SECRETION OF LIPOPROTEINS BY ISOLATED RAT HEPATOCYTES - INFLUENCE OF DIETARY AND HORMONAL FACTORS

by

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In Memory

of

Dr. David Rubinstein

TO MY PARENTS

A different sort of TLC

"Progress is a wonderful thing of course, and I can appreciate the lactiferins that are sprinkled on the pasture to turn the grass to cheese. And yet this lack of cows, however rational it may be, gives one the feeling that the fields and meadows, deprived of their phlegmatic, bemusedly ruminating presence, are pitifully empty."

Stanislaw Lem

"The Futurological Congress"
translated from the Polish by
Michael Kandel
Seabury Press, 1974, New York

ABSTRACT

The nature of nascent lipoproteins secreted by hepatocytes from euthyroid, hypothyroid and hypothyroid, cholesterol - fed (hypercholesterolemic) rats was investigated using suspensions of hepatocytes incubated in a lipid - deficient medium. The effects of diet and hormonal status on hepatic lipoprotein secretion was investigated to determine the contribution of the secretory products to the serum pool of abnormal lipoproteins in hypothyroid or hypercholesterolemic rats. The total lipid, apo-B and E secreted by hypercholesterolemic hepatocytes was markedly elevated. Triglyceride and phospholipid secretion were slightly increased by hypothyroid rat hepatocytes whereas, apo-B, E and AI secretion rates were unaffected. Gel filtration of the nascent lipoproteins demonstrated that compared to normal, proportionately more apo-B and E from hypercholesterolemic hepatocytes and apo-E from hypothyroid hepatocytes were secreted in association with larger lipoproteins. Hypercholesterolemic hepatocytes secreted abnormal cholesterol - rich particles, a significant proportion of which were discoidal. Hypothyroid hepatocytes secreted spherical lipoproteins having normal nascent lipoprotein lipid composition. These data are consistent with the hypothesis that in hypothyroidism the accumulation of beta-migrating lipoproteins results from impaired removal of lipoprotein catabolites from the serum, whereas in hypothyroid, cholesterol - fed rats, this defect would only serve to exacerbate the accumulation of the abnormal lipoproteins directly secreted by the liver.

RESUME

Nous avons étudié la nature des lipoprotéines naissantes sécrétées par les hépatocytes de rats euthyroidiens, hypothyroidiens et hypothyroidiens nourrit au cholestérol (hypercholestérolémique), en utilisant des hépatocytes isolées et incubées en suspension dans un milieu déficient en lipides. Les effets de la diète et de l'état hormonal sur la synthèse et la sécretion hépatique des lipoprotéines ont été evalués pour determiner jusqu'à quel point les produits sécrétés peuvent contribuer aux niveaux des lipoprotéines abnormales qui existent dans le serum chez les rats hypothyroidiens ou hypercholestérolémiques. La sécrétion de lipide, apo-B et E par les hépatocytes de rats hypercholestérolémique était trés élevée. La sécrétion de triglycérides et de phospholipides par les hépatocytes de rats hypothyroidiens était légèrement élevée mais la sécrétion des apo-B, E et AI n'était pas affectée. La séparation des lipoprotéines naissantes par filtration sur gel a demontré qu'une plus grande proportion des apo-B et E sécrétées par les hépatocytes hypercholestérolémique et de l'apo-E sécrétée par les hépatocytes hypothyroidiens était associées aux plus grosses lipoprotéines comparées aux hépatocytes normales. Les hépatocytes de rats hypercholestérolémique sécrétaient des particules abnormales, riches en cholestérol et une proportion significative des particules étaient des disques lamellaires. Les hépatocytes hypothyroidiens sécrétaient des lipoprotéines sphériques ayant une composition lipidémique normale. Ces observations appuient l'hypothèse qui suggére que l'accumulation des lipoprotéines abnormales chez les rats hypothyroidiens résulte d'un enlèvement du sang diminuée pour les catabolites de lipoprotéines du serum, une condition qui pourrait augmentée l'hypercholesterolémie associée avec le cholestérol alimentaire qui cause la synthèse et la sécrétion de lipoprotéines abnormales.

PREFACE

In 1954; the recognition that the various lipoprotein species could be isolated and separated from other serum components by ultracentrifugal flotation gave impetus to research on lipoprotein metabolism. Many aspects of lipoprotein metabolism have subsequently received wide attention and a burgeoning amount of information is now available on the composition, apolipoprotein content, structure and intravascular catabolism of the various lipoprotein species. However, research on the mechanisms of lipoprotein biosynthesis has progressed slowly in comparison. There are several reasons for this. Firstly, the function of lipoproteins as intravascular lipid - transport vehicles, requires that they be transformed and catabolized once they are released from their sites of synthesis. Therefore, the in vivo isolation and resolution of nascent lipoprotein species from partially metabolized particles is precluded. Secondly, the processes and regulation of lipoprotein biosynthesis are more complicated than that of other secretory proteins in that lipoproteins are macromolecular complexes containing various lipids, proteins and carbohydrate.

The secretion of large lipid complexes by most tissues would require that such particles pass through the cell membranes of endothelial cells which line the capillaries. This problem is circumvented in the intestine and liver by direct secretion into the lymphatic system and into the space of Disse, respectively. Unfortunately, even the lipoproteins secreted by the isolated perfused organs show evidence of post - secretory modification in the lymph and space of Disse. Serum lipoproteins or enzymes that may be present or physically localized within these compartments in the intact organ may account for such observations.

As a result of this, several researchers attempted to use the relatively novel system of isolated hepatocyte suspensions to investigate nascent

hepatic lipoprotein secretion. When I began my studies in Dr. Rubinstein's laboratory, three reports had appeared in the literature demonstrating lipoprotein secretion by isolated hepatocytes. Only one of the laboratories had successfully maintained the cells in suspension for 24 hours and the preliminary data indicated that the nascent VLDL was indeed different from that secreted by isolated perfused livers.

Dr. Rubinstein maintained a keen interest in isolated hepatocytes as an experimental model, especially in view of the information it could provide, as nascent lipoprotein secretion in this system had not, as yet, been extensively investigated. This model afforded the advantages of long - term studies as well as the potential of examining lipoprotein secretion under a variety of well - defined extracellular conditions. The nature of experiments conducted using the isolated hepatocyte model system to investigate hepatic lipoprotein synthesis and secretion are the subject of this thesis.

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Last of all, I would like to thank the faculty and staff in the Biochemistry Dept. at McGill University for being so helpful to me as a non-resident graduate student.

ABBREVIATIONS AND CONVENTIONS USED

ACAT:	acyl-CoA:cholesterol-O-acyltransferase
apo:	apolipoprotein
apo-B_h:	apolipoprotein B of higher molecular weight
apo-B_l:	apolipoprotein B of lower molecular weight
BSA:	bovine serum albumin
β-VLDL:	β-migrating very low density lipoprotein
CE:	cholesteryl ester
c.p.m.:	counts per minute
d:	density
DTNB:	5,5'-dithionitrobenzoic acid
EDTA:	ethylenediaminetetraacetic acid
ER:	endoplasmic reticulum
FC:	free or unesterified cholesterol
FFA:	free fatty acids
GLC:	gas liquid chromatography
HDL:	high density lipoprotein
HDL_c:	cholesterol-induced lipoprotein
HMG-CoA:	beta-hydroxy-beta-methylglutaryl-CoA
IDL:	intermediate density lipoprotein
LCAT:	lecithin:cholesterol acyltransferase
LDH:	lactate dehydrogenase
LDL:	low density lipoprotein
Lp:	hypothetical lipoprotein family characterized by a single apolipoprotein with associated lipid, e.g. Lp-E, containing only apo-E as protein
LPL:	lipoprotein lipase

M_r:	relative molecular weight
mRNA:	messenger ribonucleic acid
PAGE:	polyacrylamide gel electrophoresis
PL:	phospholipid
PTU:	propylthiouracil
r.p.m.:	revolutions per minute
SCP:	sterol carrier protein
SDS:	sodium dodecyl sulfate
TCA:	trichloroacetic acid
TG:	triglyceride
TLC:	thin-layer chromatography
VHDL:	very high density lipoprotein
VLDL:	very low density lipoprotein

In designating apolipoproteins, they are referred to according to the Alaupovic nomenclature which assigns a capital letter to each peptide (or family of related peptides), for example, apo-C. Numerals following the letters indicate the various related peptides of a single family, for example, apo-CI, apo-CII and apo-CIII. Subscripts denote the number of sialic acid residues attached to the oligosaccharide of a glycoprotein as in apo-$CIII_0$ and apo-$CIII_3$ (which have identical amino acid sequences but differ in charge by 3 sialic acid residues).

Occasionally in the text lipids are referred to by their abbreviations (TG, CE, etc.) for the sake of simplicity and easier reading.

INTRODUCTION

A) Serum Lipoproteins

1) Definition and Function

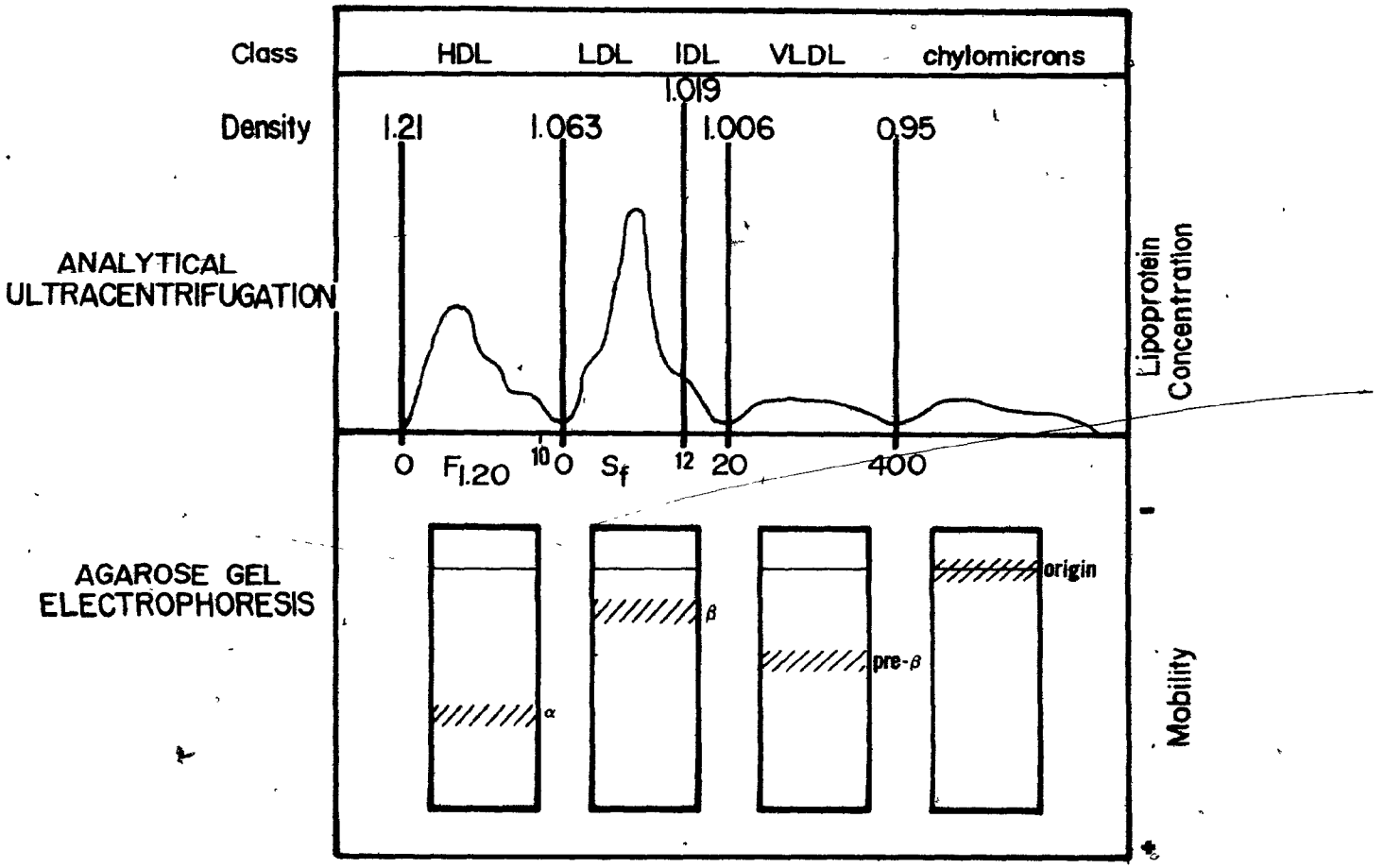
Serum lipoproteins are discrete macromolecular complexes of lipid, protein and traces of carbohydrate which are classically isolated from plasma by ultracentrifugal flotation at increasing solution densities. Lipoproteins function as transport vehicles in plasma for the water - insoluble lipids from their sites of absorption and synthesis to the various tissues in which they are stored or metabolized. The four major lipoprotein classes isolated by ultracentrifugal flotation (114,000g) at serum densities less than 1.21 $g.mL^{-1}$ are chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). The fractionation of these serum lipoproteins is based on the density ranges corresponding to intervals between peaks in the flotation profiles observed during analytical ultracentrifugation (Fig.1) (1). Within any given class of lipoprotein, the particles are not of identical size, hydrated density or protein composition, as can be inferred from Fig.1. This heterogeneity is a reflection of the nature of the relatively loose association between lipid and protein, i.e. the individual components are not present in strict stoichiometric ratios (2). The lipoprotein classes are chemically, metabolically and functionally interrelated and should be regarded as a dynamic concatenation of particles. It is becoming clear that the protein component (apolipoproteins) not only stabilizes the lipoprotein complex but plays an essential role in the metabolism of the associated lipids. The apolipoproteins together with these polar lipids form a surface "coat" around the neutral lipid core of the lipoprotein.

A schematic representation of the major pathways involved in lipo-

FIGURE 1

CORRESPONDANCE BETWEEN DENSITY, FLOTATION RATE AND ELECTROPHORETIC MOBILITY OF HUMAN LIPOPROTEIN CLASSES

Profiles are shown for lipoproteins isolated from fasted human serum and separated in the analytical ultracentrifuge. Upper panel shows the peaks of lipoprotein concentration as a function of S_f or $F_{1.20}$ (flotation rates in solution densities of 1.063 or 1.21 $g.ml^{-1}$ respectively). The Schlieren patterns are inverted for simplicity. Lower panel shows the electrophoretic mobility of the lipoprotein fractions corresponding to each of the main peaks obtained during ultracentrifugation.


Class
HDL
LDL
IDL
VLDL
chylomicrons
Density
1.21
1.063
1.019
1.006
0.95
ANALYTICAL ULTRACENTRIFUGATION
Lipoprotein Concentration
0
F1.20
100
Sf
12
20
400
AGAROSE GEL ELECTROPHORESIS
origin
β
pre-β
α
Mobility
-
+

protein metabolism is depicted in Fig.2. The absorption of dietary lipid by the intestine gives rise to the biosynthesis of triglyceride - rich chylomicrons which are not seen in fasted plasma. A smaller triglyceride - rich VLDL is also biosynthesized, however, the liver is the primary source of VLDL. Hepatic VLDL is distinguishable from the intestinal particle by virtue if its apolipoprotein composition (Fig.3) and is assembled from recycled or endogenously synthesized triglyceride and cholesterol. Low density lipoproteins are triglyceride - depleted catabolites of VLDL. High density lipoproteins are synthesized in the liver (and to an extent by the intestine) but also arise from the catabolism of the triglyceride - rich lipoproteins.

Lipoproteins within each class are heterogeneous, varying continuously in size and composition. However, in general, with increasing density of lipoproteins from chylomicrons (lowest hydrated density) to HDL, the size, molecular weight and triglyceride content of the particles decrease, whereas the relative cholesterol, cholesteryl ester and apolipoprotein content increase. The apolipoproteins play integral roles in the degradation, transformation and uptake of the lipoproteins and their catabolites, by activating specific enzymes and/or serving as recognition sites for tissue receptors.

Three major enzymes involved in the degradation and transformation of plasma lipoproteins are lipoprotein lipase (LPL), lecithin cholesterol acyltransferase (LCAT) and hepatic lipase. The first of these, LPL, resides on the capillary endothelium of all tissues utilizing triglyceride (3), and hydrolyzes this lipid in chylomicrons and VLDL to produce fatty acids and 2-monoglycerides. The liver is the primary source of the second enzyme, LCAT (4), which is secreted into the plasma and is responsible for the conversion of surface phospholipid and cholesterol of HDL into cholesteryl ester and lysolecithin (5). The relatively more hydrophobic cholesteryl ester migrates to the core of the HDL particle while the lysolecithin is largely bound by

FIGURE 2

CONCEPTUAL OVERVIEW OF LIPOPROTEIN METABOLISM

Absorption of dietary lipids occurs in the intestinal lumen. Lipoprotein assembly in the intestinal mucosa from dietary lipid and in the hepatocyte from endogenous lipid is carried out. Transformation, catabolism and uptake of the secreted lipoproteins occurs within or on the surface of cells lining the vascular compartment. Question marks indicate pathways that have not been firmly established experimentally.

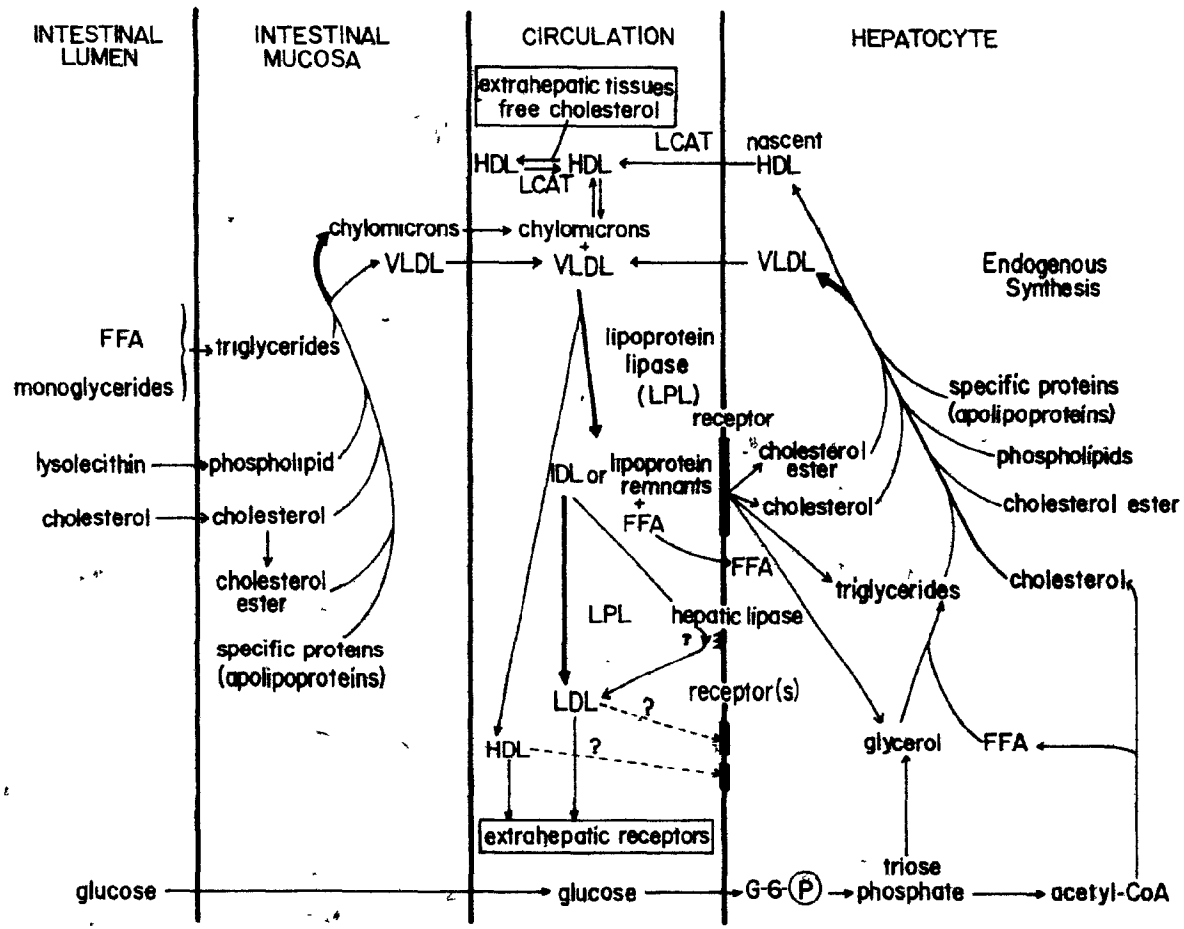
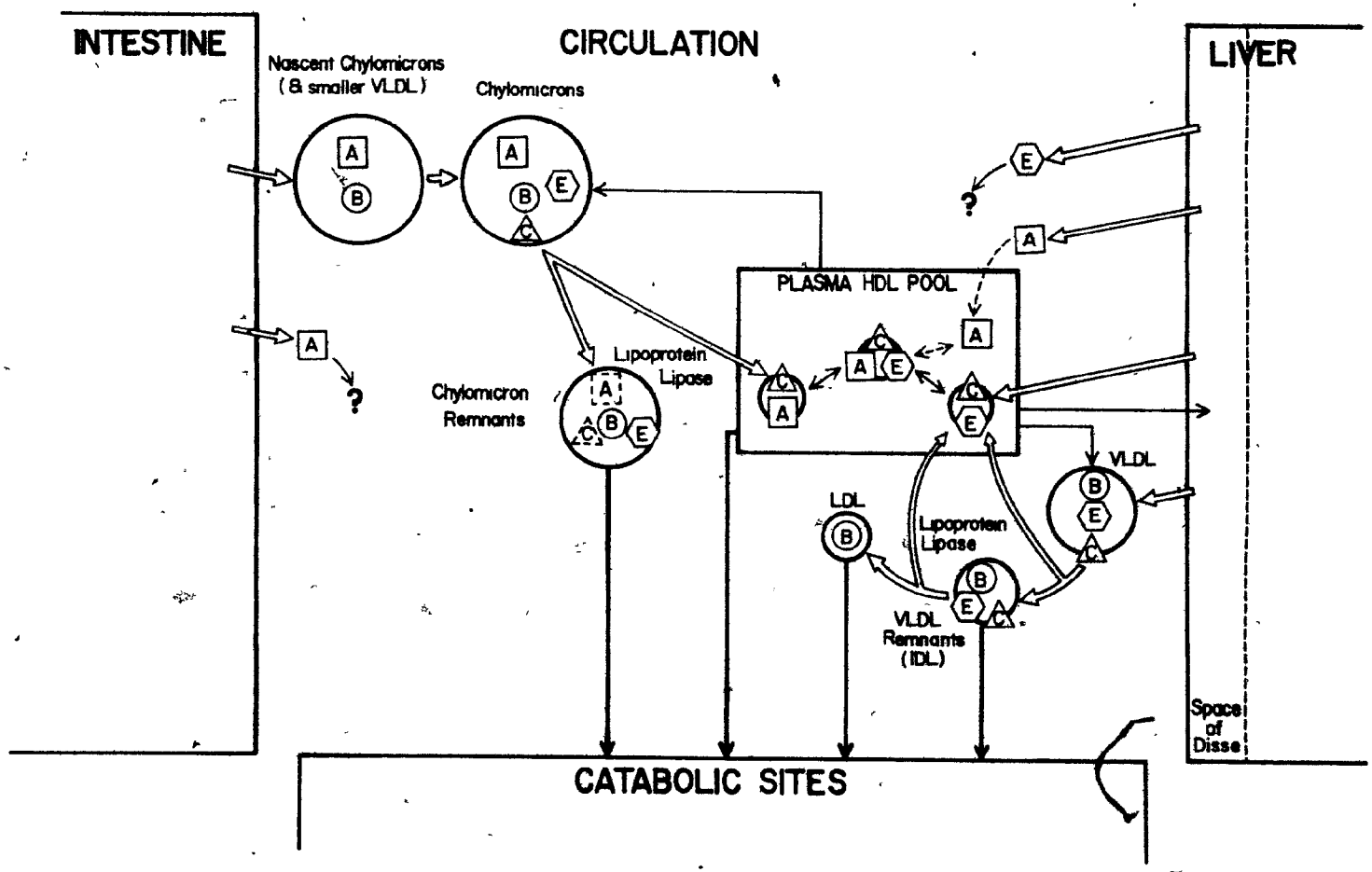

INTESTINAL LUMEN
INTESTINAL MUCOSA
CIRCULATION
HEPATOCYTE
extrahepatic tissues free cholesterol
LCAT
nascent HDL
HDL
HDL
LCAT
chylomicrons
chylomicrons
VLDL
VLDL
VLDL
Endogenous Synthesis
FFA
monoglycerides
triglycerides
lipoprotein lipase (LPL)
specific proteins (apolipoproteins)
receptor
lysolecithin
phospholipid
IDL or lipoprotein remnants
cholesterol ester
phospholipids
cholesterol
cholesterol
cholesterol ester
FFA
FFA
triglycerides
cholesterol
cholesterol ester
LPL
hepatic lipase
?
specific proteins (apolipoproteins)
LDL
?
receptor(s)
HDL
?
glycerol
FFA
extrahepatic receptors
triose phosphate
glucose
glucose
G-6-P
acetyl-CoA

FIGURE 3

CONCEPTUAL OVERVIEW OF APOLIPOPROTEIN METABOLISM

The individual apolipoproteins are indicated by their designated letter within specific symbols. For simplicity, the various related proteins of the LP-A and LP-C families have been considered as one (apo-A and apo-C) as the metabolic pathways for the individual peptides (i.e. apo-AI, AII and AIV or apo-CI, CII and CIII) have not been adequately delineated experimentally. Open arrows indicates the secretion or derivation of an intact lipoprotein particle. Thin lines indicate apolipoprotein transfer and/or exchange (probably accompanied by a small amount of lipid). Dotted lines indicate that the pathways have not been firmly established experimentally. Incompletely enclosed symbols indicate only low amounts of the apolipoprotein are associated with the lipoprotein particle.


INTESTINE
CIRCULATION
LIVER
Nascent Chylomicrons
(& smaller VLDL)
Chylomicrons
PLASMA HDL POOL
Lipoprotein
Lipase
Chylomicron
Remnants
VLDL
LDL
Lipoprotein
Lipase
VLDL
Remnants
(IDL)
Space
of
Disse
CATABOLIC SITES

serum albumin (6). The third enzyme, hepatic lipase, resides on the surface of endothelial cells of hepatic sinusoids and appears to be involved in regulating cholesterol and phospholipid concentration in LDL and HDL (7), however, it can also hydrolyze the triglyceride and phospholipid of chylomicrons (8). Receptor - mediated uptake of lipoprotein catabolites or "remnants" is accomplished by the recognition of apolipoprotein B (apo-B) and / or apo-E by specific cellular receptors (9, 10).

As shown in Fig.3, the entry of nascent, catabolically inert chylomicrons and VLDL into the plasma results in the rapid, non - enzymatic transfer from HDL of apolipoproteins C, and E. Lipolysis of these catabolically activated particles results in the return of the apo-C and some apo-E to HDL. Lipid - free apolipoproteins are probably secreted into the plasma and along with HDL serve as a pool of apolipoprotein activators which regulate chylomicron and VLDL catabolism.

2) Classification of Lipoproteins

a) Density Classes

The nomenclature of serum lipoproteins is still largely based on ultracentrifugal methods developed by deLalla and Gofman in 1954 (1) based on the analytical ultracentrifugal profiles obtained from fasted normal human sera. However, altered profiles in normal human serum can be observed after acute carbohydrate, fat or ethanol ingestion (11, 12) so that any one particular class of lipoprotein may not have the identical range of hydrated densities observed in the fasted state. This apparent problem becomes more complex when attempting to isolate defined classes of lipoprotein from patients or animals in hyperlipemic or other abnormal metabolic states.

Despite these drawbacks, however, the preparative fractionation of the major serum lipoprotein classes is achieved satisfactorily

by ultracentrifugal methods (13). Generally, the problems generated by separating the lipoproteins based on a single physical property such as hydrated density, have been dealt with by characterizing each of the normal operational classes with respect to their protein and lipid content.

b) Electrophoretic mobility

Another system of classification of lipoproteins is based on their electrophoretic mobility on supporting media such as paper (14), agarose (15), cellulose acetate (16) or Geon - Pevicon (17). This technique is generally employed as a rapid and visual means of identifying serum lipoprotein species and is generally not suitable for preparative purposes. The electrophoretic mobilities of ultracentrifugally isolated normal human serum lipoproteins gives rise to the following nomenclature: chylomicrons (no migration), β (LDL), pre-β (VLDL) and α -lipoproteins (HDL) (Fig.1).

Abnormal metabolic states can give rise to lipoproteins which isolate in a specific density class but exhibit non - characteristic electrophoretic mobility (18). This indicates that an operational classification based on the relative mobilities of the lipoproteins offers no advantage to the density classification.

c) ABC Nomenclature

Alaupovic and his-coworkers proposed an alternative system of lipoprotein classification (19, 20). They initially recommended assigning the apolipoproteins capital letters so that the major apolipoprotein of the alpha - migrating lipoprotein (HDL) was designated apo-A, while that of the beta - migrating lipoprotein (LDL) was apo-B, and other apolipoproteins were assigned letters in order of their discovery. The lipoproteins of normal human plasma were then defined in terms of the presence of a single, distinct

apolipoprotein moiety (which may be a single peptide or one of the groups of two or more related polypeptides) which resulted in the differentiation of lipoprotein "families" (20). Initially, three lipoprotein families were designated: lipoprotein family A (LP-A) characterized by the exclusive presence of apolipoprotein A (apo-A); LP-B by apo-B and LP-C by apo-C. Two additional distinct lipoprotein families, LP-D (21) and LP-E (22, 23) have subsequently been included. It is proposed that these lipoprotein families exist primarily as association complexes in the VLDL density range and mainly as discrete, free forms in the HDL density range (20, 24). As the existence of all the lipoprotein particles in serum as defined by Alaupovic (20) have not been confirmed experimentally, this nomenclature system for lipoprotein classes has not gained wide acceptance (2, 13), although the alphabetic nomenclature for the individual apolipoproteins is commonly used.

In this thesis, the ultracentrifugal classification of lipoproteins is used as this is still the most widely accepted system. The reader is reminded that in the present work, lipoproteins fractionated by non - conventional means (i.e. gel filtration) are referred to according to their density classifications. All attempts have been made, however, to clearly and unambiguously define the lipoprotein species so that differences or similarities from the serum analogues are evident.

3) The Lipoproteins of Normal Human and Rat Sera

a) Density classes

The lipoproteins of normal human serum can be separated into density classes as shown in Fig.1. The properties of the major classes of individual lipoproteins are indicated in Table I. The low and high density lipoproteins have been further divided into subclasses. The LDL of d = 1.006

TABLE I

PROPERTIES OF NORMAL HUMAN PLASMA LIPOPROTEINS[a]

	DENSITY RANGE FOR ISOLATION (g/ml)	FLOTATION RATE S_f[b]	$F_{1.20}$[c]	ELECTROPHORETIC MOBILITY[d]	APPROXIMATE PARTICLE DIAMETER (Å)
CHYLOMICRONS	1.006	>400	-	ORIGIN	>700
VLDL	1.006	20-400 OR >20	-	PRE-β OR PRE-β_1	250 - 700
LDL_1 (IDL)	1.006 - 1.019	12 - 20	-	β	220 - 240
LDL_2	1.019 - 1.063	0 - 12	-	β	196 - 227
HDL_1	1.05*	0 - 2	-	α	-
HDL_2	1.063 - 1.125	-	3.5 - 9	α	70 - 120
HDL_3	1.125 - 1.210	-	0 - 3.5	α	50 - 100
VHDL	1.210 - 1.250	-	-	-	-

* HDL_1 refers to a minor component in the highest density region of the LDL.

a Adapted from ref. 2

b S_f = flotation rate of lipoproteins in a solution density of 1.063 $g.mL^{-1}$.

c $F_{1.20}$ = flotation rate of lipoproteins in a solution of density 1.21 $g.mL^{-1}$.

d Mobility on agarose gel.

- 1.019 $g.mL^{-1}$ and LDL of d = 1.019 - 1.063 $g.mL^{-1}$ are metabolically and functionally distinct, possessing marked differences in their chemical composition, physico - chemical properties, distribution in plasma and ultimate roles in atherogenesis (25 and refs. cited therein). As a result, many researchers have adopted the convention designating normal human lipoproteins of d = 1.006 - 1.019 $g.mL^{-1}$ (IDL) as LDL_1 and those of d = 1.019 - 1.063 $g.ml^{-1}$ as LDL_2 (26). At least six ultracentrifugally separable subfractions of LDL_2 have been demonstrated (27). In the analytical ultracentrifuge a shoulder appears in the high density region of the spectrum of LDL (Fig.1) corresponding to a minor component referred to as HDL_1, whose physico - chemical characteristics resemble LDL more closely than the other HDL subfractions (28).

Schlieren profiles of HDL in the analytical ultracentrifuge provided the basis for the further subclassification of HDL into HDL_3 (1.125 - 1.21 $g.mL^{-1}$), HDL_{2a} (1.10 - 1.125 $g.mL^{-1}$) and HDL_{2b} (1.063 - 1.10 $g.mL^{-1}$) (29, 30). Other subclasses have been described within the HDL_3 subfraction (30, 31) and by affinity chromatography of HDL (32).

An additional lipoprotein fraction, very high density lipoprotein (VHDL), has been isolated from normal human plasma between d = 1.21 - 1.25 $g.mL^{-1}$ (33). Similarily, the presence in normal human serum of essentially lipid - free apolipoproteins (d > 1.25 $g.mL^{-1}$) has been reported (34, 35), although at least a percentage of these may be derived from the lower density lipoproteins by detachment of the apolipoproteins during ultracentrifugation (36, 37, 38).

Camejo (39) found that fractionation of rat serum lipoproteins by the conventional methods applied for human serum yielded pure lipoprotein classes. Rat LDL isolated at d < 1.063 $g.mL^{-1}$ is not homogeneous (40, 41). Lusk et al. (42) characterized a lipoprotein species within this class

analogous to the HDL_1 of normal human serum (28).

The total concentration of LDL in the rat is much lower than in human and is probably due to a more efficient removal and degradation of catabolized serum VLDL by the rat liver (43). Also rats (and dogs) possess a relatively higher proportion of high density lipoproteins as opposed to human (44), a property which may be associated with these animals' relative resistance to the development of atherosclerosis (45).

b) Electrophoretic mobility

Rat lipoprotein fractions have similar relative mobilities to the analogous human lipoprotein classes (Table I). Fifty percent of normolipemic humans demonstrate two discrete populations of very low density lipoproteins with fast and slow pre-β electrophoretic mobility, the properties of the slow component closely resembling those of "remnant" very low density lipoproteins (46). That such a lipoprotein does not accumulate in normal rats is also indicative of the relatively more efficient uptake of VLDL remnants by the rat liver (47).

c) Composition

The composition of lipoproteins in human and rat are shown in Table II. The two species exhibit a great deal of similarity, with the only significant exception being the higher proportion of esterified cholesterol present in rat high density lipoproteins.

The fatty acid composition of lipoprotein cholesteryl esters differs considerably between human and rat as shown in Table III, the rat having a high content of cholesteryl arachidonate. Swell et al. (48) noted a correlation between a high level of cholesteryl arachidonate and a species' resistance to atherosclerosis. The higher proportion of cholesteryl arachi-

TABLE II

WEIGHT PERCENTAGE COMPOSITION OF LIPOPROTEINS IN HUMAN AND RAT SERUM[a]

DENSITY	SPECIES	PROTEIN	PHOSPHOLIPID	TRIGLYCERIDE	CHOLESTERYL ESTER	CHOLESTEROL	EC/UC[b]
D > 1.007	MAN	7.7	18.6	49.9	14.9	6.7	1.3
	RAT (WISTAR)	5.4	11.2	75.0	4.3	4.0	0.7
1.007 < D > 1.063	MAN	20.9	22.1	11.2	38.8	9.0	2.5
	RAT (WISTAR)	24.9	21.2	18.5	26.8	8.6	1.5
D > 1.063	MAN	51.9	22.7	8.0	15.0	2.9	3.1
	RAT (WISTAR)	32.8	28.1	1.7	33.6	3.6	5.6

[a] Adapted from ref. 364.
[b] Esterified cholesterol/unesterified cholesterol.

TABLE III

FATTY ACID COMPOSITION OF THE MAJOR CHOLESTERYL ESTERS OF FASTED HUMAN AND RAT SERUM[a]

	LIPOPROTEIN OR TISSUE	FATTY ACID (% TOTAL)			
		PALMITIC (16:0)	OLEIC (18:1)	LINOLEIC (18:2)	ARACHIDONATE (20:4)
MAN	VLDL	12	26	52	6
	LDL	11	22	55	7
	HDL	11	22	55	6
	LIVER	24	37	16	3
RAT	VLDL	16	38	25	10
	LDL	12	15	34	34
	HDL	10	5	35	46
	LIVER	17	39	25	7

[a] Taken from ref.2.

donate in the rat may be partially accounted for by the preferential utilization of arachidonyl-CoA for hepatic phosphatidylcholine biosynthesis (49). Further to this, Holub and Kuksis (49) provided evidence to suggest the operation of a plasma - liver lipid cycle mediated by a specific arachidonyl-lecithin : cholesterol acyltransferase in rat plasma with the arachidonyl-CoA : lysolecithin transacylase in the liver.

