin, and sphingomyelin (Sakagami, (164)) and because lipoprotein lipase plays a role in the exchange of cholesterol esters between low and high density lipoproteins.

In summary, failure to form adequate lecithin may seriously impair the ability of orotic acid fed rats to form cholesterol esters which are
subsequently transferred to other lipoprotein species.

**EXPERIMENT 3: The Effects of Orotic Acid and Cytidine-5'-Triphosphate on the Incorporation of Amino Acids into Lipoproteins by Microsomes.**

In experiments such as that shown in Table IV, the relative amounts of proteins and lipoproteins assayed may not truly be representative of the ratio actually synthesized due to the fact that only those non-lipoproteins approaching sedimentation equilibrium in the bottom ml. of the ultracentrifuge tube were counted. Serum albumin, which accounts for approximately fifty per cent of the protein made under these conditions, is not concentrated in the bottom region of the tube. Although 100 counts per minute in the table corresponds to only 3.6 μg. of protein, conclusions can still be drawn from the results.

First, the relatively small stimulation, if any, caused by the addition of cytidine-5'-triphosphate to the control preparation is reminiscent of the results of previous experiments. This may indicate that this coenzyme is not limiting in phospholipid synthesis in the normal rat. Secondly, 0.015 M orotic acid, a concentration close to saturation in this system, did not significantly inhibit the formation of high density lipoproteins. The results support the earlier observation of Smith, Jr.
TABLE IV

The Effects of Orotic Acid and Cytidine-5'-Triphosphate on the Incorporation of Amino Acids into Lipoproteins by Microsomes. (Experiment 3)

<table>
<thead>
<tr>
<th>System studied</th>
<th>Lipoproteins</th>
<th>Non-Lipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>den. 1.063-1.210 (c.p.m.)</td>
<td>den. 1.210 (c.p.m.)</td>
</tr>
<tr>
<td>Control</td>
<td>91.3</td>
<td>82.0</td>
</tr>
<tr>
<td>Control</td>
<td>92.4</td>
<td>87.5</td>
</tr>
<tr>
<td>Control and CTP</td>
<td>97.1</td>
<td>69.0</td>
</tr>
<tr>
<td>Control and Orotic Acid</td>
<td>92.1</td>
<td>67.9</td>
</tr>
<tr>
<td>Control and Orotic Acid</td>
<td>90.3</td>
<td>61.0</td>
</tr>
</tbody>
</table>
et al (178) showing no incidence of fatty liver in individuals afflicted with congenital orotic aciduria. Therefore, it is likely that a derivative of orotic acid formed \textit{in vivo} is responsible for the decrease in phospholipid synthesis and the development of fatty liver in orotic acid fed rats. Orotidylic acid formed through the condensation of orotic acid with 5-phosphoribosyl-1-pyrophosphate appears to be the compound more directly responsible for the fatty liver, although the difficulty in obtaining this compound has made direct observations of its effects difficult. Notwithstanding, the presence of high concentrations of orotidylic acid in orotic acid fed rats are indicated by the presence of orotidine in the urine. (Habermann, (63)). This orotidine could only have arisen by the action of a phosphatase on orotidylic acid. Possible implications of this effect during early growth will be discussed later.

**EXPERIMENT 4:** The Effect of 0.02 M Phosphatidyl Choline on the Incorporation of Amino Acids into High Density Lipoproteins.

In order to define the effect of added phospholipids on lipoprotein biosynthesis \textit{in vitro} lecithin was added to the microsomal amino acid incorporating system from rat liver.

The lecithin used in the experiment, reported in Table V, was a commercial grade purified by repeated precipitation from ether solution
TABLE V

The Effect of 0.02 M Phosphatidyl Choline on the Incorporation of Amino Acids into High Density Lipoproteins. (Experiment 4)

<table>
<thead>
<tr>
<th>System</th>
<th>den. 1.063-1.210 (c.p.m.)</th>
<th>den. 1.210 (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.2</td>
<td>265.8</td>
</tr>
<tr>
<td>Control</td>
<td>7.7</td>
<td>232.4</td>
</tr>
<tr>
<td></td>
<td>13.7</td>
<td>216.2</td>
</tr>
<tr>
<td>Control and</td>
<td>31.6</td>
<td>32.5</td>
</tr>
<tr>
<td>Lecithin</td>
<td>34.4</td>
<td>37.7</td>
</tr>
<tr>
<td></td>
<td>35.3</td>
<td>37.9</td>
</tr>
</tbody>
</table>
with acetone. The lecithin concentration was determined as dipalmitoyl phosphatidyl choline. Immediately apparent is the striking effect of lecithin on the disappearance of protein from the apolipoprotein fraction and the conversion of part of this apolipoprotein into high density lipoprotein. Presumably, the remaining apolipoprotein is converted into other lipoprotein classes although not all of the label could be accounted for experimentally in these fractions. More importantly, lecithin stimulated conversion in normal systems while previous experiments showed cytidine triphosphate to exert only a nominal effect under similar circumstances. This apparent discrepancy may be explained in terms of the limiting factors which control phospholipid synthesis in normal and orotic acid fed rats. In orotic acid fed rats cytidine-5'-triphosphate appears to be limiting and the addition of this compound greatly stimulates the conversion of apolipoprotein into lipoprotein. In normal rats, however, diglyceride also appears to be a limiting factor in the production of lecithin from cytidine diphosphate choline. The above considerations no doubt apply to the synthesis of sphingomyelin though they have not been verified experimentally. They support the findings of Peterson (141a) that sphingomyelin predominantly in low density lipoproteins is the major phospholipid to increase in starvation in man when large amounts of adipose tissue are being mobilized as triglycerides and de novo triglyceride synthesis is reduced. The limiting role of phosphatidic acid
has been referred to earlier (Dawson, (38)). The fact that phosphatidic acid accumulates in fatty liver is in agreement with the findings of Windmüller and Spaeth (210) showing decreased triglyceride synthesis in orotic acid fatty liver despite triglyceride accumulation. This adaptation seems to be another example of a compensatory mechanism operating in orotic acid fatty liver.

EXPERIMENT 5: The Effects of Phosphatidyl Choline and Phosphatidyl Ethanolamine on the Incorporation of Amino Acids into Lipoproteins by Microsomal Preparations from Orotic Acid Fed Rats.

Having observed the beneficial effects of added lecithin on lipoprotein formation, a comparison of the effects of different phospholipids seemed to be in order. In Table VI, the outcome of such an experiment is shown.

