LIPOPROTEIN SECRETION BY THE REGENERATING RAT LIVER

ABSTRACT

Ph.D. Biochemistry

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Lipoprotein Synthesis and Secretion by
Rat Liver Following Partial Hepatectomy

Incorporation of intravenously administered palmitic acid 9,10-^{3}H into ß-lipoprotein steadily increases for the first 20 hours of hepatic regeneration. Thirteen hours after partial hepatectomy the concentration of circulating ß-lipoprotein is at a normal level, but the specific activities of liver and ß-lipoprotein-triglyceride were significantly diminished. During perfusion the 13 hour regenerating liver secretes quantities of ß-lipoprotein-triglyceride which exceed those released by a zero hour liver although there is no difference in liver weight. Equal quantities of protein were released by both livers. Triglyceride specific activity in the liver and perfusate ß-lipoprotein was diminished, but the protein was not affected. Prelabelling of the accumulated triglyceride with palmitate-1-^{14}C *in vivo* showed that the per cent release by the 13 hour regenerating liver was equal to that of the control tissue, while that of a CCl_4 treated liver was 1/20 of that level. Liver and perfusate triglyceride specific activities were similar for normal and regenerating livers, indicating one continuous triglyceride pool.

The 13 hour regenerating liver was secreting quantities of lipid which were on a par with that of the larger normal liver. This was accomplished by increasing the lipid to protein ratio in the ß-lipoprotein molecule. The results are discussed in relation to the possible controlling mechanisms affecting lipid storage and secretion in the premitotic stage of regeneration.

LIPOPROTEIN SYNTHESIS AND SECRETION BY RAT LIVER FOLLOWING PARTIAL HEPATECTOMY

by

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TABLE OF CONTENTS

PREFACE

The investigations concerning the steatosis of regeneration which will be described in these pages form part of the program of lipoprotein research carried out in this laboratory. Dr. Rubinstein first became involved in this general area of study some 6 years ago when investigations into the problems of carbon tetrachloride intoxication revealed that the hepatotoxin caused an inhibition in the secretion of lipoprotein. Since that time several graduate students under his direction have attempted to elucidate some of the basic mechanisms involved in the assembly and release of lipoprotein. Radioactive precursors have been extensively employed in these studies for measuring synthesis and secretion of the various lipoprotein species. The well known inhibit or of protein synthesis, puromycin, has proved to be an invaluable tool for investigating the coupling of the lipid and protein moieties within the liver cell.

By extending these investigations to a study of the underlying causes of the lipid accumulation following partial hepatectomy, it was hoped to obtain some further insights into the mechanism of lipoprotein secretion. The experiments to be described in this thesis will compare the regenerating liver with a normal and carbon tetrachloride treated liver with regard to the metabolism of both lipid and protein. The findings will be discussed in relation to current concepts of lipoprotein formation and secretion.

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ABBREVIATIONS

VLDL	Very low density lipoprotein
LDL	Low density lipoprotein
HDL	High density lipoprotein
d < 1.063	Density less than 1.063
TG	Triglyceride
PL	Phospholipid
CE	Cholesterol esters
CH	Cholesterol
FFA	Free fatty acid
GLY	Glycerol
AcCoA	Acetyl Coenzyme A
CCl_4	Carbon Tetrachloride
NAD	Nicotine Adenine Dinucleotide
RNA	Ribonucleic Acid
ATP	Adenosine Triphosphate
ACP	Acyl Carrier Protein
ACTH	Adrenal corticotropic hormone
TSH	Thyroid stimulating hormone
TCA cycle	Tricarboxylic Acid Cycle
CDP	Cytidine diphosphate
TCA	Trichloroacetic acid
PEP	Phosphoenolpyruvate
PE	Phosphatidyl ethanolamine

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

It has long been recognised that the liver plays a central role in lipid metabolism (review, 1). Consequently, the uptake, transformation, synthesis and secretion of lipid with its affiliated protein and carbohydrate components have been the subject of intensive investigation in recent years (review, 2). In many of these investigations the pathologically fatty liver has proved to be an invaluable tool both for elucidating the basic mechanisms on a molecular level as well as correlating these processes with the morphology of the cell (review, 3). Since the experiments to be described below concerning the steatosis of regeneration are closely related to investigations in the literature concerning other types of fatty liver , a discussion of fatty liver pathogenesis will be undertaken. As a preliminary to this, the metabolism of lipid in the normal liver will first be reviewed with particular reference to the problem of transport. The Introduction will be completed with a synopsis of what is currently known about the fate of lipids in the regenerating liver.

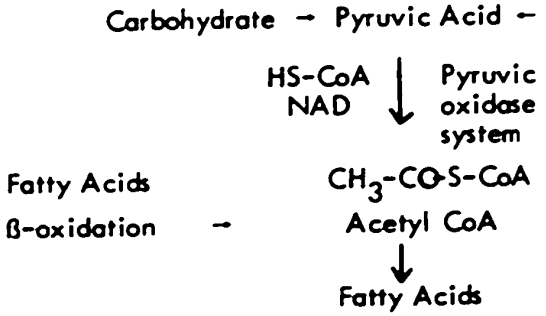
1. Metabolism of Lipid in the Liver

Free fatty acid is a primary precursor in the synthesis of triglyceride and phospholipid (4). Since the concentration of glycerol and fatty acid in the cell directly affects the formation of glycerides (5-8), the origin and metabolic fate of free fatty acids in the liver will first be discussed. The endogenous source of fatty acid can either be actual synthesis (lipogenesis) in the liver or uptake from the circulation as a result of mobilisation from adipose tissue. The metabolic fate of the free fatty acids will later be dealt with in relation to their oxidation and esterification. Finally the secretion from the liver of the completed product in the form of lipoprotein will be considered with particular reference to the controlling factors involved.

a) Sources of Fatty Acids

Lipogenesis

Fatty acids can be synthesised in mammalian tissues from any substance in the body capable of forming acetate. These include carbohydrates, some amino acids, ethyl alcohol as well as other fatty acids:



The conversion of acetyl CoA to fatty acid is achieved by a series of stepwise reactions which involve bicarbonate and a number of different enzymes (9). A critical appraisal of the experiments which elucidated these reactions has already been made (9). The scope of this introduction therefore will be confined to emphasising some possible controlling factors involved in lipogenesis.

Acetyl CoA carboxylase catalyses the conversion of acetyl CoA to malonyl CoA in the presence of bicarbonate, ATP, Mn^{++} and biotin. This is the initial step in the conversion of acetate to long chain fatty acids. The reaction has been shown to be rate limiting *in vitro* thus suggesting that the effectors of the enzyme may regulate fatty acid synthesis (10).

Stimulation of the carboxylase enzyme can be achieved by the addition of tricarboxylic acid cycle intermediates to the system (11). Citrate and isocitrate are the most effective tricarboxylic acids (10). It has been shown that palmityl CoA, an inhibitor of the enzyme is competitive with citrate (12). However, there is no evidence as yet that citrate stimulation has any physiological significance *in vivo*.

Fatty acid synthetase which catalyses the condensation of malonyl CoA with acetyl CoA is also inhibited by palmityl CoA (13). However, it is difficult to interpret the physiological significance of this inhibition since the compound affects a variety of other enzymes in a similar manner (14). Of particular interest to the subject matter of this introduction is the finding that the levels of both of these enzymes decrease in starvation and diabetes to increase again with refeeding

and insulin treatment, respectively (15,16).

It has been postulated by Lynen that the reactions of fatty acid synthesis occur with the substrates bound to the synthetase enzyme (17). This is the Acyl Carrier Protein (ACP). Transacylase enzymes which catalyse the coupling of these compounds to the ACP have been isolated from E. coli (18,19). This carrier protein has been shown also to be involved in the synthesis of lyso-phosphatidic acid (20). Thus the function of a carrier protein, even at the early stages of lipid metabolism, has been well established.

Mobilisation

Free fatty acids are mobilised by lipolysis of triglycerides in adipose tissue. The rate at which the breakdown of triglyceride occurs is regulated by many nutritional and endocrin factors (21,22). The action of such lipolytic agents as epinephrine, norepinephrine, ACTH, glucagon and TSH is mediated by cyclic AMP (23). The nucleotide itself has effective activity in vitro (23).

Since the antilipolytic agent, insulin, can lower intracellular levels of cyclic AMP (24), it is possible that its activity is also mediated through this compound. The action of nicotinic acid on the lipase is similar to insulin, and is antagonistic to epinephrine.

Promotion of lipolysis by growth hormone appears to proceed by means of a different mechanism that the other hormones. The effect is secondary to an induction of RNA synthesis (25). ACTH, on the other hand, has an immediate

action. The possibility exists, therefore, that growth hormone is required for the formation of the lipase. It has been shown, for example, that the presence of an intact pituitary gland is necessary for the normal response of adipose tissue to epinephrine, corticotropin, thyrotropin and to fasting (26).

The free fatty acids thus formed by hydrolysis of the triglyceride may then leave the fat cell provided a fatty acid acceptor such as albumin is present in the medium (27). The rapidity with which the fatty acids leave the cell is determined by the availability of free fatty acid binding sites on albumin and other extracellular protein (28). Therefore, the continued mobilisation depends upon continuous uptake by the other tissues, which, in turn, is dependent on esterification (29) and is proportional to the concentration in the plasma (7,30, 31,32).

b) Metabolic Fate of Free Fatty Acid

Oxidation

Fatty acids are broken down into two carbon units as acetyl CoA by an overall process known as "ß-oxidation". The detailed mechanisms have been worked out by many investigators over a long period of time. This work has been reviewed by Green (33), Lynen (34) and Kennedy (35).

The oxidation of fatty acid takes place in the mitochondria of the cell (33). The acetyl CoA molecules split off in the oxidative steps are normally oxidised in the TCA cycle. Generally none of the intermediates of ß-oxidation

accumulate. However, when carbohydrate utilisation is severely restricted, or when fatty acid metabolism is elevated to supply the cell's energy needs, the ketone bodies acetoacetate and its reduced derivatives accumulate. This is the case, for example, in starvation (36), high fat diets and diabetes (37).

An important stimulant in fatty acid oxidation is carnitine (38,39). It has been shown that fatty acyl esters of carnitine serve as intermediates in this stimulation (39). The theory may therefore be brought forward that perhaps the carnitine is acting as a carrier for the acyl chain to transport it from the soluble portion of the cell into the mitochondria where oxidation occurs. In support of this hypothesis, it has been found that mitochondria membrane is impermeable to esters of coenzyme A. It has been shown (40) in fact, that the rate-limiting step in palmitate oxidation is the conversion of palmityl-CoA to palmityl-carnitine. The activity of the transferase enzyme involved is elevated in fat-fed, starved or diabetic rats (41). Under these conditions fatty acid oxidation is increased.

Esterification

Esterification is the predominant mechanism by which free fatty acids are assimilated by most tissues (42). Figure 1 shows the main pathways by which fatty acid is incorporated into triglyceride and phospholipid. It can be seen that before esterification can occur the fatty acids must first be activated by combination with a CoA molecule and the glycerol moiety must be attached to a phosphate group.

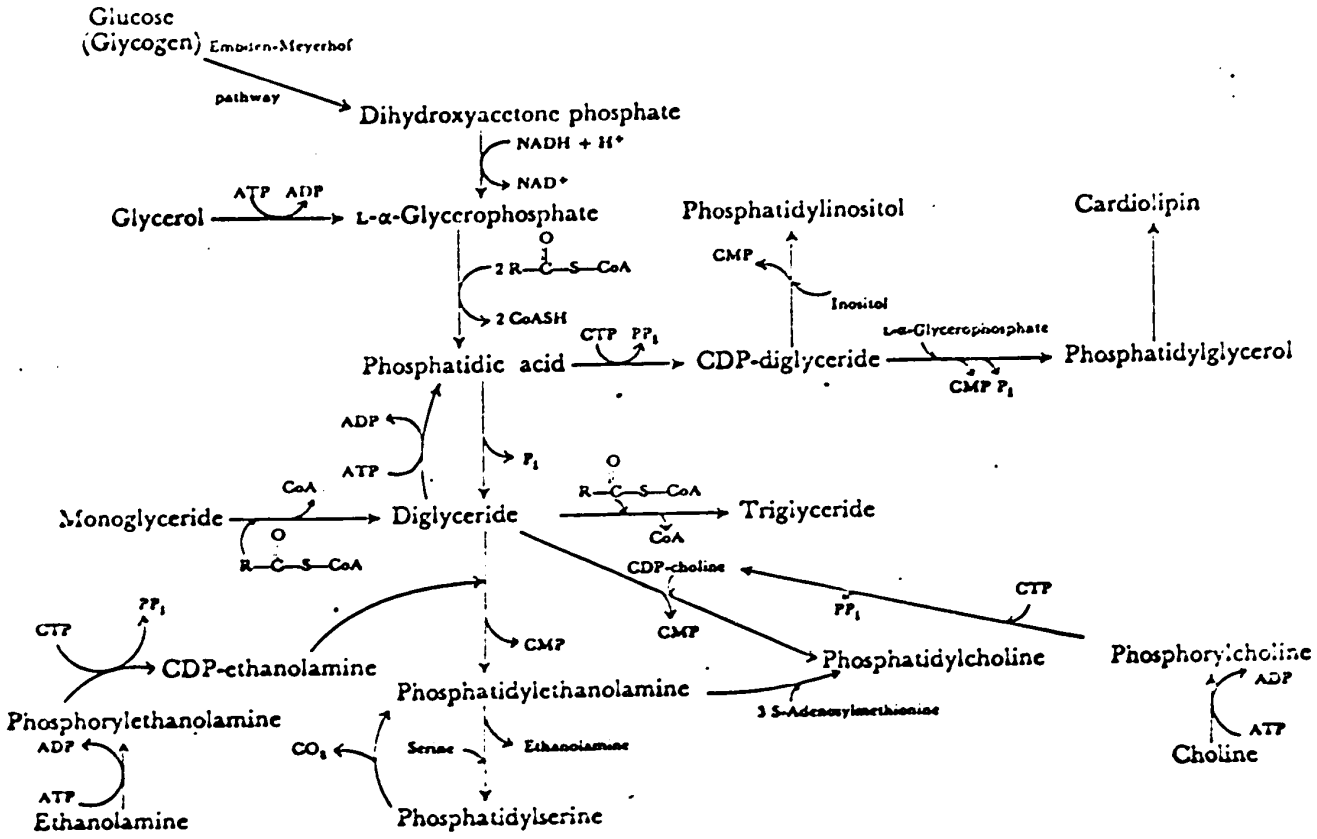


Figure -1. Summary of the pathway involved in the biosynthesis of glycerides.

Esterification then proceeds at two of the glycerolphosphate carbons. The phosphatidic acid can either be activated by combination with CDP for formation of phosphatidyl serine or can proceed directly to the diglyceride by elimination of the phosphate. The diglyceride may then be converted to lecithin, phosphatidyl ethanolamine or triglyceride. The former is made by the intervention of CDP-choline while the latter involves CDP-ethanolamine. The phosphatidyl ethanolamine can be methylated to form lecithin via S-adenosylmethionine.

In the liver triglycerides are synthesised mainly in the microsomal fraction (43,44). Synthesis seems to be mainly confined to the smooth portion of the endoplasmic reticulum (44). As will be discussed below, in certain instances of fat infiltration triglyceride appears free in the cytoplasm. The phosphatides, on the other hand, are concentrated in the nuclear, mitochondrial and microsomal fractions; phospholipid synthesis can proceed in any of these fractions (45). Esterification of cholesterol occurs mainly in the mitochondria or microsomes (46). Unlike triglyceride,synthesis of cholesterol or phospholipid can occur in either the smooth or rough portions of the endoplasmic reticulum (44).

Concentration of a-glycerophosphate and free fatty acid within the cell can exert a profound influence on esterification (47). For example, in the perfused rat liver the quantity of triglyceride appearing in the perfusate has been shown to be directly proportional to the free fatty acid content of the medium (7,47). Therefore, in the normal situation there appears to be no rate-limiting step in esterification

other than the presence of substrate. Of particular relevance to the present introduction is the finding that enhancement or reduction of plasma-free fatty acid by the presence of epinephrine or nicotinic acid, respectively will cause corresponding changes in triglyceride production (48,49). However, in certain abnormal situations a direct correlation between plasma free fatty acid and esterification cannot be shown. For example, in the fasting state although free fatty concentration in the plasma is at a high level, esterification is decreased (50). Carbohydrate feeding results in an elevation of triglyceride synthesis although plasma fatty acids are depressed (51). This could result either from activation of the enzymes involved in esterification and lipogenesis (52) or from the increased quantities of glycerophosphate present (53).

c) Secretion of Liver Lipids

In order to complete this brief review of the metabolic fate of fatty acids, some of the controlling mechanisms involved in triglyceride secretion by the liver will be discussed. As can be seen in Figure 2, the secretion of hepatic lipid is one of the major steps involved in the triglyceride cycle of the intact animal. Indeed, secretion into the plasma as a constituent of lipoproteins is the major pathway of disposal of liver triglycerides (54,55).

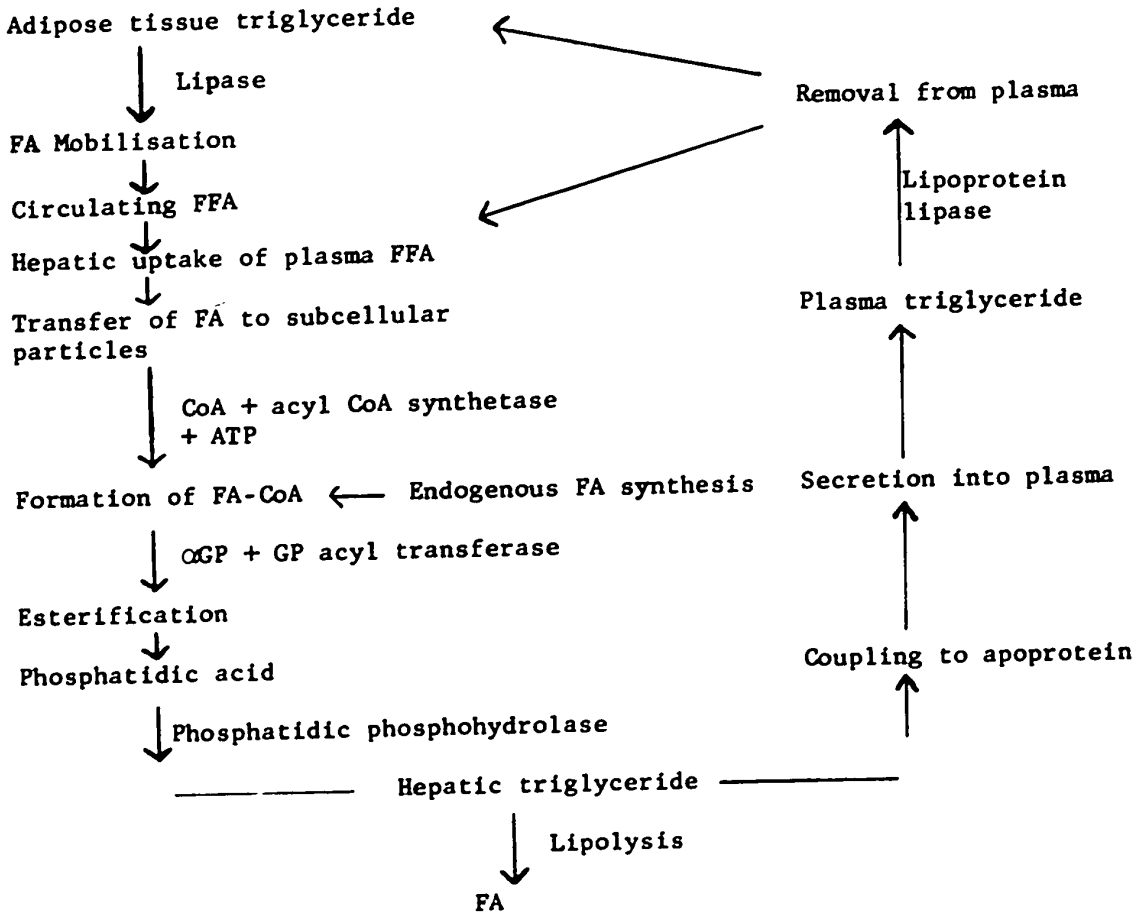


Figure 2: The Triglyceride Cycle.