Rat VLDL, in contrast to human VLDL, has a cholesteryl ester fatty acid composition which reflects that of the liver even under strongly different dietary conditions (50). This finding, as well as the lower proportion of total cholesteryl ester in the VLDL of rat (Table II) may also be explained by the observation that no significant cholesterol ester exchange from HDL to VLDL occurs in rat plasma (50). This is attributed to the absence of a cholesterol ester transfer protein in rat (51). In human plasma, the ability of HDL cholesteryl ester to transfer to VLDL and LDL (52) probably accounts for the similar cholesteryl ester composition of all human serum lipoproteins (Table III).

The rat is also unusual in that the composition of rat serum cholesterol esters changes substantially upon fasting (53). Fasted rats demonstrate an increase in cholesteryl arachidonate with a decrease in cholesteryl linoleate when compared to normal fed rats. Cholesterol feeding results in a decrease in the proportion of cholesteryl arachidonate (54). It would appear that, in the rat, the production of arachidonic acid from the essential precursor linoleic acid is diminished as the latter is being used to meet the requirements for cholesterol absorption (54).

The phospholipid compositions of human and rat serum lipoproteins are shown in Table IV. Lecithin and sphingomyelin are the predominant phospholipids in both species. The fatty acid compositions of the lecithins amongst the lipoproteins are similar within each species, a finding

TABLE IV

CHOLINE PHOSPHOLIPID COMPOSITION OF HUMAN AND RAT SERUM PHOSPHOLIPIDS

		LECITHIN	SPHINGOMYELIN	LECITHIN:SPHINGOMYELIN RATIO
CHYLOMICRONS	HUMAN[a]	81	19	4.3
	RAT[b]	79	21	3.8
VLDL + LDL	HUMAN	75	25	3.0
	RAT	95	5	19
HDL	HUMAN	87	13	6.7
	RAT	92	8	11.5

[a] Adapted from ref.2.
[b] Adapted from ref.56.
Values are mean weight percent of total lecithin and sphingomyelin.

which is probably attributable to the rapid equilibration of these lipids among the lipoproteins (55, 56). Rats have relatively low proportions of sphingomyelin in the very low and high density lipoproteins compared to human. A similar condition prevails in patients with LCAT deficiency implicating this enzyme in sphingomyelin metabolism (57). The observation that the sphingomyelin content of rat erythrocytes is only 60% that of normal humans (58), paralleling a similar observation in LCAT deficiency (57), leaves open the possibility that LCAT activity in the rat differs from that in man. A relationship between LCAT and sphingomyelin content is also substantiated by the observations of Illingworth and Portman (59).

4) Structure of Lipoproteins : Physico - Chemical Interactions

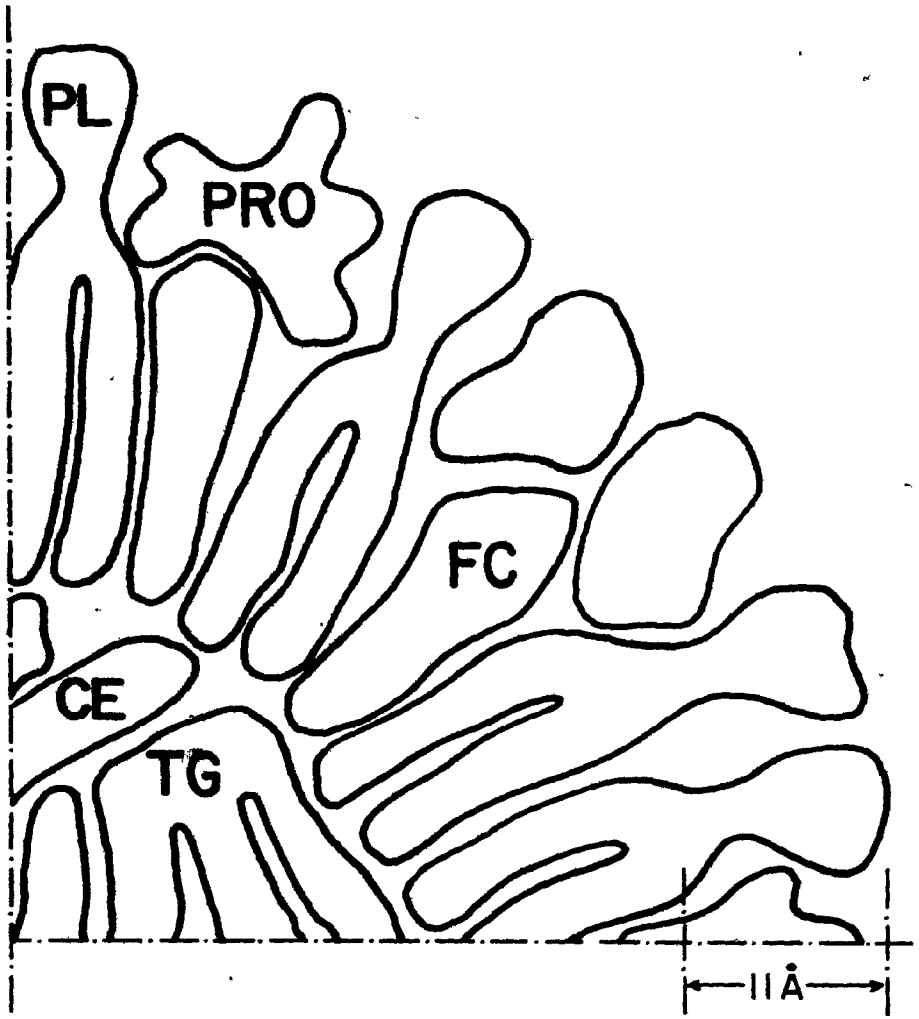
A systematic comparison of the relationship between diameter and chemical composition of all normal serum lipoproteins demonstrates a precise correlation consistent with a spherical model for these particles in which the polar components, phospholipids, free cholesterol and protein, occupy a surface monolayer with a thickness of 20 Å surrounding a "liquid" core of TG and CE (60, 61). The thickness of the monolayer corresponds to the length of the fatty acyl chains of a phosphatidylcholine molecule (60). A model for the surface structure of lipoproteins proposed by Shen et al. (61) that is consistent with recent observations is shown in Fig.4.

The polar head groups of the phospholipids reside on the external surface of HDL and LDL particles as indicated by the observation that phospholipase A_2 (which cleaves the 2-fatty acid) hydrolyzes the phospholipid without significant loss of structural integrity of these particles (62, 63). Similarly for the protein moiety, water soluble reagents such as succinic anhydride cause extensive modifications of the apolipoproteins in native HDL (64) and LDL (65). In contrast, reagents incorporated into the

FIGURE 4

MODEL OF LIPOPROTEIN STRUCTURE

The structure model of human lipoproteins was adapted from ref. 61. The dimensions of all components are derived from space - filling atomic models. The proposed location of each molecule should be considered as a statistically predominant position (due to the fluidity of all components). Such a model allows exchange reactions of molecules to occur. PRO, apolipoprotein; FC, free cholesterol; PL, phospholipid; CE, esterified cholesterol.


PL
PRO
FC
CE
TG
11 Å

apolar core of an HDL particle do not react as readily with the protein (66). Additional evidence for the surface localization of the apolipoproteins includes the observation that neuraminidase treatment can remove sialic acid from these glycoproteins in the intact VLDL particle without loss of structural integrity (67) and that native VLDL and LDL interact specifically with concanavalin A (68, 69).

Native serum lipoproteins can react specifically with antisera raised against their delipidated protein moieties, however, the lower immunoreactivity of apolipoproteins when present in native lipoprotein particles (70, 71) suggest that some antigenic sites are masked by lipids adjacent to the apolipoprotein. Henderson et al. (72) conducted ^{31}P-NMR studies with native HDL and noted that 20% of the phospholipid polar head groups interact strongly with the HDL apolipoproteins.

Unlike membrane proteins, the amino acid compositions and primary structures of the apolipoproteins do not reveal significant regions of hydrophobicity which would afford some information as to the nature of the apolipoprotein - phospholipid interaction (2). However, the construction of space - filling models of several apolipoproteins based on their primary sequences reveals novel structural features (73). Extensive helical segments exist within the proteins that have a full 180^{0} of the cylindrical surface of the helix exposing only hydrophobic amino acid side chains while on the opposite face only charged residues are exposed (73). Such an amphipathic helix is shown in Fig.5,A. Inspection of the helical sections reveals that hydrophobic amino acids on the non - polar side appear to be randomly distributed, unlike the polar side where the amino acid disposition is non - random (74). Negatively charged acidic residues are clustered towards the middle of the polar face whereas the positively charged basic side chains are directed toward the lateral edges of the polar - non - polar interface. The

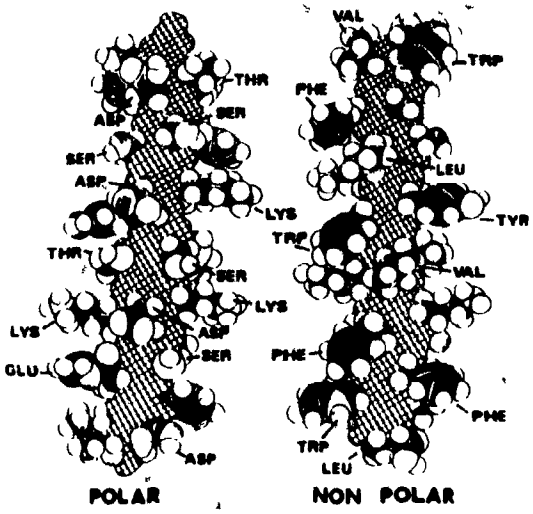
FIGURE 5

STRUCTURE OF THE AMPHIPATHIC HELIX AND MECHANISMS OF ITS BINDING TO PHOSPHOLIPID

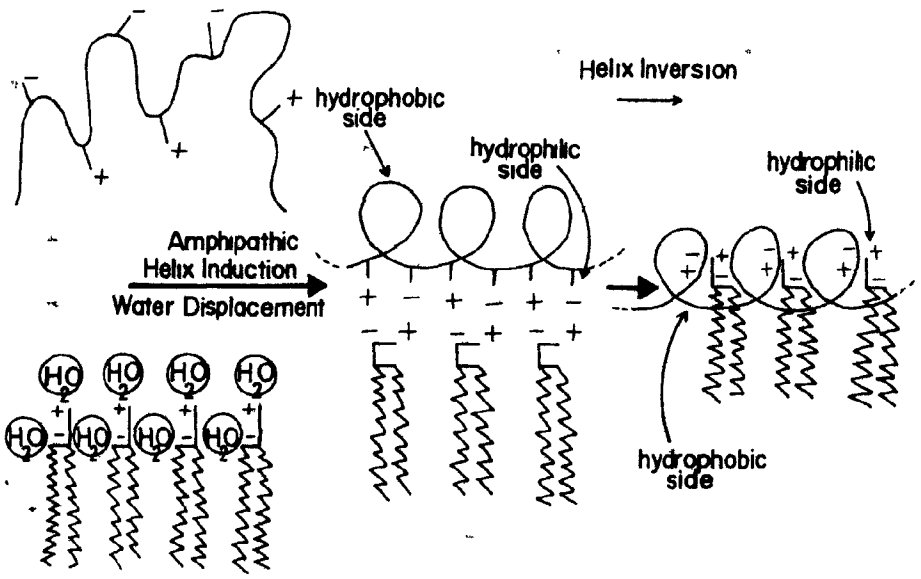
A) Space - filling model of amino acid residues 40 - 67 of apo-CIII demonstrating amphipathic helix structure. The helix is shown with its axis parallel to the plane of the page and its N-terminal end towards the top of the page. Two views of the helix rotated around the helix axis by 180 degrees relative to one another are shown, revealing the polar and non - polar faces of the helix respectively. (From ref. 73).

B) Sequence of steps probably involved in the binding of a disordered but potentially amphipathic helical segment of a peptide to a phosphatidylcholine surface. The sequence reflects experimental data (cited in ref. 74) which indicate water displacement, helix induction and transfer of non - polar residues to a more hydrophobic environment.

A


THR
SER
ASP
SER
ASP
LYS
THR
SER
LYS
LYS
ASP
SER
GLU
ASP
POLAR
VAL
TRP
PHE
LEU
TYR
TRP
VAL
PHE
PHE
TRP
LEU
NON POLAR

B


+ hydrophobic side
Helix Inversion
hydrophilic side
hydrophilic side
Amphipathic Helix Induction
Water Displacement
hydrophobic side

resultant structures can be referred to as "steric zwitterions" (74) and it was suggested that they could interact electrostatically with oppositely charged groups of phospholipids (73). The bulk of evidence to date, however, indicates that hydrophobic interactions are the major contributors conferring stability to protein - lipid complexes and that few, if any, strong ionic interactions exist (2, 74). It has been suggested on the basis of the unique arrangement of polar residues in the helical segments that they could function as templates (during lipoprotein assembly) conferring a favorable orientation of apolipoprotein with respect to phospholipid during the initial phase of binding (Fig.5,B) (74).

Analysis of the evolutionary history of apolipoproteins indicates a strong conservation and gene duplication of the amphipathic helical segments (75, 76). Examination of human apo-AI reveals a segment of eleven amino acids that repeats itself 13 times in succession without any additional intervening amino acids, beginning at the NH_2-terminal residue of the 243 residues and running to the carboxyl terminus of the sequence (75). Although the amino acid sequences of each segment are not identical, the physico - chemical characteristics of these residues have been conserved so that all the segments yield structures with alpha helices having an amphipathic character (75).

When Barker and Dayhoff (76) compared a segment representing the repeating pattern of apo-AI with the sequences of apolipoproteins CI, CIII and AII, they found that similar segments occur in a pattern that suggests an evolutionary history of several gene duplications of a common precursor to form the contemporary sequences of all these apolipoproteins (75). Sequence analyses of apo-CII (77) and apo-E (78) indicate the prevalence of amphipathic helical segments with phospholipid - binding capacities. Therefore, it appears that apolipoproteins and probably many other components of lipid -

transport mechanisms are homologous throughout the vertebrates, although intragenic duplications in the separate lines would be expected to result in significantly altered proteins (76). However, sequences of apo-AI (and apo-AII) from several vertebrate species indicates a high degree of homology (79, 80, 81, 82, 83).

Apolipoproteins are rare amongst proteins in that their alpha - helical regions are amphipathic in nature (74). In addition, apolipoproteins lack stabilizing structural features such as ligands, prosthetic groups or disulfide links that maintain the stability and spacial dispositions of their helical segments, such as is present in other proteins (albumin, myoglobin). This would therefore enable apolipoproteins to undergo much more extensive structural changes (74).

The strong interaction of the apolipoproteins with phospholipid is evidenced by the increase in ellipticity (increase in alpha - helical content) in circular dichroic spectra upon the addition of phosphatidyl-choline (74, 84). The alpha - helical content is further increased when cholesteryl ester is incorporated into the phospholipid - apolipoprotein complexes (85).

The use of intrinsic reporter groups in NMR studies has shown that the magnetic environment and motional freedom of the polar PL head groups are not significantly altered by their interaction with apolipoproteins (86). The NMR studies by Stoffel et al. (87, 88) suggest that the apolipoproteins are primarily involved in hydrophobic interaction with the fatty acyl chains of the phospholipids. The binding of apo-AII to mixed lipid vesicles was shown to result in a decreased motional freedom for carbon atoms C-3 and C-11 in the fatty acyl chains of the surface PL with an increased motional freedom for the quaternary methyl groups (87, 88).

The adsorption properties of apo-AI, apo-E and BSA to phospholipid

monolayers indicate that BSA is totally excluded while apo-E and apo-AI are adsorbed to the lipid - water interface at initial surface tensions closely approximating those of native lipoproteins (89). Apo-E is adsorbed at a higher initial surface pressure than apo-AI, suggesting that in native lipoproteins, compression of the surface components, such as that which may occur during lipolysis, would lead to the ejection of apo-AI from the particle before apo-E (89). This possibility certainly emphasizes the potential importance of non - enzymatic interconversions of lipoproteins and their constituents.

Radial electron density profiles obtained by small angle X-ray scattering of native HDL support the findings of the other studies in that they consistently show a spherical electron - dense shell surrounding an electron - poor zone (74 and refs. cited therein). The experimentally observed thickness of this outer shell is approximately 12 Å, a value closely approximating the average diameter of an alpha - helix or the length of a phosphorylcholine head group (approx. 11 Å) (84).

Electron density distributions of LDL have been more difficult to interpret. First, the thickness of the outer shell has consistently been found to be greater than that of HDL which implies a considerable penetration of this layer by solvent (90). Second, the electron density profile is temperature dependent (91), the differences in the X-ray scattering pattern being largely due to changes in the central region of the particle. Between 20°C and 40°C a thermal transition is observed for LDL and is due to a smectic → disordered transition of the cholesteryl esters in the core of the lipoprotein particle (92, 93). The precise temperature at which this transition occurs depends on the concentration of triglyceride in the particle and not on the fatty acid composition of the CE (93). In LDL particles and HDL_c (an abnormal lipoprotein that results from cholesterol feeding), it appears that the

cholesteryl esters, together with the small amount of triglyceride, are sequestered in the lipoprotein core where they occupy a volume large enough to permit cooperative behavior. In contrast, the cholesteryl ester of VLDL cannot undergo any cooperative behavior as it is completely soluble in the excess triglyceride which remains entirely fluid at 10 - 45°C (94). In a recent report, Davis et al. (95) noted that the cholesteryl - ester - rich VLDL secreted by cholesteryl - ester loaded hepatocytes contained significant amounts of triglyceride. This suggested that a certain amount of triglyceride is probably required to disorder the VLDL cholesteryl - ester core and allow its secretion (95). Temperature - dependent phase transitions are not exhibited by HDL. This has been attributed to the lack of cooperative behavior resulting from the relatively smaller volume occupied by the cholesteryl esters in the HDL particles (96).

5) Serum apolipoproteins and their physiological functions

A summary of the lipoprotein distribution and functions of the recognized apolipoproteins of normal human and rat serum is presented in Table V. The major sites of apolipoprotein biosynthesis are the liver and intestine (3) and the relative contribution of these two organs in man and rat are shown in Table VI. The roles of the various apolipoproteins in lipoprotein metabolism will be briefly discussed here.

Apolipoprotein B. Human apolipoprotein B constitutes the major portion of the low and very low density lipoprotein protein moiety, (95 - 100%) and (40 - 50%), respectively and accounts for 20% of the protein mass in lymph chylomicrons. However, because of its susceptibility to degradation and its marked insolubility in aqueous solutions in the absence of amphiphiles or denaturants, this apolipoprotein has, until recently, remained poorly characterized. Apo-B appears to be essential for the transport of tri-

TABLE V

APOLIPOPROTEINS OF NORMAL HUMAN AND RAT SERUM

APOLIPOPROTEIN	MOLECULAR WEIGHT X 10^{-3}		SERUM LIPOPROTEIN CLASS	POSSIBLE FUNCTIONS
	HUMAN	RAT		
Apo-AI	28[365]	27[366]	chylomicrons**, HDL, d 1.21	Activator of LCAT; structural protein of HDL; regulation of hepatic lipase?
Apo-AII	17[365]	8[367]	chylomicrons**, HDL	Structural protein of HDL; regulation of LCAT and hepatic lipase?
Apo-AIV	46[148]	46[366]	chylomicrons**, HDL, d 1.21	Not determined
Apo-B: Apo-B_h	549[99]	335[100],500[105]	chylomicrons**, VLDL, LDL	Lipoprotein biosynthesis and secretion; recognition of lipoproteins by cellular receptors.
Apo-B: Apo-B_l	264[99]	240[100],250[105]		
Apo-CI	7[365]	7[367]	chylomicrons, VLDL, HDL	Activator of a specific lipoprotein lipase; activator of LCAT; inhibition of chylomicron remnant uptake and inhibition of hepatic lipase.
Apo-CII	8.5[365]	8[367]	chylomicrons, VLDL, HDL	Activator of LPL; inhibitor of chylomicron remnant uptake; inhibition of hepatic lipase.
Apo-CIII	8.5[365]	10[367]	chylomicrons, VLDL, HDL	Inhibition of hepatic uptake of lipoproteins; inhibition of hepatic lipase.
Apo-E	38[126]	35[366]	chylomicrons, VLDL, HDL, d 1.21	Recognition of lipoproteins by cell receptors; cholesterol transport.

**Apolipoproteins associated with nascent, lymph chylomicrons; others acquired after serum exposure. Superscripts indicate number of reference cited.

TABLE VI

SITES OF APOLIPOPROTEIN BIOSYNTHESIS IN MAN AND RAT*

APOLIPOPROTEIN	LIVER		INTESTINE	
	MAN	RAT	MAN	RAT
Apo-B B_h	+[103]	+[108,368]	+[99]	tr[101]
B_1	?	+[108,368]	?	+[100,102]
Apo-E	+[287]	+[308]	-[369]	-[308]
Apo-AI	+[287]	+[308]	+[369]	+[308]
Apo-AII	+[287]	?	+[369]	?
Apo-AIV	?	+[308]	+[135]	+[308]
Apo-CI	?	+[308]	-[369]	-[308]
Apo-CII	+[287]	+[308]	-[369]	tr[308]
Apo-CIII	+[287]	+[308]	-[369]	tr[308]

* The liver and the intestine are the only organs identified as synthesizing significant quantities of serum apolipoproteins (308). Superscripts indicate reference number cited.
-, not biosynthesized in this organ; +, biosynthesized in this organ; tr, trace amounts are biosynthesized.

glyceride out of the liver and intestine, as it is observed that no triglyceride enters the bloodstream of patients with genetic abetalipoproteinemia (lacking apo-B) despite excessive amounts of intracellular triglyceride (97). A similar condition can be elicited in rats by the administration of the protein (apolipoprotein) biosynthesis inhibitor, puromycin (98).

In 1980, two independent groups simultaneously demonstrated the heterogeneity of human (99) and rat apo-B (100). Adapting the nomenclature of Sparks et al. (101), two major apo-B variants in man and rat are apo-B_h (apolipoprotein of higher molecular weight) and apo-B_l (apolipoprotein of lower molecular weight).

In rat, both apo-B variants are synthesized in the liver (101) whereas the intestine gives rise solely to apo-B_l (100, 101, 102). In man, in contrast, the intestine synthesizes both apo-B variants (99, 103) while the liver produces only apo-B_h (103, 104). These observations are consistent with the hypothesis that apo-B_h and apo-B_l are under separate genetic control. These differences in the relative synthesis of apo-B_h and apo-B_l by the liver and intestine of human and rat (Table VI) could explain the metabolic diversity of VLDL catabolism in these two species.

Sparks et al. (101) noted a more rapid uptake of serum apo-B_l versus apo-B_h in rats in vivo (101, 105), with the result that apo-B_h preferentially was pooled in LDL. Other studies have demonstrated that human and rat chylomicrons (containing apo-B_l) are rapidly removed by the liver (102, 106). Although apo-B is known to be essential in lipoprotein receptor recognition (107), it remains to be determined if apo-B_h and apo-B_l reside separately or together on lipoproteins and if they are both bound by a common tissue receptor to the same or varying degrees.

As low density lipoproteins are primarily derived from VLDL and not

chylomicrons (103), it is not surprising that, in man, LDL (containing only apo-B_h) accumulates to a higher degree than in rat. In the latter species, lipoproteins of hepatic or intestinal origin contain apo-B_l permitting their catabolites to be rapidly cleared from the circulation. Hepatogenous lipoproteins in man, however, contain only the apo-B_h and would have a slower turnover rate (101, 108), consituting the sizable LDL pool.

Apolipoprotein E. This apolipoprotein occurs in several classes of serum lipoproteins in both humans (109) and rats (110), and in both species a significant proportion resides in the $d > 1.21$ g.mL^{-1} fraction of serum after flotation of the lipoproteins by ultracentrifugation (36, 111). Total serum apo-E concentrations in rat (112) are four times higher than in man (113, 114). This apolipoprotein has been implicated in serum cholesterol transport (115) and, like apo-B, appears to be a key protein mediating lipoprotein recognition and uptake by fibroblasts (10) and liver cells (116, 117). The role of apo-E in cholesterol transport is suggested by the tendency of this apolipoprotein, in rats, to redistribute to cholesterol - rich lipoproteins in vivo (118, 119) and in vitro (112, 120, 121). Similarily, redistribution of apo-E is observed in incubations of LCAT - deficient patients' plasma with LCAT, in that an increase in the apo-E and cholesteryl ester content of VLDL occurs, presumably by the coordinated transfer of both components from HDL (5). Apo-E is elevated in VLDL isolated from patients with type III hyperlipoproteinemia (122) and is also increased in abnormal lipoproteins (β-VLDL and HDL_c) from cholesterol - fed rats (See "Abnormal Lipoproteins).

Heterogeneity of human and rat apo-E has been demonstrated (123) by isoelectric focussing (124). In man, the isoforms of apo-E have isoelectric points between pH 5.4 and 6.1, corresponding to apo-EI, apo-EII, apo-EIII and apo-EIV. The three major isoforms (apo-EII, apo-EIII and apo-EIV) differ from one another by a single unit of charge (125) due to the substitution of

arginine at two specific cysteine residues. Zannis and Breslow (123) have postulated that 3 alleles at a single gene locus are responsible for the 3 major isoforms and that the minor isoforms are derived from the major isoforms by post - translational glycosylation.

Rats appear to possess 2 major different molecular weight isoforms of apo-E on 2-dimensional gel electrophoresis, each having 3 differently charged (possibly allelic) forms that are more negatively charged that any human isoform (123). The two molecular weight isoforms may account for the double apo-E bands observed on urea polyacrylamide gels of rat nascent VLDL (120, 126) and serum VLDL (111). Several sialic acid derivatives of apo-E in rat have been reported (118).

Lipoproteins from several animals species including man that contain either apo-B or apo-E can bind apo-B,E cell surface receptors on human fibroblasts which, in turn, regulate intracellular cholesterol metabolism (9, 10). It has been established that a significant homology exists in the apolipoproteins and receptors of different species (10, 116, 127). A hepatic apo-E receptor has been identified in dog (98) and rat (116). In addition, distinct apo-B,E receptors have been demonstrated in the livers of puppies (98), which are absent, but inducible in the livers of adult dogs (98) and rats (128).

Arginyl (129) and lysyl residues (130, 131) clearly are involved in the recognition of lipoproteins by tissue lipoprotein receptors. Modification of these residues in apo-B and apo-E results in almost total abolition of lipoprotein - receptor binding, supporting earlier speculation that a structural sequence or similarily charged (stereospecific) region may be common to both apolipoproteins (10, 129). The interaction of lipoproteins with the fibroblast receptor sites is not determined simply by the presence of positive charges, but appears to depend on other highly specific

properties of the recognition site of apo-B and apo-E (130). Studies have demonstrated that phospholipids clearly are involved in receptor recognition (132), apparently conferring to the apo-E the requisite physical state or conformation required for its binding to the cell surface receptor.

Human familial dysbetalipoproteinemia (Type III hyperlipoproteinemia) is a pathological disorder associated with an apo-EII homozygous state (124) and results in the accumulation of remnant lipoproteins due to impaired hepatic uptake of the apo-E-containing lipoproteins (133). The work of Weisgraber et al. (134), showed that conversion of one apo-EIII arginine residue to cysteine causes a shift of the modified apo-EIII to the pI point of apo-EII upon isoelectric focussing and a decrease in the binding of apo-EII-containing lipoproteins with the fibroblast LDL receptor.

Apolipoproteins AI, AII and AIV. Apo-AI and apo-AII have been the most extensively investigated and characterized of the LP-A apolipoproteins. Apo-AI is the major apolipoprotein of HDL in man and rat. In humans, it accounts for approx. 15% of total chylomicron protein (135) while significant amounts have been isolated from the $d > 1.21$ g.mL^{-1} fraction of serum (5, 70). Apo-AII constitutes about 20% and 50% of the HDL and chylomicron protein respectively. Rats possess a higher proportion of apo-AI in their chylomicron protein (38 - 50%) (135) compared to man. apo-AII is only a minor component of rat HDL (136).

Rat and human apo-AI have similar molecular weights and amino acid compositions (137). Apo-AII, on the other hand, has been shown to be a disulfide - linked dimer in humans (138) whereas in the rat, apo-AII exists in monomeric form in the HDL, but has, nonetheless, significant homology with human apo-AII (79).

The tendency of apo-AI to self - associate in aqueous solution has made the identification of distinct forms of this protein difficult. However,

in human, two distinct polymorphic forms have been identified (139). A similar observation for rat suggests that the isoforms are chemically distinct species (140). Apo-AII, in contrast, does not demonstrate any polymorphism in human (141) or rat (79).

Human apo-AI activates the LCAT enzyme (142, 143). This property is shared with human apo-CI and while apo-AI activation of LCAT is most effective with unsaturated fatty acyl phosphatidylcholines, apo-CI shows equal activation abilities for both saturated and unsaturated phosphatidylcholine substrates (143). In contrast, apo-AII and apo-CII both inhibit the activation of LCAT by apo-AI (143). The LCAT reaction is inhibited by apo-AII via the displacement of apo-AI from the lipoprotein surface by a mechanism that does not result in any concomitant change in particle conformation or lipid composition (144).

A report by Kubo et al. (145) indicated that human apo-AI and apo-AII were independently capable of inhibiting hepatic lipase acivity, the latter having a more pronounced inhibitory effect, whereas Jahn et al. (149) suggested a specific activator role of apo-AII. Either possibility is probably of physiological significance as apo-AI and AII on the surface of HDL can readily come in contact with the lipase on the surface of hepatic endothelial cells (7).

An apo-E-AII complex can be detected in the $d < 1.006$ g.mL^{-1} lipoprotein fraction as well as in HDL_1 of normal and Type III hyperlipoproteinemic serum (147). Weisgraber and Mahley (147) also demonstrated that HDL_1 accounts for most of the high affinity binding of HDL to LDL receptors on human fibroblasts and reduction of the disulfide linkage between apo-E-AII enhances the binding of HDL_1 to the LDL receptor. It remains to be determined whether this apolipoprotein complex is of physiological significance.

Apo-AIV, was first identified in rat HDL (136). This apolipo-

protein comprises 7 - 13% of total rat chylomicron apolipoprotein (135) and accounts for approximately 20% of the HDL protein (136). Apo-AIV, in man, has been identified in plasma and lymph chylomicrons (148), but in both man and rat is mainly found free in plasma unassociated with any major lipoprotein class (135, 149). Although a definite role for this apolipoprotein has not been determined, it has been suggested that apo-AIV serves as a vehicle whereby small quantities of lipophilic substances can rapidly gain access to the blood from the intestine (150). In the absence of intestinal fat transport, a significant proportion of apo-AIV (as well as apo-AI) bypass the lymph and enter the circulation directly from the small intestine (150).

Apolipoprotein C. Apo-C (apo-CI, CII and CIII) comprises a significant proportion of the $d < 1.063$ $g.mL^{-1}$ lipoprotein protein in humans. Approximately 60% and 40% of chylomicron and VLDL apolipoprotein is accounted for by apo-C (2, 135), as opposed to < 15% of total HDL protein. In the rat, apo-C represents about 40% of chylomicron protein (151), and 60% and 20% of the total VLDL and HDL protein respectively (44).

All three apo-C proteins have molecular weights of less than 10,000, and their amino acid sequences have been determined in human (109 and refs. cited therein). The amino acid composition of human and rat apo-C's are quite similar. Apo-CI and apo-CII lack carbohydrate (2), but apo-CIII (79 amino acids) has an oligosaccharide linked to Thr-74 via an O-glycosidic bond (152). Either 0, 1 or 2 sialic residues may be present on the oligosaccharide in man (153) and a species having 3 residues has been reported (154). Rat apo-CIII has only two major sialic acid isoforms, apo-$CIII_0$ and apo-$CIII_3$ (79, 155).

The apo-C proteins play a prominent role in the metabolism of triglyceride - rich lipoproteins, probably the most important of these is the role of apo-CII as an activator for lipoprotein lipase (156, 157, 158). A

significant correlation between serum triglycerides and apo-CII levels in normo- and hyperlipidemic subjects has been reported (159). The specific activation of another unique triglyceride lipase by apo-CI has been reported (160). This apo-CI - activated lipase is absent in patients with Type I hyperlipoproteinemia (160).

Hypertriglyceridemia associated with a defective lipolysis of lipoprotein triglyceride has been identified in humans having apo-CII deficiency (161). Apo-CIII levels are elevated in the homozygous individuals (162) which may be related to the increased serum apo-CIII concentrations observed in primary Type IIb, III, IV and V hyperlipidemic patients who also exhibit impaired VLDL catabolism (163).

In normal individuals, total net lipoprotein lipase (LPL) activator property in serum does not change after acute fat ingestion, however, the apo-C's are observed to transfer from HDL to chylomicrons postprandially and subsequently back to HDL while fasting (164). Recently it has been shown that apo-CII, $CIII_1$ and $CIII_2$ are transferred to HDL and metabolized similarily (165). This is of significant interest, as the function of apo-$CIII_1$, which inhibits the hepatic removal of apo-E - containing lipoproteins (166), is different to that of apo-CII (165, 167). Huff et al. (165) suggested that apo-CII and apo-$CIII_1$ might act in unison within the lipoprotein particle, the former initiating the hydrolysis of triglyceride and the latter insuring that the particle remains in the circulation until an optimal amount of triglyceride has been removed.

Hepatic lipase is thought to be involved in the removal of chylomicron remnants from plasma (168). This enzyme is inhibited by apo-CI, CII, CIII, AI or AII and it is activated by low concentrations of serum (145, 169). The concentration of apo-C in remnants is lower than that in intact lipoproteins (170) and it is possible that this affords another regulatory

mechanism in lipoprotein uptake, by directing the activity of hepatic lipase towards remnants as opposed to intact particles.

Another role of apo-CIII in triglyceride metabolism has been suggested by the findings of Ostlund and Iverius (171) who noted that although apo-CI and apo-CIII did not possess any lipoprotein lipase activating power, both could independently influence the activation by apo-CII. Combinations of substrate and apo-CII giving maximum activation were mainly inhibited when either apo-CI or apo-CIII were added, whereas in conditions of substrate excess, the addition of apo-CI or apo-CIII enhanced lipase activity (171). The substrate : activator ratio is therefore clearly of importance in determining lipase activation (172).

Recently, Alpert and Beaudet (173) demonstrated the in vitro activation of lysosomal sphingomyelinase by apo-$CIII_1$. This suggests that apolipoproteins that enter lysosomes as part of a lipoprotein complex can also regulate the activity of lysosomal enzymes that metabolize lipoprotein particles.

Apolipoprotein D. Apo-D was first identified as a "thin-line" peptide by Kook et al. (174). It was subsequently designated as apo-AIII by Kostner et al. (175) but as this peptide was separable from the LP-A proteins of human HDL (176), the name apo-D was adopted. Relatively little is known about apo-D. Kostner (177) reported that this protein, present mainly in HDL, was the single most important activator of LCAT in vitro using lecithin : cholesterol emulsions. However, the precise role of apo-D in lipoprotein metabolism remains to be elucidated.

6) Abnormal Lipoproteins

a) The LP(a) Lipoprotein (Sinking Pre-β Lipoprotein)

Allison and Blumberg (178) described in 1961 the existence of alloptypes of human serum lipoproteins. The LP(a) antigen was thought to be one such allotype of LDL and the presence of this antigen has been positively correlated with coronary artery disease (179).

Genetic studies reveal a wide variation in the gene frequencies of Lp^a (the gene expressing the LP(a) antigen) and this observation has been explained by Hravie and Shultz (180) who demonstrated that LP(a) is expressed by a quantitative genetic trait. More sensitive assay conditions presently indicate that most individuals possess this antigen (179).

Purified LP(a) lipoprotein has a hydrated density of 1.0855 $g.mL^{-1}$, a molecular weight of $5.2 \pm 0.2 \times 10^6$ daltons and exhibits fast β migration in agarose - gel electrophoresis (179). Examination of the lipid composition of LP(a) lipoproteins discloses a similar composition to LDL with regard to neutral lipids or individual phospholipids (179). In contrast, LP(a) has a sialic acid content that is about six times higher than that of LDL, as well as higher hexose and hexosamine values (181, 182). The former may explain the higher agarose gel migration rate of LP(a) compared to LDL.

The LP(a) lipoprotein was shown not to be a true allotype of LP-B by the observation that the LP(a) antigen was dissociable from LP-B or LDL by various treatments (181; 183) and was found to have a significantly different amino acid composition from LP-B (179).

The presence of the LP(a) polypeptide amongst apo-B - containing lipoproteins is not surprising in view of its apparent affinity for LP-B (179). Studies attempting to elucidate the origin and metabolism of the

LP(a) lipoprotein indicate that it is not a metabolic product of chylomicrons or VLDL (184, 185) and that no exchange of LP(a) apolipoproteins with other lipoproteins occurs. The observation that ^{125}I-LDL degradation by the fibroblasts is inhibited in the presence of LP(a) (186) suggests a mechanism by which LP(a) lipoprotein can participate in the pathogenesis of atherosclerosis. Also, Lp(a) cholesterol taken up by fibroblasts is less efficient in its ability to suppress HMG-CoA reductase than LDL cholesterol (186).

The occurrence of LP(a) in species other than man has been documented for primates only (179), although an animal model, if found, would certainly help to elucidate the biological function of this enigmatic particle.

b) Discoidal Lipoproteins (Abnormal Lipoproteins in Cholestasis and LCAT Deficiency)

The characteristic hypercholesterolemia and hyperphospholipidemia accompanying pathological or experimental biliary obstruction in humans or animals are primarily due to the presence of an abnormal plasma lipoprotein, designated LP-X, having a hydrated density within the LDL density class (187, 188, 189, 190, 191).