The actual quantity of phospholipid added to each 2.0 ml. incubation flask was 25 mg. Phosphatidyl ethanolamine alone did not produce any significant stimulation of lipoprotein synthesis. Phosphatidyl choline alone produced a definite stimulation of both low and high density lipoprotein formation. Finally, when both phospholipids were added, the synthesis of low density lipoproteins more than doubled concomitant with a decrease in the apolipoprotein fraction.
TABLE VI

The Effects of Phosphatidyl Choline and Phosphatidyl Ethanolamine on the Incorporation of Amino Acids into Lipoproteins by Microsomal Preparations from Orotic Acid Fed Rats. (Experiment 5)

Amino Acid Incorporation into Lipoprotein Density Classes

<table>
<thead>
<tr>
<th>Density 1.063</th>
<th>Density 1.063-1.210</th>
<th>Density 1.210</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23.1 c.p.m.</td>
<td>18.9 c.p.m.</td>
<td>680 c.p.m.</td>
</tr>
<tr>
<td>36.8 c.p.m.</td>
<td>11.4 c.p.m.</td>
<td>708 c.p.m.</td>
</tr>
<tr>
<td><strong>LECITHIN (c.p.m.)</strong></td>
<td><strong>LECITHIN (c.p.m.)</strong></td>
<td><strong>LECITHIN (c.p.m.)</strong></td>
</tr>
<tr>
<td>55.5</td>
<td>24.6</td>
<td>748</td>
</tr>
<tr>
<td>46.7</td>
<td>22.5</td>
<td>785</td>
</tr>
<tr>
<td>27.5</td>
<td>27.8</td>
<td>909</td>
</tr>
<tr>
<td><strong>CEPHALIN (c.p.m.)</strong></td>
<td><strong>CEPHALIN (c.p.m.)</strong></td>
<td><strong>CEPHALIN (c.p.m.)</strong></td>
</tr>
<tr>
<td>31.3</td>
<td>18.3</td>
<td>897</td>
</tr>
<tr>
<td>19.9</td>
<td>15.9</td>
<td>354</td>
</tr>
<tr>
<td>11.6</td>
<td>----</td>
<td>675</td>
</tr>
<tr>
<td><strong>BOTH (c.p.m.)</strong></td>
<td><strong>BOTH (c.p.m.)</strong></td>
<td><strong>BOTH (c.p.m.)</strong></td>
</tr>
<tr>
<td>86.9</td>
<td>18.8</td>
<td>859</td>
</tr>
<tr>
<td>109.9</td>
<td>37.2</td>
<td>424</td>
</tr>
<tr>
<td>57.9</td>
<td>26.6</td>
<td>385</td>
</tr>
</tbody>
</table>
The preferential uptake of phospholipid by high density lipoproteins observed in this experiment has been noticed in previous experiments. Only when the high density apolipoproteins have taken up their quota of phospholipid is the synthesis of low density lipoproteins stimulated. Also, the data seem to indicate that lecithin is required for the incorporation of cephalin into low density lipoproteins but that both fulfill individual roles in lipoprotein structure. The two phospholipids differ primarily in basicity although the possibility that differences in fatty acid composition may have been primarily responsible for the differences in the results was not ruled out. Phosphatidyl choline bears a stronger positive charge than phosphatidyl ethanolamine and may be capable of ionic bonding with the aspartic and glutamic residues of the apolipoprotein. Such ionic bonds may be weakened at high ionic strengths employed in the ultracentrifuge leading to lipoprotein decomposition due to the differential separation forces acting on the low density lipid component and the high density protein component of the intact lipoprotein (Scanu and Granada, (167)).

EXPERIMENT 6: The Effect of 0.002 Molar Cytidine-5'-Triphosphate on Amino Acid Incorporation in Orotic Acid Fed Rats.

If the decreased synthesis of phospholipids found in orotic acid-fatty liver is due to the lack of cytidine-5'-triphosphate, then addition of this
compound in vitro should restore conditions to normal. The data shown in Table VII substantiate this expected effect.

The data indicate that the orotic acid system supplemented with an excess of cytidine-5'-triphosphate is capable of synthesizing lipoproteins and non-lipoproteins in a ratio almost identical to that found with the control system. The concentration of cytidine triphosphate employed is over forty times that required to produce a measurable effect in the rat liver system (Kennedy and Weiss, 91). Therefore, although it is impossible to say from the above results exactly how much of the stimulation was due to the direct utilization of cytidine triphosphate and how much was due to rephosphorylation of its break-down product, cytidylic acid, the large excess of cytidine triphosphate employed probably directly saturated the enzymes involved during the relatively short hour-incubation. The results of the above experiment indicate that there is no interference in the utilization of cytidine-5'-triphosphate in orotic acid-fed rats, in agreement with the findings by Marchetti et al (108a) of near-normal levels of liver uridine-5'-triphosphate, but rather that there is a decrease in cytidine-5'-triphosphate production. A more informative modification of this particular experiment is the study of phospholipid stimulation produced by the combined addition of cytidine-5'-diphosphate and adenosine-5'-triphosphate to be reported later.

The necessity of adding detergent to obtain cytidine diphosphate
TABLE VII

The Effect of 0.002 Molar Cytidine-5'-Triphosphate on Amino Acid Incorporation in Orotic Acid Fed Rats. (Experiment 6)

<table>
<thead>
<tr>
<th>System</th>
<th>Total lipoproteins (c.p.m.)</th>
<th>Non-lipoproteins (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3220</td>
<td>15,100</td>
</tr>
<tr>
<td></td>
<td>2990</td>
<td>15,250</td>
</tr>
<tr>
<td></td>
<td>2600</td>
<td>14,110</td>
</tr>
<tr>
<td></td>
<td>2880</td>
<td>16,440</td>
</tr>
<tr>
<td></td>
<td>2920</td>
<td><strong>Average</strong> 14,980</td>
</tr>
<tr>
<td>Orotic</td>
<td>2860</td>
<td>16,350</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>16,050</td>
</tr>
<tr>
<td></td>
<td>3490</td>
<td>17,190</td>
</tr>
<tr>
<td></td>
<td>3100</td>
<td>14,780</td>
</tr>
<tr>
<td></td>
<td><strong>Average</strong> 16,090</td>
<td></td>
</tr>
<tr>
<td>Acid</td>
<td>3110</td>
<td></td>
</tr>
</tbody>
</table>


choline-glyceride transferase activity with added diglyceride does not imply that the enzyme is saturated. Rather, as in the case of lipases, the detergent facilitates the approach of the water insoluble substrate to the enzyme. The possibility remains that the supply of diglyceride which partially controls the amount of phospholipid synthesized in the normal rat. Such a control system would have the advantage of producing lipoproteins-stabilizing phospholipids in direct proportion to the amount of triglyceride synthesized.

EXPERIMENT 7: The Effects of 0.02 Millimolar Cytidine-5'-Triphosphate on the Conversion of Iodine-131-Apolipoproteins to Lipoproteins by the Mitochondrial and Microsomal Fractions of Control and Orotic Acid-Fed Rats.

Having observed the effects of enzyme-saturating levels of cytidine-5'-triphosphate, it was of interest to compare the effects of catalytic quantities of this coenzyme under conditions where it is not regenerated. The results expressed in Table VIII are, indeed, different from those of the previous experiment.