Chemistry of Lipoproteins

In the fasted animal the liver is the main source of circulating esterified lipid (1,2,8,55-61). These lipids are secreted as part of a loosly united conglomerate of triglyceride, phospholipid, cholesterol ester, cholesterol, carbohydrate and protein, known collectively as lipoprotein (62). Lipoproteins with different percentages of the above moieties can be separated by a variety of physical, chemical and immunochemical techniques. Figure 3 described the four main classes with their constituent lipid and protein. In ascending order of density these are: chylomicra, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). These lipoprotein fractions contain decreasing ratios of lipid to protein in the molecule (62).

Since chylomicra are of extrahepatic origin (1,63) and only appear in the circulation after feeding, they need not concern us here (64,65). The triglyceride of hepatic origin is carried mostly in the VLDL with lesser amounts in the LDL and HDL fractions (62). Phospholipid, on the other hand, is carried in all fractions with the highest percentage in the HDL (62).

On paper electrophoresis three distinct bands of lipoprotein can be distinguished by lipid and protein staining (66). These are the α, β and pre-β bands. LDL moves only to the β position while HDL has the α mobility (67,68). The pre-β band, on the other hand, arises exclusively from VLDL (66,69,70). This lipoprotein is designated as having an α_2 mobility in starch block electrophoresis. It should be pointed out here that these bands do not represent discrete protein species but rather each contains a collection of peptides with differing immunochemical

Lipoprotein Class.	Density	Protein Composition.			Lipid Composition.					
		total %	apo-protein	electro-phoretic mobility.	total %	% of lipids. TG	PL	CE	CH	FA
Chylomicra	0.94.	1-3	A,B,C.	origin.	98	88	8	3	1	-
VLDL	0.96-1.006.	7	A,B,C.	pre-β.	93	56	20	15	8	1
LDL	1.006-1.063.	32	B.	β.	85	21	26	41	10	1
HDL	1.063-1.21.	48	A,(C).	α.	52	15	45	30	8	3

Figure 3- Constitution of the Plasma Lipoproteins.

and electrophoretic properties (71). The advent of relatively mild delipidation procedures permitted the apoprotein to be examined without interference from the lipid component. The apo-VLDL for example has immunochemical reactivity to both anti-LDL and anti-HDL while the native lipoprotein shows reactivity only to anti-VLDL and anti-LDL (72,73). Polyacrylamide gel electrophoresis of the VLDL apoprotein showed the presence of several peptides(74). The protein components of the HDL and LDL fractions show a similar heterogeneity (75,76).

It has been shown that the serum ß-lipoprotein will precipitate in the presence of a sulphated polysaccharide and divalent cations (77,78,79,80). A recent report from our laboratory shows that the sulphated polysaccharide, heparin, is capable of separating the α and ß moieties of native VLDL (81). Most of the triglyceride however is precipitated with the ß fraction. Electrophoretic, immunodiffusion and immunoelectrophoretic criteria have shown the relative homogeneity of this ß-lipoprotein (77,81). The immunoelectrophoretic pattern which the precipitated lipoprotein exhibits will be illustrated in the experimental section. While small quantities of other proteins may be detected by more sensitive techniques such as radio-autography of labelled proteins on the electrophoresis plate,it is reasonably certain that the heparin precipitate contains no α-component. In consideration of this finding and the high triglyceride content, the heparin precipitable lipoprotein was chosen for the metabolic studies which will be described in this thesis.

As will be discussed below in the section on fatty livers, VLDL with immunochemical identity to plasma VLDL has been isolated from liver Golgi

preparations (82). Delipidation of this lipoprotein shows it to contain the same major apoproteins as plasma VLDL (83). However, disc gel electrophoresis showed three extra bands which are not visible in normal VLDL. These bands, which are also seen in perfusate VLDL may be contamination from tissue components.

Synthesis and Secretion of Lipoprotein

The ultracentrifugation and polysaccharide separation techniques have been used as convenient tools for studying lipoprotein metabolism *in vivo* and *in vitro*. The site of synthesis of VLDL and LDL is to a very large extent confined to the liver (59). Confirmatory evidence was provided by Bungenberg de Jong *et al*. (58) who showed that rat liver ribosomes can incorporate labelled amino acids into a peptide which is immunochemically similar to HDL and LDL. Ribosomes from other tissues cannot produce this product. The site of synthesis of the HDL fraction occurs mainly in the liver although a small degree of extrahepatic synthesis seems to occur in the intestine (84,85). As will be discussed below, an increase in HDL synthesis has been indicated for 20 hour regenerating liver (86).

Control of Synthesis and Release

Perfusion of the rat liver with high concentrations of fatty acids results in a stimulation of VLDL production as measured by amino acid incorporation into the protein moiety (87). A converse controlling mechanism was indicated when a marked stimulation in esterification was obtained on adding the macromolecule

to liver microsomal incubations. The involvement of carbohydrate in lipoprotein synthesis has been suggested by the work of some investigators (88,89). For example, Eaton and Kipnis showed that glucose feeding can increase the synthesis of VLD-LD-protein in the fasted rat (89). Similar stimulations in the lipid moiety can be obtained by perfusion of alloxan-diabetic livers pretreated with insulin (88). As will be described below, orotic acid severely inhibits production of the ß-protein (90,91). Synthesis of the ß-protein by the gut is unaffected however, as is the synthesis of all other proteins. While the above metabolites are controlling factors in the synthesis or release of the ß-protein under the conditions mentioned, it is not known at present whether they have any physiological role in this regard under normal conditions.

Lipoprotein Secretion Studies

Since the apoprotein analysis indicated that VLDL and HDL have specific peptides which are common to both fractions, the studies of Roheim et al. (92) are of considerable importance. These workers have shown that a marked stimulation in VLDL production can be obtained when a density > 1.21 plasma protein fraction is added to the medium in the isolated liver perfusion system. This protein was apparently capable of picking up lipid even in the presence of puromycin. Increased release of phospholipid from liver slices incubated in vitro was obtained when serum was added to the medium (93).

This mechanism for the secretion of VLDL does not appear to be of very great significance, however, since perfusion studies in this laboratory (77) indicated

adequate release of both lipid and protein moieties into a lipoprotein-free medium. This release was readily blocked by puromycin. It was further shown (94) that there is an exchange of triglyceride between the liver and lipoprotein, which could account for some of the findings of Roheim et al. (92). The in vivo and in vitro experiments also showed (77) a considerable lag in incorporation of amino acid into the secreted lipoprotein-protein. A similar lag period was not obtained with the lipid moiety. The presence of a pool of lipoprotein-protein in the endoplasmic reticulum was therefore indicated; no mobile pool of lipid, on the other hand was observed.

Theoretical Considerations

As will be discussed in the section on fatty livers, the lipid in the normal liver appears to exist in two separate compartments; one active pool having a fast turnover rate, while the second immobile pool is relatively inert. Normally the immobile pool represents only a small percentage of the total liver lipid. However, in certain types of fatty liver conditions such as CCl_4 poisoning this pool is vastly increased. That this is in fact the situation in the liver is indicated by the in vivo incorporation studies of Schotz et al. (55). These authors administered ^{14}C-palmitate to a group of rats and investigated the time course of incorporation into the liver and plasma triglyceride. A multi-compartmental computer analysis of the data enabled estimates to be made of the quantities of triglyceride in the two main pools as well as amounts secreted by the liver per unit time. The calculations indicated that the immobile pool of hepatic triglyceride was greatly increased in

size following CCl_4 administration. The possible application of this type of analysis to the study of partially hepatectomised animals will be discussed.

II. The Fatty Liver

The mammalian liver can be induced to accumulate fat by a wide variety of experimental procedures (3,55,56,90,95-100). A large increment in hepatic triglyceride concentration is a characteristic feature of every type of steatosis so far studied. Increases in phospholipid and cholesterol concentrations, when present, are never of the same magnitude. Thus it seems that the primary effect is on the triglyceride metabolism. Consequently, in order to adequately review the literature, the various types of fatty liver will be grouped according to whether triglyceride synthesis (b) or secretion (a) is affected. This however, is not to suggest that phospholipid and cholesterol do not play important roles in the fatty liver development.

These categories are chosen arbitrarily for the purpose of discussion and do not imply that the causes of every type of fatty liver fall neatly into one or other of these two groups. For example, it is conceivable that some fatty livers may be the result of a combination of both (a) and (b).

a) Inhibition of Triglyceride Secretion

The utilisation of hepatic triglyceride can be depressed either by an

inhibition in secretion or by a decrease in the rate of oxidation in the liver itself. However, in the fed animal secretion of triglyceride is the main pathway of utilisation (54). Short term inhibition of triglyceride oxidation therefore would probably not have a significant effect on hepatic concentration.

Lipoprotein Production

Fatty livers which are known to be a direct result of a blockage in triglyceride secretion can be caused by a variety of toxic agents, the more common of which are CCl_4 (101), ethionine (102), puromycin (103), and orotic acid (90). A decrease in secretion is also indicated for animals suffering from choline deficiency (98).

All of these agents appear to exert their primary effect by inhibiting lipoprotein secretion rates in one way or another. Preliminary studies with CCl_4 and ethionine showed that quantities of circulating very low density lipoprotein (VLDL) were severely reduced after several hours and in fact preceded the triglyceride accumulation in the liver (102,104). These conclusions were later confirmed in a variety of ways.

One standard method which has been frequently used is the triton test which gives a good estimate of triglyceride secretion rates, since it has the capability of blocking the normal uptake of the circulating lipid by the extrahepatic tissues. The detergent has been successfully used in the case of CCl_4 (105), ethionine (106), and choline deficiency (107). The triton test has also shown that certain types of fatty liver e.g. regenerating (108) or colchicine treated livers do not belong to

this class (109).

Perfusion of the isolated liver with a medium devoid of lipoprotein has been equally valuable in demonstrating inhibition of secretion particularily in the case of orotic acid treatment where an inhibition of ß-lipoprotein secretion was clearly shown by immunochemical techniques (91).

Other more sophisticated methods for demonstrating lipoprotein inhibition in vivo have been recently developed (54,55,110,111). These involve a multi-compartmental analysis of radioactive fatty acid incorporation into liver and plasma triglyceride. By comparing turnover rates with those of the theoretical model, Baker et al. (55) have demonstrated a reduction in triglyceride secretion in the intact CCl_4 treated animal.

Secretion Studies

Although the inhibition of release of the triglyceride containing lipoprotein (VLDL) can readily be demonstrated by the above methods, the issue as to whether synthesis or secretion was being specifically inhibited remains a major stumbling block.

This point might be resolved by turnover studies on the protein and lipid moieties in vivo. Incorporation experiments in this laboratory have examined (77) the secretion rates of the ß-lipoprotein in the normal animal. Similar investigations with the choline deficient rat have been reported by Oler and Lombardi (112). These authors conclude that since there is no impairment in incorporation of labelled

leucine into total protein in vivo and in vitro, the impaired release of lipoprotein cannot be due to a inhibition of protein synthesis.

The authors found that in the case of albumin there was a delay in the appearance of the label in the plasma, but specific activity reached the control level after 2 hours. This indicated that the albumin was secreted at a normal rate in choline deficiency, but was affected by a slower rate of intracellular transport. Since the lipoprotein specific activity on the other hand, was persistently lower with choline deficiency, a defect in the secretion rate was indicated.

Intracellular Lipoprotein

A direct way to demonstrate inhibition of lipoprotein secretion with normal intracellular synthesis would be to isolate the lipoprotein from the liver cell in order to see if in fact there is an intracellular accumulation of the macromolecule in fatty livers. Since the membranes of the cell itself are composed largely of a lipid - protein complex (113), separation of lipoprotein designed for export from the structural components in a liver homogenate presents a major problem.

Some progress has recently been made in this direction by Mahley et al. (82). These workers showed that VLDL particles isolated from Golgi subcellular particles [cf section below (c)] are immunochemically identical to the d< 1.006 lipoprotein of the plasma. A similar identity has since been obtained on the delipidated "apoprotein" by both immunodiffusion and disc gel electrophoresis (83). Preliminary chemical analysis of the "liposomes" [cf section below (c)] which were isolated by Schluck and Lombardi from the ethionine fatty liver shows them to have the same

general composition as plasma lipoprotein (114). Further purification and more extensive analysis of these particles may show them to be in fact accumulated lipoprotein. Should this be the case, analysis of the lipid and protein moieties would tell whether the synthesis or secretion was being affected in the steatosis.

Further information could be obtained by turnover studies of these isolated hepatic particles. For example, rates of incorporation of precursors into these liver particles can be compared with those of circulating VLDL incorporation. In this manner difficulties in secretion as distinct from synthesis could readily be recognised for any given type of steatosis. A further discussion of the sub-cellular particles will be undertaken below when the work of the electron microscopists is considered.

The possibility of such agents as CCl_4 having some kind of direct effect upon the lipid - protein interaction has been raised by Lombardi (115). A rapid reduction in triglyceride secretion would result if the toxin were disrupting the extracellular combination of lipid with the circulating apoprotein as proposed by Roheim et al. (92). In this connection, it has been shown that CCl_4 can alter the structure of the lipid moiety by direct initiation of peroxidation (116). A defect in the conversion of the apolipoprotein to the completed lipoprotein has been suggested following orotic acid treatment (100).

Studies on Synthesis of Lipoprotein

The various biochemical steps which lead eventually to the release of lipoprotein by the liver include the synthesis of the different components. A

shortage of protein, cholesterol, triglyceride or phospholipid could conceivably exert an inhibitory influence on the formation of the lipoprotein complex. The implications of reduced protein and phospholipid synthesis in the development of certain types of fatty liver will now be described.

i) Inhibition of Protein Synthesis

Amino acid incorporation studies have conclusively shown that CCl_4, ethionine, puromycin, diabetes and orotic acid inhibit the synthesis and/or secretion of the protein moiety (77,91,103,117-119). However, the possibility must also be kept in mind that the agents may also be affecting the liver in other ways.

Only in the case of puromycin is the mechanism of protein inhibition moderately well understood. The antibiotic appears to compete with the amino acyl-sRNA complex at the site of peptide bond synthesis on the ribosome (120). No other direct effect is known. On the other hand the mechanism of inhibition caused by CCl_4, ethionine, orotic acid etc is still largely a matter of speculation.

Since CCl_4 has an early effect upon the endoplasmic reticulum, it may alter the function of the attached ribosomes (117) and thus cause a reduction in protein synthesis. In the case of ethionine the inhibition in protein synthesis appears to be mediated by a reduced level of hepatic ATP (121). The change in ATP concentration is followed rapidly by an inhibition of RNA and protein synthesis (122).

These changes are completely prevented by the administration of methionine, adenine or ATP (121,122,123). CCl_4 fatty livers, on the other hand, are not prevented by nucleotide precursors although ATP injected subcutaneously does reverse the accumulation of triglycerides.

The reduced ATP level in ethionine treated animals is due to an adenine trapping effect of the analog (124). The ethionine competes with methionine to form S-adenosylethionine instead of the S-adenosylmethionine which would normally be formed as a precursor for ATP.

The mechanism whereby the low ATP causes the inhibited protein synthesis could be one of the following: (a) reduced activation of amino acids for combination with transfer RNA; (b) inhibition of messenger RNA synthesis; (c) a direct effect on the structure of the polyribosomes. No evidence is currently available that conclusively indicates which mechanism is operating in the case of ethionine fatty liver. However, the reformation of the polysomes by adenine administration following their disaggregation within the cell (3) suggests that (c) is the operative mechanism.

The mechanism of reversal by adenine of the orotic acid fatty liver (90,91) is puzzling since total hepatic protein synthesis is unaffected by the drug (125). Even the inhibited production of the ß-protein may not be due to a decreased synthesis of the protein moiety, since the orotic acid may be blocking either the coupling or secretion of the lipoprotein molecule (126).

However, the work of Rajalakshmi (127) suggests a defect at the ribosomal level. This author isolated structures of low density with ribosomes attached from orotic acid treated liver homogenates. Although the proportion of 18S to 29S RNA was similar to normal ribosomes, the distribution pattern of ^{14}C-adenine radioactivity between the two RNA fractions was different, indicating that the ribosomes were not identical to the controls. The experiments of Faloona et al. (128) on the other hand, indicate that the stability of the mRNA template may be the critical factor in this type of inhibition. These workers demonstrated that administration of actinomycin D, which blocks RNA synthesis, will cause a severe inhibition of low density lipoprotein synthesis with only a moderate inhibition in the synthesis of the high density fraction. The apparent heterogeneity of hepatic template stability suggests that the LDL is subject to more sensitive control than the HDL. If the orotic acid was affecting the mRNA stability in a similar manner, this would explain the specific inhibition of the ß-lipoprotein. Incorporation studies with isolated ribosomes from orotic acid treated livers to be described below will attempt to clarify some of these problems.

An inhibited incorporation of labelled amino acids into serum proteins was demonstrated following perfusion of alloxan diabetic rat livers (129). The inhibition was reflected in all of the major lipoprotein classes. This impairment corresponds to an inhibited release of the triglyceride and phospholipid moieties shown by the same research group (88). However, the two effects may not be causally related since insulin administration does not restore the amino acid incorporation to normal levels even though release of triglyceride is stimulated (129). It is not known

whether synthesis or secretion is the major factor involved in the inhibited protein incorporation. However, it appears that at least in the case of the chronically treated animals, the alloxan reduces protein synthesis since incorporation into total hepatic protein is also inhibited. No information is apparently available concerning the mechanism of the action of the drug. The effect could be either a result of insulin depletion or a direct action of the alloxan on the liver cell.

ii) Inhibition of Phospholipid Synthesis

Recent findings by at least four independent research workers have placed new emphasis on the role of phospholipid synthesis in the removal of lipid from the hepatic cell (88,107,130,131). Shimizu, for example, clearly showed (130) that 4-5 hours after CCl_4 treatment phospholipid synthesis was markedly depressed in the liver, the inhibited step being the conversion of phosphoryl-choline to CDP-choline.

Lombardi et al. (107) have obtained confirmatory results in rats fed a choline deficient diet where both depressed liver phospholipids and decreased release of plasma phospholipid were obtained. In the case of alloxan diabetic livers also, there is a reduction in the synthesis or secretion of $d < 1.02$ lipoprotein with a concomitant decrease in phospholipid synthesis, while formation of the triglyceride did not appear to be depressed (88).

Olmsted (131) has recently shown a similar effect 3 hours after ethionine administration. In this case both the quantities and specific activities of liver and plasma phospholipid were depressed. The ratio of plasma to liver specific activity was reduced at early periods to increase again later as the effect of the drug was diminishing. Thus, there appears to be increased mobilisation of phospholipid following the period of inhibition, thereby indicating a possible role for phospholipids in the triglyceride cycle i.e. the synthesis, transport and uptake of triglycerides by the body.

Methionine, which is a precursor for choline in the cell, prevents the lipid accumulation following ethionine administration (132). It would appear therefore, that another major action of the drug, besides inhibiting formation of ATP, is as an antimetabolite in the synthesis of phospholipid. The fact that administration of choline does not protect rats against the ethionine fatty liver (132,133) suggests that the methylation of PE by methionine is the operative route under these conditions. Increased use of this pathway is suggested by recent experiments on the choline deficient animal (134).

In vitro recombination studies with the HDL-apoprotein in the presence of plasma lipid emphasise the necessity for the presence of phospholipid in the maintenance of the stability of the native lipoprotein (135,136). Although phospholipid readily combined with the apoprotein when presented in the form of micelles, the neutral lipid did not combine unless the phospholipid was also present. From these experiments it can be readily appreciated that a lack of phospholipid could result in

a depressed production of the intact HDL molecule. However, no evidence has as yet been reported for a similar controlling mechanism in the case of the VLDL.

b) Increases in Neutral Lipid Synthesis by the Liver

In this section certain factors which cause increased triglyceride synthesis in the liver will be discussed. Figure 4 shows a diagram of the various pathways involved in the synthesis and secretion of lipids by the liver cell. Sites of action of the various hepatotoxins under discussion here are indicated.

These pathways are briefly summarised as follows: Free fatty acids (FFA), mobilised from extrahepatic tissues are taken up by the hepatocyte, converted to their CoA derivative and combined with L-α glycerophosphate to form diglycerides. These can then be converted either to triglyceride by combination with another molecule of fatty acid or to phospholipid by the addition of a base such as choline. In the case of the fatty livers which we are discussing here, the flow rate of fatty acids to triglyceride is very much increased, while the conversion of diglyceride to phospholipid is unaffected. As was mentioned in Section I, triglyceride synthesis will proceed in direct proportion to the concentration of glycerophosphate and fatty acid in the cell (5-8). There appears to be no other rate limiting step in the esterification.