In negative stain electron microscopy, LP-X particles appear as pale staining discs that form rouleaux with overlapping images (192). Thin - sections of LP-X preparations reveal 400 Å spherical vesicles having an internal water compartment and characterized by a trilaminate staining of the outer edge (193). This trilaminar appearance upon staining is also observed with synthetic vesicles produced by sonicating phospholipids and cholesterol (194).

The chemical composition of LP-X is unique: 6% protein, 66% phospholipids, 22% unesterified cholesterol, 3% esterified cholesterol and 3% triglyceride (189). In addition, varying amounts of bile acids are bound to

this lipoprotein giving rise to unique physico - chemical properties. The protein moiety consists of albumin (approx. 40%) located in the core (or masked by lipids) and apo-C and apo-D which are thought to reside on the surface of the particle (187, 189). A small amount of apo-A may also be associated with LP-X (195). The presence of LP-X in serum is easily determined by virtue of its characteristic cathodic mobility on agar gel, in contrast to all normal serum lipoproteins which migrate to the anode (196).

Lipoprotein-X is also observed in the LDL fraction of serum from patients with LCAT deficiency (197) although the levels of this lipoprotein are lower than those observed in cholestasis (198). The most striking observation in LCAT - deficient patients, however, is that the majority of high density lipoproteins are disc - shaped structures 150 - 200 Å in diameter and 50 - 55 Å wide and also tend to form rouleaux in negatively stained preparations (197). The composition of the discoidal HDL fraction is approximately 21% unesterified cholesterol, 38% phospholipid, 10% triglyceride and 30% protein with virtually no cholesterol ester (199).

The discoidal HDL can be subfractionated giving rise to distinct particles rich in apo-E (LP-E or apo-E-HDL) and those containing apo-AI and apo-AII, but no apo-E (200, 201). The studies of Glomset (201) demonstrated that both apo-E-HDL and apo-A-HDL were converted to spherical HDL during in vitro incubations with LCAT but apo-E-HDL was singularly responsible for the redistribution of apo-E from HDL to VLDL. The newly converted spherical HDL had a decreased affinity for apo-E and an increased affinity for apo-AI (201).

Discoidal LDL_2 and HDL appear in rat serum in experimentally - induced LCAT deficiency (202) and the presence of discoidal apo-E rich HDL in the plasma of guinea pigs fed a high cholesterol diet has been reported (203, 204).

Isolated perfused rat livers secrete discoidal HDL resembling that of human LCAT deficiency when LCAT is inhibited (205). These discoidal HDL prove to be better substrates for LCAT than spherical nascent or serum HDL (205). An LCAT inhibitor was not added to isolated perfused livers from normal or hypercholesterolemic African green monkeys which, nonetheless, secreted discoidal HDL (206).

Such observations led to the hypothesis that discoidal HDL represent the nascent form of high density lipoprotein and are converted by LCAT action to spherical particles after secretion by the liver cell (205). A model of nascent discoidal HDL is shown in Fig. 6.

c) β-VLDL and HDL_c

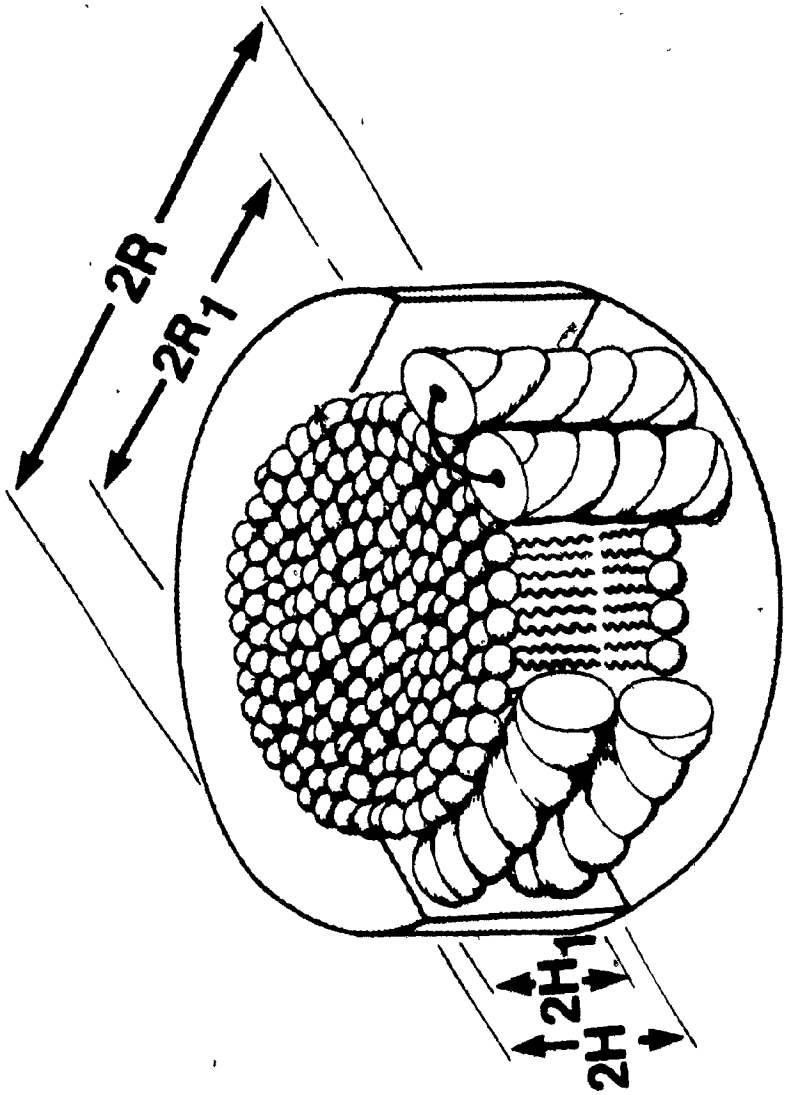
Type III hyperlipoproteinemia, in humans, is characterized by the accumulation in the plasma of cholesteryl - ester rich VLDL that have β rather than pre-β electrophoretic mobility (18). These particles resemble normal VLDL in that they are polydisperse, spherical particles, however, their content of cholesteryl esters and apo-E is significantly higher (18). These particles in Type III patients appear to be degradation products or "remnants" of normal triglyceride - rich lipoproteins that accumulate as a result of these patients' lack of the apo-E isoform responsible for lipoprotein receptor recognition and uptake (133, 134). Although this defective removal accounts for at least a part of the accumulation of these particles, the direct secretion of β-VLDL by perfused liver from hypercholesterolemic rats has been demonstrated (120). The observed cholesteryl ester accumulation in macrophages resulting from a specific receptor - mediated uptake and degradation of β- VLDL implies a role of these abnormal lipoproteins in the development of atherosclerosis (207).

The feeding of cholesterol to man (208, 209) and several

FIGURE 6

MODEL OF NASCENT HDL STRUCTURE

This annular protein - bilayer disc model is proposed by Brouillette et al. (ref. 376) for apo-AI : dimyristoylphosphatidylcholine recombinants and nascent HDL.


2R
2R1
2H1
2H

animal species (swine (81); rat (110); rabbit (210); dog (211)) also results in the accumulation of β-VLDL and, in some cases (man, dog, rat and swine) there is an additional appearance of a cholesterol - rich, α - migrating particle designated HDL_c (211). The latter particle is similar in size and composition to LDL but contains large amounts of apo-E and lacks apo-B (208, 211).

It has been suggested that HDL_c represents an expanded pool of HDL_1 in hypercholesterolemia (42, 118). The hypothesis proposed by Lusk et al. (42) suggests that HDL_1 functions as a readily detectable recycling pool of apo-E involved in chylomicron cholesterol metabolism. In cholesterol feeding an expanded pool of apo-E-rich HDL_1 (HDL_c) would consequently be observed. As HDL_c is effectively taken up by lipoprotein receptors by virtue of its apo-E content (107), it is evident that this lipoprotein may exert a direct or indirect effect on the peripheral uptake of cholesterol or the genesis of hypercholesterolemia and atherosclerosis.

B) Biosynthesis of Serum Lipoproteins

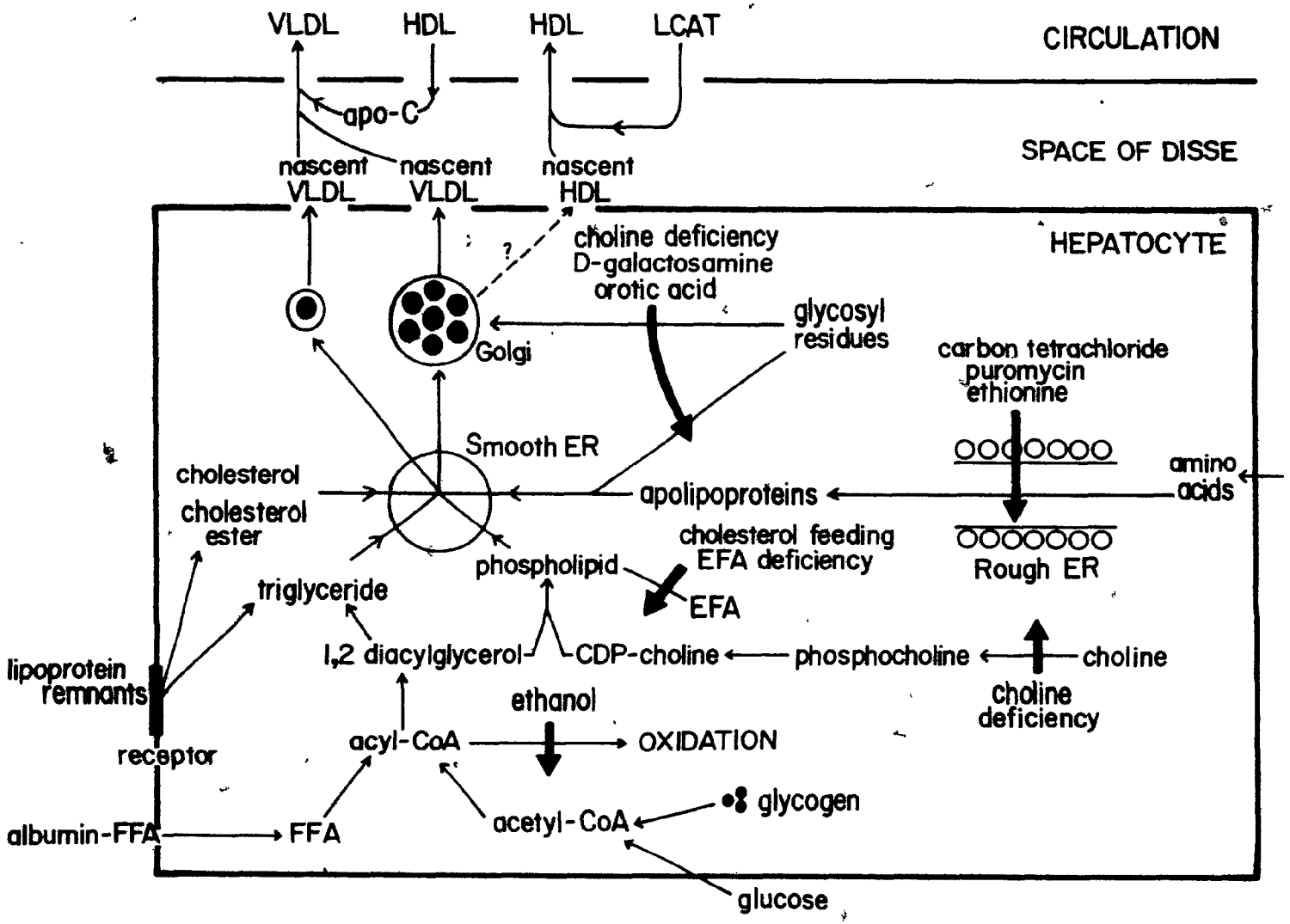
Systematically, the biogenesis of lipoproteins in the intestine and liver share many common features (44), the liver being the major source of lipoproteins in the absence of dietary fat (3). In addition, the liver has the capacity to take up lipoproteins whose lipid components may, in turn, regulate or contribute to hepatic lipoprotein secretion (212, 213, 214). A brief summary of the complex process of hepatic lipoprotein assembly and secretion will be discussed here, as it relates to the work presented in this thesis. A schematic summary is presented in Fig. 7.

As an introduction, the generally conceived pathway of lipoprotein secretion in the liver is as follows. The apolipoproteins, as other proteins, are synthesized in the RER. The lipids are synthesized in the ER and combine

FIGURE 7

PROPOSED SCHEME FOR THE BIOSYNTHESIS AND SECRETION OF VLDL (AND HDL) BY THE HEPATOCYTE IN SITU

The possible loci of action of factors causing the accumulation of triglyceride and a fatty liver are indicated by the heavy arrows. Dotted lines indicate that the pathways have not been firmly established experimentally. EFA, essential fatty acids; CDP, cytidine diphosphate. Other abbreviations as indicated in Abbreviations and Conventions Used.


VLDL
HDL
HDL
LCAT
CIRCULATION
apo-C
SPACE OF DISSE
nascent VLDL
nascent VLDL
nascent HDL
HEPATOCYTE
choline deficiency
D-galactosamine
orotic acid
glycosyl residues
Golgi
carbon tetrachloride
puromycin
ethionine
Smooth ER
amino acids
cholesterol
cholesterol ester
apolipoproteins
cholesterol feeding
EFA deficiency
phospholipid
EFA
Rough ER
triglyceride
lipoprotein remnants
1,2 diacylglycerol
CDP-choline
phosphocholine
choline
choline deficiency
ethanol
receptor
acyl-CoA
OXIDATION
glycogen
albumin-FFA
FFA
acetyl-CoA
glucose

with apolipoprotein at the junction of the SER and RER (215) at which point 70 to 100 nm particles can be visualized by electron microscopy (98). The particles move into the Golgi apparatus (216) where modifications of the nascent lipoproteins occur. Mature vesicles of the Golgi apparatus then serve as transport vehicles which eventually migrate to and fuse with the cell plasma membrane and expel the lipoprotein particles into the space of Disse (Fig.8) (216). This space lies between the liver parenchymal cells and the endothelial cells of the liver sinusoid and may contain a local concentration of hepatic secretory products.

1) Synthesis of Lipid Components

Triglyceride. The synthesis of triglyceride - rich lipoproteins by the liver is closely linked to that of triglyceride. Conditions eliciting increased triglyceride synthesis usually result in increased VLDL production whereas alterations in cholesterol biosynthesis do not affect this rate (2). In the normal animal, the phospholipids and cholesterol are most likely required to stabilize the structure of TG - rich lipoproteins and to facilitate their solubility in the serum (3). The other major lipid component of lipoproteins, CE, is largely formed in the serum compartment from cholesterol and phospholipid by the action of LCAT (6).

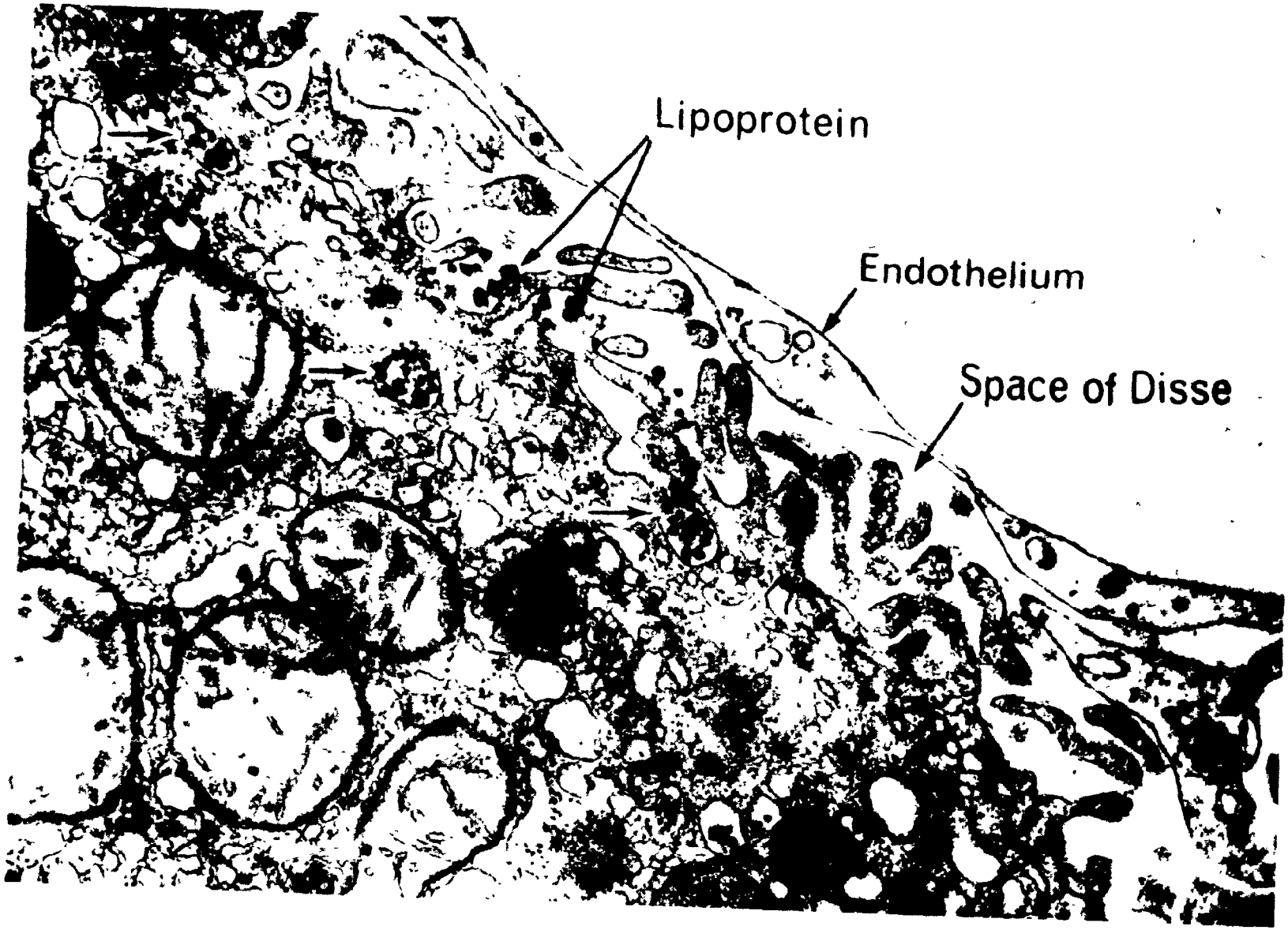
The enzymes primarily involved in triglyceride synthesis are localized in the intracellular microsomal fraction which consists of smooth and rough endoplasmic reticulum (98). Although a variety of different dietary states may significantly alter TG synthesis, the effects are not usually manifested by alterations in the activities of these enzymes, but rather it appears that the availability of substrate and/or intracellular levels of metabolites largely determines net TG synthesis (217, 218).

Fatty acids taken up from the plasma (free or as constituents of

FIGURE 8

ELECTRON MICROGRAPH OF LIVER PARENCHYMAL CELL SURFACE SHOWING THE SPACE OF DISSE

Electron micrograph of part of the surface of a rat liver parenchymal cell bordering on a sinusoid. The perivascular space is referred to as the Space of Disse (From ref. 377).


Lipoprotein
Endothelium
Space of Disse

lipoprotein remnant triglyceride) are the major precursors of VLDL - triglyceride secreted by the liver (214, 219) although endogenously synthesized fatty acids may contribute significantly, especially in carbohydrate feeding, where this becomes a major determinant of hepatic triglyceride secretion (220, 221). The preferred substrates for endogenous lipogenesis are glycogen and lactate (222).

The liver is remarkable in that in the transition from the fed to the fasted state the liver switches (in a brief period of several hours) from an organ of carbohydrate utilization and fatty acid synthesis, to one of fatty acid oxidation and ketone body production (217), the changes largely orchestrated by the two hormones insulin and glucagon. In the carbohydrate fed state (low (glucagon) : high (insulin)), intracellular concentrations of malonyl - CoA are elevated, which inhibit the action of carnitine acyltransferase I, the committal step in fatty acid oxidation (217), with the result that lipogenesis is favored. Conversely, upon fasting, the glucagon levels are elevated and malonyl - CoA production is suppressed with the concomitant activation of fatty acid oxidation and ketogenesis. Ide et al. (223) demonstrated that the rate of fatty acid oxidation is a major determinant in the production of VLDL.

As the uptake of free fatty acids by perfused rat liver does not exhibit any marked selectivity towards saturation or chain - length, differences in the output of TG with different fatty acids result from a specific metabolic disposition for the individual molecules (219). In general, however, increased availability of FFA dramatically stimulates VLDL secretion in perfused rat livers (219) and isolated hepatocytes (224). Unsaturated fatty acids stimulate TG release to a larger degree than the saturates (225, 226, 227). The molecular species of secreted TG closely resemble the cellular TG composition (225, 228), however, the TG secretion

rates are dependent on fatty acid chain length (but not on degree of saturation) (225). Palmer et al. (228) observed a significant change in the composition of cellular TG from livers of fed and fasted rats in that there was an increase in longer - chain, unsaturated FA in the latter group. These observations are probably attributable to differences in dietary and adipose tissue fatty acid composition (228).

Nascent VLDL secreted by perfused rat liver (229) or isolated hepatocytes (225) after exposure to unsaturated fatty acids were larger than those secreted after incubation with saturated fatty acids and had lower ratios of PL and cholesterol relative to TG (226). The larger size of these particles may, in part, be due to the stimulation of TG output, however, the increased volume of the VLDL is also related to the greater volume occupied by TG having more unsaturated FA (230).

The ratio of CE relative to TG in nascent VLDL is larger when saturated versus unsaturated FA are infused through a perfused liver (226). Wilcox et al. (226) postulated that CE, transported as a core lipid, is displaced by TG when the synthesis of the latter is increased. The greater proportions of PL and FC relative to TG in nascent VLDL from livers infused with saturated FA suggests a hypercholesterolemic effect of the latter (226). More surface components may be required for stability of lipoproteins having a high saturated fatty acid content (231).

Although glucose can provide a source of glyceride - glycerol, exogenous serum glycerol is primarily utilized (227). A decreased lipid production correlates directly with glycerol - 3 - phosphate acyltransferase and glycerol kinase activity and can be increased in hepatocytes after exposure to estradiol and ethanol, both of which increase VLDL secretion (227).

About 75% of the total turnover of serum TG in the rat involves TG

returning to liver via lipoproteins (214). Triglyceride - rich chylomicrons, VLDL, and their remnants cause a significant inhibition of fatty acid synthesis in hepatocytes from fed rats (232), whereas LDL and HDL show no inhibitory effect. The liver has the capacity to metabolize the lipoprotein - bound TG (214, 233, 234). Controversy still exists as to whether or not intact TG, diacylglycerol or monoglycerols are taken up, however evidence to suggest all three possibilities is available (214, 233, 234).

The evidence to date would suggest that recycling of serum TG by the liver involves intracellular events, i.e. uptake and internalization of TG - rich remnants (235, 236, 237). A single TG lipase present in lysosomes or autophagic vacuoles is believed to be responsible for the breakdown of exogenous and endogenous triglyceride, respectively (238). Radiolabelled exogenous lipoprotein products eventually migrate to the SER (237) where possible pooling with endogenous substrate can occur, since the autophagic vacuoles are continuous with the SER (239).

Two pools of TG within the cell have been predicted mathematically. Physically these can be separated into microsomal and cytosolic (hepatic stores) fractions and theoretically only the former gives rise to serum TG (228), supporting the proposed scheme of endogenous and exogenous substrate pooling in the microsomes.

Phospholipid. Little is presently known concerning the coordination of phospholipid synthesis with that of the serum lipoproteins. Whereas TG synthesis and release is strongly influenced by the nature and relative availability of fatty acids, PL synthesis is not (220, 227, 240).

Some information on the role of phospholipid metabolism as it relates to lipoprotein production has been obtained from studies of rats fed choline - deficient diets. Choline is a lipotropic agent and a major precursor for PL synthesis. Deficiency of this factor results in the intra-

cellular accumulation of TG in the liver (241) with a concomitant decrease in plasma TG and PL within 2 days (242, 243). The intracellular phosphatidylcholine to phosphatidylethanolamine (PC / PE) ratio is decreased (242) as would be expected. Triglyceride accumulation in the livers of choline - deficient rats has been demonstrated to be due to a) increased fatty acid synthetase activity in choline deficiency (241) and b) impaired lipoprotein secretion. The latter has been attributed to several possibilities. The decreased PC / PE ratio may result in alterations in microsomal membrane conformation which may in turn affect enzymatic activity of any associated proteins (242) such as is observed for UDP-N-acetylglucosamine : glycoprotein N-acetylglucosaminyl transferase, a membrane - bound enzyme involved in the glycosylation of lipoproteins, whose activity is reduced in choline - deficiency (244). Also, the altered phospholipid composition of the nascent lipoproteins may confer a lesser degree of stability to the lipid - protein complex (74).

Cholesterol and cholesteryl ester. The liver is the major site for endogenous cholesterol synthesis (245). In the absence of available exogenous cholesterol, the regulatory enzyme of cholesterol biosynthesis, HMG-CoA reductase, provides the liver cell with a constant supply of free cholesterol for hepatic TG secretion (246). The coordinate activation of this enzyme and TG synthesis by FFA has been observed (246), however, the ability of hepatocytes to take up cholesterol and presumably incorporate it into nascent VLDL indicates that lipoprotein production and cholesterol synthesis are not necessarily closely linked (246, 247).

The role of dietary cholesterol in influencing nascent lipoprotein synthesis and secretion is evidenced in experimentally - induced hypercholesterolemia, a condition which results in depressed endogenous cholesterol biosynthesis (248). The VLDL secreted by isolated perfused livers

(120) and hepatocytes (95, 112) from cholesterol - fed rats have increased proportions of core lipid CE relative to triglyceride.

The key enzymes involved in hepatic cholesterol metabolism, HMG-CoA reductase (cholesterol biosynthesis), cholesterol 7 α-hydroxylase (bile acid biosynthesis) and acyl-CoA - cholesterol acyltransferase (ACAT) (esterification of cholesterol) are all confined to membranes of the endoplasmic reticulum (249, 250). Recently, other microsomal enzymes involved in cholesterol biosynthesis have been implicated as potential sites of biosynthetic regulation, governing the flow of carbon to cholesterol (251), and of these, 4 α- methyl sterol oxidase is known to exhibit parallel changes in activity to that of HMG-CoA reductase (252). Diurnal rhythmic variation of both HMG-CoA reductase and 4 α- methyl sterol oxidase are observed (252, 253). The half - life estimated for HMG-CoA reductase is approximately 4 hours (254).

Hepatic cholesterogenesis is inhibited by exogenous cholesterol and related sterols, especially oxygenated cholesterol derivatives, whether they be presented in solution, as synthetic vesicles or as native lipoproteins (254, 255, 256). Net cholesterol uptake from lipoproteins by hepatocytes occurs readily (213), however it was shown that net uptake requires that the molar ratio of FC : PL in the lipid donor is greater than 1 (257). The uptake of cholesterol is associated with an increase in intracellular cholesterol ester content (213, 257).

In vivo studies demonstrated that cholesterol feeding, infusion of chylomicron remnants, VLDL or LDL depressed hepatic HMG-CoA reductase activity (213, 249, 256) whereas HDL or apo-HDL infused into animals resulted in an increase in reductase activity (256). Studies with isolated rat hepatocytes also demonstrated depressed HMG-CoA reductase activity in the presence of LDL or cholesterol - phospholipid dispersions and enhanced activity in

lipid - deficient media and in the presence of HDL or lecithin dispersions (254, 258). Lecithin dispersions increase the rate of cholesterol efflux from the cell, indicating that the relative rates of efflux and influx of cholesterol can regulate the activity of HMG-CoA reductase (254). Similar effects on reductase activity are not observed using liver homogenates or isolated microsomes (258), indicating that a regulatory pool of cholesterol (or sterols) probably exists in the immediate lipid environment of the enzyme, a possibility that is substantiated by kinetic studies (249).

The stimulatory effect of HDL or apo-HDL on reductase activity remains enigmatic. However, some interesting observations suggest a role for apolipoproteins themselves in the regulation of cholesterogenesis (259). Soluble proteins that stimulate microsomal enzymes in the cholesterol biosynthetic pathway have been reported (252, 259, 260) and have been referred to as sterol carrier proteins (SCP). They all have affinity for cholesterol and related sterols, the affinity for the latter being more pronounced for cholesterol precursors than for other cholesterol derivatives (259). In the presence of SCP, the reaction rates of HMG-CoA reductase and 4 alpha- methyl sterol oxidase are enhanced (259, 260) while the extent of inhibition by FC, CE or other sterol oxygenated derivatives is greater (252, 260). Ritter and Dempsey (259) noted that human or rat apo-HDL was the only serum protein tested that substituted for the binding and activation functions of SCP suggesting that a component of HDL may be identical to apo-SCP. The observations of Jakoi and Quarfordt (256) that apo-HDL (containing apo-AI, AII and C) is capable of stimulating the HMG-CoA reductase is consistent with this hypothesis. As apo-VLDL has no effect, apo-AI and / or apo-AII are probably responsible for the cholesterogenic activation (256, 259, 260). On the other hand, Boyd and Onajobi (261) reported that some apolipoproteins (not identified) caused the accumulation of squalene, a cholesterol

precursor, with the net result that cholesterol biosynthesis was reduced. It remains to be determined whether apolipoprotein(s) or related protein(s) are involved in cholesterogenic regulation.

Regulation of hepatic cholesterol biosynthesis is further complicated by the finding that HMG-CoA reductase exists in a phosphorylated (inactive) and dephosphorylated (active) form (262). It appears that under normal physiological conditions approx. 75 - 90% of the reductase is in a phosphorylated form and hence is inactive (262). Brown et al. (262) suggested that the inactive enzyme serves as a reservoir of reductase which can be activated by dephosphorylation (by phosphatase) when hepatocytes are faced with demands for cholesterol. Long - term alterations (e.g. as a result of chronic diet) of hepatic cholesterol synthesis in the rat are due to changes in the total amount of enzyme protein (262, 263) and not due to changes in the proportion of phosphorylated reductase (262). These findings and studies suggesting direct inactivation of reductase by cholesterol and its catabolites (252, 258) indicate that the latter may regulate dephosphorylation with possible involvement of SCP in short - term cholesterogenic regulation.

It has been observed that hypothyroidism is associated with decreased hepatic cholesterol biosynthesis (264, 265) as a result of decreased HMG-CoA reductase activity (266).

Esterified cholesterol in rat VLDL is largely derived from the liver as a result of the action of ACAT on FC and FFA (250). The ester composition of hepatic microsomal fractions and that of serum VLDL are identical (250). However, man possesses only 25% of the (ACAT) activity present in rats (250) so that the bulk of CE in humans is derived in the vascular compartment by the action of LCAT (5).

The cholesterol substrate for the ACAT reaction in rats constitutes a pool distinct from that destined for bile acid synthesis (250).

The ACAT enzyme in rats efficiently utilizes exogenous cholesterol added as substrate, whereas the enzyme in human liver does not (267).

Oxygenated sterols, have more marked regulatory effects on ACAT enzyme activity than cholesterol (268, 269). Substances such as 25-hydroxy cholesterol have acute stimulatory effects on ACAT which are independent of new protein synthesis, suggesting a direct interaction of the sterol with the enzyme itself (268). Incorporation of labelled oleic acid into CE, but not into TG or PL is increased 3- to 6-fold in intact hepatocytes exposed to 25-hydroxy cholesterol for several hours while the incorporation of exogenously labelled cholesterol or mevalonolactone (endogenous cholesterol) into CE is increased (268). This results in the accumulation of CE with a concomitant decrease of FC in the cell (268).

It has been demonstrated that cholesterol - feeding promotes increased hepatic ACAT activity in guinea pigs (270) and rats (271). Incubation of rat hepatoma cells with hyperlipemic serum is sufficient to activate ACAT (272). The activity of ACAT in reconstituted synthetic liposomes is found to be dependent on the amount of cholesterol used in the liposomes or on the amount of free cholesterol transferred from other membranes during experimental incubations (273). This finding and the observation that protein synthesis is not required for ACAT activation (268, 274) implies that sufficient ACAT is present in normal cells to accomodate any acute (e.g. postprandial) changes in cholesterol load.

Chronic exposure to high cholesterol levels (high cholesterol diets) leads to the secretion of cholesterol - ester rich nascent VLDL (95, 112, 120). Short - term (several hours) exposure to 25-hydroxy cholesterol gives rise to relatively CE - rich nascent VLDL, however, the total cholesterol content relative to TG in these lipoproteins is unchanged from that secreted by unstimulated cells in that only the proportion of

cholesterol in esterified form is increased (269).

2) Synthesis of Apolipoproteins

The synthesis of apolipoprotein precursors, like those of other secretory proteins, begins in the cytosol on polyribosomes which become attached to the cisternal membrane of the endoplasmic reticulum (275, 276, 277). Apolipoprotein mRNA from chicken and rat have been successfully translated in vitro in heterologous protein synthesis systems (276, 277, 278, 279). In vivo (279) and in vitro (278) studies with rat liver indicate that roughly 2% of the total liver protein synthesis in the normal animal is devoted to apo-VLDL proteins while about 1.5% alone is devoted to apo-E (279). The small proportion of total apo-B mRNA and the observed heterodispersity of anti-apo-B binding to polysomes has hampered investigation of the biosynthesis of this apolipoprotein in rat (279). However, studies on avian apo-B, which is antigenically related to human apo-B, indicate that neither protein synthesis, attachment of the polypeptide to the ribosome nor glycosylation is required for extrusion of the nascent chain through the ER membrane (280). A component, recently characterized by Meyer and Dobberstein (281) that is responsible for vectorial translocation of proteins across the ER membrane, therefore, may be primarily involved in apo-B translocation.

Avian apo-AI and a unique protein, apo-VLDL-II, were both found to be synthesized initially as larger preproteins (282). The N - terminal sequence of apo-VLDL-II consisted of a 23 amino acid extension (276) which was similar to signal sequences reported for other proteins (283). The translocation of this phospholipid - binding apolipoprotein (77) through the ER membrane is of particular interest as some mechanism would be required to prevent interaction of the protein's amphipathic region with the outer membrane surface (77, 276). It is unlikely that the signal sequence would

serve this function (283). Blobel (284) proposed that unique "topogenic" sequences in proteins may initially serve to regulate apolipoprotein - lipid interaction.

The primary translation product of rat intestinal apo-AI mRNA was found to be a novel preproprotein (277). Following the signal sequence, an unusual hexapeptide prosegment having paired glutamines rather than arginines at the COOH- terminal of the segment is present on the apo-AI (277). It has been speculated that the prosegment of apo-AI might serve as such a "topogenic" sequence preventing the interaction of proapo-AI with lipid, until this apolipoprotein is required for nascent HDL formation (277). Protein prosegments identified to date have been shown to be proteolytically cleaved at highly conserved terminal dibasic residues (285). Therefore, the observation of novel terminal residues for proapo-AI suggests that post-translational processing of this apolipoprotein occurs via currently undefined pathways and not within the Golgi as for other proproteins (286).

Translation of rat apo-E mRNA in the presence of microsomal membranes in vitro results in the formation of preapo-E which is sensitive to endoglycosidase H whereas the product formed in the absence of microsomal membranes is not (278). This indicates that the former has undergone co-translational signal peptide removal and core glycosylation (278). Plasma apo-E is not cleaved by endoglycosidase H, which implies that further modification (e.g. addition of sialic acid) of preapo-E occurs prior to its secretion by the liver cell (278). Studies on apolipoproteins synthesized by human fetal liver cultures support this concept (287). Newly synthesized apo-E consists of an array of isoforms that have higher molecular weights and more acidic isoelectric points than their serum counterparts, however, the nascent apo-E become indistinguishable from serum apo-E after treatment with neuraminidase (287). These studies suggest that rat and human apo-E are

initially synthesized as sialo apo-E and are desialated in plasma (278, 287).

In contrast, apo-AI isoforms synthesized by human fetal liver cultures are more basic than the two serum isoforms suggesting that preapo-AI isoforms undergo post - secretory modification to their plasma forms (287). A defective conversion of these preapo-AI isoforms may explain the barely detectable levels of plasma apo-AI in Tangier disease (287). The intestinal mucosa of patients with diagnosed Tangier disease secrete the preapo-AI isoforms (287), however, a defective conversion of apo-AI could result in its rapid clearance from the circulation perhaps by an asialo glycoprotein receptor (288).

Stimulation of hepatic lipogenesis by FFA is also accompanied by increased de novo apolipoproteins mRNA synthesis (289). Similarily, the increased secretion rate of apo-E by livers from rats fed an atherogenic diet (120) is mediated to a certain extent by an accumulation of translatable apo-E mRNA in liver cells (290). Hormonal stimulation by estrogen of specific apo-VLDL-II mRNA in avian liver has also been demonstrated (282).

3) Formation of Lipid - Apolipoprotein Complexes

The mechanisms whereby nascent apolipoproteins bind lipid to form lipoproteins within the cell are not known, although studies investigating the interaction of isolated apolipoproteins with phospholipid bilayers have provided useful information that may have relevance with respect to the formation of native lipoproteins. The first possible interaction of apolipoproteins with lipid can occur at the rough ER and this possibility is supported by the finding that the enzyme ACAT co-isolates with RNA suggesting that the product of ACAT (CE) is synthesized and assembled on to either preformed apolipoprotein (apo-B) or the nascent peptide (250). As apo-B is almost insoluble in aqueous solution in the absence of amphiphiles, it is

reasonable to conceive that in the formation of TG - rich lipoproteins a phospholipid apo-B complex is formed initially on to which TG is subsequently added. Studies by Bar-On et al. (291) lend support to this concept as this group noted that newly synthesized TG and PL continued to be added onto pre-existing apolipoprotein even after cycloheximide treatment. The existence of preformed pools of apo-B in the intestine (292) and of apo-E in rat liver (290) have been demonstrated and may confer to the organs a certain degree of adaptability when challenged with conditions of increased lipid synthesis or absorption. Intracellular apolipoprotein pools do not contain all the components in the proportions present in the secreted VLDL (291).