The iodinated lipoprotein fraction used in the experiment consisted of the entire protein fraction of serum with density greater than 1.210. Apolipoproteins account for approximately 12 per cent of this fraction.
TABLE VIII

The Effects of 0.02 Millimolar Cytidine-5' -Triphosphate on the Conversion of Iodine-131-Apolipoproteins to Lipoproteins by the Mitochondrial and Microsomal Fractions of Control and Orotic Acid-Fed Rats. (Experiment 7)

<table>
<thead>
<tr>
<th>System</th>
<th>Total lipoproteins (c.p.m.)</th>
<th>Non-lipoproteins (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>963</td>
<td>85,100</td>
</tr>
<tr>
<td></td>
<td>935</td>
<td>82,900</td>
</tr>
<tr>
<td></td>
<td>996</td>
<td>81,400</td>
</tr>
<tr>
<td></td>
<td>965 Average 83,000</td>
<td></td>
</tr>
<tr>
<td>Orotic</td>
<td>656</td>
<td>83,200</td>
</tr>
<tr>
<td>Acid</td>
<td>635</td>
<td>83,200</td>
</tr>
<tr>
<td></td>
<td>720 Average 83,900</td>
<td></td>
</tr>
</tbody>
</table>
Phosphoryl choline was added to a concentration of 1.5 mm. and was not limiting in the above experiment. Also, the removal of nucleoside diphosphate kinase with the microsomal supernatant effectively blocks the regeneration of cytidine-5'-triphosphate from cytidylic acid in the above experiment.

The above enzyme system contains all of the enzymes required for the cytidine pathway of phospholipid synthesis based on the intracellular localization of these enzymes as elucidated by Wilgram and Kennedy (205). The very low concentration of cytidine-5'-triphosphate employed, approximately 1 per cent that used in the previous experiment, was hardly high enough to saturate the enzymes throughout the incubation. Furthermore, because mitochondria are incapable of converting cytidylic acid to cytidine diphosphate (Herbert and Potter, (74)), the results primarily reflect the influence of endogenous cytidine-5'-triphosphate.

The lower levels of cytidine triphosphate in orotic acid-fed rats, or the presence of a competitive inhibitor of cytidine triphosphate formation might be expected to exert an effect on ribonucleic acid synthesis. However, Handschumacher et al (68) found no change in liver ribonucleic acid per unit liver weight in orotic acid-fed rats. In addition, Windmueller (209a) found no change in the turnover of soluble ribonucleic acid as evidenced by pseudo-uridine excretion. Therefore, the amount of cellular cytidine triphosphate used for phospholipid synthesis appears to be
much greater than that used for ribonucleic acid synthesis or the affinity of ribonucleic acid polymerase for this substrate is greater than that of phosphorylcholine-cytidyl transferase.

**EXPERIMENT 8: The Effects of Uridine-5'-Triphosphate on the Conversion of Iodine-131-HDL₃ to HDL₂ by the Mitochondrial and Microsomal Fractions of Orotic Acid-Fed Rats.**

The similarities in structure between uridine-5'-triphosphate and cytidine-5'-triphosphate and the marked increase in the ratio of the former to the latter which occurs in orotic acid-fatty liver prompted the investigation of this abnormal nucleotide ratio in vitro.

Reference to Table IX reveals that the use of an isolated high density lipoprotein fraction to study the effects of phospholipid stimulation greatly improved the percentage conversion, which approached 50 percent in this experiment. As in the previous experiment, no soluble enzymes were added and the system was supplemented with phosphorylcholine. The lack of soluble enzymes in this system prevented the transfer of phosphate from uridine triphosphate to cytidine diphosphate. Furthermore, the direct amination of uridine triphosphate to cytidine triphosphate in the presence of guanosine nucleotides and glutamine as donor as described by Kammer and Hurlbert (85) was not possible in this system.
### TABLE IX

The Effects of Uridine-5'—Triphosphate on the Conversion of Iodine-131—HDL$_3$ to HDL$_2$ by the Mitochondrial and Microsomal Fractions of Orotic Acid—Fed Rats. (Experiment 8)

<table>
<thead>
<tr>
<th>Iodine-131 Lipoprotein</th>
<th>Orotic Acid Control system (c.p.m.)</th>
<th>Uridine-5'-triphosphate Orotic Acid system (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL$_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>586</td>
<td>544</td>
</tr>
<tr>
<td></td>
<td>618</td>
<td>583</td>
</tr>
<tr>
<td></td>
<td>599</td>
<td>503</td>
</tr>
<tr>
<td></td>
<td>643</td>
<td>485</td>
</tr>
<tr>
<td></td>
<td>641</td>
<td>------- Average ------- 529</td>
</tr>
<tr>
<td></td>
<td>1210</td>
<td>2230</td>
</tr>
<tr>
<td>HDL$_3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1170</td>
<td>1860</td>
</tr>
<tr>
<td></td>
<td>1190</td>
<td>------- Average ------- 2050</td>
</tr>
</tbody>
</table>
The particular conversion studied is well suited to monitor the effect of the stimulated synthesis of phospholipids. The percentage composition of these lipoproteins from human serum is presented in the following table:

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Protein</th>
<th>Phospholipid</th>
<th>Cholesterol</th>
<th>Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL₂</td>
<td>40.6</td>
<td>36.2</td>
<td>18.7</td>
<td>4.5</td>
</tr>
<tr>
<td>HDL₃</td>
<td>56.0</td>
<td>26.2</td>
<td>12.9</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Scanu and Granada (167)

As can be seen from the above table, the phospholipid composition of these two lipoproteins differs by 10 per cent. In the absence of added uridine-5'-triphosphate it is likely that the conversion of HDL₃ to HDL₂ was limited primarily by microsomal cholesterol synthesis (Goodman et al. (59)). In the presence of added uridine triphosphate, however, significant inhibition of this conversion was found. In these experiments, the initial concentrations of uridine triphosphate and cytidine triphosphate were each 0.2 millimolar.

Von Euler et al. (199) reported increased levels of uridine nucleotides in the livers of orotic acid-fed rats. The increase was predominantly in uridylic acid though significant increases in uridine-5'-diphos-
phate were also found. Uridine-5'-triphosphate was not determined directly, but increased levels of uridine diphosphate glucose were found. The possible mechanism of the inhibition caused by uridine triphosphate may be related to the findings of Kennedy and Weiss (91) who discovered that uridine triphosphate could be converted in very small yields to uridine diphosphate choline by the same enzyme system responsible for the formation of cytidine diphosphate choline at much faster rates. Adenosine triphosphate and guanosine triphosphate were completely inactive in the system. Although Natori (126) found that tracer amounts of ethionine could be converted to ethyl-containing phospholipids, Stekol et al (185) found that ethionine was significantly inhibitory to normal methionine incorporation. Therefore, although tracer amounts of uridine-5'-triphosphate may be slowly converted to uridine diphosphate choline in these systems, when elevated ratios of uridine-5'-triphosphate to cytidine-5'-triphosphate are present there may be significant inhibition of the normal conversion of cytidine triphosphate to cytidine diphosphate choline. Also, any uridine diphosphate choline that is formed may inhibit the further reactions of cytidine diphosphate choline which lead to phospholipid formation.
EXPERIMENT 9: The Effects of Dietary Caffeine on Protein and Lipoprotein Synthesis.

Reported antagonism between caffeine and cytosine in the treatment of stomach ulcers as reported by Akinov and Pazarev (4) suggested that caffeine might also have some effect on lipoprotein biosynthesis in the liver. Accordingly, rats were maintained on a 1% caffeine diet for a period of a week after which the incorporation of amino acids into lipoproteins was determined.