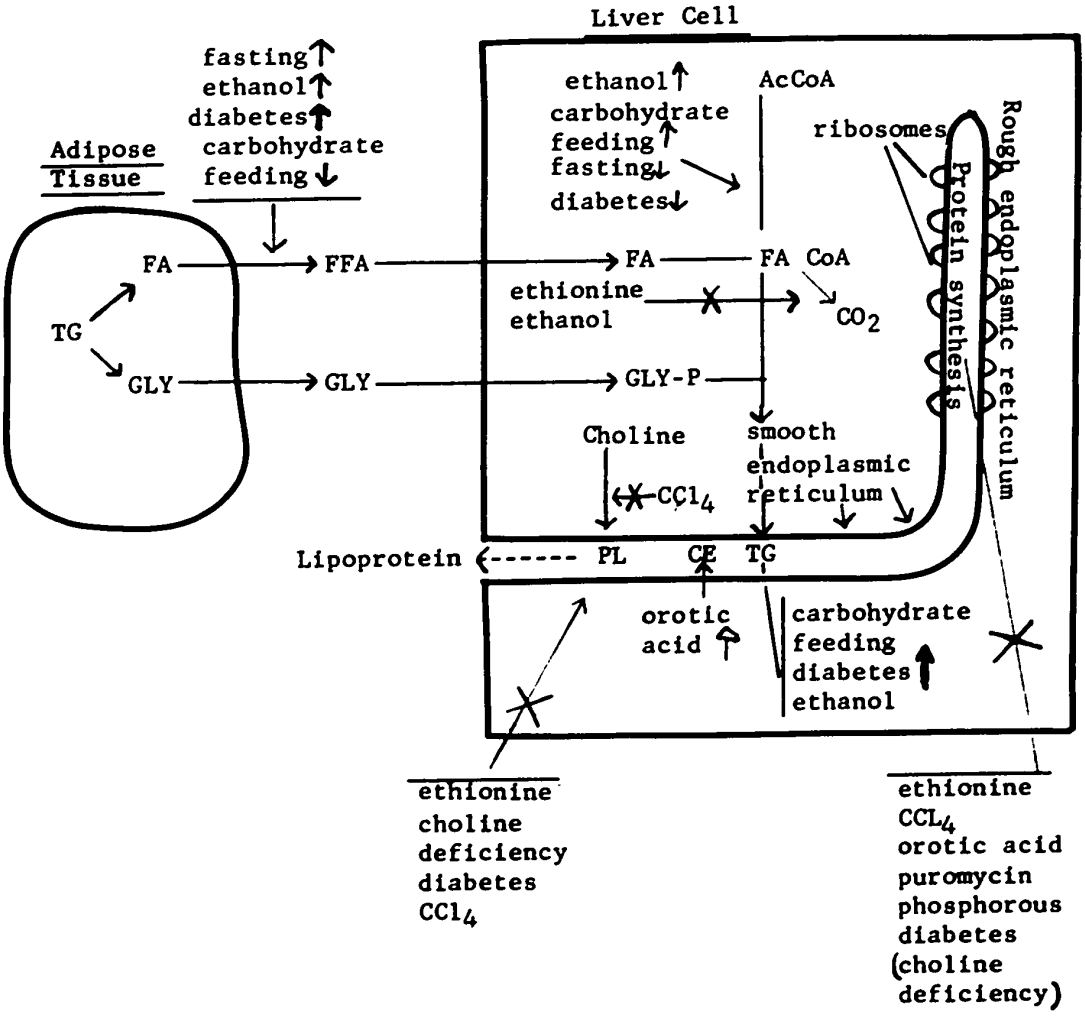


Figure 4 : Sites of action of some steatotic agents in the TG cycle.

If we consider what was discussed in Section I concerning the metabolic fate of fatty acid, factors which could conceivably regulate fatty acid concentration in the hepatocyte are decreased oxidation, increased synthesis, impaired esterification or increased uptake from the circulation.

Decreased Oxidation

Oxidation of fatty acids in the liver has been shown to be depressed after the administration of ethanol (137) or ethionine (138). It has been suggested by Lieber and Schmid (137) that the inhibition of palmitate-1-^{14}C oxidation to $^{14}CO_2$ caused by ethanol *in vitro* may be due to decreased oxidation of tricarboxylic acid cycle intermediates. This decreased oxidation could be due to the increased levels of NADH in the liver. The depressed oxidation of fatty acids seen *in vivo* after alcohol administration however is not considered likely to be of sufficient magnitude to affect the overall liver triglyceride concentration (139). In support of this it has been shown that administration of isocaloric amounts of glucose resulted in a depression in fatty acid oxidation which was even greater than that observed with ethanol; yet there was no increase in liver fat in the glucose treated rats (139). Also, the interference with fatty acid oxidation caused by ethionine poisoning, for example, is a late phenomenon and may represent a response of the liver to the accumulating triglyceride (138).

Lipogenesis in Fatty Livers

Increases in fatty acid synthesis with carbohydrate feeding has been shown by many investigators (140-143). An increased incorporation of acetate-^{14}C was clearly shown in liver preparations from rats fed a diet which contained glucose, fructose or sucrose as the main carbohydrate (142,144,145,146). Under similar conditions the in vivo incorporation was also increased (147).

The mode of stimulation by dietary sugars is not completely understood. The effect has been considered to be mediated largely by insulin (148). However, stimulation of acetate incorporation by glucose has been observed in vitro (149, 150). Furthermore, dietary fructose is at least as active as glucose despite its weak action on insulin secretion, and remains effective even in the absence of insulin (151).

Since labelled acetyl CoA and malonyl CoA are incorporated more rapidly into liver fatty acids after chronic glucose feeding (142,152), it appears that all steps in the synthesis are stimulated. A direct action of carbohydrate on the enzymes has been indicated since acetyl CoA carboxylase (143) and many other hepatic enzymes concerned with lipogenesis (140,153,154) are induced by dietary hexoses. However, time sequence studies show that enzyme induction and onset of lipogenesis are not synchronised (140,142,155), hence it is doubtful if enzyme induction represents the primary factor in increased synthesis.

The reduced lipogenesis in alloxan-diabetes seems to be part of a mechanism for controlling triglyceride accumulation in the liver. Several hours of insulin

treatment are necessary to remove the inhibition, indicating that the peptide does not act directly (156). On the other hand, many lipogenic enzymes are decreased in diabetes (153,157-159). Control of lipogenesis may be exercised via the negative feedback of high intracellular concentration of acyl-CoA (160), since these compounds inhibit the acetyl-CoA carboxylase enzyme (161). It thus appears that the increased intracellular concentration of fatty acid caused by the elevated mobilisation in diabetes exerts a negative feedback control on the lipid synthesising capacity of the hepatocyte.

Ethanol administration has been shown to cause an elevation in hepatic lipogenesis in vivo (147) and in vitro (152). Alexander et al. (152) demonstrated the enhancement in fatty acid synthesis from malonyl-CoA by cell sap preparations following prolonged ingestion of ethanol. The stimulation in the intact animal therefore is probably not due solely to the increased reducing power of the liver since optimum concentrations of activators and cofactors, including NADPH, were present in the artificial medium. An increase in the concentration of the lipogenic enzymes has been suggested by the authors. At the dose level used by these workers, however, no fatty liver resulted; hence, it does not appear likely that the stimulation in fatty acid synthesis is a major factor in ethanol steatosis.

Esterification in Fatty Livers

Increases in hepatic synthesis of triglyceride after carbohydrate feeding has been shown in vivo by many workers (162,163). In vitro incubations of liver homogenates from rats fed high carbohydrate diets provided further evidence for

this increased esterification (164). Since the carbohydrate of the diet normally constitutes the main precursor supply for lipogenesis, the total amount of fatty acid and glycerol phosphate synthesised can be expected to increase in parallel with the increased intake of the precursor. Thus, the fatty liver resulting from high carbohydrate feeding is most likely to be a direct result of increased hepatic synthesis of triglycerides. Since there is also an elevated secretion of triglyceride in the form of VLDL in this condition (50,165), an increase in the overall turnover of hepatic triglyceride is indicated. It should be emphasised that because of the greater secretion of hepatic triglyceride the lipid accumulation is not large in comparison to most other types of fatty livers.

Although the situation with regard to diabetes is far from clear, in vitro observations indicate that under this condition esterification is at least not less than normal. Heimberg et al. (88) have shown that radioactivity of liver triglycerides after perfusion with palmitate-1-^{14}C was similar in diabetic and control rats.

Studies with the intact rat have shown an elevation in the esterification of radioactive palmitate after a single large dose of ethanol in comparison to isocaloric amounts of glucose (166). A similar stimulation was noticed by the same authors in vitro when ethanol was added to the incubation medium. The finding by Nikkila and O jala of an increased glycerol phosphate content in the liver (167) suggests a possible mechanism for the elevated esterification, namely, that the glycerol phosphate may be acting as a stimulus for increasing the utilisation of the fatty acid within the cell. The alcohol-induced hypertriglyceridemia in man (168) and rabbits (169) is in confirmation with the results obtained in the rat. For example,

the magnitude of esterification in the human as measured by palmitate-^{3}H incorporation into plasma triglyceride is proportional to the hypertriglyceridemia.

As has been discussed above, there is an impairment in the synthesis of phospholipids in certain types of fatty liver conditions, for example, after CCl_4, ethionine or choline deficiency (88,107,130,131). This could conceivably result in the availability of increased quantities of substrate for triglyceride formation. The finding by Shimizu of a block at the CDP-transferase step with CCl_4 (130) supports this concept since more diglyceride would then be available for triglyceride formation. It is the opinion of the author, however, that the order of magnitude of the decreases is not sufficient to account for the comparatively large quantities of triglyceride that accumulate.

Free Fatty Acid Mobilisation in Steatosis

A large variety of steatotic agents cause increases in circulating free fatty acid levels. These include fasting (170), ethanol (171), ethionine (172), partial hepatectomy (173), diabetes (174), as well as the lipotropic drugs such as epinephrine (175) and growth hormone (176). All of these agents have a direct or indirect effect upon the stored fat of the adipose tissue, causing it to be broken down to glycerol and free fatty acids (177). These fatty acids are then circulated in the plasma as an albumin-bound complex (178), to be taken up directly by the liver.

Epinephrine and growth hormone have a direct effect upon the adipose tissue (179,180), while the other agents probably exert their influence either by increasing epinephrine secretion (181), or decreasing quantities of circulating insulin, which has

an inhibitory effect on free fatty acid mobilisation (170,182).

It is important to emphasise that uptake of fatty acid by the liver is proportional to the concentration in the plasma (7,30,183,184). Consequently, an increased flux of fatty acid through the liver can reasonably be expected to result in a net increase in lipid uptake. Nestel and Steinberg (7) have clearly shown that perfusion of the isolated liver with a heavy load of fatty acid causes large accumulations of triglyceride in the liver. Similar perfusion studies by Weinstein et al. have shown a definite requirement for fatty acid in the perfusion medium in order for the triglyceride to build up in the presence of carbon tetrachloride (30).

As indicated above, ethanol poisoning causes many changes in hepatic lipid metabolism (137,167,171). However, excessive mobilisation of fatty acids from the fat stores is believed to be a major factor involved in fatty liver development (185). In support of this, many hormonal and experimental manipulations that interfere with the mobilisation of fatty acids are effective in blocking the alcohol-induced fatty liver. For example, hypophysectomy (139), adrenalectomy (186), and cordotomy (139,171) can effectively prevent the fat accumulation; however, similar manoeuvers reduce the steatotic action of CCl_4 (187,188) which mainly acts upon lipoprotein secretion. Thus a direct relationship with the circulating catecholamines has been established. Further, the accumulated triglyceride after ethanol administration contains large amounts of the essential fatty acid, linoleic acid, which could not have come from de novo synthesis in the liver (189). The increased synthesis and decreased oxidation of fatty acids as well as impaired release

of the triglyceride found by some investigators do not appear to be major factors in the development of the alcohol-induced fatty liver (56,185).

Contrary evidence has recently been advanced however by Lieber et al. (190) who have shown that the potentiation by ethanol of the dietary deficient fatty liver results in an accumulation of triglyceride which appears to originate largely from endogenous synthesis.

Rabbits fed a high carbohydrate diet accumulate lipid in the liver with increasing proportions of unsaturated fatty acid (191). This experiment seemed to indicate an extrahepatic origin for the lipid since fatty acids synthesised de novo in the liver are mostly saturated (152). However, the inhibition of peripheral fatty acid mobilisation caused by acute glucose loads of short duration (170,182,192), seems also to continue with long term carbohydrate feeding since free fatty acid levels are considerably lower than with high fat diets (51).

The situation with the diabetic fatty livers is of considerable complexity. The accumulation of the triglyceride however appears to be due mainly to an increased flux of free fatty acids coming from the adipose tissue (193,194). The increased flux is due primarily to an elevated free fatty concentration in the plasma (195), the major cause of which is increased lipolysis of adipose tissue triglycerides (193,194). The total turnover rates of the free fatty acids and glycerol are increased in proportion to the plasma levels (192,196,197), indicating increased uptake by the liver. Normal flux rates of free fatty acid are rapidly restored by insulin treatment (198).

The experimental fatty livers obtained by perfusion with large quantities of fatty acid (7) can also be classified in this section in view of their similarity to lipid mobilisation in the in vivo state. (For example, after constant infusion into the circulation of norepinephrine, which is capable of maintaining the serum free fatty acid at a high level, there is a progressive and marked increase in liver triglyceride concentration (175)). The same pattern of accumulation applies in the in vitro perfusions as in the intact animal. For example, with high free fatty acid concentrations in the medium relatively large amounts of triglyceride appear in the liver while the quantities of cholesterol and phospholipid were perhaps even a little decreased (30). Further indications that the in vitro perfusions reflect the situation in vivo can be seen from the pattern of incorporation of linoleate versus palmitate. In vivo studies show that linoleate is incorporated more rapidly than palmitate into serum lipoprotein (119). The perfusions of Nestel and Steinberg (7) also showed that linoleate had a much greater effect upon the perfusate glyceride than palmitate, however, the effect upon the liver glycerides was exactly the reverse.

c) Ultrastructural Changes in Fatty Liver

Fat infiltrated liver has been studied under the electron microscope by a number of investigators under a variety of conditions (98-107). Of the fatty livers studied, CCl_4 (199-201), ethionine (200), orotic acid (202), choline deficiency (203), regeneration (204) and ethanol feeding (205) are characterised by

the appearance at an early stage in the process of lipid accumulation of an increasing number of osmiophilic bodies with diameters of 300-1000 Å and which appear to be surrounded by a membrane in continuity with the endoplasmic reticulum. At later times these bodies (also called "liposomes") are seen to coalesce forming larger bodies or dioplets (200). This process is illustrated in Figure 5 which shows the morphology of the cell 4 hours after ethionine treatment.

The close association of the osmiophilic bodies with the endoplasmic reticulum has significant implications for lipid transport since esterification of fatty acids, synthesis of protein as well as assembly and secretion of lipoprotein are known to occur in this region of the cell (8,206).

Since adenine can reverse the lipid accumulation after ethionine (200) or orotic acid (90,91) treatment, the location of the lipid bodies after adenine provides a means of studying their movement out of the cell. Following such treatment there is a rapid disappearance of the liposomes from the endoplasmic reticulum with simultaneous appearance of the particles in the spaces of Disse (200). Similar observations were made by Trotter in the fasted mouse liver where these bodies were found in both intracellular and extracellular locations (204,207). This author postulates that the osmiophilic particles seen after fasting represent lipid mobilised from adipose tissue. In support of this argument it was found that the inhibited mobilisation after glucose administration resulted in the number of cytoplasmic bodies being reduced to the control level. The extracellular particles were not membrane bounded and were located at indented hepatocyte plasma

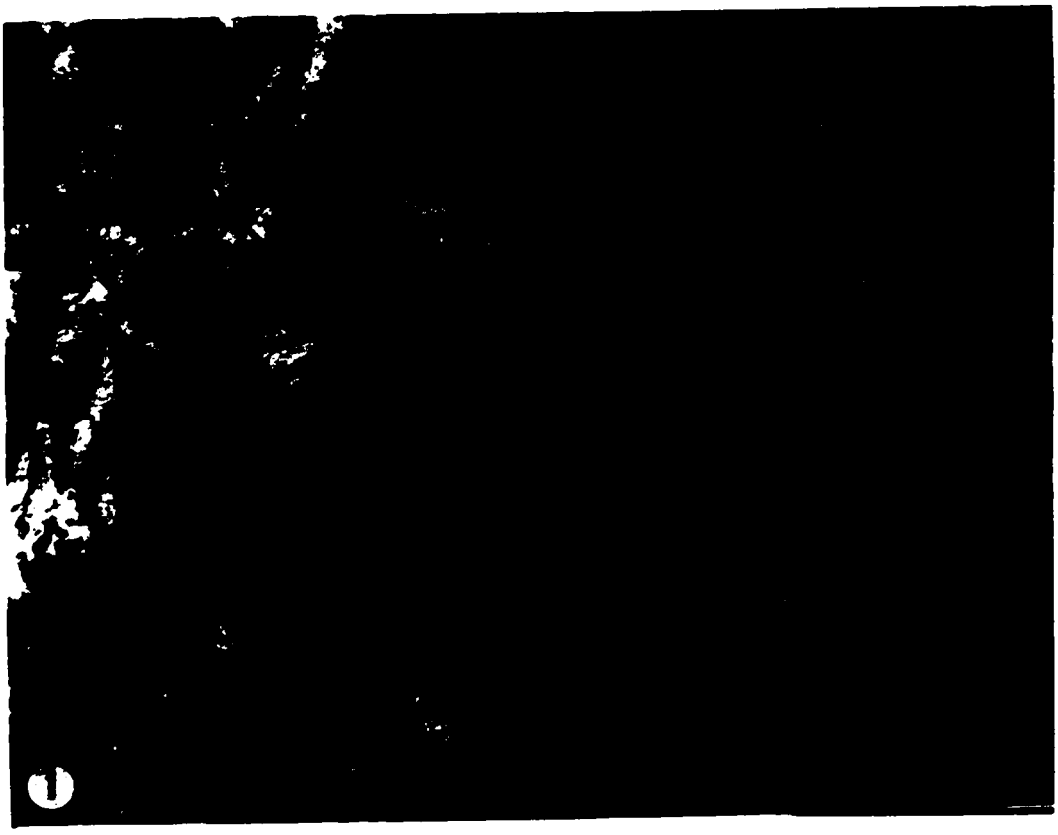


Figure 5 : Morphology of the liver cell 5 hours after ethionine administration.

Legend: Lp - liposomes

ER - endoplasmic reticulum

Ly - lysozomes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membranes or in the spaces of Disse.

The sequential electron microscopic studies that Jones et al. (208) have made with livers perfused with large quantities of linoleate gives a further insight into the process of lipoprotein secretion. These authors showed that the accumulating electron opaque particles first appear in the smooth surfaced terminal ends of the rough endoplasmic reticulum, then the smooth endoplasmic reticulum proper and finally the Golgi apparatus and spaces of Disse. Radio-autographic studies at various times after injection of tritiated palmitate or glycerol showed a similar sequence of events in the fasted and ethanol-treated rat (205). At two minutes the label was found to be concentrated over rough and smooth endoplasmic reticulum, while at five minutes it appeared over the lipid droplets in the cytoplasm. By 10 minutes there was a concentration of the label over the osmiophilic bodies in the Golgi apparatus and space of Disse border. Isolated Golgi apparatus as mentioned earlier, has been shown to contain $d < 1.006$ lipoproteins which are immunochemically similar to serum VLDL (82).