Electron microscopy using peroxidase - conjugated antibodies against apo-B and apo-C have shown that these apolipoproteins are acquired by the nascent lipoprotein particles at the junction between the RER and SER (215, 293).

The lipid - binding regions of the characterized apolipoproteins have been found to reside at the COOH - terminal (74, 77). This as well as the above observations, suggests that the apolipoproteins must be fully synthesized and translocated into the luminal aspect of the ER before they acquire their full lipid - binding capacity.

4) Transport and Secretion of Lipoproteins by the Hepatocyte

The pathway of lipoprotein secretion includes movement from the smooth ER through the Golgi apparatus via Golgi secretory vesicles and entry into the circulation after fusion of the secretory vesicles with the plasma membrane (294 and refs. cited therein). A precursor - product relationship between VLDL isolated from the Golgi apparatus and the serum was demonstrated by Nestruck and Rubinstein (295). It is evident that nascent lipoprotein particles are dynamically modified during the course of their passage through

the ER to the Golgi apparatus, the major function of the Golgi being the glycosylation of the apolipoprotein moieties (296). Addition of oligosaccharide cores to apolipoproteins can occur at the rough ER whereas the sites of addition of terminal galactose, sialic acid or fucose moieties occurs primarily in the Golgi membranes (297). Evidence presented recently also suggests an active exchange process of polar lipids between Golgi membranes and Golgi secretory lipoproteins (298).

The particles passing through the Golgi apparatus undergo a reduction in volume of about 50% with an attendant decrease in TG and an increase in CE content (299). A morphological structure (GERL) closely associated with the Golgi apparatus, but chemically distinct (hydrolase - rich), may be involved in such lipoprotein transformations (300).

Studies of glycosylation of rat apo-VLDL indicated that apo-B incorporated a major proportion of added ^{3}H-glucosamine while apo-E and apo-$CIII_3$ incorporated the remainder (301). It has also been demonstrated that additional apo-C peptides can be acquired by the nascent VLDL in the Golgi (215, 301). Difficulty in obtaining adequate sample sizes in part accounts for the paucity of information on this aspect of lipoprotein assembly.

The accumulation of nascent lipoproteins in rat hepatic Golgi during fatty liver induction by orotic acid (OA) suggests a role of glycosylation in lipoprotein secretion (302). Fatty livers induced by orotic acid feeding are characterized by an accumulation of VLDL-size particles in the Golgi and the ER within 72 hours (302). Pottenger et al. (303, 304) showed that VLDL apolipoproteins derived from orotic acid fed rat liver Golgi were deficient in apo-$CIII_3$ and N- acetylglucosamines, galactose and N- acetylneuraminic acid, the latter being attributed to a lack of exposure of the apolipoproteins to the Golgi glycosylating enzymes. As orotic acid produces a decrease in adenine and cytidine nucleotides (305) a deficiency in CMP -

substrates for glycosylation could be envisaged and could have important implications in the enzymatic incorporation of sialic acid into apo-C in particular (306). It is pertinent to note that the addition of adenine to the orotic acid diet reverses the hepatic TG accumulation (306).

A similar situation occurs in fatty liver induction by D-galactosamine in rats, where 24 - 48 hours after a single injection of this agent there is a complete disappearance of apo-C from serum VLDL and HDL (307). As apo-C is primarily derived from the liver in rats (308) this loss indicates a defective hepatic synthesis or secretion of apo-C. Sirowej and Katterman (307) proposed that an alteration in the glycosylation of this protein by D-galactosamine (309) accounted for its absence.

A clue as to the mechanism of TG accumulation in the livers of orotic acid fed rats is the observation that OA does not affect intestinal lipoprotein secretion (310). This same organ in the normal rat does not synthesize apo-C to any significant degree (308). Therefore, the impaired secretion of lipoproteins by the liver could arise from a defect in apo-C glycosylation. Glycosylation of apo-B and apo-E may not be as critical for secretion. That glycosylation is not important for lipoprotein secretion has been suggested (311, 312), however, these short - term studies do not rule out long - term effects.

To date no electron microscopic evidence is available demonstrating the presence of nascent HDL within the Golgi apparatus. Discoidal nascent HDL is secreted by isolated livers from rats in the presence of an inhibitor to LCAT (205) and from normal or hypercholesterolemic monkeys (206) in the absence of inhibitor. The presence of nascent discoidal HDL in human splanchnic circulation has been demonstrated (313). Therefore, the failure to detect Golgi HDL may be a result of the low levels of this lipoprotein (314) and the inherent difficulty of distinguishing nascent HDL from membrane

fragments which occur during Golgi isolation (298).

Recently, morphological evidence for a second pathway of lipoprotein secretion by rat liver cells was reported (216) indicating a direct route of lipoprotein particle transport via smooth ER - derived vesicles which appear to bypass the Golgi apparatus en route to the plasma membrane. Such a mechanism resembles secretory processes of evolutionarily more primitive cells (216). Nascent VLDL particles transported via this alternate pathway occur singly and are slightly larger than those in Golgi vesicles which are found as clusters (216).

The finding that colchicine, vincristine, phalloidin and cytochalasin D inhibit TG release by liver cells suggests a role for microtubules in the transport and secretion of lipoproteins (315, 316, 317) although this has been questioned (318). In a recent report, Yedgar et al. (319) observed that the synthesis and secretion of VLDL by hepatocyte monolayers decreased with increasing medium viscosity. An explanation of this phenomenon, which appears to be responsible for the hyperlipidemia observed in hypoalbuminemic patients, is not readily apparent (319). However, that the synthesis and secretion of albumin is not significantly affected by medium viscosity, phalloidin or cytochalasin suggests that the mechanisms involved in its secretion are different to those of lipoprotein synthesis and secretion (317, 319).

C) <u>The Use of Isolated Hepatocytes in the Investigation of Lipoprotein Synthesis and Secretion</u>

Studies on the synthesis and secretion of hepatic lipoproteins prior to 1975 were confined to the use of the isolated perfused liver (320, 321, 322, 323). Isolated hepatocytes incubated in suspension were used as early as 1971 (324, 325) however, the short - term incubations possible (3 hours) limited the amount of material available for analyses. In 1975, Jeejeebhoy et al.

(326) reported a method which permitted long - term incubations of isolated hepatocytes which secreted VLDL over a 24 hour period (326). The nascent VLDL gave a precipitin reaction against anti - rat- serum - VLDL, however, the urea PAGE profile indicated an absence of the apo-C peptides (327). As VLDL obtained from liver perfusions were shown to contain all the serum VLDL apolipoproteins including apo-C (320, 321, 322, 323), this was one of the first clear demonstrations that lipoproteins could undergo post - secretory modification once released from the hepatocyte. Such reactions could occur in the space of Disse (Fig.8). It had been proposed (328) that apo-C could be acquired by the VLDL after its secretion into the space of Disse, (i.e. transfer from HDL) on the basis that patients with abetalipoproteinemia (who lack VLDL) still had apo-C in their HDL. This implied that nascent HDL contained apo-C. In 1976, Nestruck and Rubinstein (295) demonstrated that Golgi VLDL could acquire apo-C in incubations with serum HDL.

The advantages of isolated hepatocytes over liver perfusions to investigate certain aspects of lipoprotein synthesis and secretion are apparent: a) the longer incubation times possible, b) the space of Disse is eliminated, reducing the possibility of post - secretory modification by preventing the localization of potential modifiers in the extracellular space and c) differences between livers can be reduced by i) incubating hepatocytes from one liver separately under different experimental conditions or ii) pooling hepatocytes from more than one liver in a single incubation. Suspensions of hepatocytes were used for the studies conducted in this thesis.

During the course of the present work, developments in culturing techniques led to the establishment of non - proliferating hepatocyte monolayer cultures (329) to investigate lipoprotein uptake. This system was subsequently used to study lipoprotein synthesis and secretion (224, 330, 331). Ultracentrifugation of the incubation medium at classical serum

densities has resulted in discrepancies in the flotation properties reported for the nascent lipoproteins (224, 330, 332). The essentially lipid - free apo-AI and apo-E found in the $d > 1.21$ $g.mL^{-1}$ fraction (331) also raises the ubiquitous possibility that these were ultracentrifugal artifacts (36). Similar observations in our laboratory, prompted us to investigate other possible modes of separating nascent lipoproteins. Agarose gel filtration of perfusate lipoproteins had proven successful in this laboratory (333) and did not subject the lipoproteins to high physical forces. As a result, this method was adapted to examine the nascent lipoprotein products secreted by isolated hepatocytes.

Serum lipoproteins represent a dynamic spectrum of particle sizes. It was reasonable to assume that the nascent particles may share such features and that dietary and hormonal factors could easily influence average particle size and apolipoprotein distribution. Gel filtration affords a more rapid and simpler means to detect such changes than ultracentrifugation.

1) Objectives of the Present Study

The objectives of the work described herein were twofold. Firstly, the nature of lipoproteins secreted by normal rat hepatocytes was investigated. The second objective dealt with an aspect of lipoprotein metabolism that has been a subject of much interest in this laboratory, namely, hypercholesterolemia.

The hypercholesterolemic rat has been used as a model to investigate various aspects of altered lipoprotein metabolism, in that abnormal lipoproteins accumulate in the sera of these animals which have similar properties to those of lipoproteins present in the sera of patients with familial Type III hyperlipoproteinemia (110, 120). However, this species, like the dog, is particularly resistant to the development of hyper-

cholesterolemia and requires the concomitant induction of a hypothyroid state. Thus the antithyroid drug propylthiouracil (PTU) is generally added to the high fat, high cholesterol diet used to induce hypercholesterolemia in these animals.

The accumulation of abnormal, β-VLDL having a high cholesterol and apo-E content in the sera of hypercholesterolemic rats was found to be due, in part, to the direct synthesis and secretion of these particles by the liver (120, 334). In order to understand the biosynthetic mechanisms responsible for the production of the abnormal lipoproteins, it was necessary to differentiate between the effects of the various components of the hypercholesterolemic diet. Dory et al. (335) observed an accumulation of LDL in the plasma of chow-fed hypothyroid rats. Other studies have also indicated that the thyroid hormones may play direct roles in regulating lipoprotein metabolism in rat (264, 336) and hypothyroidism is a well - established cause of secondary hyperlipoproteinemia in man (337). Elevated serum levels of apo-E were observed in hypercholesterolemic rats (110, 119) whereas in hypothyroid rats maintained on regular chow diets, normal (110) and elevated (335) serum levels of apo-E have been reported.

Therefore, the objective of our work was to evaluate the effects of diet and hormonal status on hepatic lipoprotein synthesis and secretion and to ascertain to what extent the products directly contributed to the serum pool of abnormal lipoproteins in hypothyroid or hypercholesterolemic rats.

MATERIALS AND METHODS

1) Animals

a) Studies on normal rats

Male Long-Evans rats (250-300g)(Canadian Breeding Farms, St. Constant,PQ) were used for all studies. The normal (control) group of rats were maintained on a commercial chow diet (Ralston Purina Co., St. Louis, MO).

b) Dietary and hormonal manipulations

A second group of rats was rendered hypothyroid by the addition of 0.1% PTU (w/v)(Sigma Chemical Co.,St.Louis,MO) to the drinking water. The third group (hypercholesterolemic) was fed a thrombogenic diet (ICN Biochemicals, Montreal,PQ) containing 40% butter fat, 5% cholesterol, 2% sodium cholate and 0.3% PTU. Rats were maintained on the PTU - treated water for 21 to 25 days and on the thrombogenic diet for 45 days. Serum thyroxine (T_4) was determined to monitor the effects of the PTU - treatment on these animals (Thyroxine Radioimmunoassay Kit,Bio-RIA, Montreal,PQ). All groups of rats (normal, hypothyroid and hypercholesterolemic) were comparable in weight at the time of the experiments.

2) Isolation and incubation of rat hepatocytes

Hepatocytes were prepared by a modification of the method of Jeejeebhoy et al.(326).The solutions used throughout the isolation procedure as well as the incubation medium were sterile - filtered and contained penicillin (10,000 $U.mL^{-1}$) and streptomycin (0.05mM). Tubing, connectors, filters and beakers were of the disposable type, whereas the glassware used for incubating was acid - cleansed and UV - irradiated overnight.

All rats were fasted overnight and isolation of the hepatocytes from the first liver was started at 9:00 A.M. the following morning. Each animal was anaethetized with 20-25 mg Nembutal (Abbott Laboratories,Montreal,PQ). The liver was prepared for perfusion according to the methods of Miller (338) using a 16-gauge Argyle Medicut intravenous plastic cannula (Sherwood Medical Ind., St.Louis, MO) and Intramedic PE-90 Tubing (Becton, Dickinson and Co., Parsippany, NJ) to cannulate the afferent portal vein and efferent supra-diaphragmatic inferior vena cava respectively. The liver was preperfused with about 50-60 mL of a non - recirculating solution of calcium - free Krebs - Henseleit buffer, pH 7.4 containing 15 mM citrate and 15 mM glucose. The liver was then perfused at a flow rate of 50 mL per min at 37^{o}C with 100 mL of a re-circulating solution of the same buffer containing collagenase (Sigma Chemical Co.,St.Louis,MO) at a concentration of 75 U.mL^{-1} for 5 mins. The flow rate was occasionally increased to facilitate dispersion of the liver cells. The buffer was constantly oxygenated by passage through a Silastic tubing oxygenator (339). Calcium chloride was then added to a final concentration of 2.5 mM to enhance enzymatic activity and accelerate dispersion (340). After 7-10 mins the liver was removed from the animal, cut into smaller pieces with surgical scissors and incubated with oxygenation and gentle agitation for 5 mins in 100 mL of a fresh collagenase solution containing 0.5% fatty - acid - free BSA. The solution was then passed through four layers of surgical gauze and the parenchymal cells were isolated and washed by gravity sedimentation or low speed centrifugation (100 x g for 5 mins). Normally cells were washed twice with Krebs - Henseleit buffer, however, preparations of hypercholesterolemic rat hepatocytes required 5 or 6 washes to remove visible particulate fat.

Hepatocytes from two livers were incubated in 600 mL of Waymouth's MB 752/1 medium (Gibco Laboratory Supplies, Grand Island, NY) supplemented with 17.5% (v/v) horse serum (Flow Laboratories, McLean, VA), 10,000 U.mL^{-1}

penicillin and 0.05 mM streptomycin sulfate. The donor horse serum was heat - inactivated at 56°C for 30 mins and delipoproteinated by one of two methods. In the first set of studies, the horse serum was delipoproteinated according to the procedure of Jeejeebhoy et al.(327) by adjusting the serum density to 1.21 $g.mL^{-1}$ with NaBr and subjecting it to ultracentrifugation for 40 hours. The serum was extensively dialyzed against saline prior to use. During the course of this work a second method of delipoproteinating horse serum was developed by Weinstein (341) and was employed for all experiments described in Part C of the Results section. Essentially all lipid and apolipoproteins B and E are removed from serum by this method (341, 342) which consists of incubating 3g Cab-O-Sil (Eastman Kodak, Rochester, NY) per 100 mL of serum overnight at 4°C with shaking. The Cab-O-Sil is removed by centrifugation and subsequent filtration of the serum. Figure 9 shows gas chromatographic profiles of horse serum used in these studies before and after Cab-O-Sil treatment.

Suspensions of hepatocytes were maintained at pH 7.1 and 37°C for 24 h or longer in a single 1-L spinner flask (Bellco Biological Glassware, Vineland, NJ) or equal parts were divided and incubated in several 250 mL spinner flasks (Johns Scientific, Toronto, Ont.). Oxygenation of the suspensions was achieved by continuous gassing with $95\%O_2:5\%CO_2$ at a flow rate of $2L.min^{-1}$. Evaporation was retarded by the attachment of water - cooled condensers as described by Jeejeebhoy et al.(326).

Zero time was taken as the time of addition of a neutralized solution of 1mCi ^{3}H-labelled amino acid mixture or 50uCi ^{14}C-leucine, 100uCi ^{3}H-mevalonolactone and 0.5mCi ^{3}H-palmitate (New England Nuclear Corp., Boston, MA) to the incubation medium. Palmitate was coupled to fatty - acid - free BSA by a modification of the method of Milstein et al.(343). Addition of the radioisotopes occurred, due to experimental procedure, at one-half hour after the

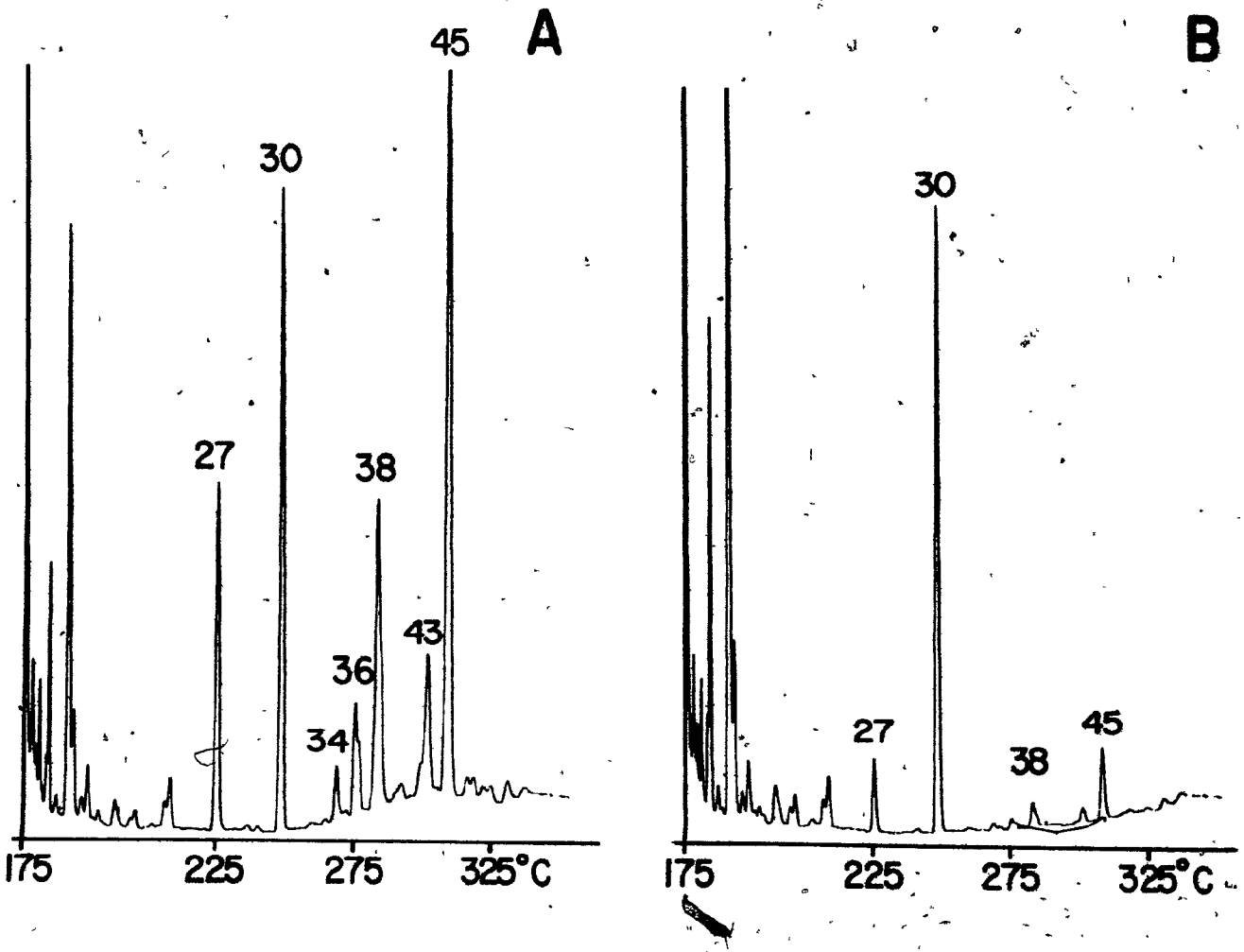
FIGURE 9

TOTAL LIPID PROFILES OF HORSE SERUM BEFORE AND AFTER INCUBATION WITH CAB-O-SIL*

Heat-inactivated, sterile-filtered horse serum (A) was delipoproteinated (B) with Cab-O-Sil* as described in the Materials and Methods. For (A) and (B), 0.3mL and 1.0mL serum samples were taken for lipid analyses respectively. The lipid profiles were obtained using low - temperature gradient GLC. Peak 30 is the tridecanoylglycerol internal standard and the identification of the other peaks is as in Fig. 31. Ninety-eight percent of the lipids were removed by treatment with Cab-O-Sil*.

GAS CHROMATOGRAPHIC CONDITIONS:

The gas chromatograph was equipped with stainless-steel columns (50cm X 2mm I.D.) containing 3% OV-1 on 100-120 mesh Gas-Chrom Q (Applied Science Labs.) and an automatic liquid sample injector. The GLC separations were conducted with temperature programming from 175 to 400^0 at approx. 4^0/min with dry nitrogen as the carrier gas (40 ml/min). The data was processed as described in ref. 361.


A
45
30
27
38
43
36
34
175
225
275
325°C
B
30
27
38
45
175
225
275
325°C

addition of hepatocytes to the incubation medium in all cases.

Total cell protein was determined on a 200 x g pellet of the suspension medium after three washes with Krebs - Henseleit buffer according to the method of Ricca et al.(344).

3) Criteria of hepatocyte viability

a) Trypan blue dye exclusion

An aliquot (0.5 mL) of the cell suspension was added to 0.1 mL of a 0.5% solution of Trypan Blue in saline and mixed thoroughly. The dye was allowed to stand with the cell suspension for 5 mins. A sample was used to fill a hemacytometer and the percent of stained cells was determined after viewing in a light microscope. At least 500 cells were measured for each sample.

b) Intracellular ATP content

Twenty mL of cell suspension was centrifuged at 200 x g for 10 mins to remove the hepatocytes. The supernatant was decanted and 2 mL of cold 5% TCA was added to the cell pellet with thorough mixing. This mixture was centrifuged at 10,000 x g and the supernatant analyzed for ATP by measuring the bioluminescence of added ATP-dependent luciferase enzyme in an LS counter (345).

c) Intracellular Potassium Content

All Corex tubes (15 mL)(Corning Glassworks, Corning, NY) were acid - cleansed and rinsed in deionized water prior to use. Ten mL of cell suspension was centrifuged at 200 x g for 10 mins and the supernatant was decanted. The pellets were dried at 105°C to constant weight. Intracellular K^+ was determined after electrolyte extraction of the dried cell pellet with 0.1N HNO_3 and subsequent analysis by flame photometry using a Beckman Klina-flame

according to the method of Flear et al.(346).

d) Lactate dehydrogenase leakage

Two mL of cell suspension was centrifuged at 200 x g for 10 mins. The supernatant was decanted and reserved and 2 mL of fresh, cell-free incubation medium was added to the pellet with mixing. This latter sample was sonicated at a setting of 60 on a Sonic Dismembrator (Artek Systems Corp., Farmingdale, NY) for 15 secs to disrupt the hepatocytes. Lactate dehydrogenase was determined in the supernatant and pellet fractions by monitoring the conversion of added NADH to NAD^+ at 340 nm according to Bergmeyer and Bernt (347).

4) Lipoprotein isolation

a) Ultracentrifugation

Rat serum lipoproteins and nascent lipoproteins secreted by suspensions of rat hepatocytes were isolated ultracentrifugally according to a modification (348) of the method of Havel,Eder and Bragdon (349) in a Beckman L5-50 or L5-65B ultracentrifuge. An SW 41 rotor was used at 33,000 r.p.m. (134,400g) to isolate lipoproteins from volumes of serum or medium less than 60 mL, whereas the SW 27 or 50.2Ti rotors were used at 27,000 r.p.m. (96,600g) and 33,000 r.p.m. (99,050g) respectively, for larger volumes. Isolated lipoproteins were removed from the tops of the tubes by aspiration with Pasteur pipettes and by tube - slicing when the swinging-bucket and fixed-angle rotors were used respectively.

Lipoproteins were isolated after adjusting the sample solutions to the appropriate densities with solid NaBr (350) and overlayering with salt solutions of the same density containing 0.01% EDTA and 0.02% NaN_3. Generally, for rat serum lipoproteins, VLDL, LDL and HDL were isolated between density ranges $d < 1.006$, 1.006 - 1.063 and 1.063 - 1.21 $g.mL^{-1}$ respectively, unless

otherwise indicated. All isolated lipoproteins were exhaustively dialyzed against 0.15M NaCl (pH 7.3) before subsequent utilization or analysis. Incorporation of radioactive amino acids into VLDL protein was measured by TCA precipitation onto 2.3 cm filter - paper discs according to the method of Mans and Novelli (351).

b) Gel filtration

Lipoproteins were also separated by gel filtration of rat serum or the hepatocyte incubation medium on a 1.25m x 2.5cm Sephacryl-300 column (Pharmacia Fine Chemicals, Upsalla, Sweden). Samples of the incubation medium (300 mL) were taken at the indicated times and centrifuged at 200 x g for 10 mins to remove the hepatocytes. The cell - free medium was overlayered with a NaBr solution (d = 1.006 $g.mL^{-1}$) and particulate fat (352) and membrane fragments (225) were removed by ultracentrifugation for 30 mins in an SW27 rotor at 27,000 r.p.m. (48,300 g.hrs). Ethylenediaminetetraacetic acid (EDTA) and NaN_3 were then added to a final concentration of 0.01% and 0.02% respectively, before concentrating the medium approximately eight - fold against icing sugar (Atlantic Sugar Ltd., St.John, NB). This method was rapid and resulted in far less protein denaturation than occasionally observed using Aquacide II-A (Calbiochem - Behring Corp., American Hoechst Corp., CA) as a concentrating agent. Twenty mL of the concentrated medium sample was applied to the column and was eluted with 0.15M NaCl, 0.01% EDTA and 0.02% NaN_3, pH 7.3 at a flow rate of 1.5 $mL.min^{-1}$.

In order to compare elution profiles of nascent to serum lipoproteins, the sera of two rats was pooled and mixed with fresh incubation medium and prepared as indicated above before being applied to the Sephacryl-300 column. Fractions (5.3 mL) were collected and pooled as indicated and then concentrated for apolipoprotein quantitation or used for

subsequent lipid and electron microscopic analyses. Isolation of lipoproteins from the column eluates for lipid analysis and electron microscopy was achieved by ultracentrifugation as described above except that the pooled fractions were adjusted to a density of 1.21 $g.mL^{-1}$ and spun once for 40 h in order to retrieve all lipoproteins secreted and eluted within the individual fractions pooled.

5) Electron microscopy

a) Hepatocytes

Hepatocytes were isolated after 12 hours of incubation and washed as for total cell protein determination except that after the last wash the pellets were fixed for 2-4 hours in a solution of 2.5% glutaraldehyde at 4°C and post-fixed in 1% osmium tetroxide for 2-4 hours at 4°C. The fixatives were buffered in 0.1M sodium cacodylate (pH 7.3). Hepatocytes were left overnight in 0.5% aqueous uranyl acetate at 4°C prior to dehydration and embedding. Thin sections were cut on an LKB - Huxley ultratome and double - stained with 2% aqueous uranyl acetate and lead citrate.

b) Lipoproteins

Freshly isolated lipoproteins from pooled column eluates were negatively stained with 2% sodium phosphotungstate, pH 7.2, on Formvar-coated 200-mesh copper grids. Photographs of the hepatocytes and isolated lipoproteins were taken with a Philips 200 electron microscope. Mean lipoprotein particle diameters and distributions were calculated after measuring a minimum of 400 particles per sample.

6) Apolipoprotein quantitation

a) Purification of apolipoproteins and production of antibodies

Native rat serum VLDL was used to elicit antibody production against apo-B as described by Dolphin (334). Rat apo-E was purified according to the procedure of Wong et al. (119). Purification of rat apo-AI was accomplished by preparative isoelectric focussing of apo-HDL (155). Antisera were prepared by injecting the apolipoproteins, emulsified in Freund's complete adjuvant into 2 kg. white New Zealand rabbits (apo-B and apo-AI) or a young male goat (apo-E). The animals were given an intramuscular injection of Pertussis vaccine (Connaught Laboratories, Willowdale, Ont.) with the first apolipoprotein injection to enhance antibody response. Rabbits were injected with 100 ug of either apo-B or apo-AI in a total volume of 1 mL subcutaneously in several spots along the back of the animal once a week for three or four weeks. The goat was injected with 150 ug of apolipoprotein subcutaneously in several spots along the rear flank once a week for five weeks. The titers were checked after the indicated immunization periods before exsanguination of the animals. Rabbits were bled by cardiac puncture whereas the goat was bled by cannulation of the carotid artery.

Gammaglobulins were isolated by $(NH_4)_2SO_4$ precipitation (353). Anti-apo-B was cross - adsorbed with HDL to remove apo-E and apo-C titers (334). Antibodies directed towards apo-E and apo-AI were passed through a column of rat albumin linked to Sepharose to remove any possible anti-albumin titer. Specificity of the antibodies was then checked by double immuno-diffusion and no precipitin reactions were observed for any of the other rat apolipoproteins, albumin or horse serum proteins. The antibodies were dialyzed against 5 mM NH_4HCO_3 and lyophilized (anti-apo-B and anti-apo-AI) or stored in small aliquots at -20°C (anti-apo-E).

b) Electroimmunoassays

The conditions and validation of the apo-B electroimmunoassay were

as described by Dolphin (334).

The apo-E electroimmunoassay was validated as per Wong et al.(I19). For the apo-E assay, gels consisted of 1.8% agarose (SeaKem LE, Marine Colloids, Rockland, ME), 5% Dextran T-10 (Pharmacia Fine Chemicals, Upsalla, Sweden) and 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in 0.06M barbital buffer, pH 8.4, containing 2.5 mM calcium lactate. The same buffer without the Dextran was used as the electrode buffer as well as sample diluent. Electrophoresis was performed at 12°C for 18 h at 2.8 V/cm.

The apo-AI assay was carried out as follows. The gel was prepared with 0.06M barbital buffer, pH 8.4, containing calcium lactate, 1% agarose and 5% Dextran T-10. Sample diluent and electrode buffers consisted of the same buffer without the Dextran. Electrophoresis was conducted at 2.5 V/cm for 18 h at 12°C. The purified apo-AI whose protein content had been determined by the method of Lowry et al. (354) was dissolved in the barbital buffer and was initially used as standard. Apo-AI content in lyophilized reference serum samples was quantitated against the standard in the presence or absence of urea (355). No difference in apo-AI concentration as determined by these two methods was observed.

Standard curves of reference serum samples for each of the electro-immunoassays are shown in Figs. 10 - 12 and were routinely run for every assay.

7) Immunodiffusion and immunoaffinity chromatography

Double immunodiffusion was carried out as described previously (24). For immunoaffinity chromatography rat albumin or purified anti-apo-E IgG was linked to Sepharose CL6B (Pharmacia Fine Chemicals, Upsalla, Sweden) using cyanogen bromide according to a modification of the method of March et al.(356). Isolated lipoproteins were delipidated whereas other fractions were

FIGURE 10

STANDARD CURVE FOR THE ELECTROIMMUNOASSAY OF RAT APOLIPOPROTEIN B

Preparation of the antibody and the assay conditions are as described in Materials and Methods. Each point represents the area under each peak calculated for the "rockets" shown in the inset. A standard curve accompanied each assay and was generated with a reference serum standard which had been quantitated against isopropyl - extracted VLDL (ref. 372).

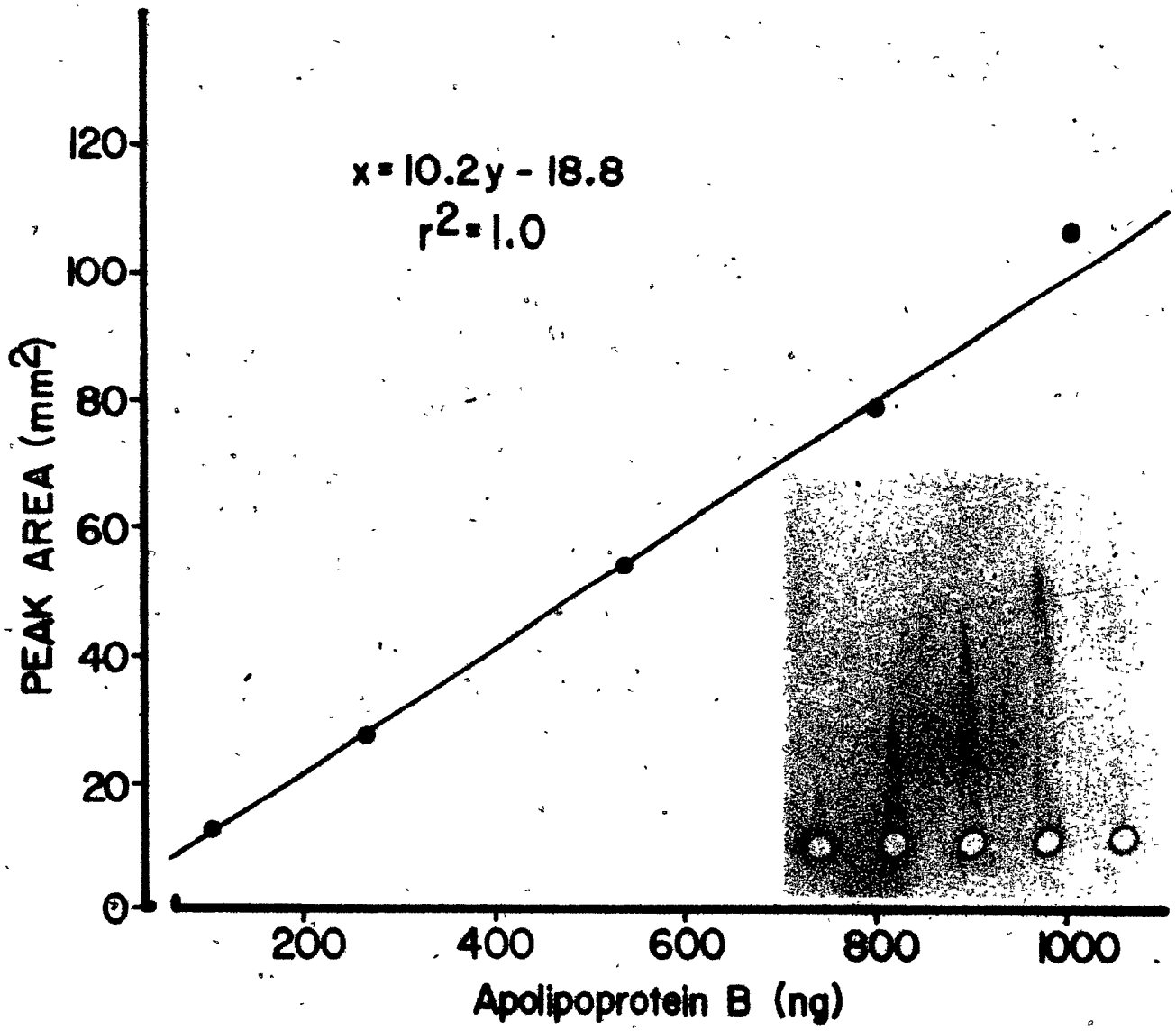

x = 10.2y - 18.8
r2 = 1.0
PEAK AREA (mm2)
0
20
40
60
80
100
120
200
400
600
800
1000
Apolipoprotein B (ng)

FIGURE 11

STANDARD CURVE FOR THE ELECTROIMMUNOASSAY OF RAT APOLIPOPROTEIN E

Preparation of the antibody and the assay conditions are as described in Materials and Methods. Each point represents the area under each peak calculated for the "rockets" shown in the inset. A standard curve accompanied each assay and was generated with a reference serum standard which had been quantitated against purified apo-E (ref. 119).