Application of Students t-test of significance to the data presented in Table X indicates that there is an 85 per cent probability that the slight apparent stimulation of lipoprotein formation per unit liver weight in caffeine-fed rats is due solely to chance. In this experiment the rats fed 1 per cent caffeine along with the control diet for a period of one week weighed an average of 109 grams compared to the control weight of 187 grams. The livers from these animals also weighed only two-thirds of the control weight. The generally lower incorporation observed in this experiment may have been to several causes. The 0.04 M triethanolamine buffer employed at pH 7.8 may be inhibitory to some reaction in protein synthesis but this seems unlikely due to its similarity to tris (hydroxymethyl)aminomethane which is commonly employed. However, the buffer does have complexing ability towards divalent cations which may have been responsible for the effect. Alternately, the 0.14 M
TABLE X

The Effects of Dietary Caffeine on Protein and Lipoprotein Synthesis.
(Experiment 9)

<table>
<thead>
<tr>
<th>System</th>
<th>Total lipoproteins (c. p. m.)</th>
<th>Non-lipoproteins (c. p. m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>501</td>
<td>2200</td>
</tr>
<tr>
<td></td>
<td>466</td>
<td>2170</td>
</tr>
<tr>
<td></td>
<td>477</td>
<td>1860</td>
</tr>
<tr>
<td></td>
<td>507</td>
<td>Average 2055</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>536</td>
<td>2110</td>
</tr>
<tr>
<td></td>
<td>462</td>
<td>2140</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2090</td>
</tr>
<tr>
<td></td>
<td>Caffeine</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>539</td>
<td>2070</td>
</tr>
<tr>
<td></td>
<td>542</td>
<td>1950</td>
</tr>
<tr>
<td></td>
<td>542</td>
<td>1880</td>
</tr>
<tr>
<td></td>
<td>520</td>
<td>Average 2040</td>
</tr>
</tbody>
</table>
ammonium chloride employed after the method of Noll (130) may not have been as optimal for this nicotinamide adenine dinucleotide-containing system as the high potassium concentration used in previous experiments after the method of Webster and Lingrel (200).

The lack of a significant effect of caffeine on synthesis is not consistent with the observed failure of the caffeine-fed rats to grow and gain weight. The animals were not pair-fed so that the possibility that the rats refused to eat the caffeine diet cannot be eliminated. Also, the possibility that observed nervous disturbances were responsible for the failure to grow has not been eliminated. Peters (141) also observed weight losses in caffeine-fed rats in excess of that in pair-fed controls when food intake was reduced 43 per cent. He also found that the effect of caffeine in reducing growth became more pronounced as food intake was further reduced. This latter observation would seem to indicate that the caffeine is interfering with the normal utilization of both exogenous and endogenous purine or pyrimidine bases. The failure to find any significant of caffeine on either hepatic protein or lipoprotein synthesis may be explained by the findings of Henderson (73) who reported the presence of enzymes in liver microsomes capable of demethylating purine analogs. Whether these enzymes are also present in other tissues is not known. The findings of Akinov and Pazarev (4) showing that gastric ulcers can be induced in rats given repeated doses of caf-
feine by gastric sondi would seem to indicate that they are not. Furthermore, the remarkable reduction of ulceration following cytosine treatment may mean that interference primarily with pyrimidine utilization is involved. The effect of caffeine on the development of orotic acid fatty liver has not yet been tested, but should prove interesting. Until then the possible beneficial effects of adding cream which contains orotic acid to coffee cannot be determined.

EXPERIMENT 10: The Effects of Added Apolipoprotein on High Density Serum Lipoprotein Distribution.

That lipoproteins readily exchange lipid has been known since the work of Kunkel and Bearn (98) with phospholipids, that of Lindgren (102) with triglycerides, and that of Fredrickson et al (55) with cholesterol and cholesterol esters. Protein exchange in human high-density lipoproteins was first observed by Scanu and Page (170) and has been confirmed by Alaupovic et al (3). In contrast, the carbohydrates of high density serum lipoproteins have been found to be tightly bound to the protein moiety even after ethanol-ether extraction (Scanu et al (169a). The function of the carbohydrate in lipoproteins is unknown but it may serve to protect the lipoprotein from degradation by proteolytic enzymes just as the carbohydrate bound to the deoxyribonucleic acid of the T-even bacteriophages
protects them from host deoxyribonuclease. This experiment was de-
signed to show why amino acid incorporation into high-density lipoproteins
in orotic acid-fed rats is only minimally affected compared to the other
lipoprotein classes.

Using 50 ml. of rat serum as starting material, background solu-
tion density was raised to 1.063 through the addition, an approximately
equal volume, of concentrated potassium bromide solution. After ultra-
centrifugation for 24 hours at 36,000 r.p.m., the top ml. was removed
and the infra-natant was adjusted to density 1.250 with a sodium bromide
solution of density 1.420. Following ultracentrifugation for 40 hours at
40,000 r.p.m. the top ml. was collected. To each two ml. of this
fraction was added 10 ml. of density 1.210 cesium chloride solution.
Following further ultracentrifugation for 40 hours at 40,000 r.p.m. an
equilibrium density gradient was established which was estimated to vary
from density 1.120 at the meniscus to density 1.250 at the bottom of the
gradient tube. (Ifft et al. (82).) The top ml. of this gradient comprised
the HDL₂ fraction while the bottom 5.0 ml. of the gradient was taken as
the VHDL₁ fraction. Half of the total VHDL₁ fraction, which prior to
the work of Alaupovic et al. (3) might have been considered an apolipopro-
tein fraction, was dialyzed and labeled with iodine-131 by the procedure
mentioned earlier. Following removal of inorganic iodide the labeled
VHDL₁ was equilibrated for 1 hour at 37° C. with the unlabeled HDL₂
fraction. The mixture was then subjected to ultracentrifugation for 40 hours at 40,000 r.p.m. at density 1.125 potassium bromide. Equal amounts of the labeled HDL₂ so obtained were equilibrated at 37°C with increasing amounts of unlabeled VHDL₁ apolipoprotein for 1 hour and then separated again at density 1.125.