Further evidence linking the liposomes with plasma lipoprotein has been obtained in a variety of ways. For example, when puromycin, a well-known inhibitor of protein synthesis, was added to the perfusion system of Jones et al. (208), production of the osmiophilic particles was depressed but on the other hand, there was no interference with the formation of large cytoplasmic fat droplets, thereby indicating

the importance of protein for the formation of the particles. The work of Hamilton et al. (209) as well as that of Trotter (204,207,210) shows further similarities between the liposomes and plasma lipoprotein. These research groups have shown by negative staining that the osmiophilic particles have diameters with a range of 300-1200 Å. This agrees with other estimates of plasma VLDL particle size (211,212). That the osmiophilic bodies or lipcsomes accumulating after ethionine treatment are indeed lipoprotein molecules, complete or incomplete and situated within endoplasmic reticulum membranes is indicated by the chemical analysis that Schluck and Lombardi have performed on their isolated liposome fractions (114). Electron micrographs of the isolated liposomes show them to be similar in magnitude and appearance to those seen in the intact cell. The membranes can be separated from the isolated particles by sonication. These liposomal membranes have a protein to RNA ratio that is similar to the microsomal fraction, while the liposomes themselves contain very low quantities of RNA relative to protein. This would therefore suggest that the liposomes are surrounded by membranes of the endoplasmic reticulum. Also the high concentration of triglyceride and other fatty acid esters in the liposomes is a possible indication of the presence of the lipid moiety of lipoprotein. The histological evidence of Novikoff et al. and Ashworth et al. also indicated a high content of lipid (202,213). While cholesterol is present mostly as the ester form in the liposomes, in the membranes it exists mainly in the free state; a finding which is compatible with the above concept since plasma lipoproteins contain low amounts of unesterified cholesterol (104). Structural lipoproteins of membranes, on the other

hand, show the opposite pattern (214). Rajalakshmi et al. (127) have demonstrated a close connection between the lipid containing bodies and the endoplasmic reticulum. The authors showed that low density structures isolated from orotic acid induced fatty livers resembled portions of endoplasmic reticulum since they were shown to have protein synthesising ribosomes attached.

Synthesis of the lipid and protein are known to occur in the microsomal fraction of the cell (8,206) which is composed largely of endoplasmic reticulum. Evidence for the synthesis of the protein moiety was provided by Bungenberg de Jong and Marsh (58). They showed that ribosomes will synthesise peptides in vitro which show immunological behavior of the plasma lipoprotein which are then capable of binding lipid. Working with the microsomal fraction, Tzur and Shapiro have been able to demonstrate (8) that not only does esterification of fatty acid occur in the endoplasmic reticulum but that synthesis of triglyceride and phospholipid could be augmented by the addition of either albumin or lipoprotein.

The theory can therefore be formulated that perhaps an acceptor protein is needed for efficient lipid synthesis which would combine with the product for the transport through the endoplasmic reticulum to the releasing sites at the surface of the cell. However, one difficulty with this hypothesis is that triglyceride synthesis apparently proceeds at at least a normal rate when protein synthesis is blocked with puromycin or CCl_4 (30,208). A further interelationship between the lipid and protein synthesis has been shown by Ruderman et al. (87) who demonstrated that perfusion with high concentrations of fatty acid can greatly enhance the synthesis and/or release of the protein moiety of VLDL. Thus some interdependence

between esterification on the one hand and protein synthesis on the other in the secretory channels of the cell has been reasonably well established.

In normal liver the endoplasmic reticulum consists of a basophilic network of membranous tubules and flattened vesicles to which are attached the granular particles or ribosomes. Examination of electron micrographs of fatty liver cells shows that the parallel stacks of tubules have become irregular and multitudes of small aggregates or "rosettes" of ribosomes (polysomes) lie free in the cytoplasm (117,200,202,215-218). Observations on a wide variety of cells indicate that ribosomes lying free in the cytoplasm predominate when protein synthesis is required for intracellular use, the ribosomes being attached to membranes when synthesis of proteins for export is required (219).

In the light of these findings it is interesting to speculate that perhaps in certain types of fatty liver synthesis of the protein moiety occurs at a normal rate but being formed in a cytoplasmic location the peptides are unable to reach the lipid moieties within the endoplasmic reticulum. This could very well be the situation in the orotic acid induced fatty liver, for example, where total protein synthesis is unimpaired but release of the ß-lipoprotein is completely blocked (90,91). Ethanol induced fatty liver is an important exception here since the electron microscope photographs of Stein and Stein (205) show clearly that there is no interference in the integrity of the endoplasmic reticulum. This finding is compatible with the above hypothesis since as mentioned above (page 32) the ethanol fatty liver is not considered to be due to a defect in the protein moiety but rather to an increased synthesis of the lipid.

II. Lipids and Regenerating Liver

a) Ultrastructure Changes Following Partial Hepotectomy

The ability of liver tissue to regenerate after 2/3 its mass has been removed is a well documented phenomenon. In the rat the remaining lobe can double its size within forty-eight hours, approaching normal liver size in seven days. This rapid regeneration has provided research workers with a valuable tool for studying the phenomenon of mitosis since normally cellular division occurs very infrequently. (One hepatocyte in 15,000 is undergoing division at any given time). A variety of reviews is available covering the vast quantity of research which has been done on regenerating liver (220-222). Therefore, this Introduction will merely cover those aspects of partial hepatectomy which are directly related to the problem of lipid transport. The motivation for the subsequent experimental studies will thereby be explained.

Under the light microscope changes can be seen in the liver tissue one hour after operation. The basophilic bodies (i.e. clumps of endoplasmic reticulum) appear to break up in the cells at the periphery of the lobule, the effect spreading gradually throughout the lobe in the following couple of hours (219). Also within a few hours numerous fat globules appear in the cytoplasm to reach a maximum after the first day (207,215). This accumulated fat turns the enlarging remnant quite yellowish giving it the gross appearance of a typical fatty liver. Glycogen granules are greatly diminished by ten hours as are the sinusoidal spaces between the enlarged

hepatocytes (222).

Various electronmicroscopists have made detailed studies of the morphology of the cell after partial hepatectomy (204,207,215,216). For our purpose the most relevant features can be observed in the photographs of Jordan (215) and of Trotter (204,207). The ultrastructural changes in the regenerating cell are strongly reminescent of a typical fatty liver such as the ethionine treated (200).

For example, Trotter finds (204) that as early as 10 minutes after partial hepatectomy osmiophilic bodies appear in the cytoplasm; these are also seen with sham operated animals. At 3 1/2 hours after partial hepatectomy these bodies, which have diameters ranging from 300–1,000 Å, are much more numerous and appear throughout the cytoplasm, each located within a vesicle. Histochemical evidence indicates that these bodies may contain lipid and protein components. After fixation with potassium permanganate, the bodies display a peripheral rim of electron-opaque material surrounding a clear core. After fixation with osmium tetroxide however, they are electron opaque throughout. Since osmium tetroxide, but not potassium permanganate stains lipid, some lipid component is indicated. After several hours the osmiophilic bodies can be seen to coalesce to form larger globules. With the passage of time the number of these large droplets increases. There is a concomitant reduction however in the number of the osmiophilic particles (i.e. 600–1,200 Å).

It has been suggested by both Jordan (215) and Trotter (204) that the electron-opaque bodies enter the cell from the circulation by a process of pinocytosis.

In support of this it was found that 10 minutes after partial hepatectomy the bodies were more numerous in the spaces of Disse than at later intervals. Also at the cell membrane numerous invaginations, each containing a lipid body, have been observed again indicating pinocytosis. However, since the sham operated animals also show this phenomenon it is difficult to visualise this process as being the cause of the fat infiltrated liver. Also since the animals used by Trotter were not fasted before operation it is quite possible that these very early appearances of the osmiophilic bodies in the spaces of Disse represent trapped chylomicra. This does not explain however why the livers get fatty in the fasted partially hepatectomised animal. The extent of the infiltration is in fact as great as in fed animals (223). Very recent studies by Olivecrona and Fex (204) give credibility to the theory that the early osmiophilic bodies in the space of Disse are chylomicra. They have shown an increased uptake of labelled chylomicra on a gram liver basis in 4 hour partially hepatectomised rats and postulate that there is an increased trapping of chylomicra in the extracellular spaces of these livers.

An examination of the ultrastructure of the regenerating liver cell shows that the endoplasmic reticulum membranes are no longer arranged in normal stacks (215). This is in agreement with the morphological findings in most other fatty livers. As has been described above (200–202), ribosomes of the rough endoplasmic reticulum are dissociated from the membranes and lie in clusters in the cytoplasm. At approximately 12 hours the reformation of the

regular ordering of the endoplasmic reticulum has begun probably through synthesis of new membranes (225). This new structure is invariably found closely associated with the mitochondria and usually consists of smooth membranes since the ribosomes do not reform until later. The parenchymal cells do not appear completely normal with respect to the endoplasmic reticulum for 3-4 days when the greatest burst of cellular division has ceased.

Of further significance to the problem of lipid transport after partial hepotectomy is the "streamlining" of the cell surface which is evident at six to nine hours (226). However, by 24 hours the invaginations or "microvilli" have reappeared around the space of Disse (216). These ultrastructural changes in the subcellular organelles which are ordinarily concerned with the uptake and secretion of complex molecules such as lipoprotein are of considerable importance to the lipid metabolism of the liver and will be further discussed below in the light of the biochemical findings.

b) Alterations in Lipid Metabolism Following Partial Hepatectomy

Hepatic Lipids in Regeneration

The accumulated lipid of partial hepatectomy has been recorded and examined by many early research workers (220,221,227). Significant increases in the lipid content of the liver can be detected by six hours, the concentration being double the normal value by 10 hours. The total lipid content reaches a maximum after one day (223) but remains stable thereafter for 21 days (220).

Bengmark et al. (221) have performed a detailed analysis on the infiltrating fat. They showed that on the basis of fat-free dry tissue the increase in triglyceride content amounted to two thousand per cent. Even at four weeks after operation the triglyceride content was still 53 per cent above normal. However, later studies show that on a gram wet liver basis, the triglyceride increases are more modest (86,173,228), being two to five times the value of sham operated controls at 20 or 18 hours, respectively.

Quantities of cholesterol and phospholipid have been shown by Bengmark et al. to be elevated after several days of regeneration (221), however, the alterations are not as pronounced as those of the triglyceride fraction. At the pre-mitotic stage, however there seems to be a certain amount of disagreement in the literature concerning phospholipid concentrations. For example, at 18 hours Levin et al. (229) showed a 50 per cent phospholipid increase in the mitochondria and microsomal fractions. Other investigators (86,173,228,230) could detect no increase in phospholipid concentration when expressed on a gram liver basis and compared to sham operated controls. This large infiltration of triglyceride into the regeneration liver with concomitant small increases of the other lipids closely corresponds to the effect seen in most other types of fatty liver as discussed above (page 17). Some further similarities and differences will now be considered.

The finding by Fex and Olivecrona of an increase in plasma free fatty acids in 18 hour partially hepatectomised rats (173) when compared to laparotomised animals is interpreted as being a contributary factor in the hepatic lipid buildup.

This increase in all likelihood involves an elevated mobilisation of fatty acid from the adipose tissue. However Camargo et al. (223) have been unable to detect any difference in free fatty acid levels between 70 per cent partially hepatectomised rats and sham operated controls at the 18 or 24 hour intervals. Significant differences were obtained however between operated and non-operated controls.

It appears therefore that the effect of the surgical procedure and fasting contributes to an increased mobilisation of fatty acids. This mobilisation could be brought about by the action of pituitary or adrenal hormones (231,232). It has been shown for example that no lipid accumulation will occur in adrenalectomised animals following CCl_4 treatment (188). Similar experiments by Camargo et al. (223) with hypophysectomised, adrenalectomised or reserpine-treated rats showed that these procedures were ineffective in preventing the triglyceride accumulation of regenerating liver. The authors thereby demonstrated that an increased stimulation of fatty acid mobilisation was not necessary for the steatosis and conclude that the origin of the lipid lies within the liver itself. Such conclusions would seem to be premature however, since the flux of fatty acid per gram of liver could be increased without an elevation in plasma free fatty concentration. This concept will be further examined below.

The finding of Simek et al. (223) however are difficult to reconcile with the conclusions of Camargo. They have found that continuous intravenous infusion of glucose and insulin not only inhibits the triglyceride accumulation but significantly reduced the overall regeneration activity as measured by thymidine ^3H incorporation into parenchymal nuclei. These authors speculate that perhaps the glucose and insulin

exert their effect by reducing the quantities of free fatty acid coming from adipose tissue. A further interesting speculation is that perhaps the inhibition of lipid elevation is having a direct effect on the regeneration activity by eliminating a possible energy source. However, this seems unlikely in view of the considerable quantities of glucose which are available for utilisation by the liver cells.

The uptake of fatty acid by the regenerating liver has been investigated by Fex and Olivecrona using tritated oleic acid (173). They found that even though the rate of decay of the isotope from the plasma is somewhat depressed in the case of the 18 hour partially hepatectomised rats, the higher concentration of fatty acid in the circulation equalises the flux rates through the plasma pool. However, it should be borne in mind that because the regenerating liver is one third the normal size, the uptake of fatty acid per gram tissue is elevated. The increased blood flow in the remnant after removal of the two lobes (234,235) is compatible with this argument. Examination of the liver lipids 2 minutes after the injection of the radioactive fatty acid showed a significantly elevated incorporation per gram of liver; a finding which is consistant with an increased uptake of plasma free fatty acid. As will be seen below, a confirmation of this increase was obtained concurrently in this laboratory with in vivo incorporation studies on the 13 hour partially hepatectomised rat (236,237).

Several workers have studied hepatic lipid synthesis in regeneration.

Early investigations by Johnson and Albert (230,238) showed increased incorporation of glycerol and acetate-1-^{14}C into hepatic triglycerides *in vivo* at the 18 hour interval. It is interesting to note that neither this research group nor Olivecrona's group were able to demonstrate increases in the phospholipid fraction although the glycerol incorporation was somewhat elevated. Incorporation of ^{32}P on the other hand has been shown to be increased into all phospholipid fractions in a variety of subcellular particles, including mitochondria and microsomes (229,239). The evidence of Gurr *et al*. however does not support these findings (45). Labelling of the liver phospholipid fractions *in vivo* with ^{32}P was not significantly altered after hepatectomy. These discrepencies might be resolved if the same period of incorporation of the isotope had been used. Also it is possible that there may be a certain amount of contamination from labelled non-lipid material as suggested by Gurr *et al*. (45). In view of its specificity it would seem that a labelled fatty acid is the most reliable precursor for measuring phospholipid synthesis.

In vitro incorporation experiments have been performed on the isolated perfused regenerating liver by two groups of investigators apart from the present author. Bartsch and Gerber showed (228) that while the total incorporation of acetate-1-^{14}C into triglyceride, phospholipid, and cholesterol fractions was decreased, on a gram basis there appeared to be an increased triglyceride synthesis in comparison to the larger normal liver. Cholesterol synthesis on the other hand was still somewhat depressed whereas the phospholipid remained unchanged. Similar findings were obtained by the present author (236,237) using tritiated palmitate as the precursor and will be described below.

Recently a French group, Infante et al., have examined the synthesis of hepatic lipids in the perfused regenerating liver (86). These authors found that incorporation of radioactive oleate into triglyceride was increased by a factor of three in comparison to laparotomised or normal livers. In agreement with the in vivo studies, phospholipid and cholesterol synthesis was only slightly elevated in the regenerating liver. It should be pointed out here, however, that all of these determinations were based on liver weight. The legitimacy of this is open to question, since ratio of uptake and secretion may not be in proportion to liver mass. It will be noted that total triglyceride radioactivity was found by Infante's group to be similar in all livers indicating that the same quantity of radioactive fatty acid is taken up regardless of the size of the liver.

Lipoproteins in Regeneration

In order to fully understand the controlling factors involved in the fat infiltration of the regenerating liver, it is desirable to examine the rates of synthesis and release of the various lipoproteins, with particular reference to the triglyceride containing fractions.

The plasma proteins and lipoproteins in partially hepatectomised animals have been studied by several authors. For example, Serafini (240) has examined the plasma proteins of rabbits following partial hepatectomy using electrophoresis and amido black/Sudan-Schwarz B staining techniques. Albumin was found to be depressed by 25 per cent at 24 hours after operation, the α, ß and γ protein fractions being unaltered. The α-lipoprotein was slightly decreased when refered to control

values while the ß-lipoprotein was somewhat elevated. The reliability of these estimations however is open to question since the protein bands are not well separated on paper and lipid staining may not be linear with concentration.

Recent determinations by Narayan et al. (241) have clarified the plasma protein picture somewhat. These authors have used the more reliable technique of disc gel electrophoresis and ultracentrifugation to separate their fractions. The total serum lipid and protein of 24 hour partially hepatectomised rats were about the same as sham operated controls. In comparison to normal (non-operated) rats, however, a slight decrease was detected. High density lipoprotein showed a lowering in total lipid and protein at the 24 hour period. On the other hand, quantities of lipid and protein in the very low density lipoprotein were similar to those in the sham animals while the lipid in the low density fraction was elevated.

Incorporation of radioactive precursors such as fatty acids or amino acids into the circulating lipoprotein has been used by several authors including the present one as a means of monitoring the secretion rates (77,86,92,236,237,242). A very recent paper by Narayan (242) shows the incorporation pattern of U-^{14}C-leucine into the lipoprotein fractions of 22 hour partially hepatectomised rats. Incorporation of the isotope into high density lipoprotein was considerably reduced in comparison to the sham animals, radioactivity in the low density and very low density fraction being also lowered although to a lesser extent. Sham operated animals, on the other hand, showed increased incorporation into most protein fractions in comparison to normal, making these results very difficult to interpret. Similar studies by Majumdar et al. (243) showed that synthesis of serum albumin and fibrinogen

increased steadily after partial hepatectomy to a maximun at 24 hours. The increased incorporation is probably due solely to elevated synthesis rather than secretion since the rate of appearance of the tagged proteins did not differ from untreated controls. This indicates that the actual rate of secretion was not accelerated. Similar rates of incorporation into the lipoprotein-protein of the intact animal has previously been reported from this laboratory (77).

Studies by Fex and Olivecrona (108) have shed some further light on the lipoprotein secretion by the regenerating liver. They have found that by administering triton to groups of 3,8 and 24 hours partially hepatectomised and laparotomised rats the resulting hyperlipemia was higher in the hepatectomised rats than in the laparotomised controls at 3 hours, similar at 8 hours and lower at 24 hours. Since triton is known to cause an inhibition of triglyceride uptake from the circulation, it has been used successfully to show the inhibition of lipoprotein secretion with certain types of fatty liver conditions (page 18). The experiment of Fex and Olivecrona shows that the ability of the liver to secrete triglyceride is not decreased at a time when a rapid accumulation of fat is taking place in the remaining liver. Since the liver is the main source of serum triglyceride in the fasted rat, the hyperlipemia in the presence of triton represents secreted lipoprotein. If we keep this in mind it is apparent that on a weight basis the regenerating liver is secreting approximately three times as much triglyceride as that of the laparotomised controls at three and eight hours, assuming that uptake is completely blocked by the triton. This finding is of particular significance in interpreting the experiments which will be described below.

In order to follow secretion rates of lipoprotein and also to monitor the actual quantities of lipid and protein released in relation to the normal tissue, the isolated perfused liver system is a most reliable method (77). By these means, the effects of extrahepatic organs on such factors as the rates of removal of lipoprotein from the circulation as well as hormonal effects can be eliminated.

Bartsch and Gerber measured the incorporation of ^{14}C-acetate into secreted lipids after perfusion with whole plasma (228). They found a considerable reduction in triglyceride and phospholipid specific activities, a finding which closely reflects the situation in the liver. It should be noted, however, that were these radioactivities expressed on a gram liver basis the decreases would not be so large. The recent perfusion studies of Infante et al. show a somewhat different picture (86). The release of low and high density lipoprotein by 20 hour regenerating liver was followed by perfusing with whole rat blood and Krebs-bicarbonate containing tritiated oleate and ^{14}C-leucine. While the total lipid and protein radioactivity in the density < 1.063 lipoprotein was slightly less than the laparotomised controls, when expressed on the basis of liver weight a definite increase in synthesis and/or release of these moieties was indicated. These results agree with concurrent experiments from this laboratory (236,237).

Incorporation of the fatty acid into the high density lipoprotein-lipid was also slightly elevated. In the case of the protein moiety, however, the incorporation of the labelled amino acid was eight times that of the controls. If this increase represents an elevated synthesis and/or secretion of high density lipoprotein-protein it is considerably difficult to reconcile with the findings of Narayan et al. (241,242). These authors detected a definite decrease in the total

amount of circulating high density lipoprotein in the partially hepatectomised animal. A possible explanation has been advanced by Infante et al. (86) who postulate that there is an increased catabolism of this lipoprotein fraction. A similar discrepency between amounts in the circulation and rates of synthesis exists in the case of albumin and fibrinogen (243,244). Further investigations on the turnover rates of high density lipoprotein in partial hepatectomy are warranted in view of the role which has been assigned to the apoprotein in lipid transport (92) (cf page 15).

Since none of the research groups to date have used lipid free perfusates, the actual quantities of lipid and protein secreted by the regenerating liver could not be estimated. Studies of this kind will be described in the experimental section of this thesis. An attempt will also be made to clarify some of the hitherto unknown aspects of lipid metabolism in the regenerating liver by means of in vivo and in vitro metabolic studies. It is hoped in this way to emphasise the main differences from some other types of fatty liver with regard to the mechanism of accumulation and secretion of lipid.