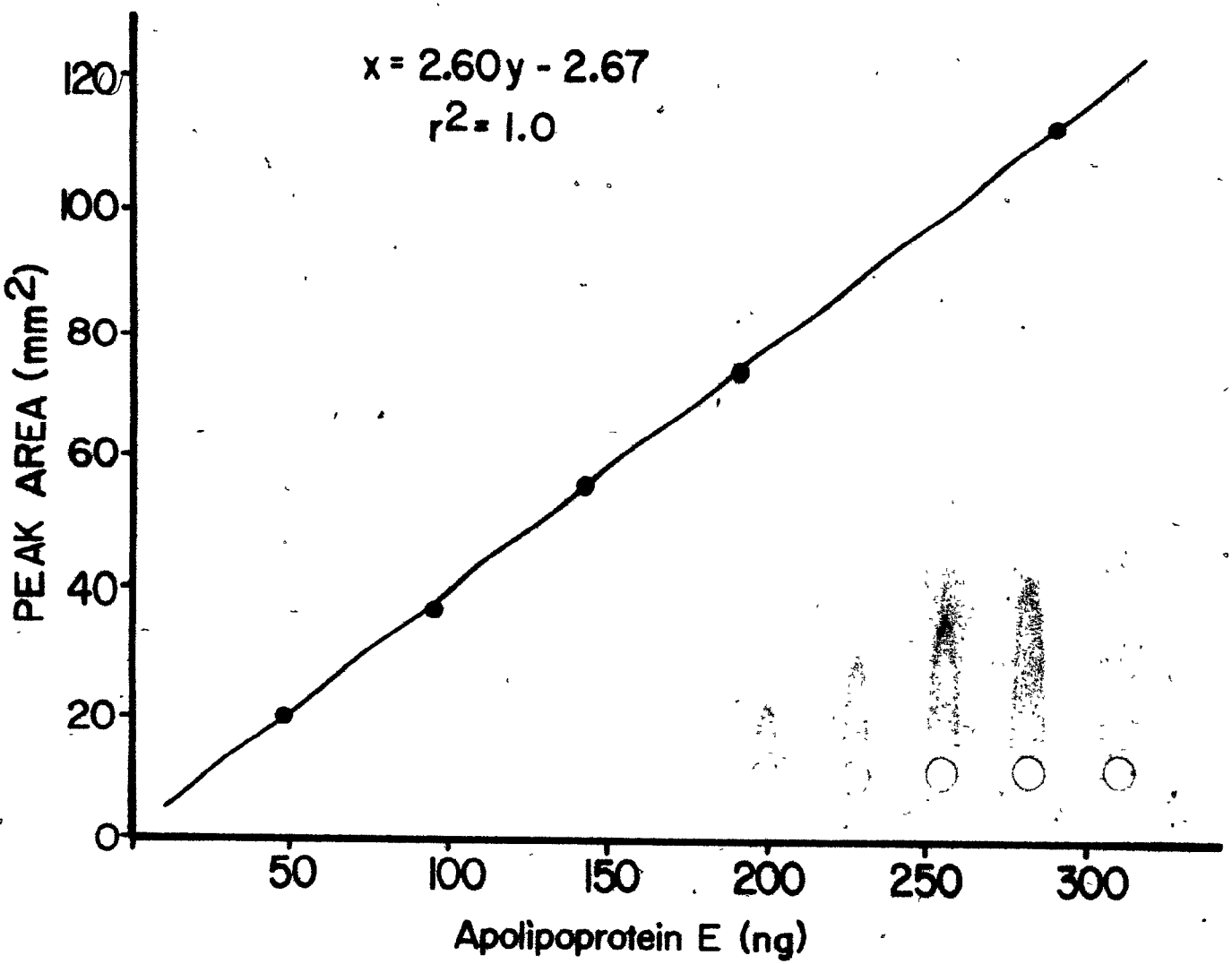
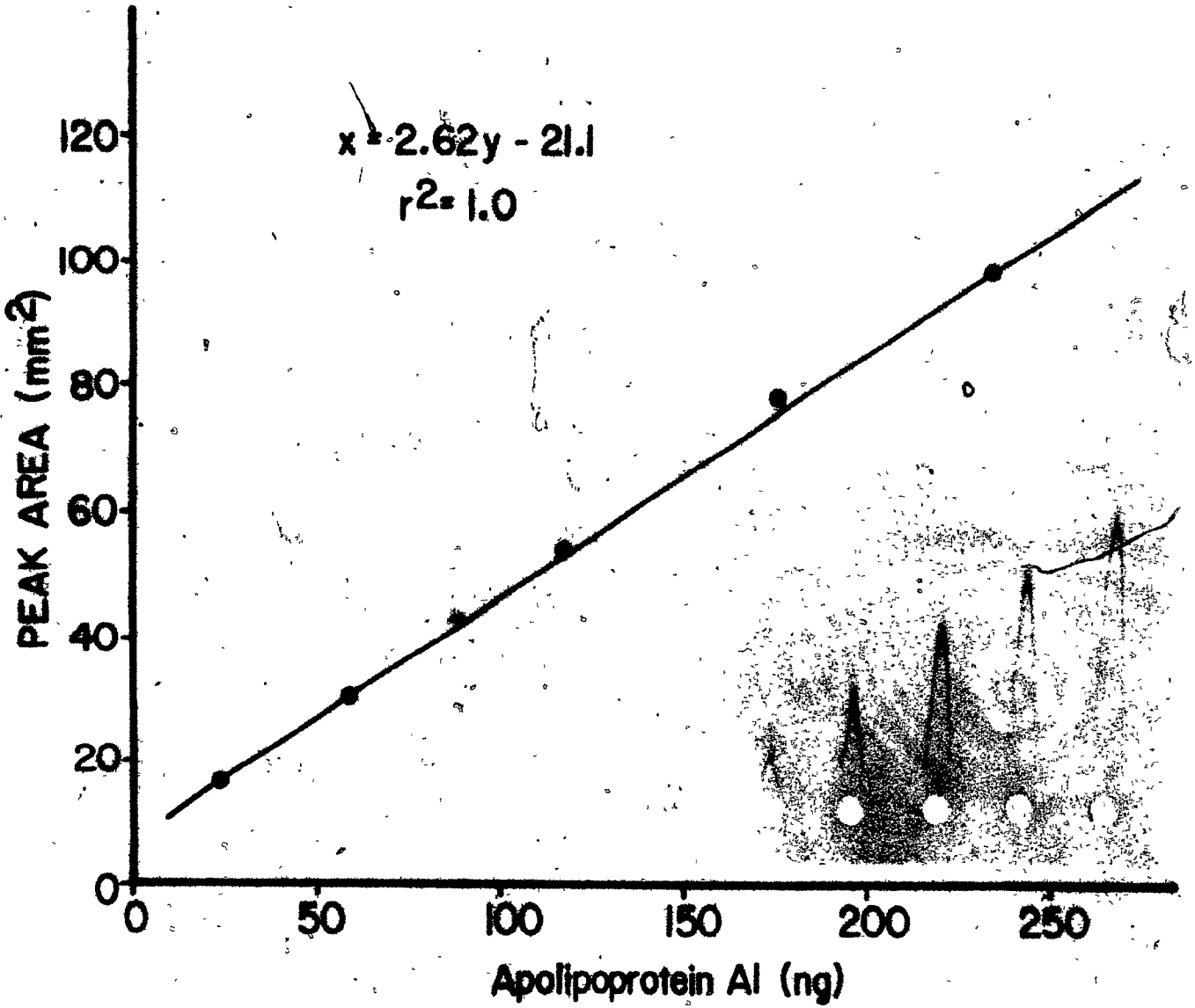

x = 2.60y - 2.67
r2 = 1.0
PEAK AREA (mm2)
120
100
80
60
40
20
0
50
100
150
200
250
300
Apolipoprotein E (ng)

FIGURE 12

STANDARD CURVE FOR THE ELECTROIMMUNOASSAY OF RAT APOLIPOPROTEIN AI

Preparation of the antibody and the assay conditions are as described in Materials and Methods. Each point represents the area under each peak calculated for the "rockets" shown in the inset. A standard curve accompanied each assay and was generated with a reference serum standard which had been quantitated against apo-AI as described in Materials and Methods. The apo-AI was purified by preparative isoelectric focussing of apo-HDL (ref. 155).


x = 2.62y - 21.1
r2 = 1.0
PEAK AREA (mm2)
120
100
80
60
40
20
0
0
50
100
150
200
250
Apolipoprotein AI (ng)

simply dialyzed against 0.05M phosphate buffer, pH 7.4,(running buffer) prior to incubation with the protein - linked Sepharose. Incubation was carried out overnight at 4°C with shaking in polypropylene Econo - columns (Bio-Rad, Richmond, CA). The columns were washed with several volumes of borate - buffered saline, pH 8.5 and the adsorbed protein was eluted from the immuno-affinity column with 6M NaSCN in 0.01M Tris-HCl buffer, pH 7.2.

8) Lipid analyses

a) Chemical methods

Lipoprotein lipids were extracted during the delipidation procedure with ethanol : ether 3:1 at -10°C (357) in the earlier studies. The lipids were separated by TLC as described previously (120). Phospholipids were determined according to the method of Fiske and SubbaRow (358), triglycerides according to Van Handel (359) and cholesterol by the method of Zlatkis et al.(360).

b) Gas liquid chromatography

The individual lipids of rat serum and isolated nascent lipoproteins were analyzed by gas liquid chromatography according to the methods of Kuksis et al.(361) on a Hewlett - Packard model 5840 A automatic gas chromatograph. Separation of lipids prior to gas chromatography when indicated was achieved by TLC (362) to separate neutral from other lipids, followed by TLC using the solvent system chloroform : methanol : acetic acid : water (74:45:12:6) to separate the neutral lipids.

9) Agarose gel electrophoresis

Electrophoretic mobilities of rat serum and nascent lipoproteins were determined by agarose gel electrophoresis according to the method of Maguire

et al.(363). Gels were stained with 0.2% Sudan Black.

10) Polyacrylamide gel electrophoresis

Delipidated apolipoproteins or serum proteins were solubilized in Tris-glycine buffer, pH 8.9 containing 7M urea, 1% β-mercaptoethanol and 1% SDS. The proteins were separated by electrophoresis on a 10% polyacrylamide gel with a 3% stacking gel both containing 7M urea as previously described (348). The gels were stained with 1% amido black in 7% acetic acid and destained in 10% acetic acid. Radioactivity associated with the apolipoprotein bands was determined after slicing the stained and intervening gel regions, drying and oxidizing the gel pieces in an Intertechnique Oxymat as previously described (295).

RESULTS

A) Criteria of Viability and Suitability of isolated Rat Hepatocytes as a Model to Investigate Lipoprotein Synthesis and Secretion

The isolation and long - term incubation of hepatocytes as suspensions has been shown to be potentially very useful as a model to investigate nascent lipoprotein synthesis and secretion (327). The purpose of these studies was to exploit this system in order to acquire information as to the nature of nascent lipoprotein secretory products in normal and experimental rats. Initially, however, it had to be established that isolated rat hepatocytes as prepared and incubated in our laboratory was a suitable and viable model for such studies.

Isolated rat hepatocytes as they appear at zero time of incubation under the light microscope are shown in Fig. 13. The percent of cells unstained by trypan blue exceeded 85% in all cases. Electron microscopy of isolated hepatocytes even after 12 hours of incubation revealed that the majority of cells had a well - preserved ultrastructure (Fig. 14) with an intact plasma membrane (Fig. 14,c) and rough endoplasmic reticulum (Fig. 14,a,d). Lipid droplets (Fig. 14,a) and glycogen particles (Fig. 14,c) could be observed within the cytoplasm. It would appear that intracellular polarity was maintained in these hepatocytes. A region corresponding to the peribiliary region of the hepatocyte is shown in Fig. 14,b. Such areas are commonly seen in freshly isolated cells and are characterized by being almost devoid of mitochondria and containing autophagic vacuoles, lysosomes and Golgi complexes (370).

A rapid and sensitive means of establishing cellular viability was needed to routinely monitor preparations of hepatocytes. Many researchers have confined themselves to the trypan blue test, however, this method requires the tedious numeration of large numbers of cells for accuracy. This method also

FIGURE 13

LIGHT MICROSCOPY OF FRESHLY ISOLATED RAT HEPATOCYTES

Cell suspensions were prepared and a sample was taken at zero time of incubation and mixed with trypan blue dye as described in Materials and Methods. Non-viable cells stain blue and routinely account for less than 15% of the total cell number. (X 310)

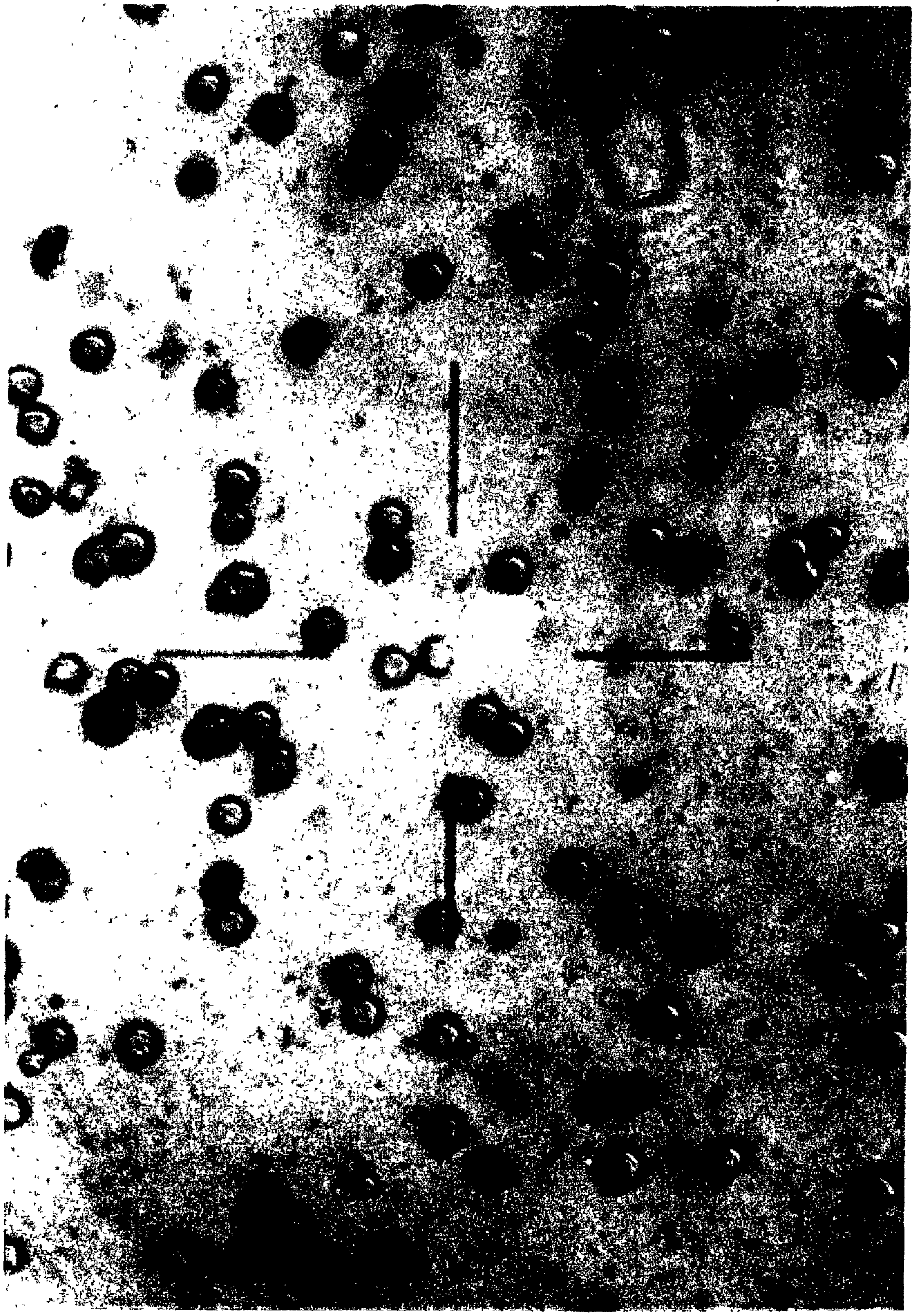
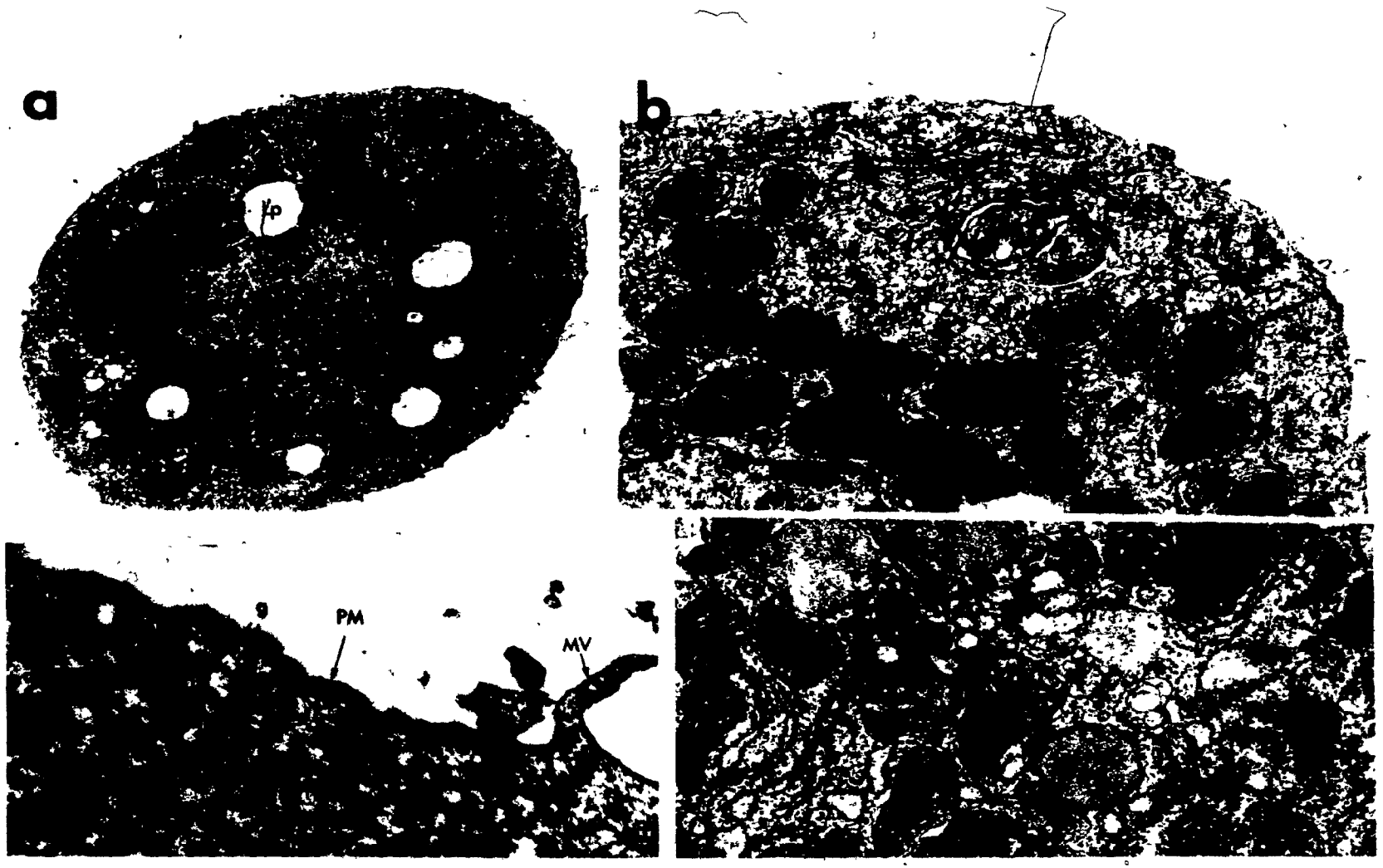

α

FIGURE 14

ELECTRON MICROGRAPHS OF ISOLATED HEPATOCYTES FROM NORMAL RAT

Cells obtained after 12 hours of incubation were fixed in glutaraldehyde and post-fixed with osmium tetroxide. Sections were stained with uranyl acetate and lead citrate. Lp,lipid droplets; Ly,lysosomes; N,nucleus; M,mitochondrion; RER,rough endoplasmic reticulum; GC,Golgi complex; AV,autophagic vacuole; g,glycogen; PM,plasma membrane; MV,microvilli;. (a) Single isolated cell prepared from normal rat liver by collagenase perfusion, X 6,490. (b) Higher magnification of section of cell in (a) showing well-preserved cell organelles, X 16,310. (c) Area of a cell showing intact plasma membrane and microvilli, X 46,570. (d) Area of a cell showing lamellar reticulum as well as numerous ribosomes. Material within reticulum is indicated by the arrowheads, X 37,990.


a
b
yp
PM
MV

has the drawback that wholly disintegrated cells cannot be detected and that medium proteins have the ability to interfere with the penetration of the trypan blue dye into the cells. Therefore, the trypan blue test could give an artificially high value for viability.

A sensitive index of cellular integrity is the amount of LDH (a cytoplasmic marker enzyme) released into the medium by the cells (371). Hepatocytes initially retaining larger proportions of the total measurable extra- and intracellular LDH, tended to retain higher proportions of LDH over the entire incubation period (Fig. 15). This indicated that LDH provided a good initial index of cellular integrity. Preparations of hepatocytes used for the studies presented here retained more than 85% of the total measurable LDH at zero time. Such preparations retained no less than 60% of the total LDH after 24 hours of incubation.

The trypan blue dye exclusion test consistently gave values for cell viability higher than those obtained by measuring LDH leakage as shown in Fig. 16,A. The ratio of viability as measured by trypan blue to that measured by LDH was always greater than 1.0 which is probably a consequence of the tendency of the former assay system to overestimate cellular viability as indicated earlier.

A good correlation between the total milligram cell protein and total units of LDH activity (determined at various time points for several experiments) was observed (Fig. 16,B). A correlation of 0.85 suggests that LDH is synthesized by the cells at a similar rate in all cases. Hence, measurement of LDH activity affords a quick and reliable index of total cell protein.

To determine whether or not the LDH assay provides any index of subtler cellular functions than simply severe irreversible damage, experiments were carried out to measure the capacity of isolated hepatocytes to synthesize ATP and retain or recapture potassium ions. Such studies indicated that these

FIGURE 15

LEAKAGE OF LDH INTO THE INCUBATION MEDIUM OF SUSPENSIONS OF ISOLATED HEPATOCYTES

LDH was determined as described in Materials and Methods. Each line represents the results of duplicate assays on a single suspension. To compare loss of LDH enzyme, the values for the percent of total LDH retained at t=0 for the 4 suspensions were arbitrarily assigned 100%. The actual initial values are also indicated for each experiment. The continued loss of cytoplasmic LDH to the medium is more marked for hepatocytes having poorer viability as initially determined by the LDH assay.

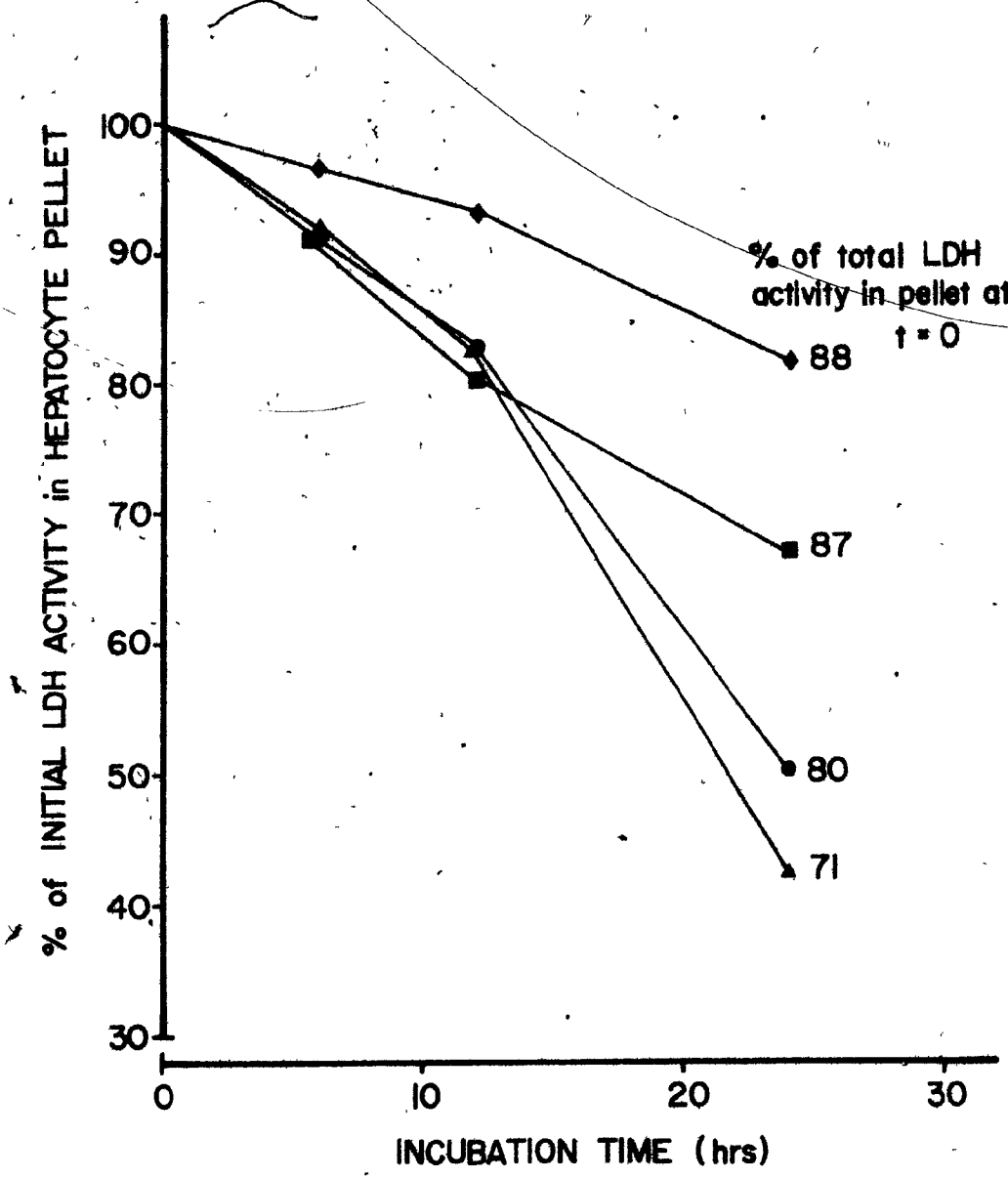
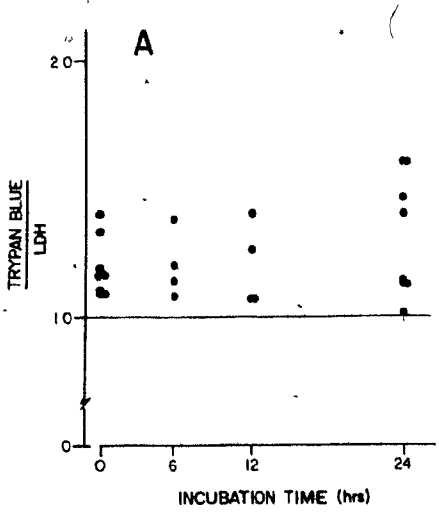
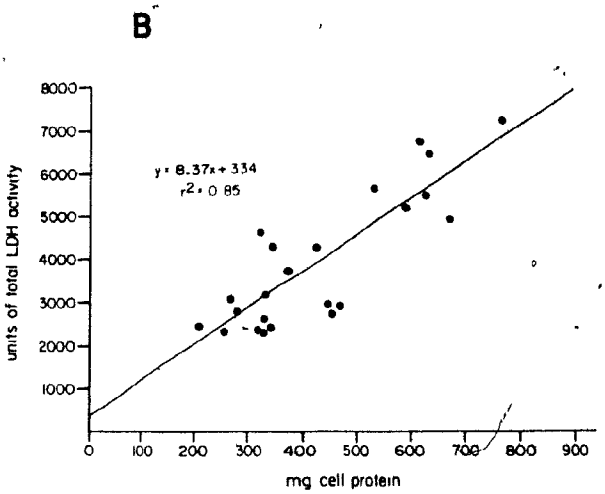

% of INITIAL LDH ACTIVITY in HEPATOCYTE PELLET
100
90
80
70
60
50
40
30
0
10
20
30
INCUBATION TIME (hrs)
% of total LDH activity in pellet at t = 0
88
87
80
71

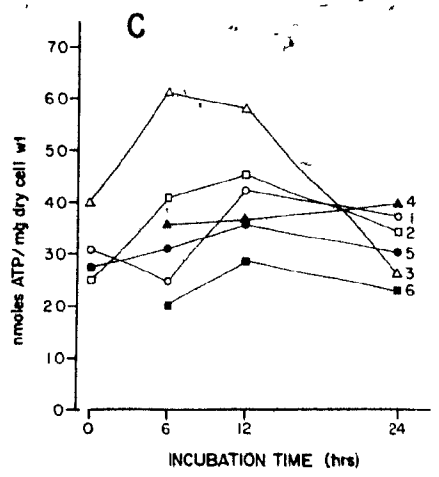
FIGURE 16

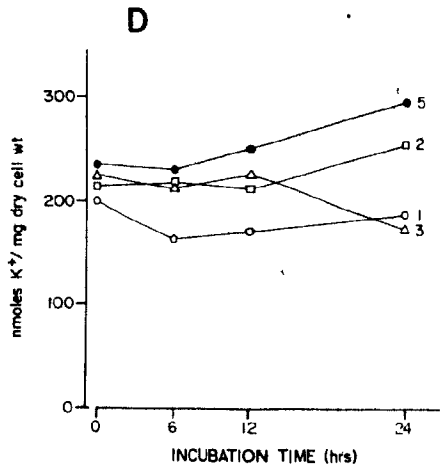
ASSAYS PERFORMED TO DETERMINE VIABILITY OF ISOLATED HEPATOCYTES AT VARIOUS TIMES OF INCUBATION

(A) Ratio of cellular viability as determined by trypan blue dye exclusion to that determined by LDH enzyme retention. At least 400 cells were counted under the light microscope for trypan dye exclusion. Values for trypan blue and LDH represent the percent of the total hepatocytes excluding dye and the percent of total LDH retained by the hepatocytes, respectively. (B) Correlation between total milligrams cell protein and units of total LDH activity in hepatocyte suspensions. (C and D) Time course of intracellular ATP and potassium levels in hepatocyte suspensions for six and four experiments respectively. Each open symbol represents the mean of duplicate assays on three separate incubations of the same hepatocyte preparation. Closed symbols represent the mean of duplicate assays on a single incubation. Levels at zero time for experiments four and six were not determined.


A
TRYPAN BLUE / LDH
20
10
0
0
6
12
24
INCUBATION TIME (hrs)


B
units of total LDH activity
y = 8.37x + 334
r2 = 0.85
8000
7000
6000
5000
4000
3000
2000
1000
0
100
200
300
400
500
600
700
800
900
mg cell protein


C
nmoles ATP/ mg dry cell wt
70
60
50
40
30
20
10
0
0
6
12
24
INCUBATION TIME (hrs)
4
1
2
5
3
6


D
nmoles K+/ mg dry cell wt
300
200
100
0
0
6
12
24
INCUBATION TIME (hrs)
5
2
1
3

cells retained or increased their levels of ATP and potassium over a 24 hour incubation period (Fig. 16,C,D). It should be noted that in experiment 3 the marked decrease in intracellular ATP after 12 hours was paralleled by a loss of potassium ions. This was similarily reflected in the low viability of the cells as determined by trypan blue dye exclusion (48%) and LDH retained (30%) by these hepatocytes (Exp't. 3) at 24 hours of incubation. Viability at this time as determined by LDH for the other experiments was not lower than 60%. Thus LDH retention correlated well with the ability of the cells to synthesize ATP and recapture potassium ions.

B) Studies on Ultracentrifugally Isolated Nascent Lipoproteins Secreted by Hepatocytes from Normal Rats

Incubation of the hepatocytes in a lipid - deficient, glucose - containing medium provided the simplest system with which to study lipoprotein secretion, as the cells are dependent on endogenous synthesis (or stores) for lipoprotein precursors. With this sytem, a) differences in the lipoprotein products secreted by hepatocytes from treated rats could be attributed to differences in the endogenous production of lipoproteins rather than the ability to utilize or be affected by external lipid substrates and b) the effects of the subsequent addition of specific components to the medium could be more readily interpreted.

For the studies reported here, VLDL was isolated from the incubation medium after adjusting the solution densities to d = 1.063 $g.mL^{-1}$. This procedure also isolates lipoproteins of the intermediate and low density range which have been shown to be secreted by hepatocytes in short - term incubations (324). Capuzzi et al.(324) noted, however, that the low density lipoproteins accounted for only a minor fraction of the total d = 1.006-1.063 $g.mL^{-1}$ lipoproteins secreted therefore no attempts were initially made in our studies to separate and isolate the low density lipoprotein species. Hence,

for convenience, VLDL refers to the range of densities 1.006-1.063 $g.mL^{-1}$ for the purposes of this section (B) of the results only.

The incorporation of ^{3}H-labelled amino acids into VLDL protein secreted by the isolated hepatocytes is shown in Fig. 17. The incorporation of radioactive amino acids and total amount of VLDL protein secreted into the medium increased over a 20 hour incubation period, with a slight reduction after 24 hours.

VLDL apolipoproteins were separated by urea polyacrylamide gel electrophoresis and ^{3}H- amino acid incorporation into the various protein bands was measured. The results of these experiments are summarized in Table VII. The bulk of the radioactivity was associated with the topmost region of the gel (stacking and running gel interface) corresponding to the high molecular weight apolipoprotein B and the only other significant proportion (which increased to approx. 20% after 20 hours) was associated with a lightly staining band with apo-E mobility (gel region 3). No stainable protein band nor significant radioactivity was detected in the gel region corresponding to apo-C (gel region 6), a normal component of serum apo-VLDL. An unexpected observation was the consistent presence of darkly stained protein bands observed in VLDL isolated at any time during the incubation which had little radioactivity associated with them (gel regions 2 and 4). We suggest that these bands were horse apolipoproteins derived from the ultracentrifugal $d > 1.21$ $g.mL^{-1}$ fraction after delipoproteination of the horse serum used in these incubations. This possibility is evaluated later.

The lightly staining protein band having a significant amount of radioactivity associated with it after 6 hours of incubation had the mobility of apo-E, however, in order to confirm this possibility, the delipidated nascent VLDL was applied to an anti-rat-apo-E immunoaffinity column. The results of such an experiment are shown in Fig. 18. The protein adsorbed to the immuno-

FIGURE 17

SECRETION AND RADIOACTIVE AMINO ACID INCORPORATION INTO VLDL BY HEPATOCYTES FROM NORMAL RATS

Each value represents the mean ± S.E.M. of duplicate assays performed on VLDL isolated from three separate hepatocyte suspensions. VLDL secreted into the hepatocyte incubation medium was isolated ultracentrifugally as described in Materials and Methods. Aliquots (200-400 μl) were applied to filter-paper discs, precipitated and delipidated according to the procedure outlined in the Materials and Methods and counted for ^{3}H-amino acid incorporation into VLDL protein. Total VLDL protein was determined by the method of Lowry et al. (354) on whole, fresh VLDL in the presence of 5% deoxycholate.