The reaction described above graphically may be represented by the following equation:

\[ ^{131}\text{I-HDL}_2 + \text{VHDL}_1 \rightleftharpoons ^{131}\text{I-VHDL}_1 + \text{HDL}_2 \]

Since a rapid equilibration takes place between these entities, the equilibrium concentrations are related by:

\[ \frac{^{131}\text{VHDL}_1}{^{131}\text{HDL}_2} \frac{\text{HDL}_2}{\text{VHDL}_1} = K_{eq} \]

Rearrangement of this equation yields the relation:

\[ \frac{^{131}\text{VHDL}_1}{^{131}\text{HDL}_2} = \frac{K_{eq}}{\text{HDL}_2} \cdot (\text{VHDL}_1) \]

This relation is largely borne out by the following graph. The situation which exists in orotic acid-fed rats can best be illustrated by reversing the labeled and non-labeled components in the above discussion. The
MICROLITERS OF NON-LABELED VHD\textsubscript{1} APOLIPOPROTEIN ADDED

C.P.M. (VHD\textsubscript{1}) / C.P.M. (VHD\textsubscript{2})
apolipoprotein synthesized normally exchanges with the high density lipoproteins in serum so that amino acid incorporation into high density lipoproteins is only marginally reduced compared to the other lipoprotein classes. Thus, Roheim et al (155) found a 56 per cent drop in the quantity of high density lipoproteins in orotic acid-fed rats but only a 9 per cent drop in the specific activity of this fraction. From their results it may be calculated that:

\[
\frac{\text{Rate of VHDL}_1 \text{ exchange}}{\text{Rate of VHDL}_1 \text{ synthesis}} = \frac{.91}{.46} \sim 2
\]

Assuming that the time required to synthesize a polypeptide in vertebrate system is about 3 minutes at 37\(^o\) C., it can be calculated that the labeled and unlabeled peptides are half-equilibrated in 1.5 minutes.

In the actual exchange process, a small lipid-rich peptide fragment is transferred. In the case of human serum lipoproteins, the minimum molecular weight of this fragment may be calculated as 207,000 of which 40,000 is protein. No attempt was made to isolate this fragment whose ratio of lipid to protein is comparable to that found in low-density lipoproteins. The role of these lipid packets in lipid transport is completely unknown and any function that may be ascribed to them in the mobilization of triglyceride from the liver is purely hypothetical. However, their isolation and the study of their exchange among other lipoproteins,
specifically chylomicrons, should prove invaluable contributions to the present knowledge of lipid transport.

**EXPERIMENT 11:** The Thermodynamics of the Conversion of VHDL₁ Lipoproteins to HDL₃ Lipoproteins.

In the previous discussion VHDL₁ was considered as an apolipoprotein because it has a hydrated density greater than 1.210 and is found in a density fraction formerly considered devoid of lipoproteins. Actually, it is a lipoprotein. The characteristic lipid compositions of these two title lipoproteins has been determined by Alaupovic et al (3) and are compared below:

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Lysolecithin</th>
<th>Sphingomyelin</th>
<th>Lecithin</th>
<th>Cephalin</th>
<th>Phos-inositol</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL₃</td>
<td>1.9</td>
<td>10.6</td>
<td>81.4</td>
<td>1.9</td>
<td>5.0</td>
</tr>
<tr>
<td>VHDL₁</td>
<td>3.5</td>
<td>11.8</td>
<td>71.6</td>
<td>2.9</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Since additional data had indicated that both lipoproteins consisted of the same peptide backbone of molecular weight close to 75,000 surrounded by different lipid shells, an attempt was made to study the thermodynamic relations involved in the conversion of VHDL₁, molecular
weight 153,000 to HDL\textsubscript{3}, molecular weight 175,000.

Rat serum was fractionated by the following technique. Its density was increased to 1.125 with potassium bromide and all floating lipoprotein were separated by ultracentrifugation for 40 hours at 40,000 r.p.m. The density of the infranatant was then increased to 1.250 with a concentrated solution of sodium bromide. Following an identical ultracentrifugation the top ml. was diluted to density 1.210 and centrifuged as before. The top ml. from this last ultracentrifugation was taken as the HDL\textsubscript{3} fraction while the bottom 5.0 ml. was taken as the VHDL\textsubscript{4} fraction. The VHDL\textsubscript{4} fraction was then dialyzed and labeled with iodine-131 as in previous experiments. This fraction was dialyzed against 0.1 M triethanolamine buffer, pH 7.4, before use.

A mixture of several commercial animal and vegetable oils including cottonseed oil, corn oil, olive oil, sperm oil, and Mazola and Wesson Oils was mixed with aqueous 0.1 N triethanolamine buffer, pH 7.4 and subjected to ultrasonication at 250 watts and 10 kilocycles for a period of 15 minutes in the Raytheon sonic oscillator, at a temperature of 15° C. The resulting mixture consisted of a fully emulsified and a partially emulsified lipid mixture which was easily separated in a separatory funnel. The rational behind the above procedure was to obtain the most stable, saturated emulsion in the buffer employed. This state was arbitrarily chosen as the standard state of the lipids in the absence
of lipoprotein. The emulsion so prepared was milky in appearance and consistency and showed no tendency to separate over a period of 48 hours.

In the next phase of the experiment 0.8 ml. of the iodinated VHDL\textsubscript{4} was mixed with 3.0 ml. of lipid emulsion at temperatures of 0.2, 10.2, and 20.2\degree C. in 4.0 ml. ultracentrifuge tubes in the Spinco number 40 rotor. After complete equilibration, usually 30 minutes after the reactants had been mixed, the emulsion was broken by centrifugation at 36,000 r.p.m. for a period of 15 minutes. The top lipid layer was removed with the aid of a tube slicer and 1.0 ml. aliquots of the infranatant were adjusted to 12.0 ml. and density 1.210 with a potassium bromide solution. Following ultracentrifugal separation again for 40 hours at 40,000 r.p.m., the top and bottom ml. were collected. The protein in these fractions was precipitated with 5.0 ml. of 6.0 per cent trichloroacetic acid and assayed by liquid scintillation spectrometry. The ratio of activity in the top ml. to that in the bottom ml. at the various temperatures employed is given below:

<table>
<thead>
<tr>
<th></th>
<th>HDL\textsubscript{3}</th>
<th>0.2\degree</th>
<th>10.2\degree</th>
<th>20.2\degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHDL\textsubscript{4}</td>
<td>0.443</td>
<td>0.670</td>
<td>0.510</td>
<td></td>
</tr>
</tbody>
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The decrease in the fraction of VHDL\textsubscript{4} converted at 20.2\degree C. was
taken as evidence of denaturation although non-ideal behavior was not eliminated. The value at this temperature was not used in the thermodynamic calculations. For simplicity, the reaction was considered to take place according to the following equation:

\[ \text{VHDL}_1 + \text{Lipid} \rightleftharpoons \text{HDL}_3 \]

The equilibrium constant for the above reaction is given by

\[ K_{eq} = \frac{(\text{HDL}_3)}{(\text{VHDL}_1)(\text{Lipid})} \]

Since the saturated lipid emulsion was chosen as the standard state, the concentration of lipid must be corrected for the dilution which occurred on mixing. Correction for this is performed by using 3.0/3.8 or 0.791 as the lipid concentration. For the calculation of the standard free energy of the reaction at 0°C:

\[ \Delta G = -2.303 \cdot RT \log \frac{0.443}{0.791} = 100 \text{ cal.} \]

The standard enthalpy change was calculated from the change of the equilibrium constant at the temperatures of 0°C and 10.2°C respectively using the integrated form of the Gibbs-Helmholtz equation:
\[
\log \frac{0.0670}{0.0443} = \frac{\Delta H}{2.303 \, RT} \left( \frac{1}{273.2^\circ} - \frac{1}{283.2^\circ} \right)
\]

from which: \( \Delta H = 6340 \text{ cal.} \)

Finally, the entropy change for the reaction at \(0.2^\circ \text{C.}\) was calculated from the relation:

\[
\Delta S = \frac{\Delta H - \Delta G}{T} = \frac{6240 \text{ cal.}}{273.2^\circ \text{C.}} = 22.8 \text{ e.u.}
\]

These thermodynamic determinations are based on the assumption that the percentage recovery of both lipoproteins during ultracentrifugation was the same. Although the tubes were not at strict equilibrium, they approached equilibrium closely. In addition, although the force exerted on the HDL_3 molecules at the meniscus is lower than that exerted on VHDL_1 molecules at the maximum radius of rotation, this difference is partially compensated by the larger molecular weight of HDL_3 versus VHDL_1.