EXPERIMENTAL

a) Materials and Methods

i) Treatment of Experimental Animals

Male hooded rats were used throughout and except where indicated weighed 240-270 gms. The animals were fasted for 16 hours before being anesthetized by an intraperitoneal injection of 5 mg Nembutal per 100 gm body weight. For the in vivo experiments, 2 μ Ci amino acid-U-^{14}C mixture and 0.5 μ Ci of albumin-bound palmitate 9,10^{3}H in a total volume of 0.5 ml saline were injected via the jugular bulb. In the first series of experiments the radioisotopes were given simultaneously, serial samples of blood being taken at intervals from the tail. After clotting, the serum was separated by centrifugation at 1000 g for 2 1/2 hours. The lipoprotein fractions were then separated and analysed as described below. In the second series of in vivo studies, the radioactive amino acids were injected 1 1/2 hours before sacrifice while 1/2 hour was allowed for incorporation of the tritiated palmitate. As will be seen, these periods were chosen in accordance with the metabolic pattern obtained in the first series.

ii) Perfusion Technique

The perfusion of the liver was performed according to the procedure of Miller et al. (245) a flow rate under 210 drops per minute being unacceptable. Blood was obtained by heart puncture of discarded breeders, combined, and defibrinated by stirring. The erythrocytes were washed twice with equal volumes of 0.9% saline. Each perfusion medium consisted of 25 ml of the packed red cells and 75 ml of Krebs-Ringer bicarbonate solution at a pH of 7.2. The bicarbonate solution contained 3% bovine serum albumin

(Cohn fraction V) 500 mg % glucose, and 150 mg % of a mixture of all the essential amino acids (246). In the experiments where large amounts of palmitic acid were circulated, the fatty acid was complexed to the albumin solution as follows: a 1% solution of palmitic acid in saline was prepared by adding 4.5 ml 1 N NaOH and boiled. Four ml of this hot solution was added to 100 ml of the Ringer-albumin perfusion medium with constant stirring. The solution was then shaken for 1/2 hour giving a concentration of 40 mg per cent fatty acid. The solution, which was constantly infused into the perfusion medium, consisted of 40 ml of a 4% albumin-saline solution containing 60 mg of palmitic acid. Infusion was carried out with a 50 ml glass syringe attached to a Multi-Speed constant infusion apparatus. The rate of flow was 20 ml per hour.

After the first 25 ml of perfusate had been collected and discarded, 12 μ Ci of albumin-bound palmitate 9,10^{3}H and 6 μ Ci amino acid-U-^{14}C were added to the medium. At the conclusion of the experiment all livers were washed by perfusion with 30 ml of saline, rinsed, blotted, weighed and homogenised in 50 ml of 0.25 M sucrose.

The observations of Miller et al. (245) showed that in the course of the perfusion oxidation of glucose proceeds for the full period. The experiments of Buckley (77) in this laboratory as well as those of Infante et al. (86) confirm that the gross appearance and bile flow do not appear to be significantly altered by the perfusion. The flow rate was within the range 210-250 drops per minute and was constant throughout the perfusion period.

iii) Induction of Fatty Livers

Partial hepatectomy, which resulted in the removal of 70% of the liver was performed according to Higgins and Anderson (247). A carbon tetrachloride induced fatty liver was produced by intraduodenal injection of 0.1 ml of the hepatotoxin per 100 g body weight 13 hours before sacrifice. A 3% orotic acid diet was prepared by homogenisation with Purina

Labena Chow after which the food was set into cakes by the addition of water and air dried.

iv) Lipoprotein Isolation

Separation of lipoprotein fractions was achieved by ultracentrifugation in the International B-60 machine using a modification of the procedure of Havel, Eder and Bradgon (248), as described previously (77). ß-lipoprotein of the serum or cell-free perfusate was isolated by the calcium-heparin precipitation method of Jordon et al. (80) and the precipitate washed twice with 0.4% calcium chloride. As shown in Figure 6, this procedure isolated the ß-lipoprotein of the VLDL and LDL and includes almost all of the serum triglycerides (81).

v) Purity of Lipoprotein Fractions

Disc gel electrophoresis (77) and paper electrophoresis (81) of $d < 1.063$ and heparin precipitated lipoprotein showed that these fractions were uncontaminated with albumin or HDL.

The heparin precipitate was subjected to further immunochemical study in order to justify its use in the metabolic experiments. The procedure was as follows: Rabbit antisera to rat heparin precipitate was prepared by four weekly injections into the foot pad of equal volumes of the antigen, solubilised in 5% saline, and Freund's

adjuvant (58). Blood was collected 10 days after the final dose and 0.01% sodium merthiolate added. Immunodiffusion studies, carried out according to Ouchterlony (249) showed a single line of identity against heparin precipitate or whole serum (81). Immunoelectrophoresis was performed by a modification of the procedure of Hirschfield (250) as previously reported from this laboratory (81). As shown in Figure 6, a single band was obtained when the anti-serum was run against the heparin precipitate or whole serum. This band was in the ß-region and stained for both lipid and protein with oil red-O and amido black respectively.

vi) Extraction and Separation of Lipids

The liver, lipoprotein and perfusate lipids were extracted by the method of Folch et al. (251). Neutral lipids and phospholipids were separated by silicic acid column chromatography using chloroform and methanol respectively as the eluting solvents (252). Free fatty acids were separated from triglycerides by the extraction procedure of Borgstrom (253). Efficiency of the separation technique was checked by development of the lipid samples on thin layer plates of Silica Gel H (254,255).

vii) Preparation and Incubation of Ribosomes

Ribosomes were prepared according to the procedure of Bungenberg de Jong

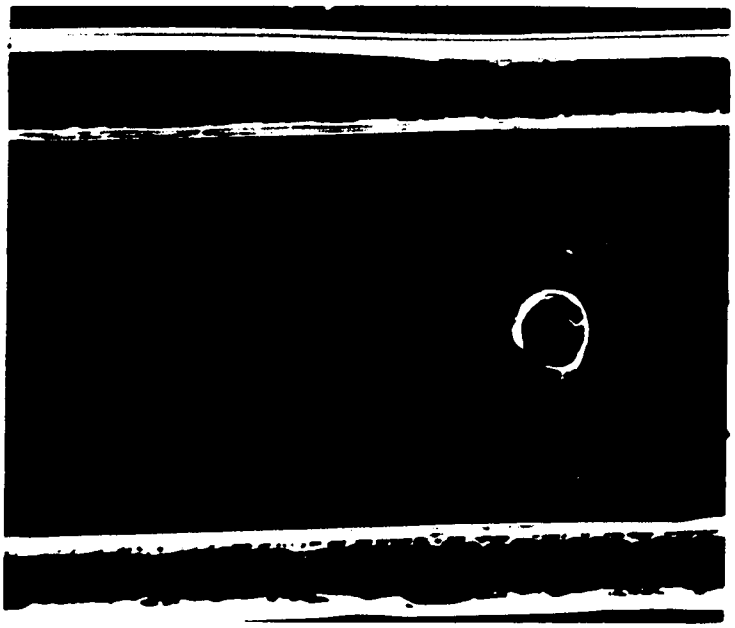

Figure 6 : Immunoelectrophoresis of rat serum βlipoprotein.
ß - lipoprotein was placed in the centre well.
Both troughs contained the anti-ß-lipoprotein.

and Marsh (58). Whole livers from fasted rats weighing 200-220 gm were homogenised in 0.44 M sucrose and spun at 15,000 g for 15 minutes. Following treatment of the supernatant with 5% Na deoxycholate, the ribosomes were precipitated at 150,000 g and washed in tris buffer pH 7.5. pH 5 enzymes were simultaneously prepared by homogenising a whole liver in 0.05 M KCl and spinning at 105,000 g for 50 minutes. After the pH had been brought to the range 5.3-5.1 by the addition of 1 N acetic acid, the precipitated enzymes were resuspended in 0.045 M tris at pH 7.5. The procedure used was exactly as described by Bungenberg de Jong and Marsh (58).

The final incubation medium consisted of 0.5 ml ribosomal suspension, 0.25 ml pH 5 enzymes, and 0.3 ml of a cofactor solution. The latter contained the cofactors ATP, PEP and GTP as outlined by Bungenberg de Jong and March (58), PEP kinase, 30 μM cold amino acid mixture and 0.2 ml of (U)-^{14}C amino acid mixture (24×10^6 cpm) in a total volume of 1.5 ml.

After the one hour period of incubation at 37°C, the ribosomes were sedimented and allowed to stand overnight in 2 M LiCl. Following centrifugation 0.5 ml of the extract was precipitated with 10% TCA. Three ml of the remaining extract solution was added to 0.5 ml of serum and 0.3 ml of ß-lipoprotein solution. The total ß-lipoprotein from this mixture was then precipitated with heparin and $CaCl_2$ in the manner described above for total serum (page 57).

TCA precipitates were washed twice with 3 ml of 5% TCA and 0.1% amino acid mixture to remove any contaminating labelled amino acid. The precipitates were then washed with ethyl ether to remove the TCA and counted on paper. Heparin precipitates were washed twice with 0.6% $CaCl_2$ containing 0.1% amino acid mixture, and dissolved in formic acid for counting on paper.

viii) Chemical Determinations

Triglyceride levels were estimated by the procedure of Van Handel (256) while lipid phosphorous was determined according to Fiske and SubbaRow (257). Protein in the tissue homogenate was precipitated with 10% trichloroacetic acid, washed twice with 10% trichloroacetic acid containing 100 mg/100 ml unlabelled leucine and finally with 2 ml of ethyl ether. The lipoprotein and liver precipitates were dissolved in N NaOH for protein estimation according to Lowry et al. (258). Radioactivity was measured by spotting and drying 0.2 ml of the protein solution on Whatman No. 2 filter paper. The paper was then placed in 15 ml of standard toluene-based phosphor solutions. Neutral lipid samples were dissolved directly in the phosphor solution while phospholipids were first solubilised in 1 ml of methanol. A Packard "Tri-Carb" liquid scintillation counter was used at double label setting when both labels were present; maximum tritium or carbon settings being used when either label was present alone. Efficiency at these settings was 38% for tritium and 85% for carbon. Corrections were made for self absorption, overlap of isotopes and for quenching using an external standard.

The radioactive fatty acid was bound to bovine albumin according to the procedure of Milstein and Driscoll (259). The radioactive isotopes were obtained from New England Nuclear Corp., Boston, the unlabelled amino acids, cofactors and puromycin HCl, from Nutritional Biochemical Corp., Cleveland and all other chemicals from Fisher Scientific Co., Montreal.

b) Results

i) Metabolism of Lipoprotein in the Normal Rat

As a preliminary to the metabolic studies with partially hepatectomised animals it was decided to investigate the synthesis and secretion of lipoprotein in the intact normal rat. The purpose of this was to get an indication of the relative turnover rates of the lipid and protein moieties. To this end 0.06 μ moles of L-leucine-1-^{14}C containing 2 μCi and 0.02 μ moles of albumin bound palmitate 9,10^{3}H containing 0.5 μCi in a total volume of 0.6 ml was given intravenously to a rat. Approximately 0.5 ml of blood was taken periodically from the tail for four hours. The very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins were separated from the serum by ultracentrifugation. Lipid and protein radioactivities were expressed as cpm/μgm protein for each fraction and plotted as a function of time.

Figure 7 shows typical incorporation curves for VLDL + LDL ($d < 1.063$) lipoprotein and is representative of the pattern obtained with 9 different animals. A maximum incorporation of ^{14}C-leucine occurred at about 1.5 hours, the specific activity at this time being approximately 4.5 cpm/μgm protein. The lipid radioactivity on the other hand was always at a maximum in the first sample taken, indicating that the radioactivity peak occurs in less than 30 minutes. By 3/4 to 1 hour the radioactivity had fallen to half the value obtained at the 30 minute interval, reflecting the rapid

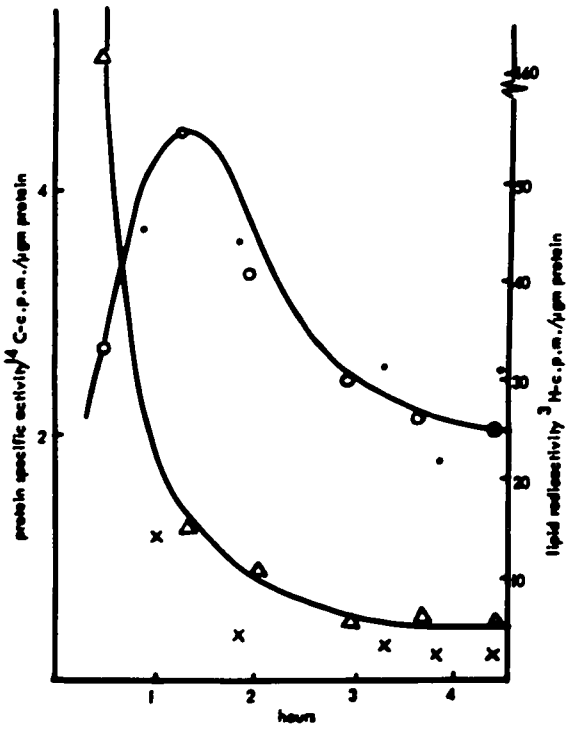

Figure 7 : Incorporation of leucine-1-^{14}C and palmitate-9,10 ^{3}H into d < 1.063 lipoprotein for the normal rat following I.V. injection of the isotopes.

Key: Δ Lipid radioactivity; × lipid radioactivity (repeat 2 weeks later).
o Protein radioactivity; • protein radioactivity (repeat 2 weeks later).

turnover of the lipid moiety in comparison to the slower rate of incorporation exhibited by the protein in this fraction. The turnover of both moieties of the HDL was also examined (not illustrated) and was shown to be less rapid than the VLDL + LDL (260). As shown in the Figure, when the experiment was repeated on the same animal 2 weeks later, a similar incorporation pattern was obtained.

In an attempt to gain some insight into the different mechanisms involved in the control of the secretion rates of the two major moieties in the $d < 1.063$ lipoprotein fraction, the influence of puromycin on the incorporation pattern was examined. One week after a time course experiment had been performed as already described on a normal rat, 20 mg of puromycin were administered. Thirty minutes before the administration of the radioactive tracers 10 mg of puromycin were given by intravenous injection; the remaining 10 mg were injected simultaneously with the radioisotopes.

Figures 8 and 9 show the effect of puromycin on the incorporation pattern of the lipid and protein respectively. It can be seen that puromycin causes not only a severe reduction in the maximum lipid radioactivity but also a delay in incorporation of the tritiated palmitate of approximately one hour. A similar delay in the appearance of the reduced protein specific activity was also observed (Figure 9). It is of interest here to note that the lipid radioactivity maximum was delayed by the puromycin for the same length of time as the protein. This type of pattern was obtained in four similar experiments. The studies indicate that the secretion of lipid and protein in the $d < 1.063$ lipoprotein are very closely tied together.

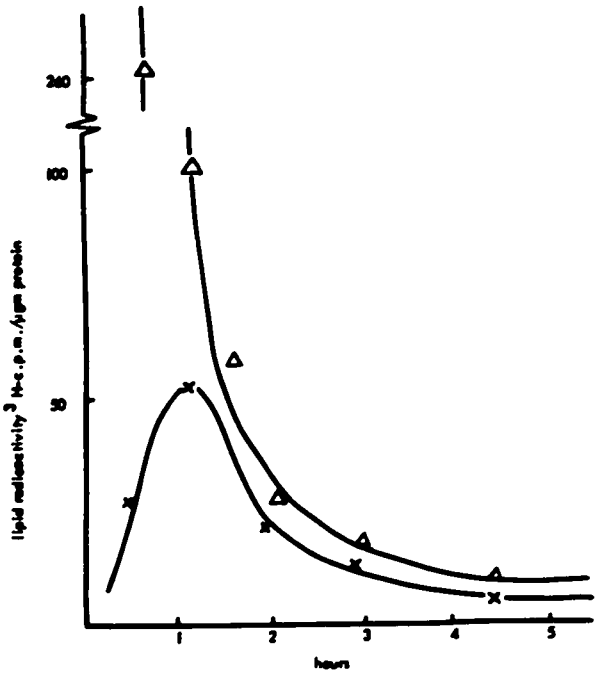

Figure 8 : Pattern of incorporation of palmitate 9, 10 ^{3}H into rat d<1.063 lipoprotein-lipid with and without puromycin administration.
Key: Δ lipid radioactivity, normal rat.
× lipid radioactivity after puromycin.

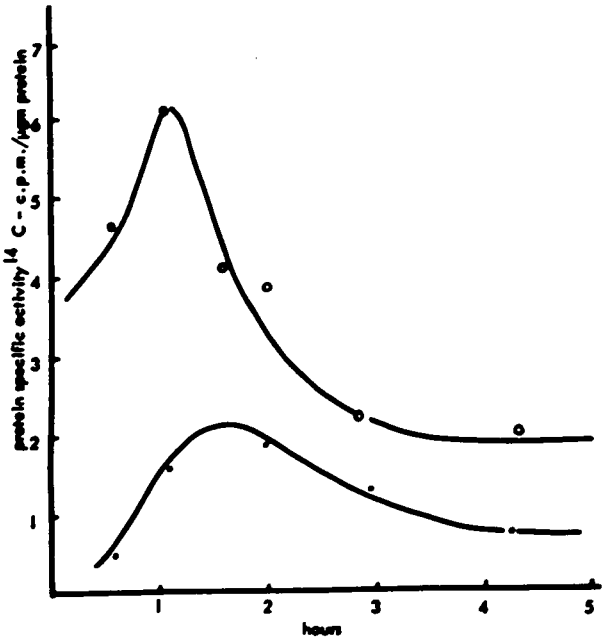

Figure 9: Pattern of incorporation of leucine-1-^{14}C into rat d<1.063 lipoprotein-protein with and without puromycin.

Key : o protein specific activity , normal rat.

• protein specific activity after puromycin administration.

ii) Secretion of Triglyceride in vivo by the Partially Hepatectomised Rat

The fatty liver of partial hepatectomy was compared to carbon tetrachloride and orotic acid treated livers with regard to quantities of hepatic triglyceride. This experiment was a preliminary to the projected studies on the accumulation and secretion of lipid by the partially hepatectomised rat. As can be seen in Figure 10, 0.1 ml CCl_4 / 100g administered intraduodenally causes a five fold elevation in the triglyceride content of the liver, while livers taken 24 hours after partial hepatectomy and from animals fed 3% orotic acid for one week show 11 1/2 and 9 mg /gm respectively.

In order to establish the optimum time for the metabolic studies, the accumulation of triglyceride was determined at various time intervals after partial hepatectomy. As shown in Figure 11, the level of triglyceride continues to rise steadily for 20 hours. This accumulation pattern is similar to that obtained by Camargo et al. (223) who studied the total lipid of the liver as a function of time after partial hepatectomy. It should be pointed out that although on a gram liver basis the quantity of triglyceride is elevated, the level at 20 hours is approximately equal to that found in the whole liver of the intact normal animal.

In consideration of this finding, a 13 hour period of regeneration was chosen as a suitable time for investigating the steatotic phenomenon since at this period there was still active lipid accumulation although no change in the liver weight had as yet occurred. In addition the first alteration in ribosomal configuration characteristic of regeneration is seen at this point (261).

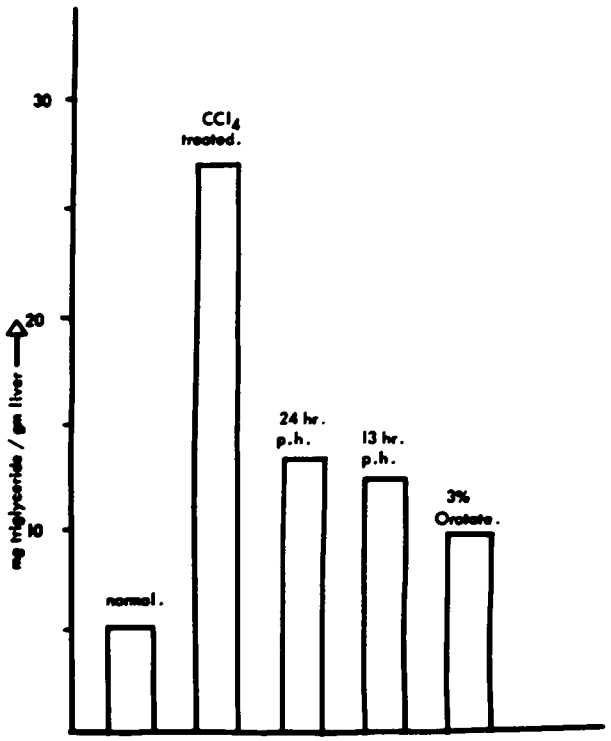

Figure 10 : Quantities of triglyceride per gram of liver tissue for normal, partially hepatectomised (ph), CCl_4 and orotic acid treated livers.