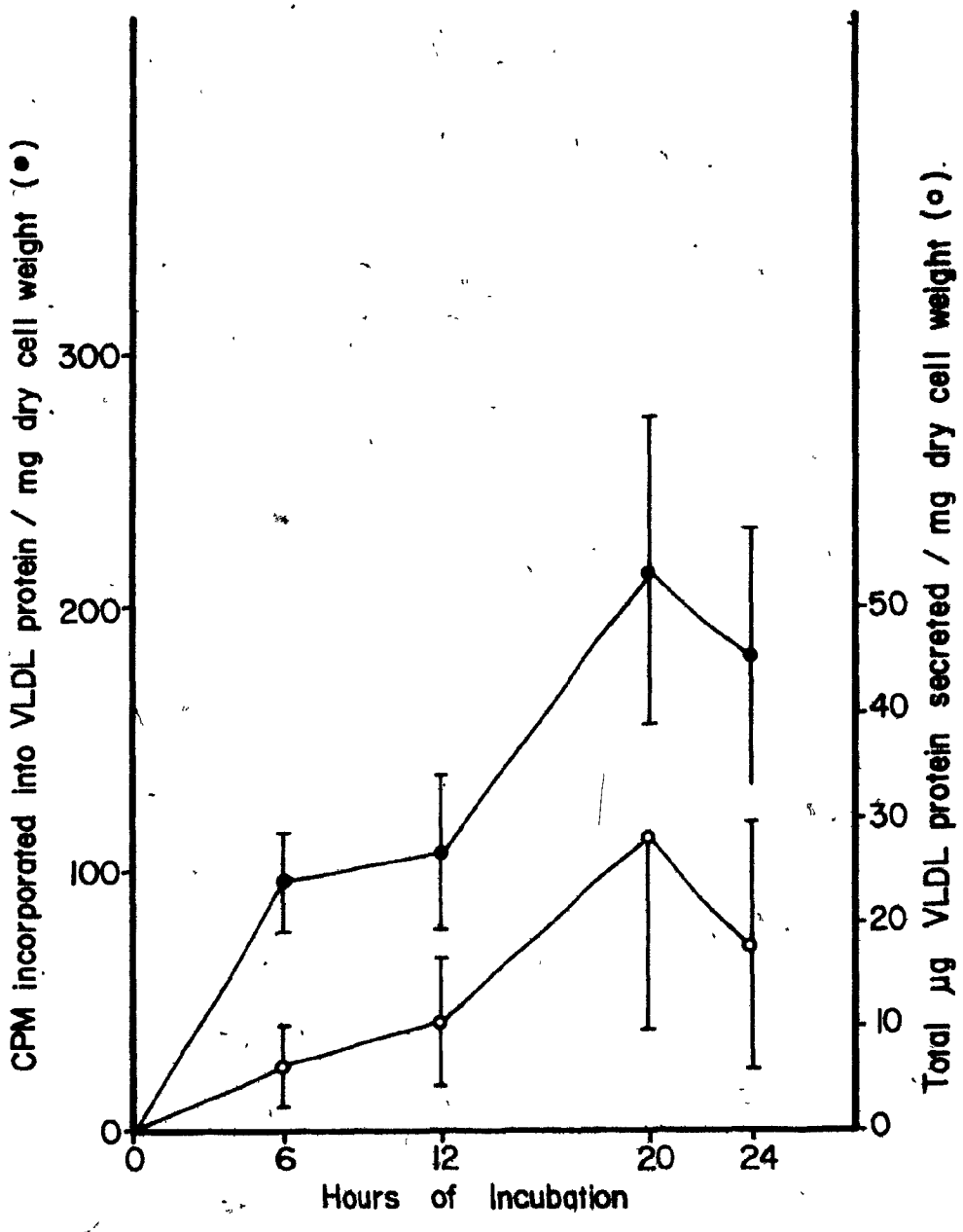
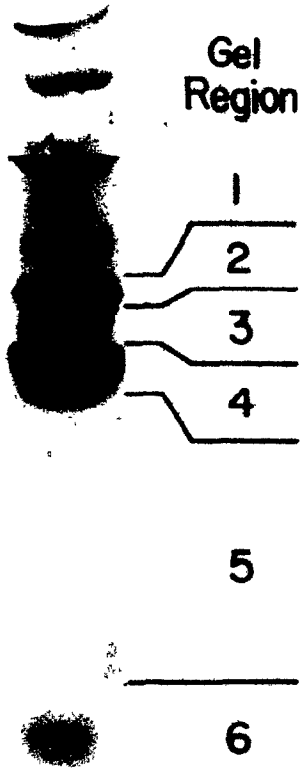

CPM incorporated into VLDL protein / mg dry cell weight (•)
Total µg VLDL protein secreted / mg dry cell weight (o)
300
200
100
0
50
40
30
20
10
0
0
6
12
20
24
Hours of Incubation

TABLE VII

% OF TOTAL RADIOACTIVITY INCORPORATED INTO VLDL PAGE GEL BANDS VERSUS TIME



GEL REGION	INCUBATION TIME (HOURS) 6 (2)	12 (3)	20 (2)	24 (3)
1	95	75 ± 9.2	69	71 ± 2.8
2	2	4.2 ± 0.8	5	6.0 ± 1.4
3	1	10 ± 1.5	22	19 ± 3.9
4	0	3.2 ± 1.7	2	2.0 ± 0.4
5	2	5.8 ± 4.4	1	2.1 ± 0.8
6	0	1.8 ± 1.4	0	0.4 ± 0.1

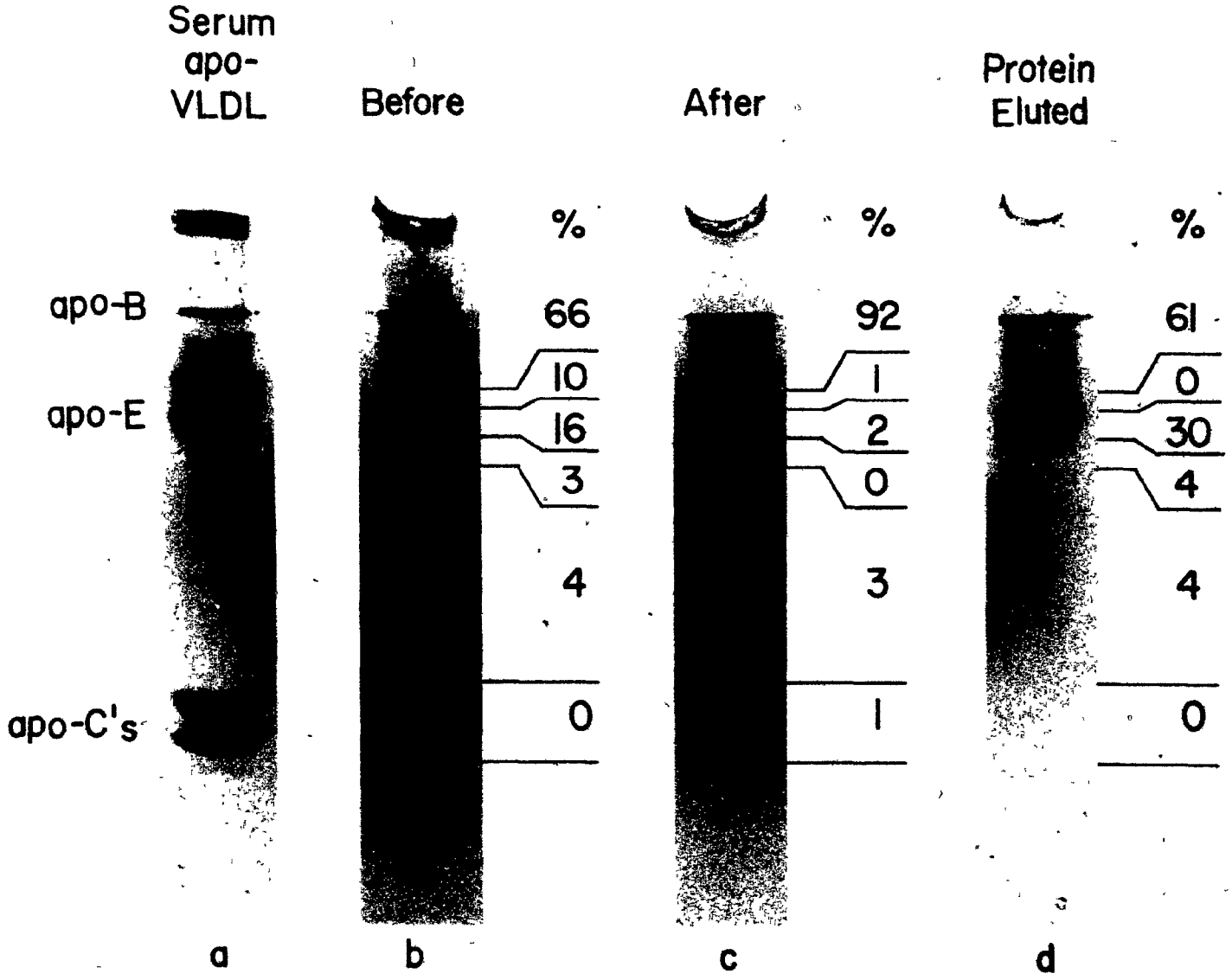
Each value represents the mean % of duplicate gels for the number of experiments in parentheses (± S.E.M. is included where the number of experiments permits). Urea gels were run and counted as described in Materials and Methods.

Average cpm applied per gel	7500	7410	25440	16910

FIGURE 18

CHROMATOGRAPHY OF NASCENT VLDL ON ANTI-RAT-APO-E IMMUNOAFFINITY COLUMN

VLDL secreted into the incubation medium was isolated by ultracentrifugation after 24 hours of incubation, delipidated and applied to an anti-rat-apo-E immunoaffinity column as described in Materials and Methods. Electrophoretic profiles on urea polyacrylamide gel are shown for VLDL at various stages of the experiment. (a) Gel pattern of delipidated rat serum VLDL ($d < 1.006$ $g.mL^{-1}$) with the major apolipoprotein species indicated. (b) Delipidated nascent VLDL before being applied to the immunoaffinity column. (c) Non-adsorbed VLDL protein eluted from column. (d) Adsorbed VLDL protein eluted from column with 6M sodium thiocyanate, pH 7.2. The numbers refer to the % of total gel radioactivity associated with the gel regions designated between the lines and was determined by oxidizing and counting the gel slices as described in the Materials and Methods. Gels were divided as in Table VII. Total cpm applied per gel track: (b), 11,000; (c), 8,000; (d), 19,000.


Serum apo-VLDL
Before
After
Protein Eluted
apo-B
apo-E
apo-C's
%
66
10
16
3
4
0
%
92
1
2
0
3
1
%
61
0
30
4
4
0
a
b
c
d

affinity column was indeed the lightly stained but radioactive band, as evidenced by the absence of any significant radioactivity associated in this gel band region after passage through the column (Fig. 18,c). The adsorbed apo-E eluted from the column gave the PAGE pattern shown in gel (d). The bulk of the radioactivity was found at the stacking and running gel interface and probably represents self - associated apolipoprotein, however, in this experiment 30% of the radioactivity was associated with the apo-E band. Upon closer examination, the apo-E band is composed of two or more narrowly separated bands, an observation that had previously been noted for VLDL apo-E obtained from perfused rat liver (120), rat serum (111) and the hepatic Golgi apparatus (334). Similar immunoaffinity studies for apo-B were not conducted in view of the insolubility of this apolipoprotein in aqueous solutions.

The secretion of VLDL lipid by normal rat hepatocytes is shown in Fig. 19. The secretion of triglyceride was significantly greater than any of the other lipids, although the concentrations of all components increased in the medium over the 24 hour incubation period and was due to de novo biosynthesis as indicated by the incorporation of the added radioactively labelled precursors, ^{3}H - palmitate and ^{3}H - mevalonolactone (Fig. 19).

Isolation of high density lipoproteins (d = 1.063-1.21 $g.mL^{-1}$) from the incubation medium gave urea polyacrylamide gel patterns shown in Fig. 20. The patterns differ significantly from those of serum HDL, in that no apo-C nor apo-E was visually detected and a darkly staining band having mobility slightly faster than serum apo-AI was observed. The bulk of the radioactivity incorporated into the nascent HDL was associated with the stacking and running gel interface and probably represents apolipoprotein B, a component found in normal serum HDL_1 (d = 1.063-1.085 $g.mL^{-1}$) (334). The remainder of the radioactivity was associated with the gel region directly below the stacking gel, a region normally devoid of any of the major serum HDL apolipoproteins with the

FIGURE 19

SECRETION OF VLDL LIPID BY HEPATOCYTES FROM NORMAL RATS

VLDL was isolated from the medium at various times of incubation and was delipidated as described in Materials and Methods. The lipids were separated by TLC and quantitated chemically. Points on each graph represent the means ± S.E.M. for the total amount of each lipid component secreted as VLDL for three experiments. The dashed line represents the ^{3}H-palmitate and ^{3}H-mevalonolactone incorporated into total VLDL lipid for a single experiment. Incorporation of ^{3}H into the individual lipid components was assayed in duplicate and each point on the dashed line represents the sum of the means to give radioactivity incorporated into total VLDL lipid.

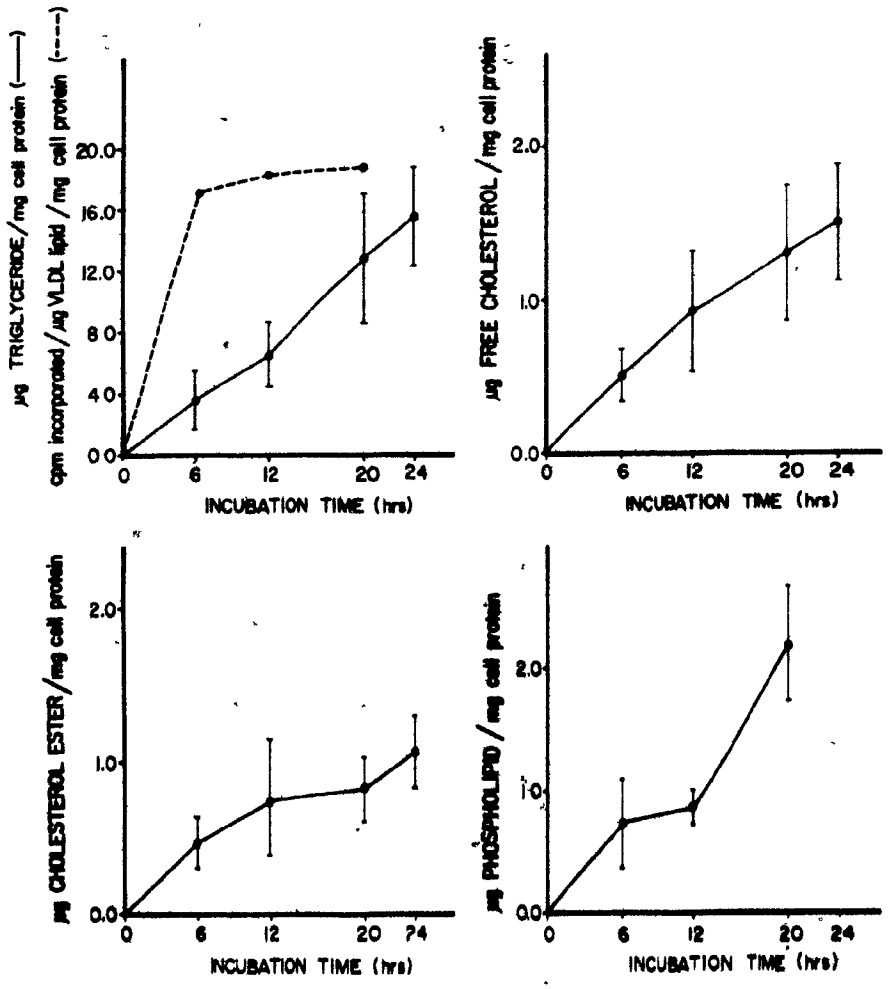
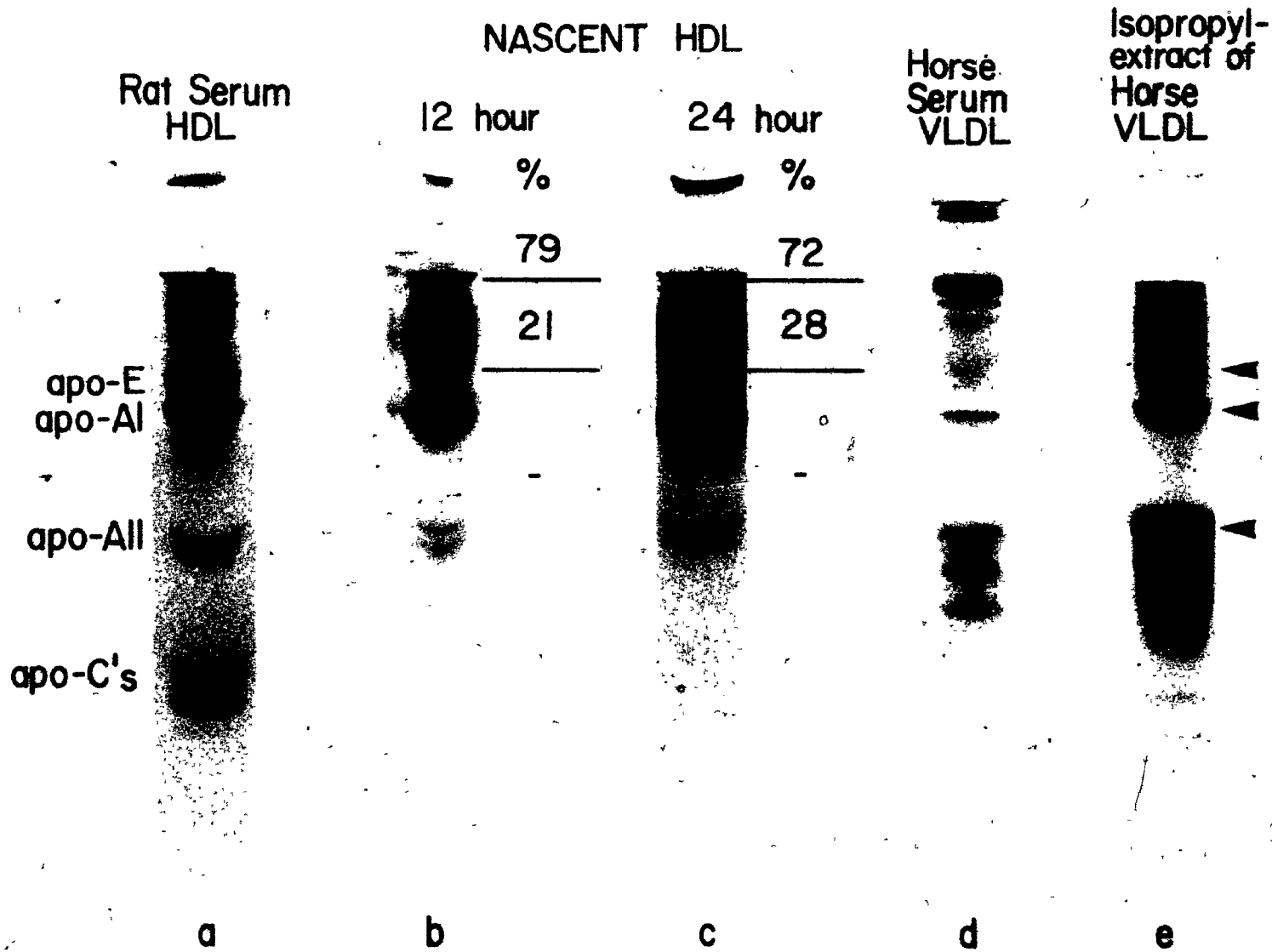

μg TRIGLYCERIDE/mg cell protein (——)
cpm incorporated/μg VLDL lipid / mg cell protein (- - - -)
20.0
16.0
12.0
8.0
4.0
0.0
0
6
12
20
24
INCUBATION TIME (hrs)
μg FREE CHOLESTEROL / mg cell protein
2.0
1.0
0.0
INCUBATION TIME (hrs)
μg CHOLESTEROL ESTER/mg cell protein
INCUBATION TIME (hrs)
μg PHOSPHOLIPID / mg cell protein
INCUBATION TIME (hrs)

FIGURE 20

UREA POLYACRYLAMIDE GEL PROFILES OF NASCENT HDL AND HORSE SERUM APOLIPOPROTEINS

HDL was isolated from the incubation medium at 12 and 24 hours of incubation, delipidated and run on urea polyacrylamide gels as described in Materials and Methods. (b,c) The numbers refer to the % of total radioactivity associated with the gel regions designated between the lines. Rat serum HDL was treated similarily and the major serum HDL apolipoproteins are designated, (a). Horse serum VLDL ($d < 1.006\ g.mL^{-1}$) was ultracentrifugally isolated from fresh, non-frozen horse serum as for rat serum VLDL. Isopropanol - extraction of horse serum VLDL was performed as described by Holmquist and Carlson (372). Urea polyacrylamide gel patterns of these preparations are shown (d,e). Total cpm applied per gel track: HDL, 12 hour (2600) and 24 hour (2500).


NASCENT HDL
Rat Serum HDL
12 hour
24 hour
Horse Serum VLDL
Isopropyl-extract of Horse VLDL
%
%
79
21
72
28
apo-E
apo-AI
apo-AII
apo-C's
a
b
c
d
e

exception of albumin, which is often found associated with unwashed preparations of serum HDL. No significant radioactivity was detectable in the lower portion of the gels, despite the presence of the darkly stained band and faint bands having apo-AI mobility. To test the possibility alluded to previously that these non-radioactive protein bands may represent horse apolipoproteins associating with the nascent rat lipoproteins, horse serum VLDL was isolated and a portion of it was extracted with isopropanol (372). The faster-migrating, lower molecular weight apolipoproteins (i.e. apo-E and apo-C) are soluble in this solvent and are selectively extracted from the insoluble higher molecular weight apo-B. We considered it reasonable to assume that the former apolipoproteins could be lost from the horse lipoproteins during the ultracentrifugal delipoproteinating procedure. Rat or human serum are found to contain essentially lipid - free low molecular weight apolipoproteins in the $d > 1.21\ g.mL^{-1}$ fraction after being subject to the ultracentrifugal forces required to isolate the lipoproteins (36). These apolipoproteins present in the $d > 1.21\ g.mL^{-1}$ fraction could be available for binding to the nascent lipoproteins, if indeed, such a mechanism did operate. It can be seen in Fig. 20 that the horse proteins designated by the arrowheads migrate to identical positions as the darkly staining proteins seen in nascent VLDL and HDL as well as the more lightly staining duplet seen in nascent HDL. These bands were not visualized when colloidal silicic acid (Cab-O-Sil*) was employed to delipoproteinate the horse serum (Fig. 35).

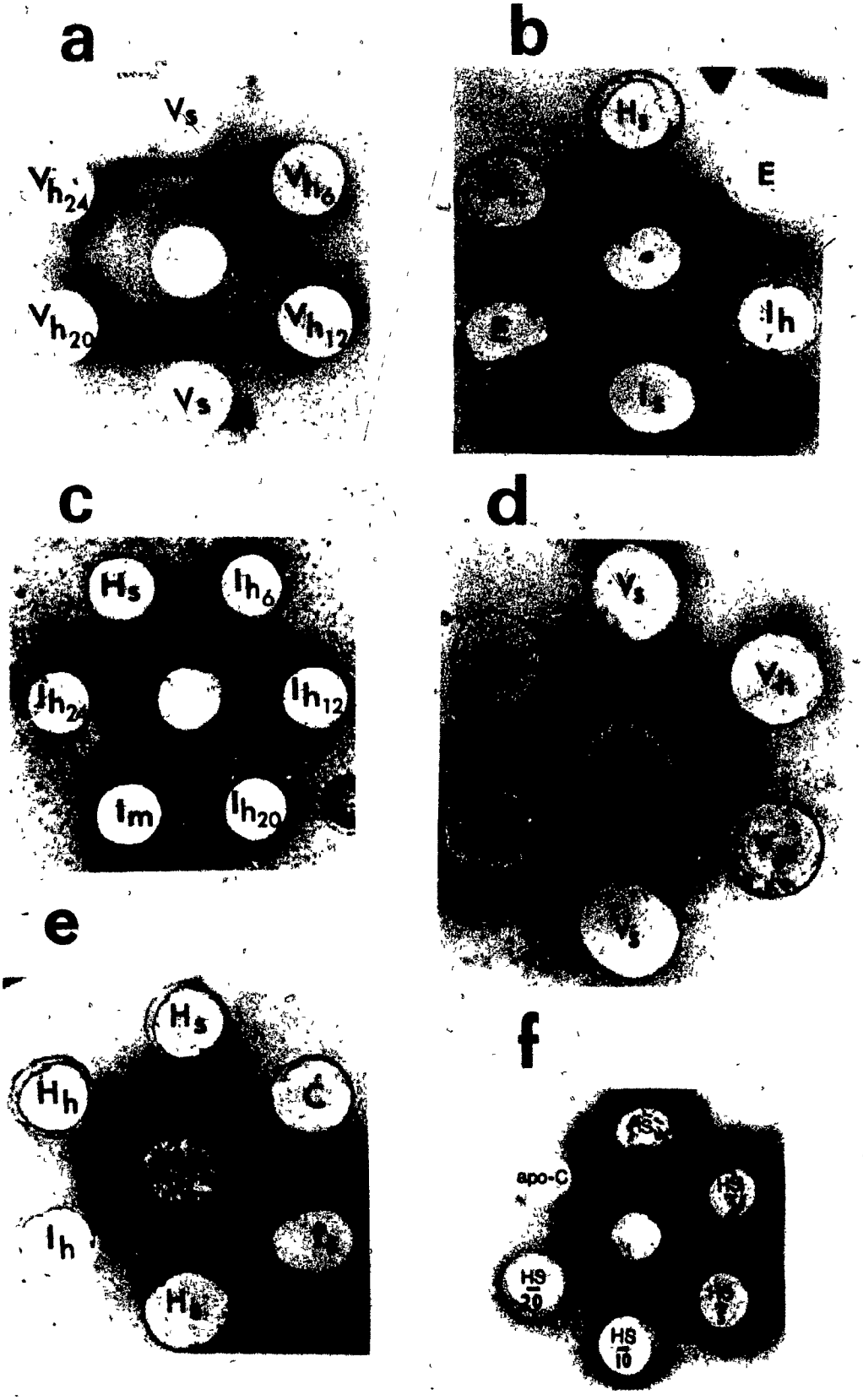
The $d > 1.21\ g.mL^{-1}$ or lipoprotein - free fraction of the incubation medium was tested for the presence of apolipoprotein by double immunodiffusion. The results of these studies are presented in Fig. 21.

Immunodiffusion of anti-rat-VLDL against VLDL isolated from the hepatocyte incubation medium at 20 and 24 hours of incubation gave reactions of identity with rat serum VLDL (Fig. 21,a). The lack of a detectable reaction

FIGURE 21

DOUBLE IMMUNODIFFUSION STUDIES ON ULTRACENTRIFUGALLY ISOLATED LIPOPROTEINS SECRETED BY NORMAL RAT HEPATOCYTES

Immunodiffusion plates were prepared from 1% ion agar and 4.0mm wells were cut with a Bio-Rad gel punch in the patterns shown. Gels were stained with Coomassie Blue. Other conditions were as described in ref. 24. Purified preparations of anti-rat-apolipoprotein IgG's were applied to the center wells and serum and incubation medium samples were allowed to diffuse from the peripheral wells. Abbreviations are as follows: V_s, normal rat serum VLDL; V_h, VLDL isolated from normal hepatocyte incubation medium; V_p, VLDL isolated from homogenized hepatocyte cell pellet; H_s, normal rat serum HDL; H_h, HDL isolated from incubation medium; I_s, $d > 1.21$ g.mL^{-1} infranatant of normal rat serum (after removal of salt); I_h, $d > 1.21$ g.mL^{-1} infranatant of hepatocyte incubation medium; I_m, $d > 1.21$ g.mL^{-1} infranatant of the incubation medium, not having been incubated with hepatocytes; E, purified rat apo-E; C or apo-C, purified rat apo-C; HS_w, delipoproteinated horse serum applied undiluted; HS/2, HS/5, HS/10, HS/20, denote horse serum diluted 2-, 5-, 10-, and 20-times, respectively. Subscripts indicate incubation time samples taken. Where no subscripts are indicated, time of incubation was 24 hours. Anti-rat apolipoprotein IgG's in center wells: (a) Anti-rat VLDL; (b,c) Anti-rat apo-E; (d,e,f) Anti-rat apo-C.


a
Vs
Vh24
Vh6
Vh20
Vh12
Vs
b
Hs
E
Ih
Is
c
Hs
Ih6
Ih24
Ih12
Im
Ih20
d
Vs
Vh
e
Hs
Hh
C
Ih
Hs
f
apo-C
HS 20
HS 10

for similarily diluted samples at 6 and 12 hours probably reflects the low amounts of VLDL present at those times, resulting in no immune complex formation in the regions of those wells at the antibody - antigen concentrations applied.

In contrast, HDL from hepatocyte incubations failed to show a precipitin reaction against anti-apo-E as is observed for rat serum HDL (Fig. 21,b). However, a faint, yet consistently observed reaction was detected against the $d > 1.21\ g.mL^{-1}$ fraction (infranatant) of the incubation medium. Upon increasing the concentrations of the infranatant applied to the immunodiffusion plates, distinct precipitin reactions formed against anti-apo-E (Fig. 21,c). Similarily treated incubation medium alone did not yield a reaction, indicating that there was no cross - reactivity with any horse apolipoproteins that may be present. Rat serum HDL appeared to exhibit a reaction of identity as well as partial identity as indicated by the spurs at either end of the precipitin arc. This is probably indicative of different exposure of antigenic sites on the HDL versus the essentially lipid - free form of $d > 1.21\ g.mL^{-1}$ apo-E.

The lack of a precipitin reaction against anti-rat-apo-C confirmed the visual absence of this apolipoprotein in PAGE gels of the nascent VLDL (Fig. 21,d) and was not a result of medium proteolytic activity (126). Although immunocytochemical evidence exists indicating that apo-C is synthesized and bound to VLDL - size particles within liver parenchymal cells (215), we failed to find immunological evidence for its presence in VLDL obtained from homogenized hepatocyte cell pellets, suggesting that the intracellular levels of this apolipoprotein are probably low. However, secreted nascent HDL gave a precipitin reaction against anti-apo-C (Fig. 21,e) when applied at high concentrations. Unlike serum HDL, only a single precipitin arc was observed. The double reactions against serum and purified apo-C occur as the antibody was

elicited in animals using apo-C purified by column chromatography which consists of apo-CII and apo-CIII. Apo-C was not detectable in the $d > 1.21$ $g.mL^{-1}$ fraction of the medium that had not been incubated with hepatocytes. Since several protein bands in the nascent HDL PAGE gel patterns were suspected to be horse apolipoproteins, the possibility was investigated that the anti-rat-apo-C reaction for the nascent HDL could, in fact, result from cross-reactivity of a horse apolipoprotein. However, no detectable precipitin reactions were observed for the ultracentrifugally delipoproteinated horse serum used in these studies (Fig. 21,f).

C) Nascent Lipoproteins Secreted by Normal, Hypothyroid and Hypothyroid, Hypercholesterolemic Rat Hepatocytes and Separated by Gel Filtration

For the remainder of the experiments, nascent lipoproteins were fractionated by gel filtration and lipid analyses were performed by gas - liquid chromatography. Hepatocytes were isolated from normal, PTU - treated and PTU - treated, cholesterol - fed rats and incubated as for the previous experiments.

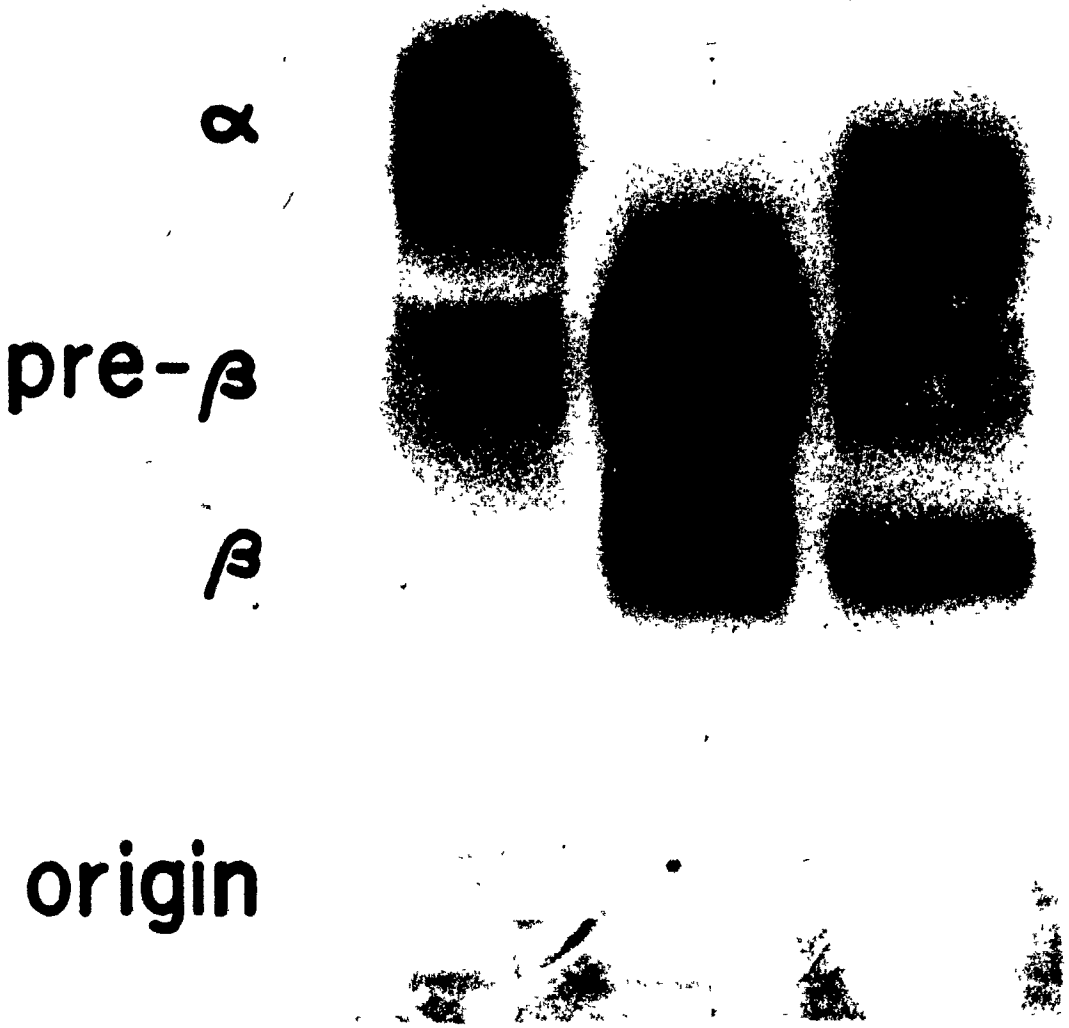
Rats administered propylthiouracil with or without cholesterol feeding became hypothyroid, with serum thyroxine levels at the times of the experiments that were barely detectable by the assay system used. Specifically the values were 0.6 ± 0.07 and 0.6 ± 0.18 µg/dl for PTU - treated and PTU - treated, cholesterol - fed rats respectively, compared to 7.4 ± 0.3 µg/dl for the normal group ($p < 0.001$).

Serum data was obtained for each of the three groups of animals in order to determine to what extent the nascent lipoproteins resembled their serum counterparts. Agarose gel electrophoresis of the serum from each of the three groups of rats gave the patterns shown in Fig. 22. Normal rat serum patterns are characterized by mainly α -migrating and pre- β -migrating lipoproteins (HDL and VLDL, respectively). In hypercholesterolemia, the pre- β lipoproteins

FIGURE 22

AGAROSE GEL ELECTROPHORESIS OF SERUM SAMPLES FROM NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RATS

Agarose gel electrophoresis of samples from normal (N), hypercholesterolemic (HC) and hypothyroid (HT) rat serum was performed as described in Materials and Methods. Serum samples were obtained from animals that had been fasted overnight. The plate was stained with 0.2% Sudan Black.


α
pre-β
β
origin
N
HC
HT

are increased and the presence of a darkly staining β -migrating band is evident. This band is also present in the sera of hypothyroid rats, however, no increase in pre- β -migrating lipoproteins was observed.

Total serum lipids were markedly increased in the PTU - cholesterol - fed (hypercholesterolemic) group (Table VIII). Treatment with the hypothyroid agent alone gave rise to higher free cholesterol and phospholipid levels with a reduction in total serum triglycerides.

The serum levels of apolipoproteins B, E and AI were markedly increased in the hypercholesterolemic group (Table IX). The hypothyroid state without cholesterol feeding was characterized by increased serum levels of apolipoproteins B and E when compared to the normal group, but these levels were significantly lower than those observed in hypercholesterolemia.

Separation of the nascent lipoproteins secreted by the isolated hepatocytes was achieved, in these studies, by gel filtration of the concentrated incubation medium on Sephacryl-300 for the reasons outlined previously. A typical elution profile of samples of the incubation medium is shown in Fig. 23. Lipoproteins having a density less than 1.21 $g.mL^{-1}$ eluted in Fractions 1 to 3a.

The apolipoprotein distribution amongst the column fractions for normal rat serum is shown in Fig. 24. Apo-B elutes only in fractions 1 and 2, with a greater proportion (55%) found in fraction 2. Apo-E is associated primarily with particles eluting in fraction 2 (60%) whereas fraction 3a contained the only other significant proportion (30%). In contrast, apo-AI is primarily associated with a particle eluting in fraction 3a (55%) with 35% eluting in fraction 2. It is significant to note that very little apo-E or apo-AI in serum could be detected as essentially lipid - free particles in fractions 3b, 4 or 5. This suggests that gel filtration is not as harsh a procedure as ultracentrifugation and does not give rise to essentially lipid - free

TABLE VIII

TOTAL SERUM LIPID LEVELS IN NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RATS

	mg / dl		
LIPID	NORMAL (11)	HYPOTHYROID (9)	HYPERCHOLESTEROLEMIC (8)
TRIGLYCERIDE	24.6 ± 4.2	8.5 ± 2.6[b]	43 ± 9[a]
PHOSPHOLIPID	52.0 ± 2.2	66.0 ± 7.0[a]	483 ± 87[c]
CHOLESTEROL ESTER	135.5 ± 6.4	132.5 ± 14.4	1421 ± 300[c]
FREE CHOLESTEROL	16.6 ± 0.6	25.1 ± 2.7[b]	331 ± 56[c]
TOTAL CHOLESTEROL	96.2 ± 3.9	103.2 ± 11.0	1191 ± 235[c]

Each value represents the mean ± S.E.M. of the number of experiments in parentheses. Lipid analyses were performed on sera of fasted animals by GLC as described in Materials and Methods. Statistical evaluation compared to normal group according to Student's t-test.

[a] $P < 0.05$

[b] $P < 0.005$

[c] $P < 0.001$

TABLE IX

SERUM APOLIPOPROTEIN LEVELS IN NORMAL, HYPOTHRYOID AND HYPERCHOLESTEROLEMIC RATS

	mg / dl		
APOLIPOPROTEIN	NORMAL	HYPOTHYROID	HYPERCHOLESTEROLEMIC
APO-B	35.5 ± 1.4 (49)	61.2 ± 2.2 (34)[a]	310 ± 72 (6)[b]
APO-E	24.2 ± 0.9 (37)	28.2 ± 1.1 (34)[a]	67.3 ± 5.0 (6)[b]
APO-AI	90.9 ± 1.8 (49)	92.4 ± 3.4 (28)	161 ± 8.0 (6)[b]

Each value represents the mean ± S.E.M. of the number of animals in parentheses. Apolipoproteins were quantitated by electroimmunoassay as described in the Materials and Methods on sera from fasted rats.

[a] $P < 0.005$

[b] $P < 0.001$

FIGURE 23

ELUTION PROFILE OF INCUBATION MEDIUM ON SEPHACRYL-300*

The hepatocyte-free incubation medium obtained at 0, 12 or 24 hours of incubation from suspensions of normal, hypothyroid or hypercholesterolemic rat hepatocytes was concentrated and applied to a 1.25m x 2.5cm Sephacryl-300 column as described in the Materials and Methods. The resulting profile shown here is essentially that of the horse serum proteins used in the incubations, the secreted rat proteins being obscured due to their relatively small contribution to the overall protein content. A volume of 5.3 mL was collected per tube. Column void volume (V_o) is indicated by the arrow. Eluates are pooled as indicated by the vertical dotted lines into Fractions 1, 2, 3a, 3b, 4 and 5. The calibration curve (Stokes radii) is shown (——). Each point represents the peak concentration of each of the proteins used for calibration: ▲, thyroglobulin; ●, aldolase; ■, human serum albumin; ◆, ribonuclease A. The bars at the top of the profile indicate the elution ranges for three separate preparations of rat serum lipoproteins isolated ultracentrifugally at the density ranges indicated.