The results indicate that the conversion of VHDL_1 to HDL_2 is essentially reversible at \(0^\circ \text{C.}\). At body temperature, the HDL_2 molecule would tend to spontaneously release its lipid load to the tissues, especially to those tissues which are actively metabolizing. In summary, although blood has properties far removed from simple buffer solution and
the state of lipids in the tissues is different from the emulsion used in the above experiment, the general behavior determined from thermodynamics is what one would expect of a physiological lipid-carrying protein.

**EXPERIMENT 12: The Effects of Cytidine-5'-Diphosphate and Cytidine-5'-Triphosphate on the Conversion of Phosphorylcholine to Cytidine Diphosphate Choline by Enzymes from the High-Speed Supernatant from Control and Orotic Acid-Fed Rats.**

In Experiment 8 it was shown that elevated ratios of hepatic uridine-5'-triphosphate to cytidine-5'-triphosphate could inhibit the utilization of the latter compound for phospholipid synthesis. This elevated ratio occurs as a result of decreased cytidine-5'-triphosphate concentration, and not as a result of increased uridine-5'-triphosphate concentration so that it cannot be the cause of the fatty liver.

The reduced concentration of cytidine-5'-triphosphate may be produced either through an interference with the de novo formation of cytidine-5'-triphosphate from uridine-5'-triphosphate, or through interference with the successive phosphorylation of cytidylic acid to cytidine-5'-triphosphate. A decision between these possibilities can be made based on the fact that the enzymes responsible for the successive phosphorylation of cytidylic acid to cytidine-5'-triphosphate are the same as those
responsible for the successive phosphorylation of uridylic acid to uridine-5'-triphosphate. The slightly decreased concentrations of hepatic uridine-5'-triphosphate found in orotic acid-fatty-liver, in spite of the increased concentrations of uridylic acid and uridine-5'-diphosphate suggest that there is an interference in the further phosphorylation of these pyrimidine nucleoside diphosphates. The fact that this interference persists when the decarboxylation of orotidylic acid is inhibited by 6-aza-uridylic acid further suggests that orotidylic acid itself can cause such an interference when present in the relatively high concentrations found in orotic acid fatty liver.

The lack of mitochondrial changes in orotic acid-fatty liver is also consistent with the inhibition of the conversion of nucleoside diphosphates to the corresponding triphosphates by the enzyme nucleoside diphosphate kinase (2.7.4.6). If the conversion of nucleoside monophosphates to the corresponding diphosphates were inhibited, then mitochondria, which do not carry out such a conversion and which are dependent upon a cytoplasmic supply of the nucleoside diphosphates, should be altered. The fact that the mitochondria from orotic acid-fatty livers are normal supports the suggested mechanism.

If it is assumed that cytidine-5'-triphosphate is limiting in normal liver, and especially in orotic acid fatty-liver in the synthesis of cytidine-diphosphate choline, and furthermore, that the phosphorylation of
cytidine-5'-diphosphate to cytidine-5'-triphosphate is inhibited in orotic acid fatty liver, then, by the previous considerations of enzyme kinetics, one would expect the addition of a constant amount of cytidine-5'-diphosphate to produce a greater percentage stimulation of cytidine diphosphate choline formation in the control rat than in the orotic acid fed rat. This expectation is valid and the effect is even enhanced in the presence of elevated concentrations of orotidylic acid, the suggested inhibitor of the phosphorylation, and is equivalent to the expectation of elevated levels of cytidine-5'-diphosphate in orotic acid fatty liver. Circumstances did not permit the presentation of the results of the proposed experiment here, but they are presented in the appendix.
Many investigators have confirmed the production of fatty liver in the rat which occurs within one week after the inclusion of one per cent orotic acid in the diet. In addition, Roheim et al (154) have shown that the apolipoprotein of beta-lipoproteins is synthesized normally in the orotic acid-fed rats. Nevertheless, triglycerides rapidly accumulate in the liver. There is increased hepatic synthesis of cholesterol and reduced synthesis of fatty acids (Windmueller and Spaeth, (210) ) probably caused by the triglyceride build-up (Tzur and Shapiro, (196) ). In spite of the increased lipids orotic acid fatty livers secrete less triglyceride, phospholipid and cholesterol than normal livers. Creasey et al (36) have further shown that phospholipid synthesis is reduced in orotic acid-fed rats.

Following a study of various types of fatty liver and cognizant of the importance of phospholipids in lipoprotein structure, a hypothesis was advanced that orotic acid fatty liver might be due to interference in the synthesis of phospholipid. A study of phospholipid synthesis in the liver revealed that cytidine-5'-triphosphate was required in all pathways of phospholipid formation. Since orotic acid is a precursor of cytidine-5'-triphosphate, their precise interrelationships were further investigated.
An in vitro experimental method enabled the elimination of orotic acid itself as the cause of the fatty liver. This result concurred with the failure of individuals affected with hereditary orotic aciduria to develop fatty livers despite the presence of a ten-fold increase of orotic acid which is found in their urine. This genetic disease is characterized by reduced levels of the enzymes, orotidine-5'-phosphate and orotidylic acid decarboxylase, which are necessary for the further metabolism of orotic acid. A metabolite of orotic acid was therefore sought as the responsible agent.

Reported high concentrations of orotidylic acid and uridylic acid, as well as elevated levels of other uridine nucleotides in orotic acid fatty livers, because of their close relationship to the cytidine nucleotides, suggested that an investigation of these nucleotides on the conversion of apolipoproteins to lipoproteins might prove fruitful. Furthermore, the ability to isolate relatively phospholipid-poor high density lipoproteins and to label them with radioactive iodine enabled the study of the effects of these nucleotides independently of the process of protein synthesis. These lipoproteins were found to respond well to net increases in phospholipid synthesis produced by the addition of cytidine-5'-triphosphate.

At this point, it was found that in vitro systems from orotic acid-fed rats responded much more to cytidine-5'-triphosphate addition than did similar control systems. From this observation it was concluded
that orotic acid fatty livers had lower levels of this nucleotide than normal livers, or that there was some interference in the utilization of cytidine-5'-triphosphate in the orotic acid fatty livers. The observed block in the utilization of cytidine-5'-triphosphate could be due to a decrease in its production, either de novo from uridine-5'-triphosphate and glutamine or through phosphorylation of cytidylic acid and cytidine-5'-diphosphate. The increase in liver uridine diphosphate glucose and the increase in liver glycogen found in orotic acid fatty liver suggested increased levels of uridine-5'-triphosphate which, all other factors being the same, should result in greater synthesis of cytidine-5'-triphosphate via the de novo pathway. The possibility that high levels of uridine-5'-triphosphate might be inhibitory to the utilization of cytidine-5'-triphosphate was investigated.