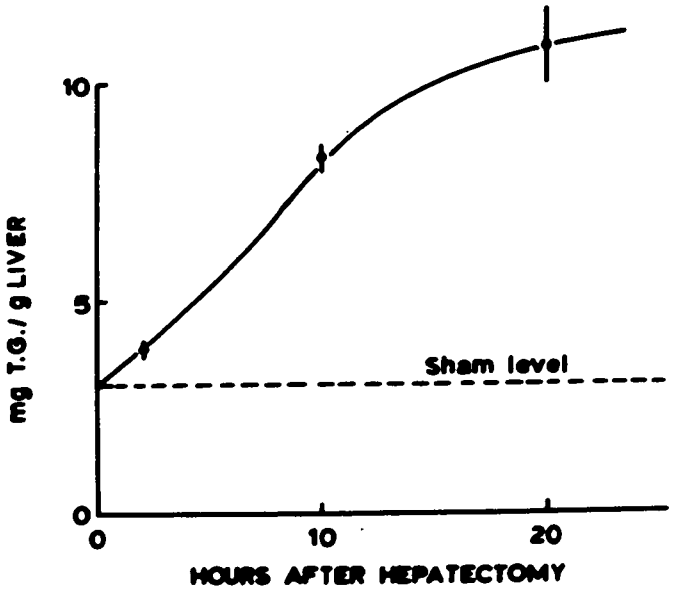

Figure 11 : Accumulation of triglyceride as a function of time after partial hepatectomy.
Each time point represents the mean of seven different animals ± Standard Error.

Intravenous injection of amino acid mixture ^{14}C and albumin bound palmitate 9,10^{3}H provided a comparison of the synthesis and secretion of the lipid and protein moieties of the ß-lipoprotein in vivo for 13 hour partially hepatectomised animals and sham operated controls. The heparin precipitation technique for ß-lipoprotein was used in all subsequent experiments both for its convenience and reliability as well as its high triglyceride content (81).

Table I shows the radioisotope incorporation into the lipid and protein of the liver following administration of the tracers. As can be seen the quantities of triglyceride per gram of liver are approximately double that of the controls while the protein is unaltered. Triglyceride radioactivity on a gram basis is considerably increased, possibly indicating an elevation in synthesis, although radioactivity per total liver is reduced, which is presumably a reflection of the smaller size of the regenerating liver. A similar increase in amino acid incorporation per gram of liver tissue was also noted. These changes result in a lower triglyceride and higher protein specific activity in the regenerating liver. Table II is an analysis of the circulating ß-lipoprotein for the same group of animals. It will be noted that the quantities of triglyceride and protein in the lipoprotein are not significantly altered by the partial hepatectomy. An increased incorporation into the protein moiety was indicated while in the case of the lipid it was significantly lower. The specific activity of the protein on the other hand was in the same range for both groups, while the specific activity of the lipid was considerably diminished in the case of the partially hepatectomised animals. The latter may be a reflection of the lower specific activity of the liver triglycerides. The fact that

TABLE I

In vivo incorporation of palmitate-9-10-^{3}H and amino acid-U-^{14}C into the triglycerides and protein respectively of liver

	Units	Triglycerides			Protein		
		Sham Operated	Partial Hepatectomy	P	Sham Operated	Partial Hepatectomy	P
Liver weights:	g	7.9 ± .2	2.2 ± .1	<.01	7.4 ± .2	2.0 ± .1	<.01
Content:	mg/g liver	5.6 ± .5	10.7 ± .5	<.01	150. ± 1.1	140 ± 4.8	N.S.
Radioactivity:	cpm x 10^{4}/g liver	64.5 ± 4.5	86.6 ± 7.7	<.03	3.3 ± .4	4.8 ± .3	<.05
Specific activity:	cpm x 10^{2}/mg	1240 ± 140	810. ± 70	<.05	2.2 ± .2	3.5 ± .2	<.05
Number of animals:		12	9		4	3	

Values represent mean ± standard error of the mean.

* N. S. not significant.

TABLE II

In vivo incorporation of palmitate-9-19-^{3}H and amino acid mixture-^{14}C into triglyceride and protein of ß-lipoproteins

	Units	Triglyceride		Protein	
		Sham	Partial Hepatectomy	Sham	Partial Hepatectomy
Content:	mgs/100 ml serum	60 ± 5	62 ± 5.5	22 ± 2.7	24 ± 1.7
Radioactivity:	cpm x 10^2/ml serum	52 ± 5	27 ± 2*	12.7 ± 1.2	15.5 ± 0.9*
Specific activity:	cpm/µg	8.5 ± 0.7	4 ± 0.3*	6.2 ± 0.8	6.5 ± 0.7
Number of animals:		11	11	7	8

* Significantly different from control ($p < .01$)

Figures represent means ± S.E.M.

Mean Liver Weights

hr	2	10	20	sham
g	2·76	2·94	2·96	9·5

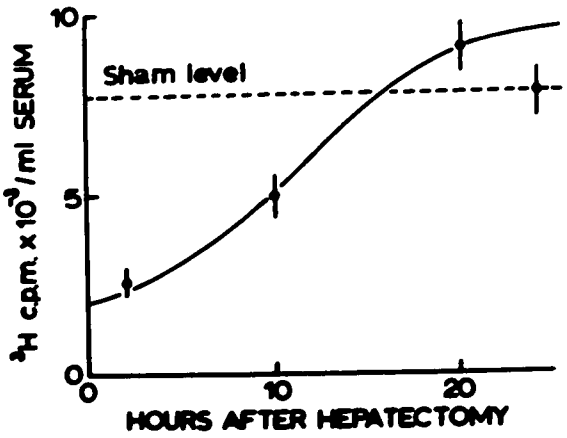

Figure 12 : Conversion of palmitate 9, 10 ^{3}H into βlipoprotein triglyceride at several time intervals after partial hepatectomy. The isotope was administered by I.V. injection 1/2 hour before exsanguination. Each time point represents the mean of seven different animals ± Standard Error of the mean.

the serum ß-lipoprotein concentration is unaltered as well as the constancy of the protein specific activity suggests that the regenerating liver retains its ability to secrete adequate quantities of lipoprotein.

In order to get a broader view of the pattern of lipid release during regeneration, conversion of palmitate 9,10^{3}H into ß-lipoprotein triglyceride was studied at several time intervals after partial hepatectomy. The incorporation pattern obtained 1/2 hour after injection of the isotope in each case is shown in Figure 12. Each time point here represents the mean of seven different animals. It can be seen that 2 hours after partial hepatectomy the livers incorporate fatty acids into lipoprotein triglyceride at about 1/3 the rate of the sham-operated animals, although on a gram liver basis the incorporation corresponds to that of the controls. As time progresses, however, the regenerating liver apparently develops an increased ability to secrete labelled ß-lipoprotein triglyceride so that by 20 hours the secretion is on a par with the controls, although the weight of the livers remains unchanged.

iii) Perfusion Studies on the Partially Hepatectomised Liver

In order to investigate the secretion phenomenon further as well as to rule out such extrahepatic factors as the removal of triglycerides by other tissues and hormonal effects, the incorporation studies were next performed on the isolated perfused liver system.

Before making a more detailed examination of the liver and lipoprotein,

the incorporation of the tritiated palmitate into the perfusate triglyceride was examined at several time periods after the start of the perfusion. This experiment was undertaken in order to get an indication of the optimum time for release as well as to compare the secretion rates after different treatments. Figure 13 shows typical incorporation patterns into perfusate triglyceride for normal, partially hepatectomised and CCl_4 treated livers. It will be noted that the most rapid incorporation occurred with normal liver while the release of labelled neutral lipid by livers which had been partially hepatectomised either immediately before perfusion or 13 hours earlier was considerably lower, although the rate of release by the two types of partially hepatectomised livers was not significantly different. A carbon tetrachloride treated liver was included in this experiment since it is known to have a decreased lipoprotein secretion rate (30). As seen in the graph the CCl_4 treated liver had an even greater diminution in labelled perfusate triglyceride than the smaller partially hepatectomised livers, and was only 1/10 that of the normal. These results demonstrate that the time taken for perfusate radioactivity to reach a maximum was similar for the normal or partially hepatectomised livers and that by 2 hours the greatest incorporation had been reached in both cases.

In order to find out whether the hepatic triglyceride radioactivity was a meaningful index of esterification, it was necessary to first determine the rates of uptake of the labelled fatty acid. The rate of uptake of the fatty acid precursor in the course of these perfusions is shown in Figure 14. Each time point represents the mean of four experiments; variations were less than 10 per cent of initial radioactivity. It can be seen that the $t_{1/2}$ for the normal liver (20 minutes) is considerably shorter than either the partially hepatectomised (50 minutes) or carbon tetrachloride treated (40 minutes) liver. This indicates that the smaller liver is not capable of taking up the same quantity of fatty acid

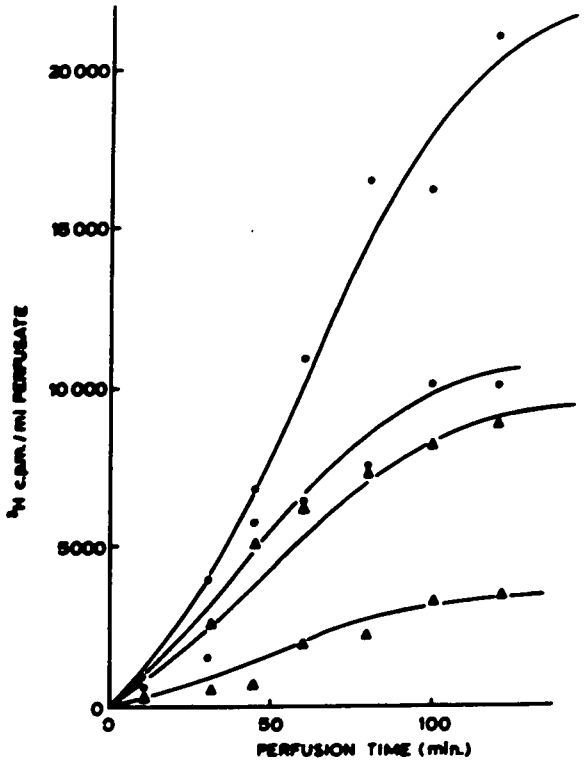

Figure 13 : pattern of incorporation of albumin bound palmitate 9, 10 ^{3}H into perfusate triglyceride for normal (•), partially hepatectomised [(o) zero hour, (▵) 13 hour] and CCl_4 (▲) treated rats.
The labelled palmitate was added to the perfusate at zero time.

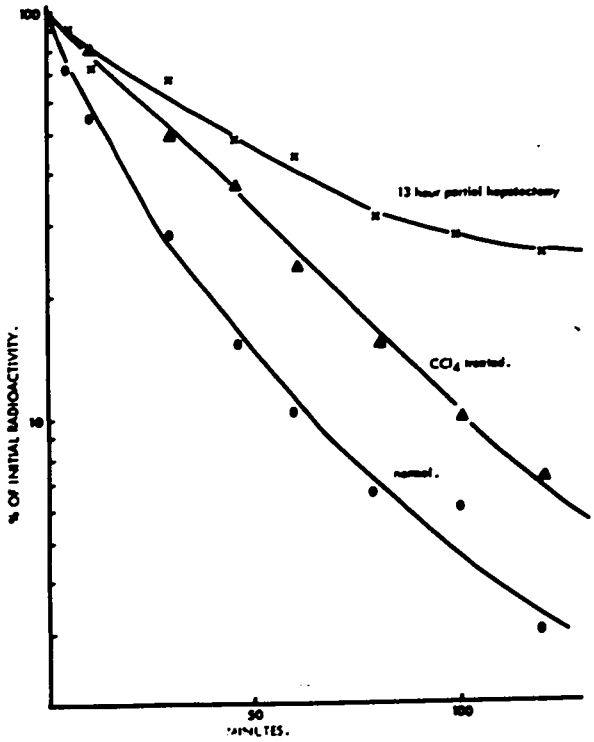

Figure 14 : uptake of palmitate 9, 10 $-^3H$ in the course of the perfusions cited in figure 13. Two ml samples of perfusate were taken.

Key : ● Normal liver.
△ CCl_4 treated liver.
× 13 hour partial hepatectomy.

as normal sized liver; however, on a gram basis uptake by the regenerating liver would still be greater. To make this comparison easier livers perfused immediately before perfusion were compared to 13 hour regenerating livers. Figure 15 shows the rate of uptake by these livers. As was the case with the full sized livers, CCl_4 caused a marked reduction in the decay of the isotope for zero hour partially hepatectomised livers.

As shown by the perfused liver experiments cited in Figure 13, on the basis of liver weight, partially hepatectomised liver retains its ability to synthesise and secrete labelled neutral lipid. However since the in vivo experiments indicated a possible increase in lipoprotein secretion at later periods after partial hepatectomy, a detailed examination of fatty acid and amino acid incorporation into the lipid and protein was undertaken after 120 minutes of perfusion. Partially hepatectomised livers were excised immediately or 13 hours after surgery and perfused with the Krebs-Ringer albumin solution, thus avoiding the complications arising from the use of livers of different weight. When the first 25 ml had flowed through and had been discarded, 12 μ Ci palmitate 9,10^{3}H and 6 μ Ci amino acid mixture (U)-^{14}C were added to the medium. Table III is an analysis of the hepatic lipid and protein content and radioactivity at the termination of the perfusion and is not unlike the pattern obtained in the liver of the intact animal (cf. Table I). The weight of both types of liver was not significantly different although the 13 hour regenerating tissue contained a great deal more triglyceride. Again, the amount of protein was apparently unaltered although an increased incorporation was detected. A significantly increased incorporation into the hepatic triglyceride was also noted.

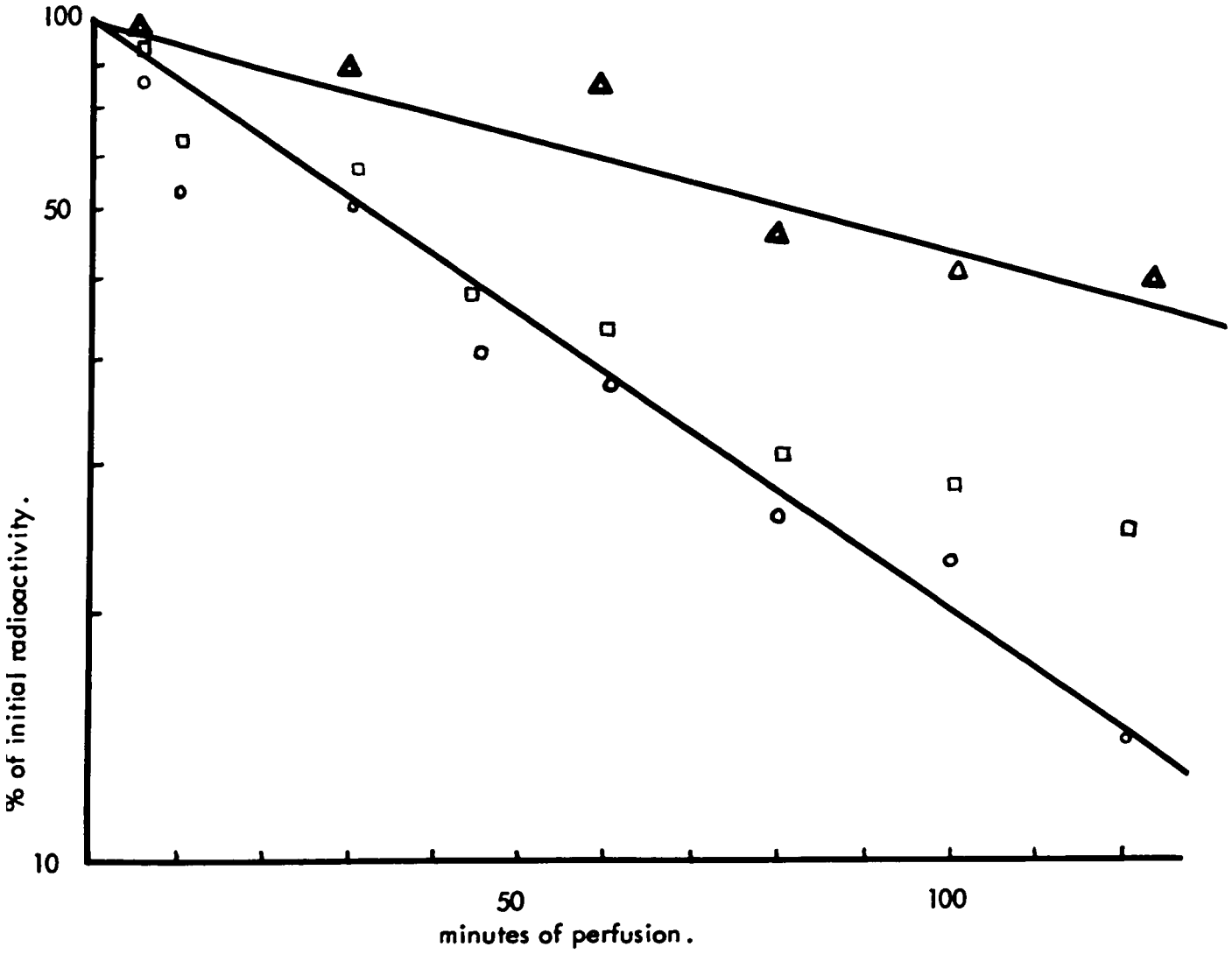

Figure 15 : Rate of uptake of palmitate 9, 10 $\frac{3}{-}$ H during perfusion of 13 hour (□), zero hour (○) and CCl_4 (△) treated partially hepatectomised rats.

TABLE III

Incorporation of palmitate-9-10-^{3}H and amino acid-U-^{14}C into hepatic triglycerides and protein respectively during perfusion of partially hepatectomized livers

	Units	Triglyceride			Protein		
		hrs. after partial hepatectomy		P	hrs. after partial hepatectomy		P
		0	13		0	13	
Liver weight:	g.	2.7 ± .5	2.9 ± .2	N.S.	2.4 ± .2	2.8 ± .2	N.S.
Content:	mg/g liver	5.97 ± .53	13.4 ± 1.8	<.01	134 ± 10	130 ± 12	N.S.
Radioactivity:	cpm x 10^3/g liver	604 ± 160	855 ± 110	<.04	54.1 ± 7.0	93.2 ± 11.7	<.05
Specific activity:	cpm/mg	117 ± 23	64 ± 20	<.05	0.39 ± .02	0.74 ± .08	<.02
Number of animals:		7	8		4	4	

Figures represent means ± S.E.M.

However, the triglyceride specific activity was again reduced which is presumably a reflection of the large amounts of lipid in the 13 hour regenerating liver.

Table IV shows an analysis of the ß-lipoprotein secreted in the course of these perfusions. It can be clearly seen that while the quantities of protein released by both livers were the same,quantities of triglyceride secreted by the 13 hour regenerating liver were considerably greater than that of the normal tissue. It thus appears that 13 hours after partial hepatectomy the lipoprotein contains a higher ratio of triglyceride to protein than that secreted by the freshly partially hepatectomised liver.

As was found with the in vivo experiments, the specific activity of the ß-lipoprotein-lipid secreted by the regenerating liver was considerably lower. This may be a reflection of the lower triglyceride specific activity seen in the liver (cf. Table III).

In view of the discrepencies in the literature concerning phospholipid synthesis in regeneration (cf. above,page 47), incorporation of the isotope into this lipid fraction was also measured. Table V shows the pattern of phospholipid synthesis in the liver and ß-lipoprotein in the course of several perfusions. It will be noted that while the regenerating liver contained approximately the same quantity of phospholipid as the control tissue, the specific activity was nevertheless lower. The lipoprotein phospholipid followed a similar pattern to the triglyceride, namely, an increased quantity of the lipid was secreted by the 13 hour liver while the specific activity was lower.