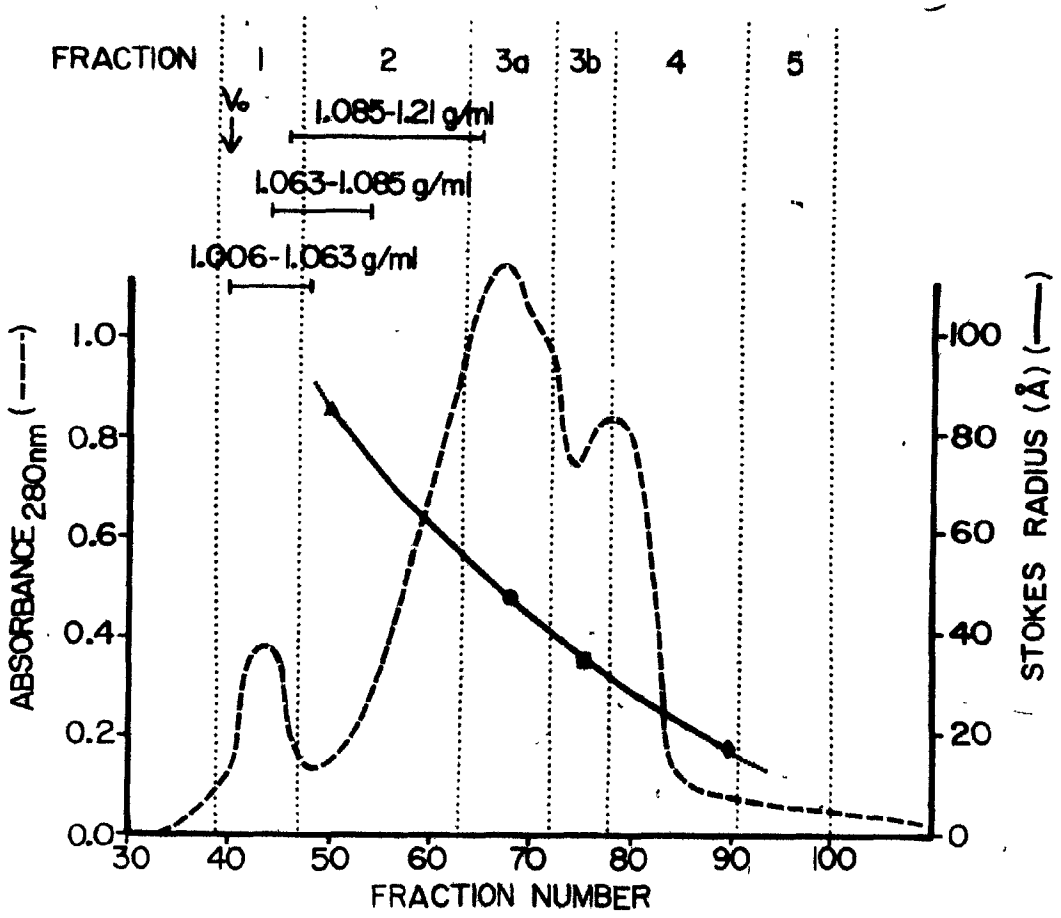
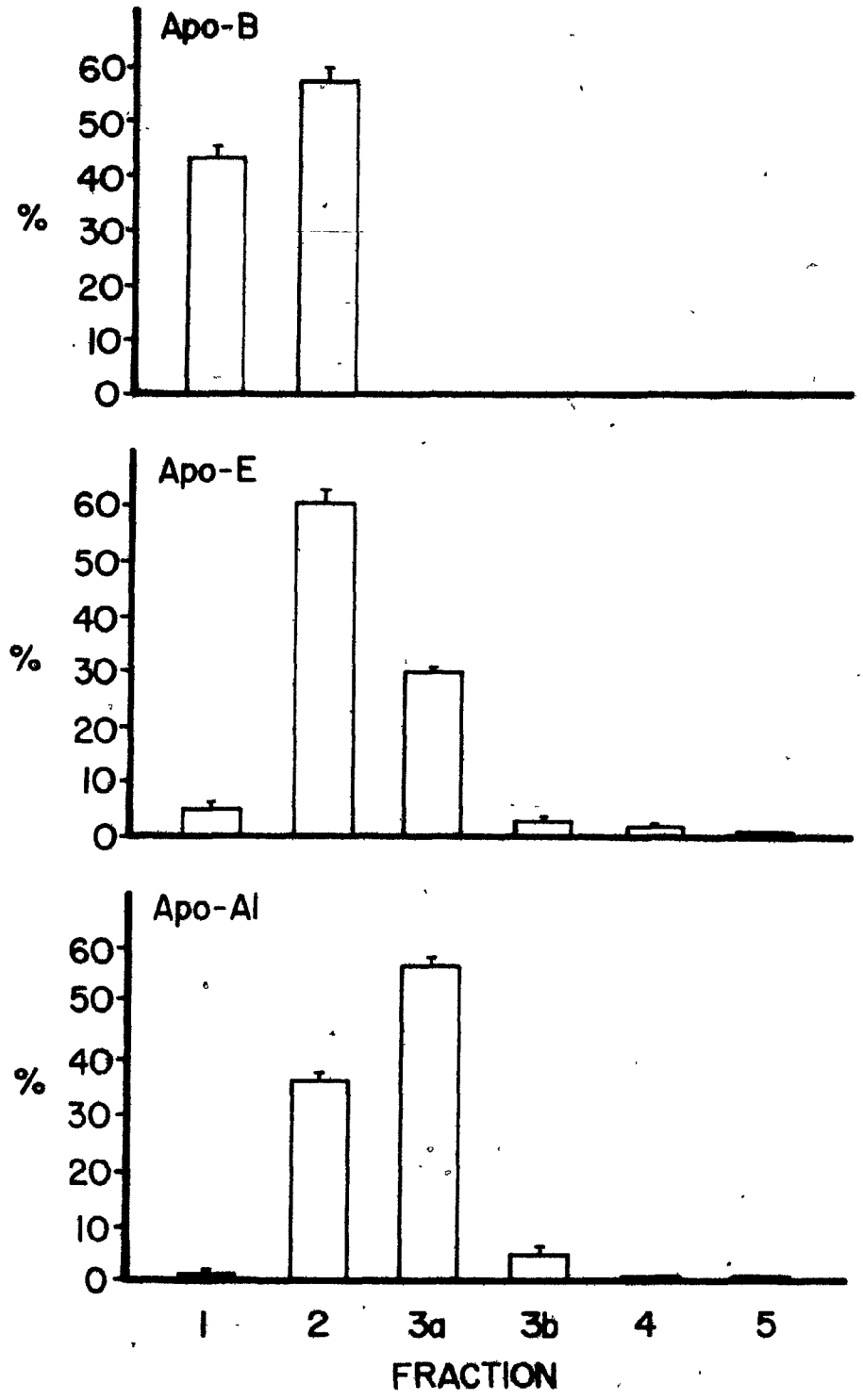

FRACTION
1
2
3a
3b
4
5
V_o
1.085-1.21 g/ml
1.063-1.085 g/ml
1.006-1.063 g/ml
ABSORBANCE 280nm (----)
1.0
0.8
0.6
0.4
0.2
0.0
STOKES RADIUS (Å) (—)
100
80
60
40
20
0
30
40
50
60
70
80
90
100
FRACTION NUMBER

FIGURE 24

DISTRIBUTION OF NORMAL RAT SERUM APOLIPOPROTEINS AMONGST SEPHACRYL-300* COLUMN FRACTIONS

The sera of two fasted rats were pooled, mixed with the incubation medium and treated like all incubation medium samples as described in Materials and Methods. The results of three such experiments ± S.E.M.'s (indicated by thin bars) are presented. Column fractions are as indicated in Fig. 23. Percentages refer to percent of total serum apolipoprotein present in a particular column fraction.


Apo-B
60
50
40
%
30
20
10
0
Apo-E
60
50
40
%
30
20
10
0
Apo-AI
60
50
40
%
30
20
10
0
1
2
3a
3b
4
5
FRACTION

apolipoproteins (36).

The total amount of apolipoproteins secreted into the incubation medium (fractions 1 to 5) is shown in Table X for incubations of hepatocytes from all three groups of rats studied. Hepatocytes from normal rats secreted all the apolipoproteins at a higher rate after 24 hours of incubation than at 12 hours, the difference in all cases, however, was not significant. An explanation for this observation is not readily apparent and is unlikely to be due to the release of apolipoprotein into the incubation medium by cell lysis as hepatocytes from hypothyroid animals secreted the apolipoproteins at similar rates at 12 and 24 hours. Although hypothyroid rat hepatocytes secreted apolipoproteins at a comparable rate to the normal group, secretion rates at 24 hours of incubation were consistently lower than that observed for normal rat hepatocytes. However, this did not reach statistical significance although a consistent trend was noted.

Hepatocytes from hypercholesterolemic rats, in contrast, secreted 5 to 10 times the amount of both apolipoproteins B and E than the normal rat hepatocytes. Apo-AI secretion was increased to a lesser extent and did not reach statistical significance. The secretion rate for apo-B and apo-E decreased after 24 hours. To determine whether the secretion rate at 12 hours for the hypercholesterolemic rat hepatocytes simply reflected either an initial release of intracellular apolipoproteins or serum contamination (as a result of inadequate washing of the hepatocytes during the isolation procedure), samples of the incubation medium were taken at time zero after the addition of hepatocytes and processed as described in Materials and Methods. (Due to experimental procedure, zero time was taken as one - half hour after the addition of hepatocytes to the incubation medium in all cases). No apolipoprotein was detectable by electroimmunoassay (Fig. 25). Therefore, the decreased secretion rate observed after 24 hours for apo's B and E, probably

TABLE X

TOTAL APOLIPOPROTEIN SECRETED INTO THE INCUBATION MEDIUM BY HEPATOCYTES FROM NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RATS

	μg apolipoprotein / g cell protein / hour		
APOLIPOPROTEIN	NORMAL	HYPOTHYROID	HYPERCHOLESTEROLEMIC
APO-B 12 hour	43.6 ± 12.7 (8)	42.0 ± 4.6 (4)	439 ± 198 (4)[b]
24 hour	56.5 ± 10.4 (10)	44.3 ± 7.1 (4)	219 ± 66 (5)[c]
APO-E 12 hour	33.5 ± 4.3 (7)	31.8 ± 3.8 (4)	309 ± 125 (4)[b]
24 hour	58.1 ± 9.0 (9)	33.1 ± 3.8 (4)	137 ± 35 (5)[a]
APO-AI 12 hour	7.7 ± 2.9 (3)	12.8 ± 6.7 (4)	24.4 ± 17.3 (2)
24 hour	16.4 ± 5.2 (4)	11.6 ± 2.3 (4)	23.2 ± 5.5 (3)

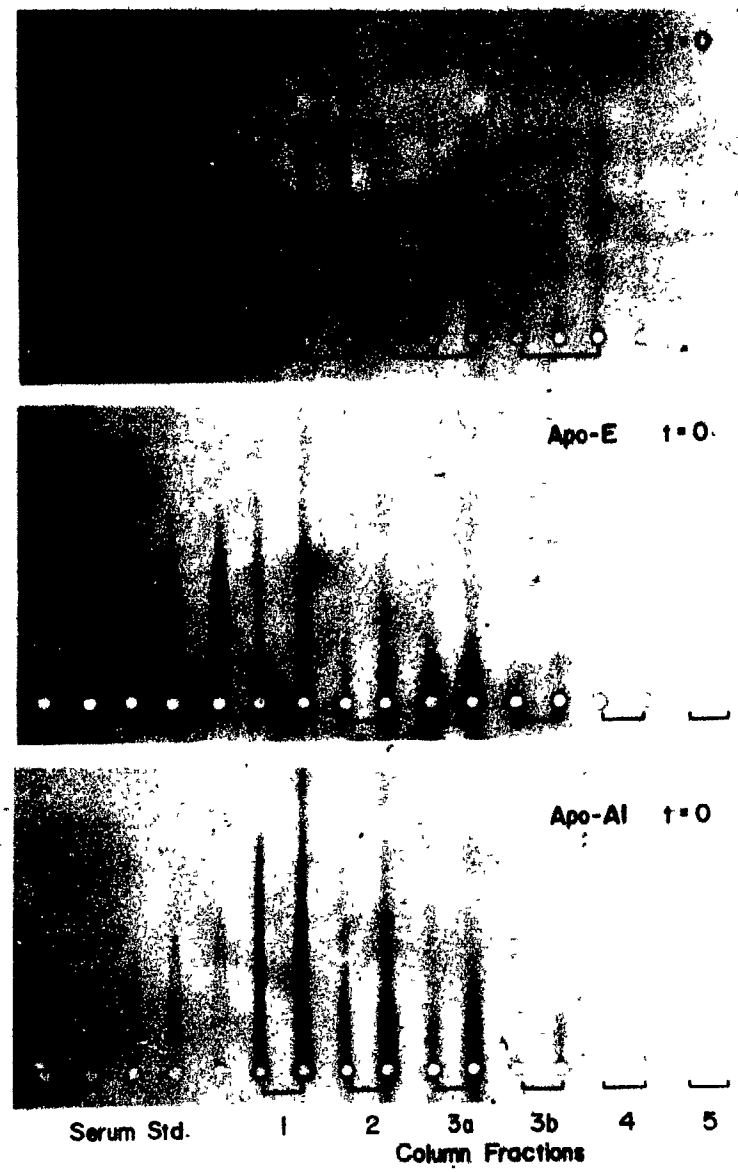
Total apolipoprotein secreted into the incubation medium was determined after 12 and 24 hours of incubation by electroimmunoassay as described in the Materials and Methods. Each value represents the mean ± S.E.M. of the number of experiments in parentheses. Where N < 3, mean ± difference / $\sqrt{N}$ is given. Statistical evaluation compared to normal group according to Student's t-test.

[a] $P < 0.025$
[b] $P < 0.01$
[c] $P < 0.005$

FIGURE 25

APOLIPOPROTEIN ELECTROIMMUNOASSAYS PERFORMED AT ZERO TIME FOR AN INCUBATION OF HYPERCHOLESTEROLEMIC RAT HEPATOCYTES

Electroimmunoassays were performed on column fraction samples from hypercholesterolemic rat hepatocyte incubations prepared as described in Materials and Methods. The first five wells (and last three in the apo-B assay) contained reference serum standard dilutions. Different dilutions (duplicates) of each sample were applied for column fractions 1 to 5 for the apo-E and apo-AI assays whereas three different dilutions of each sample were applied for column fractions 1 to 3a for the apo-B assay. Dilutions of the same sample are enclosed within square brackets. No "rockets" were detectable for any of these samples. The streaks observed result from non-specific (mainly horse serum) proteins that elute in the column fractions indicated. These normally do not interfere with rocket detection. Prolonged soaking of plates in saline prior to staining removes these streaks. For comparison, the bottom anti-apo-E plate is shown. Two different preparations of column fractions 1 and 2 (with and without asterisk) from 24 hour incubations of normal rat hepatocytes were applied to the center wells. The wells on the far right contain the designated volumes of normal rat serum that had been incubated with Cab-O-Sil, demonstrating the complete removal of apolipoprotein E by this treatment. The dots indicate heights and half - heights of "rockets" used for measuring areas.


Apo-E t=0
Apo-AI t=0
Serum Std. 1 2 3a 3b 4 5
Column Fractions

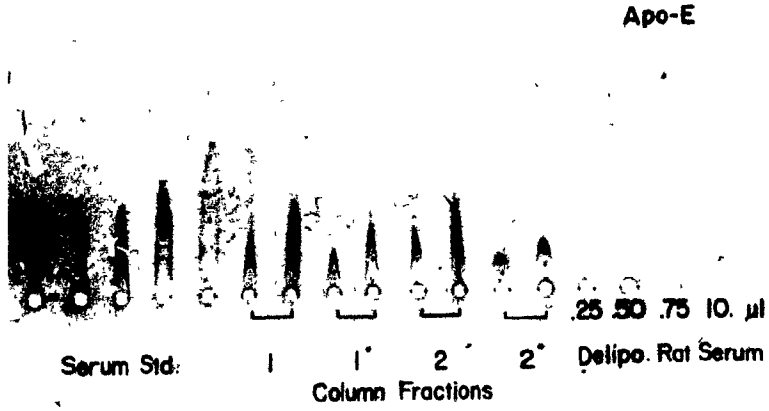
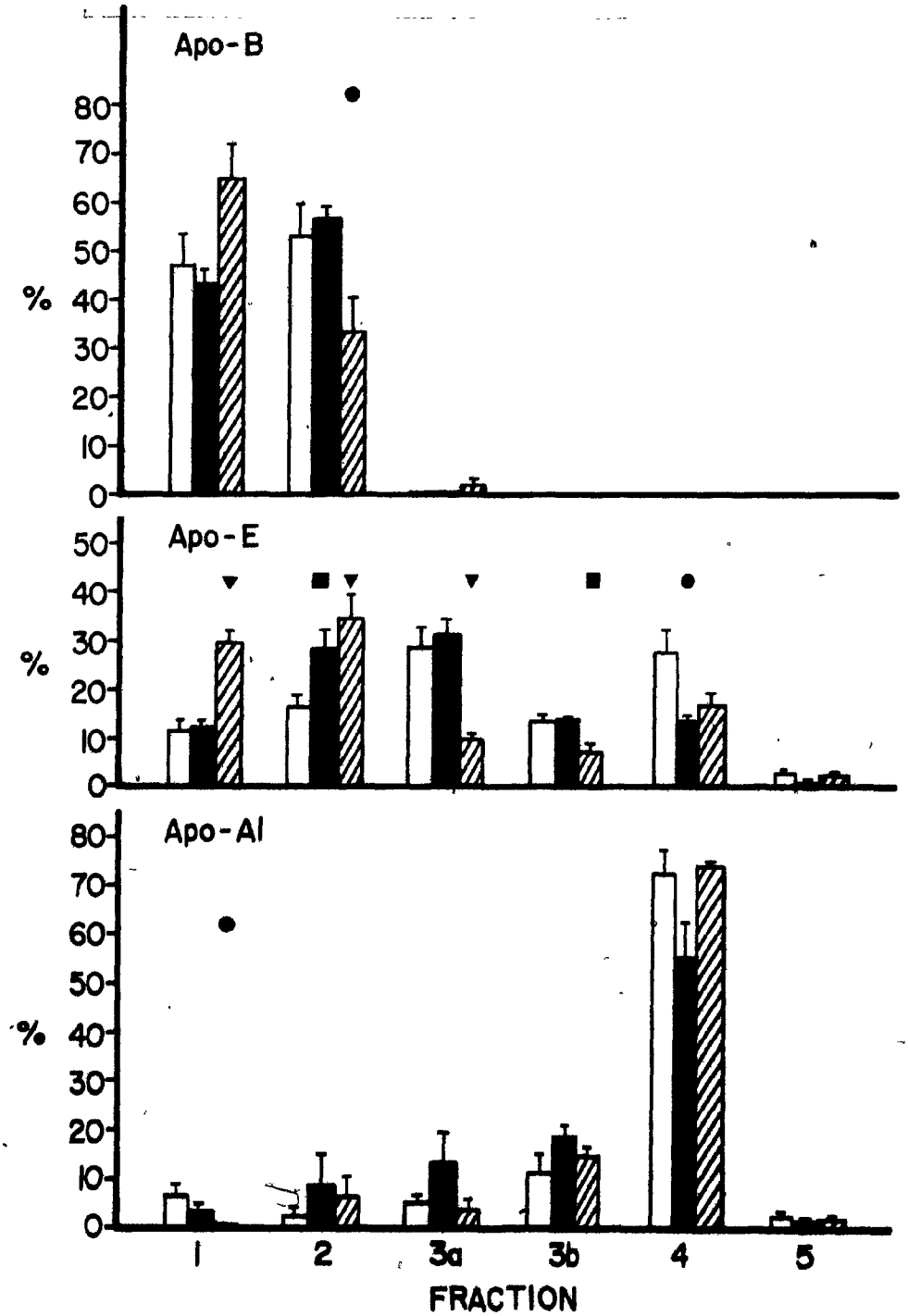

Apo-E
.25 .50 .75 10. µl
Serum Std: 1 1' 2 2' Delipo. Rat Serum
Column Fractions

FIGURE 26

PERCENT DISTRIBUTIONS OF APO-B, E AND AI VERSUS SEPHACRYL-300* COLUMN FRACTIONS AFTER INCUBATION OF HEPATOCYTES FROM NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RATS

Apolipoproteins were quantitated in the various column fractions by electro-immunoassay after 24 hours of incubation. Column fractions are as indicated in Fig. 23. S.E.M.'s of percentages are indicated by thin bars over histograms for experiments with normal (□), hypothyroid (■) and hypercholesterolemic (▨) rat hepatocytes. Significance as compared to the normal group was determined according to Student's t-test and is indicated by symbols over the histograms: ●, $p < 0.05$; ■, $p < 0.025$; ▼, $p < 0.005$.


Apo-B
80
70
60
50
%
40
30
20
10
0
Apo-E
50
40
30
%
20
10
0
Apo-Al
80
70
60
50
%
40
30
20
10
0
1
2
3a
3b
4
5
FRACTION

reflects a genuine phenomenon.

The distribution of apolipoproteins amongst serum (110) and nascent lipoproteins secreted by isolated perfused livers (120) can be altered by feeding cholesterol supplemented with PTU to the rats. The extent of the influence these factors have in regulating apolipoprotein distribution amongst nascent lipoproteins secreted by rat hepatocytes was investigated by quantitating the levels of apolipoproteins B, E and AI in the various column fractions after gel filtration of the concentrated medium from incubations of normal, PTU - treated (hypothyroid) and PTU - treated, cholesterol - fed (hypercholesterolemic) rat hepatocytes. The distributions after 24 hours of incubation are shown in Fig. 26. No significant difference in the apolipoprotein distribution profiles was observed between 12 and 24 hours of incubation for each group of hepatocyte incubations.

In incubations of normal rat hepatocytes, apo-B was secreted into fractions 1 and 2 only, with 55% secreted into the latter. No significant difference was observed for the distribution of secreted apo-B in hypothyroid rat hepatocyte incubations when compared to the normal incubations. In contrast, more apo-B secreted by hypercholesterolemic rat hepatocytes eluted as a larger particle (fraction 1) and significantly less eluted in fraction 2.

Apolipoprotein E was detectable in all column fractions after gel filtration of the medium from incubations of hepatocytes from all three groups of rats (Fig. 26). The apo-E secreted by normal rat hepatocytes showed peaks of highest concentration in fractions 3a and 4. The distribution of apo-E amongst the column fractions indicated that this apolipoprotein was secreted in association with a lipoprotein particle (fractions 1, 2 and 3a) as well as in an essentially lipid - free form (fractions 3b, 4 and 5).

A significantly increased proportion of the apo-E secreted by hypothyroid rat hepatocytes eluted in association with a large particle (fraction

2) with the result that significantly less was secreted as a particle with a Stokes radius smaller than albumin (fraction 4) when compared to normal. Cholesterol feeding appeared to augment this trend, as the proportion of apo-E secreted into both fractions 1 and 2 was significantly increased in incubations of hypercholesterolemic rat hepatocytes. Proportionately far less eluted as a lipid - free particle than observed in normal or hypothyroid rat hepatocyte incubations (fractions 3b, 4 and 5). However, it is pertinent to note that the absolute amount of apo-E secreted into these latter fractions by hypercholesterolemic rat hepatocytes was approximately the same as in normal incubations.

Apolipoprotein AI was primarily secreted by all three groups of hepatocytes as a particle having a Stokes radius smaller than albumin, eluting in fraction 4 (Fig. 26). In incubations of hypothyroid rat hepatocytes it appears that there may be a shift in apo-AI distribution towards the higher molecular weight fractions. The only significant shift in distribution was observed in incubations of hypercholesterolemic rat hepatocytes, in that there was proportionately less apo-AI in fraction 1 when compared to normal incubations.

The relative apo-E enrichment of nascent lipoproteins from hypercholesterolemic rat hepatocyte incubations is more clearly indicated in Table XI. The ratio of apo-B to apo-E in these lipoproteins is low in both fractions 1 and 2 compared to normal or hypothyroid nascent lipoproteins, although the difference is only significant for fraction 2.

The total lipid secreted into fractions 1 and 2 (corresponding to $d < 1.21\ g.mL^{-1}$) by hepatocytes from the rat groups studied is given in Table XII. Only very low amounts of lipid were obtained from fraction 3a. The lipid secreted into fraction 1 by normal and hypothyroid rat hepatocytes accounts for 90% of the total lipid secreted compared to 77% for hepatocytes from hypercholesterolemic rats. In incubations of normal rat hepatocytes, tri-

TABLE XI

RATIO OF IMMUNOASSAYABLE APO-B TO APO-E IN NASCENT LIPOPROTEINS SECRETED BY ISOLATED HEPATOCYTES

		NORMAL	HYPOTHYROID	HYPERCHOLESTEROLEMIC
FRACTION 1	12 HR	4.9 ± 1.1 (7)	6.3 ± 0.8 (4)	3.26 ± 0.2 (4)[c]
	24 HR	4.6 ± 0.7 (8)	4.8 ± 0.5 (4)	3.42 ± 0.6 (5)
FRACTION 2	12 HR	7.1 ± 2.2 (6)	4.0 ± 0.9 (4)	1.1 ± 0.2 (4)[a,c]
	24 HR	4.1 ± 0.8 (9)	2.8 ± 0.5 (4)	1.4 ± 0.2 (5)[b,c]

Values represent means ± S.E.M. for the number of experiments in parentheses. Statistical evaluation according to Student's t-test.
[a] $P < 0.05$, compared to normal
[b] $P < 0.01$, compared to normal
[c] $P < 0.01$, compared to hypothyroid

TABLE XII

TOTAL LIPOPROTEIN LIPID SECRETED INTO FRACTIONS 1 AND 2 BY HEPATOCYTES FROM NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RATS

	µg lipid / g cell protein / hr		
LIPID	NORMAL (4)	HYPOTHYROID (3)	HYPERCHOLESTEROLEMIC (3)
TRIGLYCERIDE	90.7 ± 38.5	226 ± 91[a]	317 ± 93[b]
CHOLESTEROL ESTER	10.4 ± 8.7	12.5 ± 5.5	383 ± 156[c]
FREE CHOLESTEROL	11.5 ± 4.9	18.0 ± 4.9	285 ± 143[b]
PHOSPHOLIPIDS	41.4 ± 14.0	89.2 ± 32.5[a]	370 ± 140[c]
TOTAL LIPID	154 ± 73	345 ± 133[a]	1350 ± 530[c]

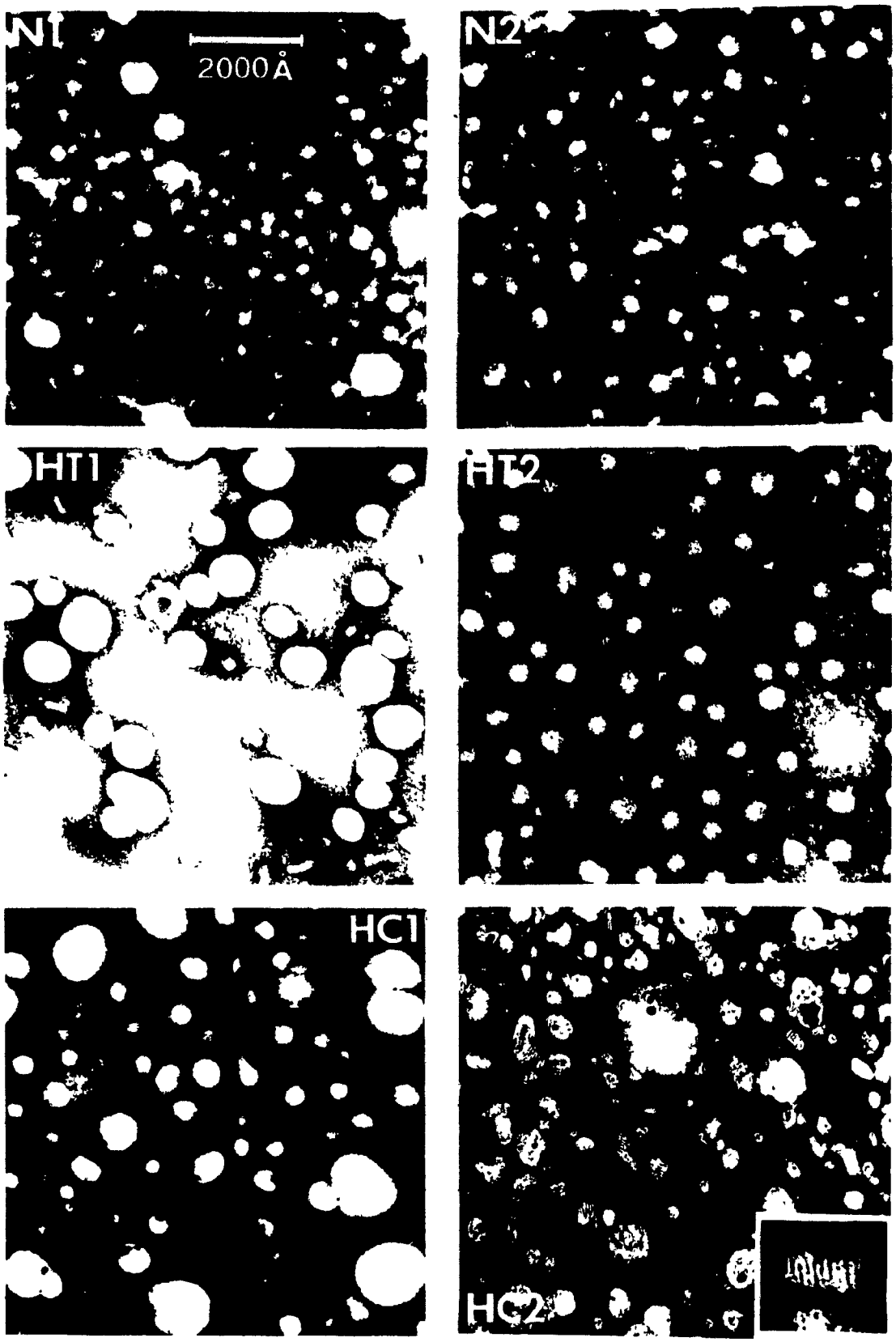
Total lipid secreted into Fractions 1 and 2 was determined by GLC after 24 hours of incubation as described in Materials and Methods. Each value represents the mean ± S.E.M. of the number of experiments in parentheses. Statistical evaluation compared to normal group according to Student's t-test.

[a] $P < 0.10$
[b] $P < 0.05$
[c] $P < 0.025$

FIGURE 27

ELECTRON MICROGRAPHS OF NASCENT LIPOPROTEINS ISOLATED FROM COLUMN FRACTIONS 1 AND 2 FROM INCUBATIONS OF HEPATOCYTES FROM NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RATS

Lipoproteins were isolated from column fractions 1 and 2 after 24 hours from incubations of normal (N1 and N2), hypothyroid (HT1 and HT2) and hypercholesterolemic (HC1 and HC2) rat hepatocytes. Lipoproteins were negatively stained with 2% sodium phosphotungstate. x 96,220. The inset in HC2 shows a higher magnification of a rouleau formation of discoidal particles. x 264,000.


N1
2000Å
N2
HT1
HT2
HC1
HC2

glyceride and phospholipid account for approximately 85% of the total lipid secreted. The total amount secreted by hypothyroid rat hepatocytes is higher than for normal cells and with a larger number of observations could probably become significant. Triglyceride and phospholipid secretion were consistently higher in these hepatocyte incubations accounting for the elevated secretion rates for total lipid. Cholesterol secretion was unaltered compared to normal rat hepatocytes.

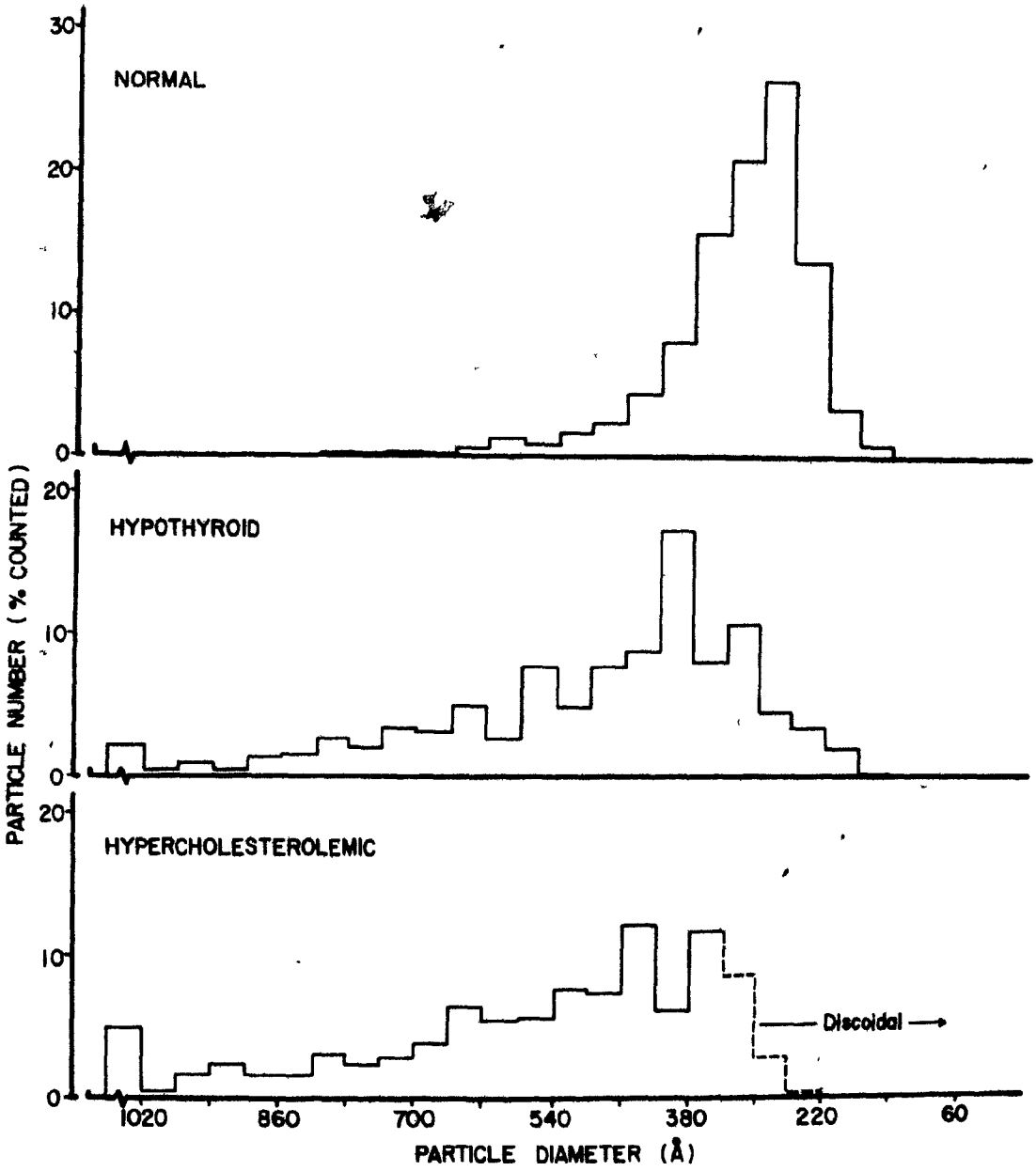
A drastically elevated secretion rate for all the individual lipid components was observed for hypercholesterolemic rat hepatocytes, in agreement with earlier studies using isolated perfused livers from these animals (120). It is especially interesting to note that compared to normal rat hepatocytes, the triglyceride and phospholipid secretion rates increased 3.5 and 9 times respectively, while the secretion of free and esterified cholesterol was 25 and 37 times greater than normal for hypercholesterolemic rat hepatocytes. In view of the observation that hepatic cholesterol biosynthesis is markedly reduced after cholesterol feeding (373) or thiouracil treatment (264), the high secretion rates for cholesterol by hypercholesterolemic hepatocytes probably reflects a release of a portion of the expanded intracellular pool that is characteristic of the livers of these animals (120, 374, 375).

Electron micrographs of the nascent lipoproteins isolated from the incubation medium are shown in Fig. 27. Nascent lipoproteins secreted by hypothyroid (HT1,2) and hypercholesterolemic (HC1,2) rat hepatocytes are larger than those from normal (N1,2) incubations. A higher degree of heterogeneity is evident in the nascent lipoproteins from hypothyroid or hypercholesterolemic rat hepatocyte incubations when compared to normal incubations, which is more clearly visualized in Fig. 28. Nascent lipoproteins secreted by normal rat hepatocytes have a fairly narrow range of particle diameters (220-460 Å) whereas the lipoproteins secreted by hypothyroid or hypercholesterolemic rat

FIGURE 28

DISTRIBUTION OF LIPOPROTEIN PARTICLE DIAMETERS FOR NASCENT LIPOPROTEINS FROM INCUBATIONS OF HEPATOCYTES FROM NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RATS

Lipoproteins were isolated from column fractions 1 and 2 after 24 hours of incubation as described in the Materials and Methods. Diameters of at least 800 particles were measured for each profile for normal, hypothyroid and hypercholesterolemic rat hepatocyte incubations. The dotted line in the hypercholesterolemic histogram indicates the limiting size range for spherical lipoprotein particles.


NORMAL
HYPOTHYROID
HYPERCHOLESTEROLEMIC
Discoidal
PARTICLE NUMBER (% COUNTED)
PARTICLE DIAMETER (Å)
30
20
10
0
1020
860
700
540
380
220
60

hepatocytes exhibit a wide spectrum of particle size, and up to 5% of the particles have diameters greater than 1000 Å.