The addition of uridine-5'-triphosphate at equimolar concentrations was found to be inhibitory to the utilization of cytidine-5'-triphosphate in vitro. This inhibition was evidenced principally through failure of apolipoprotein to be converted to lipoproteins. That this inhibition is of a competitive nature was shown in an experiment in which excess cytidine-5'-triphosphate was added to both control and orotic acid preparations. In this instance, the conversion of apolipoprotein to lipoprotein in the orotic acid system exceeded that in the control system. This result may be taken to mean that, under circumstances in which cytidine-5'-
triphosphate is not limiting, the supply of D-1,2-diglyceride also becomes a limiting factor in control rats governing phospholipid synthesis while the increased phosphatidic acid which accompanies fatty liver may be taken to mean that diglyceride is not limiting in the orotic acid system under similar conditions.

A possible mechanism of the inhibitory effect of uridine-5'-triphosphate has been suggested by the work of Kennedy and Weiss (91). They found that phosphoryl choline-cytidyl transferase could form significant amounts of uridine diphosphate choline when uridine-5'-triphosphate was substituted for its cytidine analog. Presumably, this uridine derivative could also be formed when the ratio of uridine-5'-triphosphate to cytidine-5'-triphosphate is elevated as it is in orotic acid-fatty liver. Once formed, it would then be available to inhibit competitively the conversion of cytidine diphosphate choline to lecithin and sphingomyelin, phospholipids essential to the structure of lipoproteins.
CHAPTER V

CONCLUSION

In the absence of evidence indicating that cytidine-5'-triphosphate is limiting in ribonucleic acid synthesis, the lack of an effect of orotic acid fatty liver on ribonucleic acid turnover cannot be taken as evidence that the production of cytidine-5'-triphosphate is not reduced. Reports that 6-azauridine-5'-diphosphate was a potent inhibitor of the incorporation of cytidine-5'-triphosphate into the terminal sequence of transfer RNA (Rychlik, (158)) combined with the reports of Handschumacher indicating that 6-azauridylic acid was a potent inhibitor of orotidylic acid decarboxylase suggested the possibility that orotidylic acid might be inhibitory to the phosphorylation of cytidine-5'-diphosphate and uridine-5'-diphosphate by the enzyme nucleotide diphosphate kinase. Such an inhibition of the phosphorylation of uridine-5'-diphosphate would be more than offset by the high substrate levels of this nucleotide produced from the large excess of uridylic acid through the action of the enzyme nucleoside monophosphate kinase. The resulting competitive effects of the increased orotidylic acid and uridine-5'-diphosphate would effectively reduce the phosphorylation of cytidine-5'-diphosphate. The commercial unavailability of the product has hampered the testing of this possible direct effect of orotidylic acid. The ability of mitochondria to convert uridine and cy-
tidine diphosphates to the corresponding triphosphates and their inability to phosphorylate the corresponding nucleoside monophosphates, combined with the lack of mitochondrial morphological alterations in orotic acid fatty liver, tends to eliminate any inhibition of the production of the di-phosphates by soluble cytoplasmic enzymes.

An indirect approach was therefore taken in the study of the phosphorylation of cytidine-5'-diphosphate to cytidine-5'-triphosphate in 100,000 x g. supernatants from control and orotic acid-fed rats. The cytidine-5'-triphosphate produced was assayed by enzymic conversion to radioactive cytidine diphosphate choline. An argument derived from enzyme kinetics was used to show that decreased percentage stimulation of cytidine diphosphate choline formation in orotic acid preparations compared to control preparations upon the addition of equal quantities of cytidine-5'-diphosphate to each in the presence of excess adenosine-5'-triphosphate could be taken as evidence of increased levels of cytidine-5'-diphosphate relative to cytidine-5'-triphosphate in orotic acid-fed rats and hence, of inhibition of phosphorylation of cytidine-5'-diphosphate.

The results of the phosphorylation study are not yet available. Regardless of its outcome, the inhibitory effect of uridine-5'-triphosphate on cytidine-5'-triphosphate utilization must be viewed as a new factor contributing to orotic acid-fatty liver. Assuming the absence of
phosphorylation effects, the administration of uridine in place of orotic acid should result in fatty livers as serious as those produced by orotic acid itself. This confirmatory experiment has not yet been completed. The failure of Standerfer and Handler (184) to produce fatty liver by uracil administration may have been due to the poorer utilization of the free pyrimidine bases for nucleotide formation as compared to the corresponding nucleosides (Hammarsten, et al (65).

As an adjunct to these experiments, the inhibitory effects of caffeine on the utilization of cytidine-nucleotides were investigated and found to be negligible in the liver as judged by protein and lipoprotein synthesis. Effects of caffeine on pyrimidine utilization in other tissues were not investigated.

The roles of the various classes of lipoproteins in the mobilization of liver fat has not been well defined, although the lipotropic effects of phosphatidyl choline and other phospholipids have been apply verified. Approximately all of the plasma cholesterol and phospholipid is synthesized in the liver which is also the major site of degradation of plasma phospholipids. The specific function of lipoproteins appears to be in the transport of long-chain fatty acids containing over fourteen carbons as opposed to the shorter chain fatty acids which are readily transported bound to serum albumin. The temporary storage of these long chain fatty acids as chylomicron triglycerides saves the liver the trouble of
having to break down the bulk of ingested fatty acids to meet immediate energy demands only to resynthesize them later. This mechanism results firstly in the efficient removal of the lipids from the intestinal absorption sites. Secondly, the shorter length fatty acids are degraded in preference to the long chain acids. Thirdly, the depot lipids predominantly reflect the type of long chain acid present in the diet. When presented with an excess of fatty acid over and above immediate energy demands the liver synthesizes triglycerides and the phospholipid required to stabilize them in the form of low density lipoproteins. These lipoproteins circulate in plasma where, according to the release of tissue lipases, they release the energy containing fatty acids to acceptor albumin and thence to the tissues. A similar rise in low density lipoproteins is seen during starvation and is due to the excessive hormonal release of fatty acids from adipose tissue to the liver where triglycerides are formed and secreted. Although this manner of transferring fatty acid from the depots to the tissues is indirect, it allows a balance to obtain between fatty acid release and fatty acid utilization and storage. Furthermore, when energy demands are great, the fatty acid leaving the depots may be utilized directly without going through the triglyceride transformation in the liver.