TABLE IV

Incorporation of palmitate-9-10-^{3}H and amino acid-U-^{14}C into perfusate ß-lipoproteins

	Units	Triglyceride		Protein	
		Hrs. after partial hepatectomy		Hrs. after partial hepatectomy	
		0	13	0	13
Content:	mg/gm liver	113 ± 26	217 ± 17 *	568 ± 200	663 ± 260
Radioactivity:	cpm/gm x 10^3	40.6 ± 16	34 ± 12	220 ± 12	465 ± 176
Specific activity:	cpm/mg	349 ± 68	143 ± 28 **	0.39 ± .05	0.71 ± .16 *
Number of animals:		6	8	4	4

Significantly different from control: * ($p < .01$) ** ($p < .05$)

Figures represent means ± S.E.M.

Table V

Incorporation of Palmitate 9,10 ^{3}H into Phospholipid of Liver and Perfusate β-Lipoprotein.

		Liver		β-Lipoprotein	
	units	hours after hepatectomy		hours after hepatectomy	
		0	13	0	13
Content:	mgs/gm	17 ± 1.8	21 ± 3.8	1.7 ± 0.4	3.5 ± 0.12*
Radioactivity:	cpm/gm x 10^3	1400 ± 30	1000 ± 162	7.6 ± 1.6	4.8 ± 1.1
Specific Activity:	cpm/μgm	86 ± 7	46 ± 5*	50 ± 16	14 ± 3.4*
Number of experiments:		3	3	3	3

* Significantly different from control ($p < .02$)
Figures represent means ± S.E.M.

iv) Secretion of Accumulated Lipid

The finding of a lower triglyceride specific activity in the ß-lipoprotein suggests that the accumulated lipid is not sequestered as a relatively inert pool but may be directly secreted. In order to further investigate this point, the triglyceride pool of the partially hepatectomised livers was labelled by the intravenous administration of 2.5 μCi palmitate-1-^{14}C 13 hours before the livers were excised for perfusion. A carbon tetrachloride treated liver was included for comparison, since it is well known to have an inhibited lipoprotein secretion rate. The per cent of this accumulated triglyceride pool secreted during the perfusion was compared with that of the newly synthesised lipid, the latter being monitored by the addition of albumin-bound palmitate 9,10^{3}H to the perfusate. Table VI shows a comparison of these percentages for the various types of livers. It can be seen that there is no significant difference in the release of the accumulated ^{14}C labelled stored triglyceride for either of the partially hepatectomised livers; in the case of the carbon tetrachloride treated liver , however, the per cent release is approximately 1/20 of the value obtained for the other livers. Secretion of de novo synthesised triglyceride is considerably greater than the accumulated material. It is recognised that a comparison of ^{14}C and ^{3}H per cent release is not very meaningful since the latter is based on liver radioactivity at the termination of the experiment only. However, it should be noted that in the case of CCl_4 treatment, the increase in triglyceride ^{3}H release over triglyceride ^{14}C release is considerably greater than was the case with the other livers studied.

TABLE VI

Per cent transfer of stored ^{14}C labelled and de novo synthesised ^{3}H triglyceride into perfusate

	^{14}C			^{3}H		
	13 hour partial hepatectomy	Zero hour partial hepatectomy	CCl_4 + zero hour partial hepatectomy	13 hour partial hepatectomy	Zero hour partial hepatectomy	CCl_4 + zero hour partial hepatectomy
% of liver triglyceride	5.6 ± 0.45	6.3 ± 0.33	0.27 ± 0.054	17.5 ± 2.65	27.8 ± 3.2	6.3 ± 1.1
No. of Experiments	(5)	(4)	(2)	(5)	(4)	(2)

Values represent means ± S.E.M.

Palmitate-1-^{14}C was injected intravenously 13 hours before removal of the livers in each case.

Perfusion time: 120 minutes.

The ^{14}C specific activities from the various groups of animals (Table VII) cannot be compared directly since the palmitate-1-^{14}C was injected when the livers from the control and CCl_4-treated animals were intact, but in the case of the 13 hour livers the isotope was given after the partial hepatectomy. Both zero hour and 13 hour hepatic specific activities were similar to perfusate specific activities, again suggesting that the accumulated triglyceride in the liver forms a continuous pool with the secreted lipid. On the other hand the specific activity of the perfusate triglyceride secreted by the CCl_4-treated liver was considerably lower than the hepatic specific activity suggesting that in this case little of the accumulated triglyceride contributes to the secretory pool.

Table VIII shows the quantities of triglyceride and phospholipid in the perfusion medium and in the liver after the 2 hour period of perfusion. It will be noted that the quantities of triglyceride secreted by the CCl_4-treated liver are considerably lower than the control tissue.

When the rate of release of the two types of triglyceride radioactivity was plotted with time, it was seen that the secretion rate of the accumulated material was at least as rapid as the de novo synthesised lipid even though the per cent secretion was depressed; these are shown in Figures 16 and 17, respectively. Although the total percentage release of the accumulated phospholipid is considerably less than that of the triglyceride, nevertheless, as seen in Figure 18, the slope of the release curve is steeper. This elevated rate of release of the phospholipid moiety is seen for both the zero hour, 13 hour and carbon tetrachloride treated livers. Further experiments with perfused normal livers (not illustrated) showed the same phenomenon.

TABLE VII

Hepatic and perfusate ^{14}C specific activity following perfusion of the livers discussed in Table VI

Treatment of animals:	Liver excised immediately after partial hepatectomy	Liver excised 13 hr after partial hepatectomy	CCl_4-treated 13 hr before partial hepatectomy. Liver removed immediately after surgery
No. of animals	4	5	3
Liver Triglyceride Specific Activity cpm/μgm	1.5 ± 0.5	2.3 ± 0.2	3.0 ± 0.8
Perfusate Triglyceride Specific Activity cpm/μgm	1.6 ± 0.6	1.8 ± 0.5	0.6 ± 0.2 *

Values represent means ± S.E.M.

* Significantly different from control.

TABLE VIII

Quantities of triglyceride and phospholipid in liver and perfusate after perfusion of the livers discussed in Table VI

	Liver			Perfusate		
	13 hour	zero hour	CCl_4	13 hour	zero hour	CCl_4
mgs Triglyceride/ gram liver	9.9 ± 0.85	4.4 ± 0.59	27 ± 4.0	0.84 ± 0.20	0.59 ± 0.21	0.36 ± 0.105
No. of Experiments	(4)	(4)	(2)	(4)	(4)	(2)
mgs Phospholipid/ gram liver	21.7	22.5	22.2	1.1	1.1	1.6

Values represent means ± S.E.M.

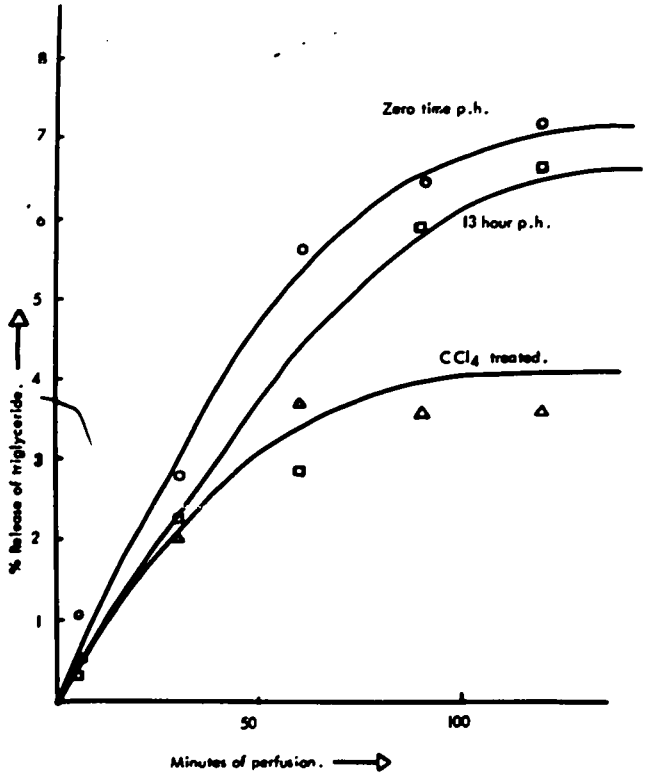

Figure 16 : Rate of release of prelabelled accumulated ^{14}C triglyceride during the perfusion of 13 hour (□) , zero hour (○) , and CCl_4 (△) treated livers. Triglyceride was labelled by I.V. injection of palmitate-1-^{14}C 13 hours before perfusion.

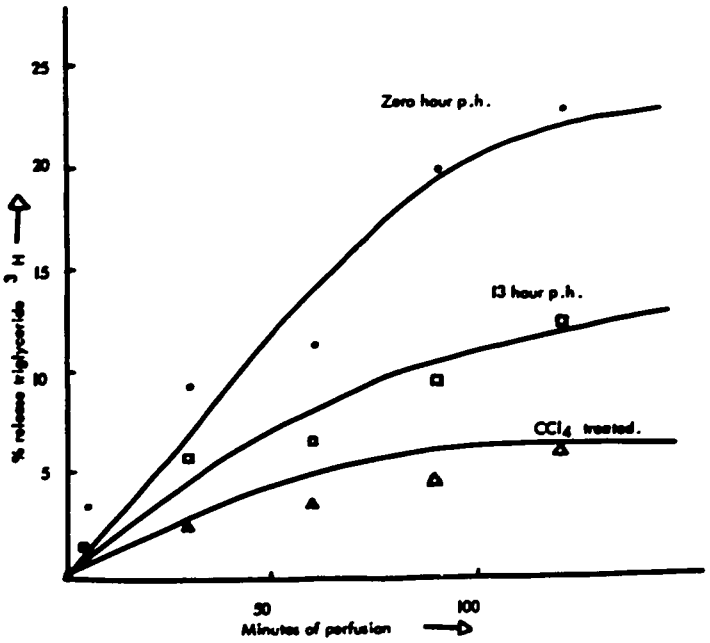

Figure 17 : rate of release of de novo synthesised ^3H-triglyceride during the perfusions cited in figure 16. Triglyceride was labelled by direct uptake of palmitate - ^3H from the perfusate.

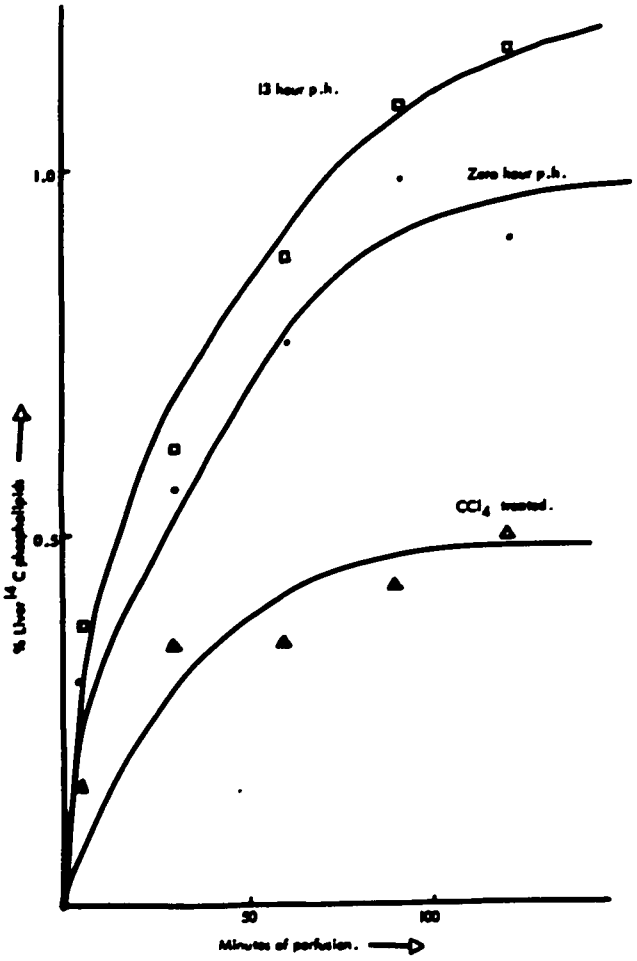

Figure 18 : Rate of release of prelabelled ^{14}C phospholipid during the perfusions cited in figs. 16 and 17. Phospholipid was labelled by I.V. injection of palmitate-1-^{14}C 13 hours before perfusion.

In an attempt to prove that a partially hepatectomised liver will synthesise more triglyceride when presented with a higher concentration of fatty acid per gram the following experiment was performed; both normal sized and zero time partially hepatectomised livers were perfused with large amounts of palmitic acid. Two experiments of this type were executed, the first had a total quantity of 300 μEq of fatty acid while the second had 200 μEq in the perfusate. One third of these amounts was present initially in the perfusate while the remaining 2/3 was added continuously in the course of the perfusion. Table IX shows the quantities of fatty acid at various time points as well as the decay of the tritiated palmitate. It can be seen that the rate of uptake by the partially hepatectomised liver is reduced in comparison to the normal liver; however, on a weight basis net uptake of the fatty acids is greater.

Table X clearly shows the elevation in triglyceride caused by the high dosage of fatty acid. It will be noted that both the quantities and incorporation of triglyceride per gram of liver at the end of the 2 hour period are vastly increased in the case of the hepatectomy for both the liver and perfusate. Table XI demonstrates that the quantities of hepatic phospholipid are unaltered by this process although that in the perfusate is somewhat increased. These results correspond to the finding in vivo that the hepatic phospholipid is unchanged after partial hepatectomy.

v) Synthesis of ß-Lipoprotein-Protein by Isolated Ribosomes from Normal and Orotate Treated Rats

In the course of the metabolic experiments with ß-lipoprotein described

TABLE IX

Concentrations and radioactivity of perfusate palmitic acid ^{3}H during perfusion of normal and partially hepatectomised livers**

Perfusion time (mins)	Experiment I *				Experiment II *			
	Normal		Zero time partial hepatectomy		Normal		Zero time partial hepatectomy	
	Palmitate		Palmitate		Palmitate		Palmitate	
	µEq	cpm ^{3}H x 10^{3}	µEq	cpm ^{3}H x 10^{3}	µEq	cpm ^{3}H x 10^{3}	µEq	cpm ^{3}H x 10^{3}
5	101	78	130	85	75	87	101	103
60	88	18	159	34	69	12	67	50
120	78	3	105	18	45	2.5	78	7

* Experiment I and II had initially 300 µEq and 200 µEq of palmitic acid in the perfusate, respectively.

** Concentration of fatty acid was maintained at a high level by continuous infusion of the unlabelled fatty acid.

TABLE X

Triglyceride concentrations and radioactivity of liver and perfusate following liver perfusion with large amounts of palmitic acid 9,10^{3}H **

		Liver		Perfusate	
		Normal	Zero time partial hepatectomy	Normal	Zero time partial hepatectomy
Experiment I *	mgs TG/ gm liver	13	26	0.81	2.37
	cpm TG/ gm liver x 10^{3}	432	1728	70	151
Experiment II *	mgs TG/ gm liver	6.4	11.4	0.62	0.76
	cpm TG/ gm liver x 10^{3}	437	1320	64	75

* Experiment I and II had initially 300 μ Eq and 200 μ Eq of palmitic acid in the perfusate, respectively.

** Concentration of fatty acid was maintained at a high level by continuous infusion of the unlabelled fatty acid.

TABLE XI

Phospholipid concentration and radioactivity of liver and perfusate following perfusion with large amounts of palmitic acid 9,19^{3}H **

		Liver		Perfusate	
		Normal	Zero time partial hepatectomy	Normal	Zero time partial hepatectomy
Experiment I *	mgs PL/ gm liver	20	27	2.2	6.1
	cpm PL/ gm liver x 10^3	93	126	11	55.8
Experiment II *	mgs PL/ gm liver	21.2	23.7	2.7	4.3
	cpm PL/ gm liver x 10^3	107	158	11	41

* Experiments I and II had initially 300 μEq and 200 μEq of palmitic acid in the perfusate, respectively.

** Concentration of fatty acid were maintained at a high level by continuous infusion of the unlabelled fatty acid.

above the author became interested in the problem of the synthesis of the protein moiety vis-a-vis total protein synthesis. The problem of synthesis and secretion by orotate treated livers was therefore of considerable interest.

Protein synthesis by isolated ribosomes from orotic acid treated rats was compared to that of normal livers. Incorporation of ^{14}C labelled amino acid mixture into the nascent peptides was determined after one hour of incubation. Total protein incorporation was determined by measuring radioactivity in the TCA precipitate while ß-lipoprotein incorporation was measured by counting the heparin precipitate of the ribosomal extract. This data is shown in Table XII. It can be seen that incorporation into the total protein and ß-lipoprotein is similar for both normal and orotate livers. This indicates that the excess orotate has no effect on the ability of the isolated ribosomes to synthesise the protein moiety of the ß-lipoprotein.

TABLE XII

Incorporation of amino acid mixture (U)-^{14}C into nascent proteins by ribosomes in vitro

cpm in total extract x 10^{-3}	Normal	Orotate treated	P Value
Total protein	15.18 ± 1.4	14.89 ± 1.6	>0.8
Heparin precipitate	2.05 ± 0.3	1.84 ± 0.2	>0.6

Each value represents the mean of 7 different incubations.

DISCUSSION

a) Lipoprotein Incorporation Studies in the Normal Rat

The in vivo experiment depicted in Figure 7 showed that the intravenous injection of trace amounts of amino and fatty acids provides a suitable method of studying the secretion rate of the lipid and protein moieties of the very low and low density lipoproteins $d < 1.063$. The rapid clearance of these precursors from the circulation has previously been shown (110,262). Havel et al. have demonstrated that the maximum incorporation into the serum neutral lipid of the rabbit occurs at 45 minutes (263). Here, in contrast, in the rat a lipoprotein lipid peak of less than 30 minutes was obtained, which however, agrees with the triglyceride curves of Baker and Schotz (54).

The finding of a maximum amino acid incorporation into VLDL + LDL at approximately 1 3/4 - 2 hours as seen in Figure 7, confirms a similar finding by Yap in this laboratory (262). This is in contrast to in vitro studies by Radding and Steinberg (264) who obtained maximum incorporation at 4 hours, and in vivo studies with rabbits (265,266) that showed a maximum incorporation of alanine-1-^{14}C into VLDL + LDL at approximately 6 hours. A similar study with methionine-^{35}S in the rooster showed a peak at 5 hours (267). Recent incorporation experiments with the choline deficient rat however (112) show the maxima of VLDL and LDL to be

approximately 1/2 and 1 hour respectively. Fried et al. (268) have found the peak incorporation to be at least one hour later in the same species.

The finding that the amino acid takes longer to incorporate into the lipoprotein than the lipid precursor could be due to a number of factors: (a) The rate of synthesis of the protein may be slower than the lipid; (b) the intracellular pathway of the protein moiety may be considerably more devious than that of the lipid, resulting in the protein taking a longer time to reach the surface of the liver cell; (c) there may be an intracellular pool of amino acid or preformed peptide which would dilute the radioactivity of the protein moiety.

The perfusion experiments of Buckley et al. (77) which confirm the in vivo findings with regard to the delay in protein incorporation, show clearly that hepatic protein synthesis was not delayed. This indicates that there was no large pool of intracellular amino acid which could dilute the label and that entry of the ^{14}C-amino acid into the precursor pool was not delayed in some other manner.

The necessity of continuous protein synthesis for lipid release by the liver cell is emphasised by the effect of puromycin depicted in Figures 8,9. It can be seen that not only is the maximum radioactivity very much reduced but also the time of incorporation is considerably delayed for both moieties. The close relationship between the lipid and protein is clearly demonstrated by the immediate release of lipid when the puromycin block is removed. In a number of experiments (not illustrated) the antibiotic had a delayed effect on the incorporation curves; however, in this case also the lipid radioactivity release closely preceded the protein peak. This effect of puromycin has also been shown with perfused liver (77).

b) The Fatty Liver of Regeneration

Comparison with carbon tetrachloride treatment shows that the fatty liver of partial hepatectomy is relatively mild, the quantities of triglyceride being only 3 times that of a normal liver. The leveling off at approximately 24 hours of regeneration is consistant with the findings of Camargo et al. (223) and Bengmark (221), who studied the regeneration for several days. It is interesting to note that the total quantity of hepatic lipid remains relatively constant throughout the regeneration period. In this respect, the phenomenon is similar to fasting (269) and quite different from acute CCl_4 or orotic acid treatments.

The in vivo incorporation studies indicate that on a gram basis the liver is synthesising a greater quantity of triglyceride and protein, findings which correspond to those of Fex and Olivecrona (173), Johnson and Albert (230,238) and Tsukada et al. (249). The former authors demonstrated an elevated incorporation of oleic acid-^3H into the hepatic non-phospholipid lipid of 18-hour partially hepatectomised rats. Radioactivity in the phospholipid fraction however was not increased. These authors also found that the half-lives were 0.55 and 0.38 minutes respectively indicating that on a gram basis the smaller liver took up a greater fraction of the tracer. The differences in half-life and liver weight make the results difficult to interpret since the incorporation cannot be based on liver weight in view of the dissimilarity between the rates of uptake of the tracer. The same difficulties arise when an appraisal is made of the protein radioactivity.

c) ß-Lipoprotein in the Partially Hepatectomised Rat

The finding of similar levels of ß-lipoprotein triglyceride and protein for the sham operated and 13 hour partially hepatectomised animals is in contrast to the results of Infante et al. who found a 1/3 lowering in the plasma triglyceride and phospholipid at 20 hours (86). As mentioned in the Introduction, Narayan found reduced quantities of VLDL and HDL protein at the 24 hour period (242), while total VLDL lipid was similar to the sham level (241). Interpretation, however, is difficult since the animals were fed ad libitum in these experiments, making it difficult to distinguish between hepatic lipoprotein and chylomicra.

The increased incorporation of the ^{14}C amino acid mixture which we have observed in the protein of the ß-lipoprotein, is in agreement with the finding of Narayan (242) for the LDL and total serum protein which was elevated in comparison to the normal animal but not, however, when compared to sham operated rats. As mentioned in the Introduction an increased incorporation into albumin and fibrinogen was also noted by Majumdar et al. (243). These authors are of the opinion that an increased efficiency of the polyribosomes is involved and that the effect is not due to secretion rates.

The observation that there is a decrease in the triglyceride radioactivity is not necessarily a reflection of a decreased secretion rate; indeed Fex and Olivecrova have shown (108) by means of the triton test that the lipoprotein secretion is maintained after partial hepatectomy. This is confirmed by the finding that the quantities of lipoprotein are not diminished in the circulation although the rates of

catabolism could conceivably be altered.

The lowering of the lipid specific activity suggests that the fatty liver is secreting part of the unlabelled accumulated triglyceride although the in vivo results alone do not prove this conclusively. The constancy of the protein specific activity, however, would indicate secretion of normal amounts of this moiety.

The time course experiment (Fig. 12) supports the theory that the regenerating liver acquires the ability to secrete accumulated lipid by 13 hours. Since the radioactivity incorporation into plasma triglyceride steadily increases following partial hepatectomy, an increased ability of the liver to secrete lipoprotein is indicated. This greater secretion capacity may be a major factor in terminating the triglyceride accumulation which levels off approximately 20 hours (Fig. 11) postoperatively.

d) Perfusion of Regenerating Liver

The finding that the secretion rate of triglyceride by perfused regenerating liver is similar to the control tissue is a further indication that there was no inhibition in the mechanism of lipoprotein release. The maximum incorporation of labelled precursor into perfusate triglyceride was considerably less than for a full sized liver, although it is noted that this occurs roughly at the same time. This indicates that the rate of lipoprotein release is the same as for a normal liver. The greatly reduced secretion of labelled triglyceride in the case of CCl_4 treated

livers, however is consistant with other findings in the literature (30,55). It would appear from this experiment that the intracellular transport of the lipid is not being affected by the partial hepatectomy since there is no delay in the appearance of the label as is the case for example with puromycin (77).

The finding of a decreased rate of uptake of the trace amounts of palmitic acid by the regenerating liver is consistant with the in vivo decay curves of Fex and Olivecrona (173) as well as the in vitro decay of acetate-^{14}C shown by Bartsch and Gerber (228). Despite this reduction, however, the net uptake of the free fatty acid on a gram basis is still greater for the regenerating liver.

Thirteen hour and zero hour partially hepatectomised livers show almost identical rates of fatty acid uptake. This indicates that the removal rate of fatty acids from the circulation is not affected by hepatic triglyceride concentration per se. The demonstration of a decreased uptake by carbon tetrachloride treated livers has not been reported before in the knowledge of the author and may be the result of a direct peroxidation action of the hepatotoxin on the unsaturated lipid in the cellular membranes as has been suggested for subcellular particles (270).

The elevated incorporation per gram tissue into hepatic triglyceride and protein as seen in both the in vivo and in vitro experiments may be manifestations of the initial stages in the regeneration process. The apparent increase in protein synthesis is presumably part of the increased formation of membranes and enzymes which precedes mitosis (222).

Synthesis of ß-protein

The elevated incorporation of amino acid also applies to the ß-lipoprotein. This may represent an increased synthesis, although no differences in amounts were detected. If the increased protein synthesis were due to the removal of an inhibitor acting on the polyribosomes as has been suggested (243,244), then it would appear that synthesis of the secretory proteins such as albumin or lipo-protein-protein is also affected by these inhibitors.

However, as mentioned in the Introduction, the work of Windmueller (90,91) suggests a different controlling mechanism for the ß-protein than for the other proteins. Secretion of ß-lipoprotein by the perfused orotic acid treated liver was totally inhibited while albumin secretion was normal. Synthesis of hepatic protein in vivo was also found by several research workers to be unaffected (271,272,273). The present finding of an apparently normal protein synthesising ability of isolated ribosomes from orotate treated livers supports the earlier results.

As also mentioned in the Introduction, the circumstantial evidence of Rajalakshmi et al. (128) and Faloona et al. (128) suggests that synthesis of the ß-protein moiety is being specifically inhibited. In contrast, Roheim et al. found (126) a decreases conversion of $d > 1.21$ apoprotein to lipoprotein and suggest that the orotate causes an inhibited formation of the lipoprotein with circulating apoprotein. The ribosomal data presented here supports the concept of an inhibited secretion rather than synthesis since the incorporation of labelled amino acid was similar to the controls. Consequently, no conclusive evidence has yet been presented which shows that synthesis of the ß-protein is being controlled in a different

manner to other proteins of hepatic origin.

Triglyceride synthesis

The increased esterification in the 13 hour regenerating liver parallels a simultaneous increase in triglyceride secretion. A comparison may be drawn here to the increased esterification and triglyceride secretion of carbohydrate feeding (274,275).

It thus appears that the accumulated triglyceride results from two basic factors: (a) an increased rate of esterification, (b) increased flux of free fatty acids per gram of liver tissue. This latter hypothesis has been raised by Fex and Olivecrona (173) who find an elevated plasma fatty acid concentration coupled with an uptake rate of radioactive oleate which is almost on a par with a full sized liver. This hypothesis will be further discussed below in the light of the experiments presented here.

The finding of increased amounts of triglyceride secreted by the regenerating liver together with the lower triglyceride ^{3}H specific activity suggests that the unlabelled accumulated lipid is being mobilised at a rapid rate and consequently does not appear to form part of a large inert pool as has been suggested for carbon tetrachloride treated livers (55). Since the protein moiety remains at the control level it is apparent that the lipid to protein ratio is increased. Differences in this ratio for VLDL have previously been suggested by the work of Ruderman et al. and Wilcox et al. (129), who showed an increase in the triglyceride to protein ratio after treatment of diabetic livers with insulin in vitro.

Phospholipid synthesis

The finding that the hepatic phospholipid content remains stable during regeneration confirms earlier investigations (86,173,228). A slightly lowered incorporation of radioactive precursor into this lipid fraction has been shown by the in vivo studies of Fex and Olivecrona (173) as well as by the perfusion studies of Bartsch and Gerber (228). However, the perfusion studies of Infante et al. (86) show similar rates of incorporation of radioactive fatty acid into hepatic phospholipid. As mentioned in the Introduction, in vivo studies by Gurr et al. (45), Levin et al. (229) and Smellie et al. (239) show conflicting results with regard to phospholipid synthesis. These confusing results may be explained by the fact that different periods of regeneration as well as different precursors were used by the various authors.

It has been suggested by Weinstein and coworkers (30) that triglyceride, phospholipid and cholesterol esters are released in discrete molar ratios in the VLDL molecule. Therefore the finding of an increased secretion of ß-lipoprotein phospholipid by regenerating liver in conjunction with the elevated triglyceride synthesis is in agreement with this hypothesis. In fact, as suggested by Olmsted (131), increased mobilisation of phospholipid may be essential for elevated triglyceride release. It is possible that the phospholipid and cholesterol are essential components for maintaining the physical-chemical composition as well as the solubility of the VLDL molecule and thus would have to be increased in order to transport large quantities of triglyceride to the extrahepatic tissues.

The lowered phospholipid specific activity again suggests secretion of unlabelled phospholipid. This interpretation is consistant with the lowered specific activity obtained in the perfusate by Bartsch and Gerber (228), as well as a lowered percentage incorporation of labelled precursor into the phospholipid moiety of the d < 1.063 lipoprotein as shown by Infante et al. (86).

e) Prelabelling of Accumulated Triglyceride

The studies on per cent release of prelabelled accumulated ^{14}C-triglyceride by perfused 13 hour regenerating liver demonstrated that the tissue acquired the capability of secreting the stored lipid. The release was similar to that of the control liver, indicating that the same percentage of intracellular triglyceride was being mobilised. Since the normal tissue contains only a very small pool of inactive lipid (54), it is probable that only a small percentage of the prelabelled triglyceride was being immobilised; this also holds for the 13 hour regenerating liver since the release is the same. Further support for this concept is obtained from the ^{14}C specific activities. Perfusate and liver specific activities were similar for both 13 hour and zero hour partially hepatectomised livers. This indicated that the hepatic triglyceride formed a continuous pool with that released into the medium.

Examination of the release by CCl_4 treated livers showed that the secretion of the prelabelled triglyceride was vastly reduced in comparison to the controls.

Furthermore, with CCl_4 the ^{14}C specific activity of the perfusate was considerably lower than that of the hepatic triglyceride. These experiments indicated that the regenerating liver was handling the accumulated lipid in a manner similar to a normal liver and totally different from a CCl_4 treated liver.

Comparison of the per cent release of de novo synthesised ^{3}H triglyceride with the ^{14}C labelled stored lipid is not very meaningful since the former was calculated on the basis of the hepatic radioactivity at one time point. However, it can be noted that in the case of CCl_4 treatment release of de novo triglyceride was greater than stored triglyceride by a factor of 20 while normal and regenerating livers were only 4 times and 3 times greater. This again emphasises the immobility of the accumulated lipid in the case of CCl_4 treatment.

The ultrastructure studies of Jordan (215) and Trotter (204,207) provide a microscopic parallel to our findings. The sequence of events after partial hepatectomy appears to be as follows: osmiophilic bodies appear in the cytoplasm surrounded by a membrane at 3 1/2 hours. At later periods these particles fuse together to form the fat droplets which are still surrounded by a vesicle. It is not unreasonable to suggest therefore that the cytoplasmic bodies represent accumulated VLDL in the endoplasmic reticulum which later amalgamate to form the large droplets but presumably still retain the protein component so that secretion of lipid can be readily achieved. This kind of arrangement would probably not apply to other types of fatty liver such as carbon tetrachloride or ethionine poisoning where protein synthesis is inhibited and where most of the accumulated lipid resides in an inactive pool.

Further support for these arguments can be obtained from the secretion rates of the de novo and stored triglyceride. Since the rate of release is equally rapid in both cases, it is apparent that the accumulated lipid takes no more time to reach the exterior of the cell than the de novo synthesise material. This is true for either the normal or regenerating livers.

Although the per cent release of the accumulated phospholipid is considerably less than accumulated triglyceride, the rate of release is greatly increased. This result indicates the possibility of a small actively turning-over pool of phospholipid which could be attached to the lipoprotein molecule in those regions of the Endoplasmic Reticulum that are closest to the Golgi apparatus and spaces of Disse. This could result in a short intracellular distance for this lipoprotein moiety to travel.

The in vivo secretion rates obtained by Lombardi et al. (107) after injection of triton and palmitic acid, 9,10^{3}H do not support our findings. These workers found that the secretion rate of phospholipid is less than that of the triglyceride for normal rats but in the case of choline deficiency it is slightly greater. However, these curves are difficult to compare with the experiments reported above since triton caused a delay of at least 30 minutes in the maximum time of incorporation into the triglyceride.

The fact that non-triglyceride esters in the liver and extrahepatic tissues are labelled more rapidly than triglyceride in the in vivo experiments of Baker and Schotz (110) supports the concept of a fast turnover rate for phospholipid. The same authors demonstrated the existance of a rapidly labelled polar lipid in the circulation. However, similar experiments by Stein and Shapiro (276) showed a faster rate of

incorporation into hepatic triglyceride than phospholipid.

In the experiments reported here the secretion rates were studied at a relatively long interval after the labelling of the lipid (13 hours). Therefore, it is unlikely that the rates of synthesis or intracellular exchange would be major factors in the appearance of radioactivity in the perfusate. Early equilibration between mitochondrial and microsomal phospholipid as well as between liver and plasma has been reported (276,277). The exchange of labelled phospholipid between liver and lipoprotein as suggested by Kook and Rubinstein (93), would not be a factor in these studies since a lipoprotein-free perfusion medium was used here.

As mentioned in the Introduction, Baker and Schotz have applied a multicompartmental analysis to their in vivo specific activity time curves for normal or carbon tetrachloride treated rats in order to monitor the intracellular movement of triglyceride (54). For the normal liver the model consists of one small slowly turning-over pool and one large mobile pool. The latter is however broken up into several adjoining small compartments.

From the above results a tentative model for phospholipid synthesis and secretion can be drawn; this would consist of one fast turning over pool representing the phospholipid moieties of the nascent lipoprotein on the one hand, and a second larger relatively immobile pool on the other. The inert compartment would of course consist mostly of membrane material. To check this model experiments of the type outlined by Baker and Schotz could be performed and a computer analysis made using the same program.

The model that these authors have designed for the carbon tetrachloride treated liver has an inert triglyceride pool which is considerably larger than that of the normal (55). By the use of this model the authors provided evidence to demonstrate considerably diminished triglyceride secretion in vivo. This finding has been confirmed in the perfusion studies above (Table VII).

A hypothetical model for regenerating liver would however be considerably different. There would have to be an expansion of each fast turning over compartment to account for the relatively mobile accumulated triglyceride. The inert pool would be the same size as normal, which would agree with the photographs of Trotter (207) who showed that the cytoplasmic droplets were not increased in regeneration. The increased fatty acid uptake, esterification and secretion would also have to be taken into account.

As was discussed in the Introduction, Fex and Olivecrona have shown that fatty acid flux rates through the plasma pool are unaltered after partial hepatectomy (173). This implies an elevated uptake per gram of liver tissue. The experiments reported above where large quantities of palmitic acid were perfused attempted to test whether this uptake was a major factor in the steatosis of regeneration.

The experiments of Nestel and Steinberg (7) as well as Heimberg et al. (278, 30) clearly showed that perfusion of a liver with large quantities of fatty acid will result in an increased concentration of hepatic triglyceride. The latter authors also demonstrated that the accumulation of triglyceride was proportional to the concentration of free fatty acid in the medium. It can be suggested therefore that perfusion of a liver of smaller size than normal will have the same net effect as

increasing fatty acid concentration, since the liver apparently takes up all fatty acids which are presented to it.

The experiments presented above (Table IX) confirm that the uptake of radioactive fatty acid is quite rapid even when large amounts of the precursor are present. This supports the argument that the liver will passively take up whatever quantity of fatty acid is presented to it.

The demonstration that the partially hepatectomised liver will take up and esterify a greater quantity of fatty acid than the normal (Table X) shows that the accumulation seen in vivo can be simulated by the in vitro conditions of the perfusion. The synthesis of accumulated triglyceride in carbon tetrachloride treated livers in the course of perfusion with large amounts of fatty acid has already been demonstrated by Weinstein et al. (30). In the case of the partial hepatectomy, the total quantity of triglyceride per liver never exceeds the control level, thus maintaining the normal amount of hepatic lipid in the body and emphasising the fact that the physiological balance is not very much disturbed by the trauma.

Corresponding with the in vivo findings, the phospholipid content in the partially hepatectomised liver does not increase in the course of these in vitro accumulation studies. The constancy of the hepatic phospholipid is a phenomenon which applies equally to all types of fatty livers so far studied. A possible exception to this is the fatty liver of essential fatty acid deficiency where a 50% increase in hepatic phospholipid was noted (279).

Since it is now reasonably well established that the liver is the major if not the sole site of synthesis and degradation of endogenous plasma phospholipid and cholesterol, it is to be expected that this tissue would have special regulatory systems which could control the metabolism of these compounds. An important controlling factor may therefore be a type of negative feedback mechanism which has already been shown to regulate cholesterol synthesis (280). Controlling systems of this type need not apply to triglyceride which is readily degraded by the extrahepatic tissues.

A study of the controlling mechanisms involved in phospholipid synthesis is complicated by the fact that synthesis can proceed via several different pathways and also that exchange of part of the molecule can occur without de novo synthesis (277,281,282,283). These complications are reflected in the heterogeneity of hepatic lecithin. While the direct synthesis from free choline is normally the main route for lecithin formation (284), under certain conditions such as choline deficiency there is indirect synthesis by means of the methylation of cephalins (285).

Two possible approaches which could be taken towards the elucidation of the controlling mechanism are as follows: (a) The effect of the addition of intermediates such as choline or phosphoryl choline to the phospholipid synthesising system may reveal the rate controlling step in the synthetic pathway; phosphoryl choline has already been shown to increase the synthesis of secretory protein in vitro (286). (b) An investigation which would elucidate the main site of action of phenobarbital on the metabolic pathway may reveal information concerning the nature of the

controlling factors, since the drug is known to have a stimulatory effect on phospholipid and cholesterol synthesis in the smooth endoplasmic reticulum (287,288).

SUMMARY and CLAIMS TO ORIGINALITY

1. Following the injection of leucine-1-^{14}C and palmitate 9,10^{3}H the maximum radioactivity of the lipid and protein in the serum d < 1.063 lipoprotein of the normal rat occured at 1/2 hour and 2 hours, respectively. A rapid turnover of the lipid moiety was indicated.
2. Administration of puromycin caused a reduction in the maximum lipid and protein radioactivity as well as a delay in incorporation of the tracers. Release from the protein block was closely preceded by the lipid maximum.
3. Uptake of palmitate 9,10^{3}H by perfused 13 hour regenerating rat liver on a gram basis was greater than for normal liver. CCl_4 treated livers showed a reduced rate of uptake of the tracer. 13 hour and zero hour partially hepatectomised livers had identical removal rates.
4. In vivo and perfusion incorporation studies indicated that synthesis of hepatic triglyceride and protein was elevated in 13 hour regenerating liver.
5. In vivo secretion studies indicate that as time proceeds after partial hepatectomy the liver acquires the capability of increasing lipoprotein lipid release into the circulation.
6. The rate of secretion of lipoprotein lipid by perfused 13 hour regenerating liver was approximately 1/2 that of a full-sized normal liver; a CCl_4 treated liver had only 1/10 the secretion rate.
7. Administration of radioactive palmitate in vivo and in perfusion indicated that 13 hour regenerating liver was secreting a greater quantity of unlabelled

triglyceride than a normal liver. This was shown by a reduced hepatic and lipoprotein triglyceride specific activity.

8. Comparison of perfused 13 hour regenerating liver with a liver partially hepatectomised immediately before perfusion showed that elevated quantities of triglyceride were being released.

9. The phospholipid of the ß-lipoprotein followed a similar pattern to the triglycerides: increased amounts and a reduced specific activity was obtained with regenerating liver.

10. ß-lipoprotein secreted by 13 hour regenerating liver contained a higher ratio of triglyceride to protein than that of the control tissue.

11. Per cent release of prelabelled accumulated triglyceride by regenerating liver was similar to controls. This indicated that the accumulated lipid was being rapidly mobilised. In contrast, % release by CCl_4 treated livers was 1/20 of the normal rate. Specific activities of liver and perfusate triglyceride were similar for normal and regenerating livers, indicating that the two triglyceride pools were continuous.

12. Although the per cent release of prelabelled phospholipid was considerably less than triglyceride, the rate of secretion was elevated. This pattern was obtained for each type of perfused liver.

13. Quantities of triglyceride per gram liver accumulating during perfusion with large amounts of palmitic acid were greater for a partially hepatectomised liver than for a normal. Increased triglyceride release per gram was also noted. Quantities of phospholipid were unaltered in the liver.

14. Synthesis of nascent total protein and ß-lipoprotein by rat liver ribosomes was monitored by means of amino acid-^{14}C incorporation. The results show no defect in the synthesising ability of ribosomes from orotate treated livers.

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