Existing evidence implicates high density lipoproteins in the transport of cholesterol esters. These esters form the predominant lipid
which accumulates in the tissues in Tangier's disease in which there is congenital absence of high density lipoproteins. This function correlates with the cholesterol esterifying activity found in serum high density lipoproteins by Lossow (104). Another function of high density lipoproteins appears to be that of serving as phospholipid sinks. In Tangier's disease, for example, there is an increase in very low density lipoproteins in addition to the absence of high density lipoproteins. This rise is probably due to the absence of the reaction:

\[ \text{VLDL} + \text{VHDL}_1 \rightarrow \text{LDL} + \text{HDL}_3 \]

This reaction corresponds to the transfer of cholesterol esters from low density to high density lipoproteins. The involvement of high density lipoproteins in chylomicron formation suggests that a similar reaction exists for the transfer of phospholipids. Such a reaction might be:

\[ \text{HDL}_2 + \text{Beta-apolipoprotein} \rightarrow \text{HDL}_3 + (\text{VHDL})_2 + \text{Beta-lipoprotein} \]

The reverse of this reaction would occur on the hydrolysis of the chylomicrons, the phospholipids involved reverting to the high density lipoproteins.

When discussing mobilization of liver lipid, some consideration must be given to the form in which the lipid leaves the site of synthesis. If the chemical potential change involved in the combination of apolipoproteins (low density) with lipid were not negative, there would be little
tendency of lipid to be mobilized from the liver. In orotic acid-fatty liver there is no deficiency of triglyceride and beta-apolipoprotein, yet there is decreased mobilization of lipid. The role of charged phospholipids in combining with neutral triglycerides and thereby raising the chemical potential of the triglycerides in the lipid phase and enabling the combination of the resulting lipid complex with lipoproteins through ionic forces to occur with a decrease in free energy must be important in lipid transport. Phospholipids appear to be more stable in low density lipoproteins than in high density lipoproteins as evidenced by the greater ease with which they may be extracted from the latter. Still, the phospholipids are more stable in the structure of high density lipoproteins than they would be in an emulsion. These differences in phospholipid stability are basic to the function of high density lipoproteins which donate their phospholipids for chylomicron formation but recover them when the chylomicrons are degraded.

The difference in the stabilities of phospholipids when bound to low and high density lipoproteins reflects structural differences in the lipoproteins. Cook and Martin (33) in an analysis of the composition of many lipoproteins concluded that there were two basic types of structures: Those with protein contents below 33 per cent (LDL) having neutral lipid-phospholipid ratios from 1:1 to 10:1 and; those having protein contents above 33 per cent in which the neutral lipid-phospholipid ratio
remains close to 1:1 (HDL). The low density structures were found to resemble micelles with a relatively large amount of occluded water and a surface covering of protein.

Substantiating the role of phospholipids in lipid mobilization, Salem (162) concluded that electrostatic forces were of prime importance in bringing and holding together the protein and lipid components of lipoproteins but that London-Van der Walls dispersion forces were of prime importance in maintaining micellar structure such as is found in low density lipoproteins. The increase in these dispersion forces with fatty acid length may partially explain the presence of such acids in low density lipoproteins. Further evidence of differences in phospholipid binding is indicated by the work of Williams (207a) who found that phospholipase D was able to attack low density lipoproteins but not high density lipoproteins. While extraction of cholesterol from the high density lipoproteins with ether does not enable phospholipase A (A Naja Naja venom) to attack them, this treatment enables phospholipase D to liberate 88 per cent of the choline present.

A subunit structure has been proposed for high density lipoproteins by Scanu and Granada (167). This structure may be visualized as ellipsoid subunits bridged by lipid molecules. HDL3 and VHDL4 contain five of these basic units while HDL2 contains seven. Attachment of phospholipids to HDL3 is associated with the concurrent gain of a peptide
fragment of approximately 40,000 molecular weight. This fragment is
decomposed of two basic subunits. That lysine residues are important in
the cohesion of the subunits is indicated by the ready disassociation of
the structure by succinic anhydride (Scanu et al (167, 171)).

The change in the structure of HDL₃ in accepting phospholipids in-
volves the change in a close packing arrangement containing five subunits
to an arrangement containing seven subunits. The exchange experiment
performed in the course of this work indicates that such a conversion is
constantly occurring. The possibility of being able to detect this molecu-
lar shift through infra-red absorption or other means would shed light on
the nature of the bond between the subunits.

In 1958 Wilgram (204) found that atherosclerosis could be produced
in rats by feeding a choline-deficient diet. Conceivably, a defect in cho-
line utilization could produce the same effect. The above study has
shown that an abnormality induced in nucleotide metabolism can affect
lipid metabolism. The precise physiological importance of the effect of
dietary orotic acid cannot be stated. However, the inhibition of liver li-
pid mobilization is probably of greatest importance to the infant who sub-
sists wholly on milk which contains significant quantities of orotic acid.
The ability of infants to synthesize orotic acid de novo may not be fully de-
veloped so that the orotic acid serves as an important nucleotide pre-
cursor. In addition, the infant has some lipid stores at birth and the inhibition of liver lipid mobilization may enable the liver to retain the lipid energy which is utilized for rapid growth.
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In contrast to the effect of orotic acid in producing fatty liver when administered in the diet, the addition of orotic acid to an in vitro preparation from rat liver was without effect on lipoprotein biosynthesis. This result indicated that some metabolite of orotic acid, rather than orotic acid itself, was primarily responsible for orotic acid-fatty liver.

Thermodynamic studies on the conjugation of lipid with protein proved the ready reversibility of the reaction thereby indicating that the lack of the equilibration of lipid, protein, and lipoprotein was due in part to the absence of some lipid required in the reaction. The fact that this lack of equilibration in orotic acid-fed rats was completely overcome in vitro by phosphatidyl choline indicates that the critical lipids are phospholipids. Furthermore, the ability of cytidine-5'-triphosphate to reverse the effect by stimulating phospholipid synthesis shows that the trace levels of cytidine-5'-triphosphate in orotic acid-fed rats are directly related to the development of the fatty liver.

From a study of the paradox in pyrimidine metabolism which occurs in orotic acid-fed rats, namely, that the oral administration of a precursor of cytidine-5'-triphosphate leads to a decrease in its hepatic con-
centration, the hypothesis was advanced that the increase in orotidyllic acid, evidenced in orotic acid-fed rats by increased excretion of orotidine, inhibits the conversion of uridine-5'-diphosphate to uridine-5'-triphosphate and the conversion of cytidine-5'-diphosphate to cytidine-5'-triphosphate by the enzyme nucleoside diphosphate kinase. The inhibition of the conversion of uridine-5'-diphosphate to uridine-5'-triphosphate is almost completely overcome by elevations in the substrate level of uridine-5'-diphosphate so that the de novo synthesis of cytidine-5'-triphosphate from uridine-5'-triphosphate is unimpaired. On the other hand, the ability of the liver to regenerate the catalytic coenzyme cytidine-5'-triphosphate is so reduced that phospholipid synthesis decreases and fatty liver develops. This hypothesis is consistent with all the experimental findings on orotic acid-fatty liver.

A direct test of the above hypothesis involving the preparation of a purified enzyme free from orotidyllic acid decarboxylase activity was impossible because of time limitations and was therefore beyond the scope of this thesis.
APPROVAL SHEET

The thesis submitted by Theodore S. Musiala has been read and approved by the undersigned member of the faculty of the Loyola University Stritch School of Medicine who served as director of the research program.

The final copies have also been examined and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

May 26, 1967

Date

Signature of Adviser

Hugh J. McDonald