Rather unexpectedly we observed discoidal particles in fraction 2 of the medium with incubations of hypercholesterolemic rat hepatocytes (Fig. 27,HC2). Discoidal particles were not detected in incubations of hepatocytes from normal or hypothyroid rats. The discoidal particles secreted by hypercholesterolemic rat hepatocytes were 227 X 54 Å, tended to aggregate in rouleau formations and were similar to discoidal particles observed in the plasma of hypercholesterolemic guinea pigs (252 X 51 Å) (203). A higher magnification of these particles showing a variety of aggregate forms is shown in Fig. 29.

Agarose gel electrophoresis of column fractions 1 and 2 from incubations of hepatocytes from all three groups of rats is shown in Fig. 30. Nascent lipoproteins in fraction 1 of the normal rat hepatocyte incubation medium show several faint bands having mobilities extending from α to β, with the most predominant but diffuse band having α mobility. Fraction 2 exhibits a darkly staining band with mobility between the normal serum pre-β and β (VLDL and LDL) respectively. The lipoproteins secreted into fraction 1 by hypothyroid rat hepatocytes had a fast pre-β mobility, however, the band tended to be diffuse but more heavily stained than lipoproteins from normal rat hepatocyte incubations. Lipoproteins in fraction 2 from these incubations had similar mobility to normal nascent lipoproteins, but did not have the β-migration characteristic of the lipoproteins which accumulate in hypothyroid rat serum. Hypercholesterolemic rat hepatocytes secreted lipoproteins which, in fraction 1, exhibited fast pre-β mobility. The amount of lipid secreted is far greater than observed for normal or hypothyroid hepatocytes as evidenced by the darker staining of the lipoprotein band. The narrowness of this band also suggests a higher degree of homogeneity with respect to charge on these

FIGURE 29

ELECTRON MICROGRAPH OF DISCOIDAL LIPOPROTEINS SECRETED BY HEPATOCYTES FROM HYPERCHOLESTEROLEMIC RATS

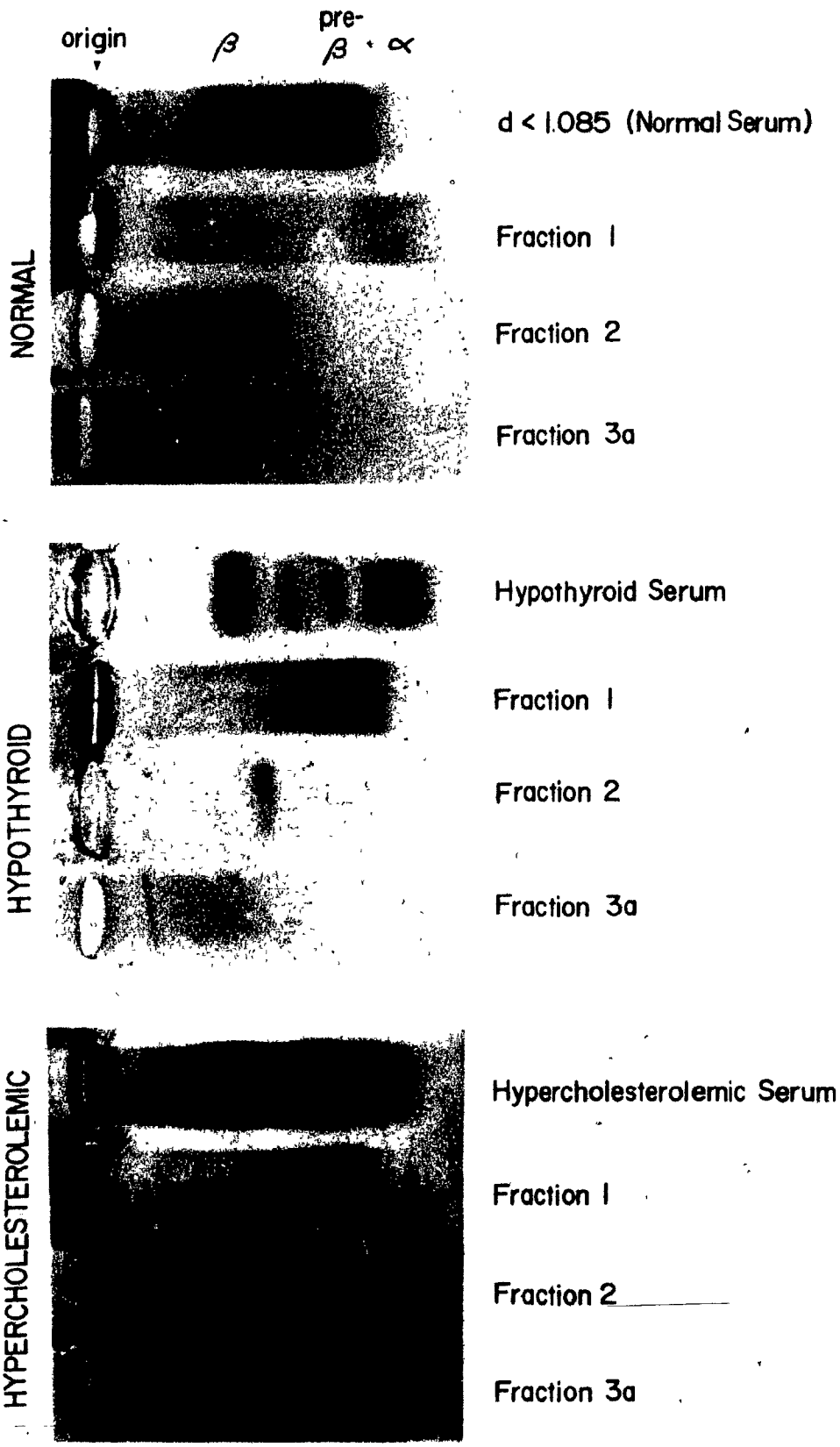
Lipoproteins were isolated from column fraction 2 of the medium from incubations of hypercholesterolemic rat hepatocytes and negatively stained with 2% sodium phosphotungstate. The small arrow designates a single discoidal particle viewed edge on. Rouleau formations of discoidal particles are indicated by medium arrows. Occasionally rouleaux of double length are observed in fraction 2 (large arrows) as well as other large aggregates. (X 270,000)


A

FIGURE 30

AGAROSE GEL ELECTROPHORESIS OF COLUMN FRACTIONS FROM INCUBATIONS OF NORMAL, HYPOTHYROID OR HYPERCHOLESTEROLEMIC RAT HEPATOCYTES

Agarose gel electrophoresis was performed on concentrated column fractions and serum samples as described in Materials and Methods. Gels were stained with 0.2% Sudan Black. Electropherograms of fractions 1, 2 and 3a from normal, hypothyroid and hypercholesterolemic rat hepatocyte incubations are shown with serum samples (or a density less than 1.085 $g.mL^{-1}$ fraction) run with each for comparison. No detectable lipid - staining band was noted for fractions 3a nor for fractions 3b, 4 and 5 (not shown).


origin
β
pre-
β
α
NORMAL
d < 1.085 (Normal Serum)
Fraction 1
Fraction 2
Fraction 3a
HYPOTHYROID
Hypothyroid Serum
Fraction 1
Fraction 2
Fraction 3a
HYPERCHOLESTEROLEMIC
Hypercholesterolemic Serum
Fraction 1
Fraction 2
Fraction 3a

nascent hypercholesterolemic lipoproteins. The discoidal particles in fraction 2 have mobility virtually identical to the same fraction from the other incubations, however, the discoidal lipoprotein band was much less intensely stained, a characteristic typical of lipoproteins containing predominantly polar lipids (205).

The lipid compositions of the nascent lipoproteins isolated after 24 hours of incubation from column fractions 1 and 2 are given in Table XIII. Nascent lipoproteins secreted into fraction 1 by normal rat hepatocytes compare favorably in lipid composition to ultracentrifugally isolated VLDL obtained from hepatocyte monolayers (330). Unlike serum VLDL, the normal nascent rat lipoproteins are relatively deficient in cholesterol ester (126). The smaller lipoproteins eluting in fraction 2 are still rich in triglyceride and deficient in cholesterol ester, indicating that the hepatocyte secretes a "small" VLDL which has no semblance to serum LDL (126).

Hypothyroid rat hepatocytes secreted nascent lipoproteins having lipid compositions that closely resembled those of lipoproteins secreted by normal cells. These lipoproteins were not enriched in cholesterol. Nascent lipoproteins from incubations of hypercholesterolemic rat hepatocytes differed significantly from both the normal and hypothyroid lipoproteins in that they were rich in cholesterol and deficient in triglyceride. Even after 24 hours of incubation in a lipid - deficient medium, the hepatocytes from hypercholesterolemic rats still secreted an abnormal lipoprotein in agreement with previous shorter - term studies using perfused livers (120).

Total lipid profiles obtained by gas liquid chromatography of the nascent lipoproteins isolated from incubations of normal, hypothyroid and hypercholesterolemic rat hepatocytes are shown in Figs. 31 - 33. When compared to normal, the nascent hypothyroid lipoproteins were enriched in triglyceride, and had relatively less free cholesterol (See also Table XIII). The striking

TABLE XIII

LIPID COMPOSITION AND PARTICLE DIAMETERS OF NASCENT LIPOPROTEINS SECRETED BY NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RAT HEPATOCYTES

COMPONENT (% of total lipid)	NORMAL (4)	HYPOTHYROID (3)	HYPERCHOLESTEROLEMIC (3)
		FRACTION 1	
TRIGLYCERIDES	62.8 ± 3.6	64.9 ± 3.1	29.7 ± 2.9
CHOLESTEROL ESTER	2.5 ± 1.3	1.9 ± 0.5	30.5 ± 0.5
FREE CHOLESTEROL	7.5 ± 0.4	6.0 ± 1.3	16.9 ± 2.3
PHOSPHOLIPIDS	27.2 ± 3.8	27.2 ± 2.2	23.0 ± 0.5
PARTICLE DIAMETER (Å)	354 ± 5[a]	613 ± 10[a]	562 ± 12[a]
		FRACTION 2	
TRIGLYCERIDES	42.0 ± 6.2	44.5 ± 4.3	10.4 ± 1.4
CHOLESTEROL ESTER	3.2 ± 1.3	6.6 ± 1.0	20.0 ± 1.5
FREE CHOLESTEROL	12.7 ± 2.6	9.2 ± 2.1	27.3 ± 1.9
PHOSPHOLIPIDS	42.2 ± 4.8	39.8 ± 1.4	42.3 ± 2.3
PARTICLE DIAMETER (Å)	303 ± 3[a]	370 ± 4[a]	227 X 54[a,b]

Each value represents the mean ± S.E.M. of the number of experiments in parentheses. Lipids were quantitated by GLC for lipoproteins isolated after 24 hours of incubation. [a] Mean particle diameter as determined by electron microscopy. [b]Discoidal particles.

FIGURE 31

GAS CHROMATOGRAPHIC LIPID PROFILES OF NASCENT LIPOPROTEINS SECRETED BY NORMAL RAT HEPATOCYTES

Total lipid profiles for nascent lipoproteins isolated from column fractions 1 (A) and 2 (B) were obtained using low - temperature gradient GLC. Peak 27, trimethylsilylether of cholesterol; peak 30, tridecanoylglycerol internal standard; peak 34, trimethylsilylether of palmitoylsphingosine; peaks 36-42, trimethylsilylethers of diacylglycerols of a total number of 34-40 acyl carbons; peaks 43-47, cholesteryl esters of fatty acids with a total number of 16-20 acyl carbons; peaks 48-56, triacylglycerols with a total number of 48-56 acyl carbons. GLC conditions as described in legend to Fig. 9.

SPECIFIC IDENTIFICATION OF PEAKS

Peak Number	Lipid (Common Nomenclature)	
27	Trimethylsilylether of unesterified cholesterol	
30	Tridecanoylglycerol Internal Standard	
34	Palmceramide	
36	Palmstearin	Trimethylsilylethers of diglycerides of phosphoglycerides resulting from phospholipase treatment.
38	Distearin	
40	Arachistearin	
42	A mixture of arachi- and behenceramide	
43	Cholesteryl palmitate	
45	Cholesteryl oleate	
47	Cholesteryl arachidonate	
48, 48+n...	Triacylglycerols having 48, 48+n... acyl carbons	

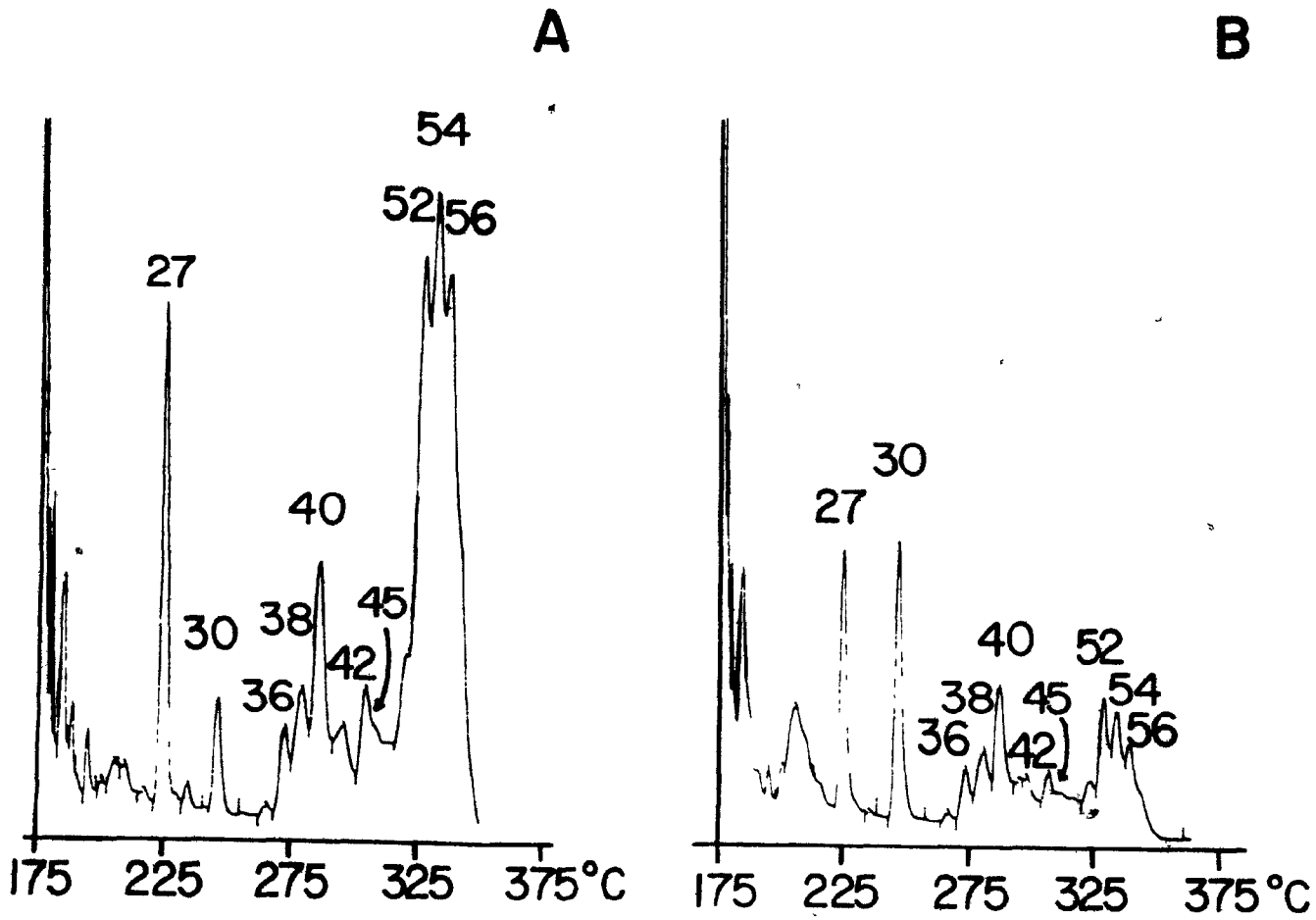
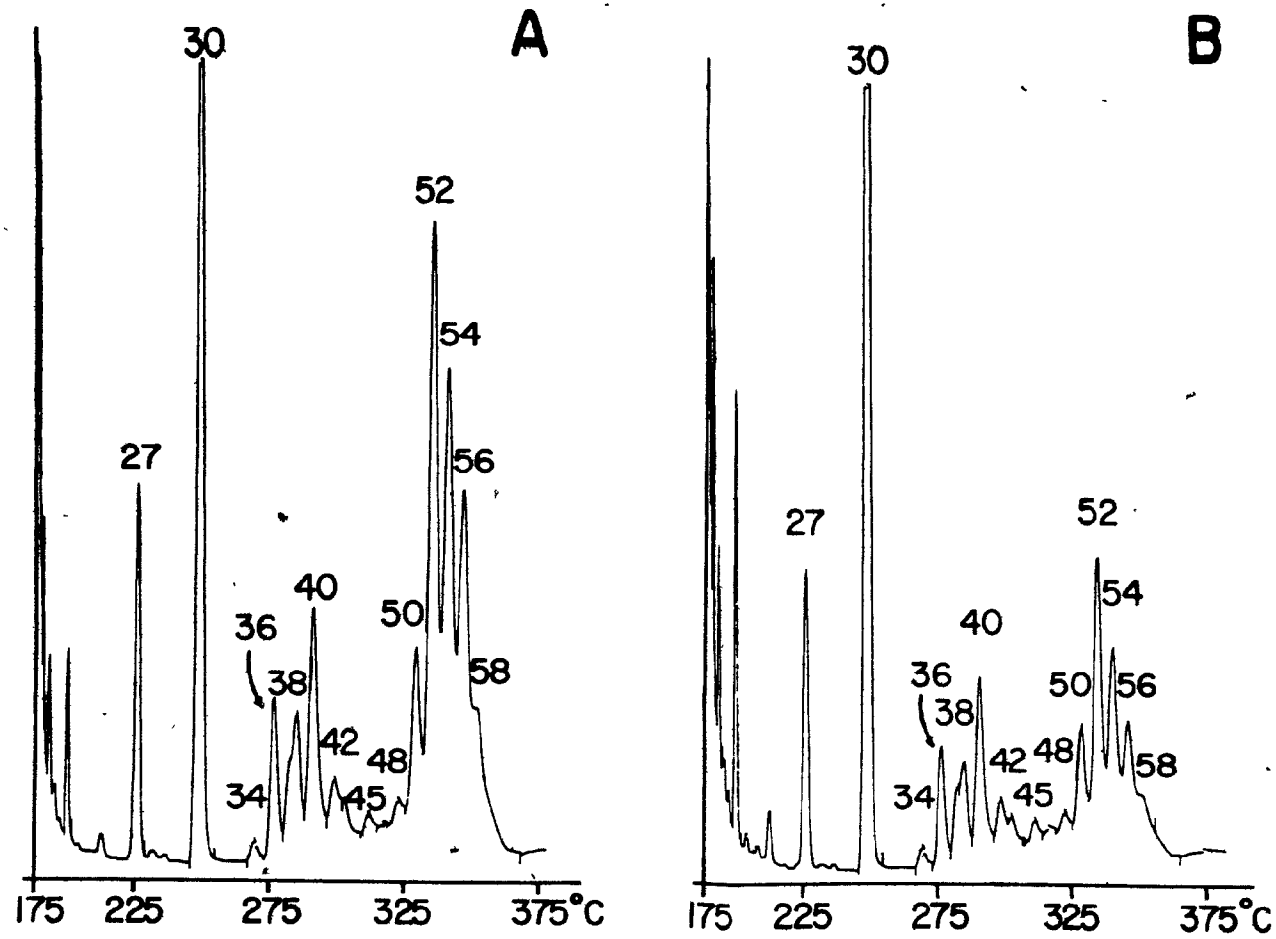

A
54
52
56
27
40
30
38
45
36
42
175
225
275
325
375°C
B
30
27
40
52
38
45
54
36
42
56
175
225
275
325
375°C

FIGURE 32

GAS CHROMATOGRAPHIC LIPID PROFILES OF NASCENT LIPOPROTEINS SECRETED BY HYPOTHYROID RAT HEPATOCYTES

Total lipid profiles for nascent lipoproteins isolated from column fractions 1 (A) and 2 (B) were obtained using low temperature gradient GLC. Peak identification as in Fig. 31.


A
30
27
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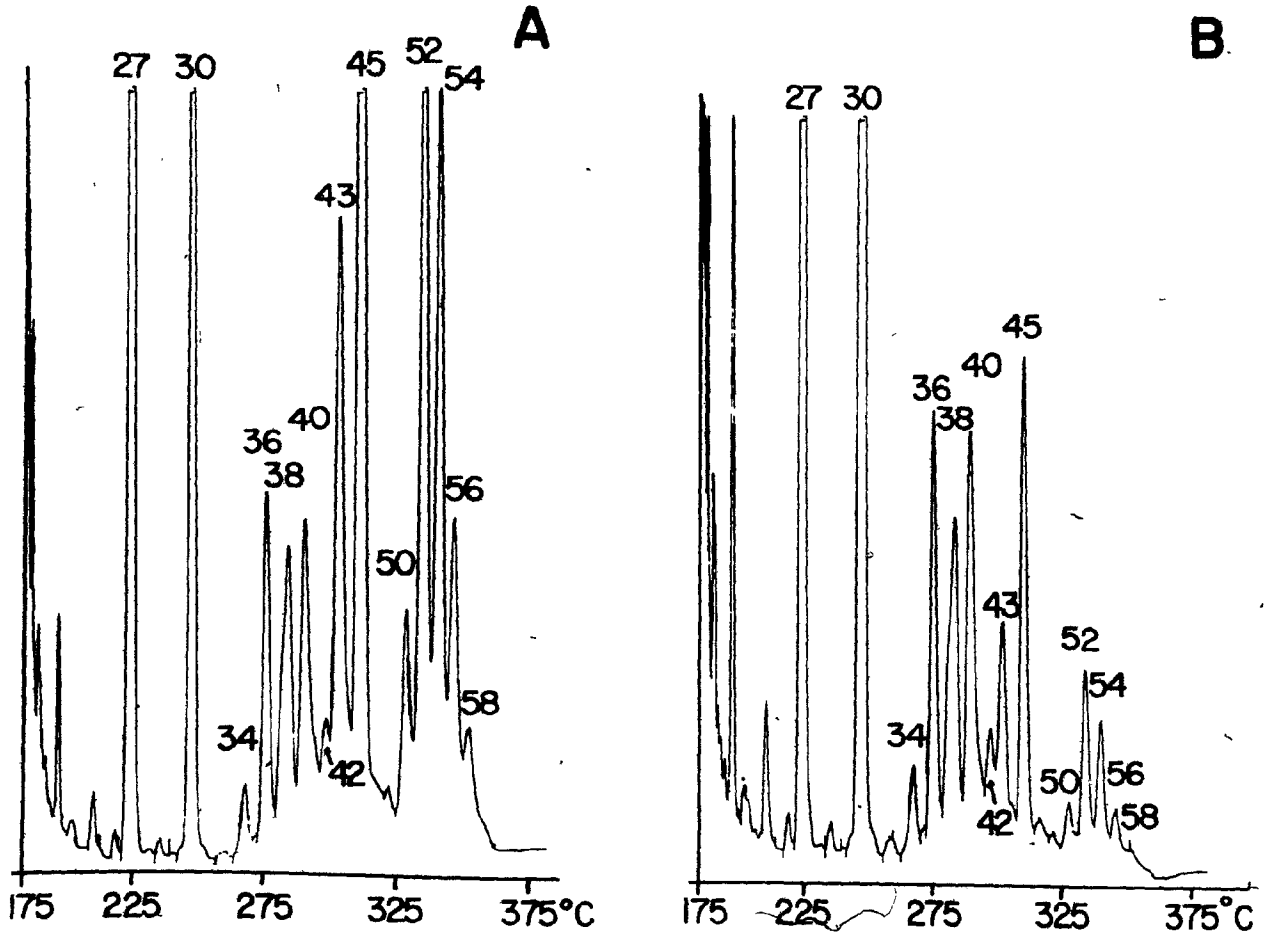
54
56
36
40
50
38
58
42
48
34
45
175
225
275
325
375°C
B
30
27
52
40
54
36
38
50
56
42
48
58
34
45
175
225
275
325
375°C

FIGURE 33

GAS CHROMATOGRAPHIC LIPID PROFILES OF NASCENT LIPOPROTEINS SECRETED BY HYPERCHOLESTEROLEMIC RAT HEPATOCYTES

Total lipid profiles for nascent lipoproteins isolated from column fractions 1 (A) and 2 (B) were obtained using low temperature gradient GLC. Peak identification as in Fig. 31.


A
27
30
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52
54
43
36
40
38
56
50
58
34
42
175
225
275
325
375°C
B
27
30
45
36
40
38
43
52
54
34
50
56
42
58
175
225
275
325
375°C

difference in profiles of nascent hypercholesterolemic lipoproteins is the significant amount of free and esterified cholesterol present, the latter being present in only very small amounts in nascent normal and hypothyroid lipoproteins. Triglycerides, as a result, account for relatively less of the total lipid in nascent hypercholesterolemic lipoproteins. It is relevant to note that the proportions of phospholipid present in the lipoproteins secreted by the hepatocytes was equivalent in all three groups of hepatocytes incubated.

Peaks 36 - 42 are derived from phospholipase C digestion of phospholipids during the sample workup for GLC (361), however, these peaks could represent diglycerides present as such in the lipoproteins, either secreted or arising perhaps from medium lipolytic activity. To test whether or not this could account for some of the peaks in the present studies, individual nascent lipoprotein lipids were separated by TLC prior to phospholipase digestion and analysis by GLC. The results of such an experiment conducted on nascent lipoproteins secreted by normal rat hepatocytes are shown in Fig. 34.

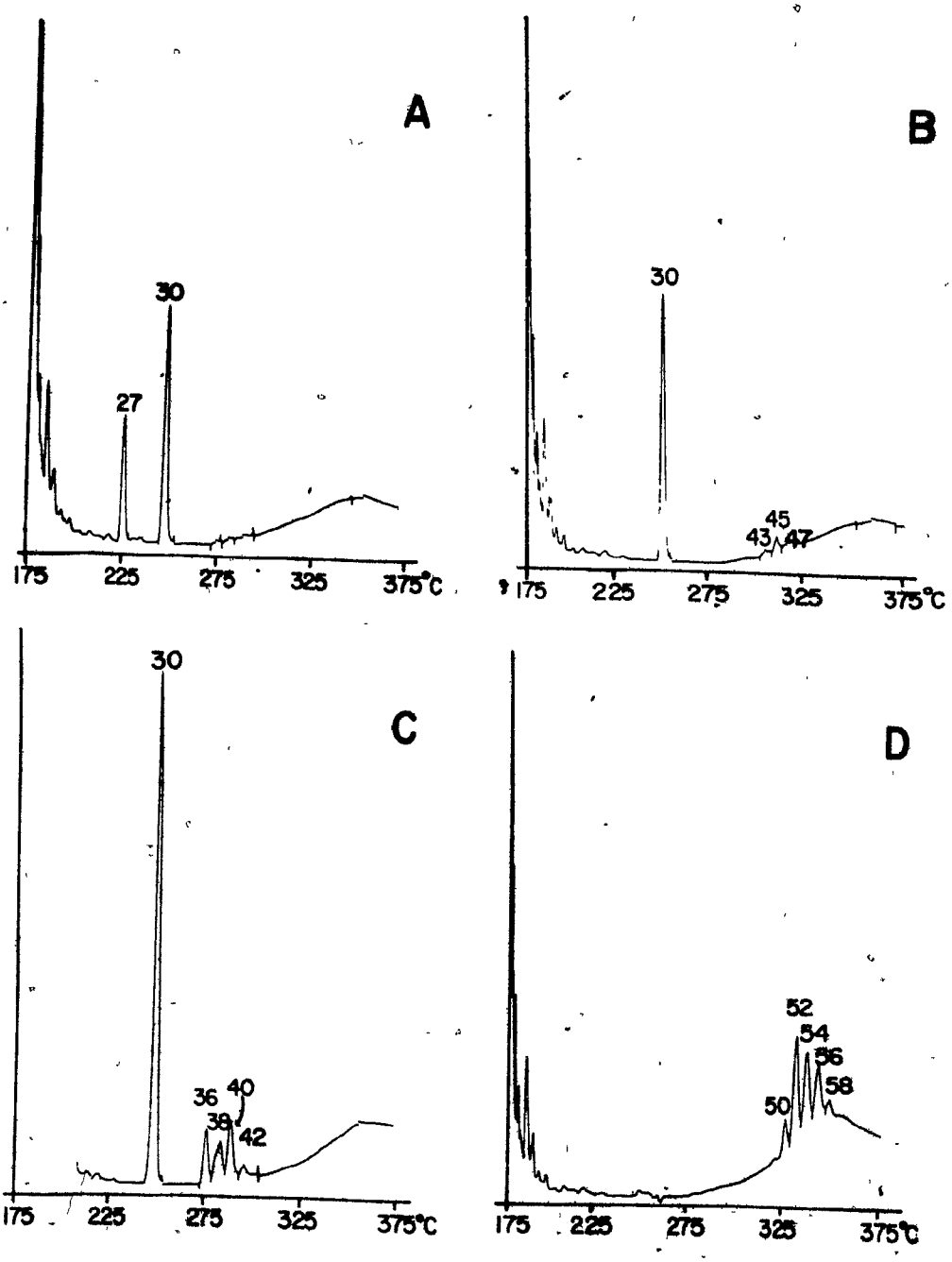
Diglycerides and free cholesterol are only marginally separated on TLC with the solvent system used, therefore the total area corresponding to these two components was eluted from the silica gel. As shown in Fig. 34,A barely detectable levels of diglyceride (peaks 36 - 42) were present in normal nascent lipoproteins, in comparison to the significant amount of phospholipid in this lipid preparation (Fig. 34,C). The cholesterol ester content was low (B) in these fractions and the GLC profile of the isolated triglycerides is shown in (D). Sphingomyelins were barely detectable. Similar results were obtained on TLC-separated lipids extracted from nascent hypothyroid hepatocytes.

Based on the proposed models of lipoprotein structure, one would expect to see significantly different molar ratios of surface lipid components for

FIGURE 34

GAS CHROMATOGRAPHIC PROFILES OF TLC-SEPARATED LIPID COMPONENTS OF NASCENT LIPOPROTEINS SECRETED BY NORMAL RAT HEPATOCYTES

Lipids were extracted from nascent lipoproteins secreted by normal rat hepatocytes and separated by TLC as described in Materials and Methods. The individual components were then prepared and analyzed by GLC as described in Materials and Methods. Peak identification as in Fig. 31. (A) Diglycerides and free cholesterol, (B) Cholesteryl esters, (C) Phospholipids and (D) Triglycerides.


A
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27
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B
30
45
43
47
175
225
275
325
375°C
C
30
36
40
38
42
175
225
275
325
375°C
D
52
54
56
58
50
175
225
275
325
375°C

the abnormal lipoproteins secreted into fraction 2 by hypercholesterolemic versus normal and hypothyroid rat hepatocytes. Free cholesterol to phospholipid molar ratios in nascent lipoproteins are shown in Table XIV. Ratios were closely reproducible in lipoproteins secreted by hepatocytes from all three groups of rats. The ratios were less than 1 for normal and hypothyroid nascent particles and were not significantly different from each other. In contrast, the ratios, exceeding 1 in both fractions of lipoproteins isolated from incubations of hypercholesterolemic rat hepatocytes were significantly different from the values obtained for nascent lipoproteins from the other groups.

Urea polyacrylamide gel electrophoretic patterns of hypercholesterolemic versus normal nascent lipoproteins are shown in Fig. 35. The normal pattern is virtually identical to that of nascent apo-VLDL obtained from hepatic Golgi (295) and that secreted by normal rat hepatocyte monolayers incubated in serum - free medium (330). The major apolipoproteins are apo-B and apo-E for fractions 1 and 2 for both the normal and hypercholesterolemic nascent lipoproteins, however significantly more apo-E is present in the latter (See also Table V). Incorporation of radioactivity into apo-E in the normal nascent lipoproteins was similar to that observed in ultracentrifugally isolated VLDL from the earlier studies. In contrast, the higher incorporation of radioactive amino acids into the apo-E of nascent hypercholesterolemic lipoproteins suggests a significantly increased biosynthesis of this apolipoprotein in these animals.

The fairly wide distribution of particle sizes of lipoproteins secreted by isolated hepatocytes from all groups of rats suggests a microheterogeneity of the nascent species. The double immunodiffusion studies depicted in Fig. 36 support this concept. Nascent lipoproteins from all groups of rat hepatocytes studied gave identical patterns. (The reader is reminded that the terms

TABLE XIV

FREE CHOLESTEROL : PHOSPHOLIPID MOLAR RATIOS
OF NASCENT LIPOPROTEINS FROM INCUBATIONS OF HEPATOCYTES FROM NORMAL,
HYPOTHYROID AND HYPERCHOLESTEROLEMIC RATS

	NORMAL	HYPOTHYROID	HYPERCHOLESTEROLEMIC
FRACTION 1	0.61 ± 0.09 (4)	0.45 ± 0.06 (3)	1.53 ± 0.18[a] (3)
FRACTION 2	0.60 ± 0.08 (4)	0.47 ± 0.09 (3)	1.36 ± 0.17[a] (3)

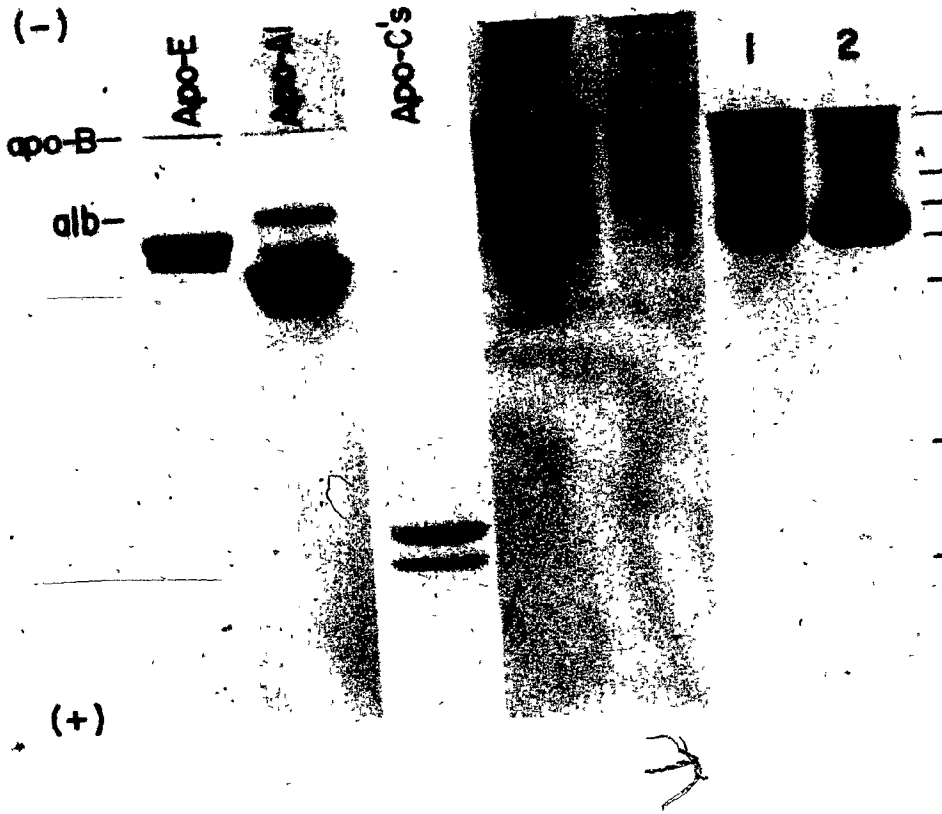
Each value represents the mean ± S.E.M. of the number of experiments in parentheses. Statistical evaluation compared to normal according to Student's t-test.

[a] $P < 0.005$

FIGURE 35

UREA POLYACRYLAMIDE GEL PROFILES OF NASCENT LIPOPROTEINS (FRACTIONS 1 AND 2) FROM NORMAL AND HYPERCHOLESTEROLEMIC RAT HEPATOCYTE INCUBATIONS

Lipoproteins were isolated from fractions 1 and 2 of the medium from 24 hour incubations of normal and hypercholesterolemic rat hepatocytes as described in Materials and Methods. Urea polyacrylamide gels were run for each fraction, sliced and counted. The % of total radioactivity associated with the gel regions designated between the lines is shown. Normal purified rat serum apolipoproteins are shown for comparison. Total c.p.m. applied per gel track: normal fractions 1 (7,300) and 2 (2,800); hypercholesterolemic fractions 1 (23,200) and 2 (31,700).



% of Total Radioactivity in Gel

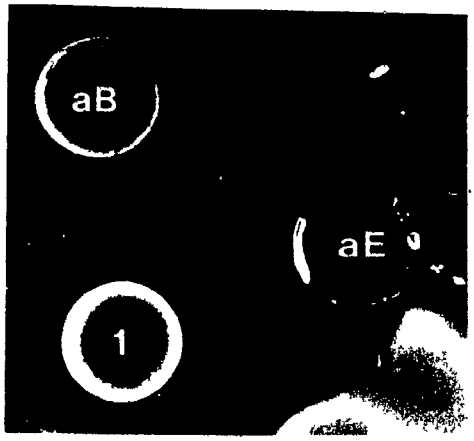
Gel Region	Normal		Hyperchol.	
1	47	47	33	7
2	18	27	21	8
3	4	13	7	8
4	26	13	35	63
5	3	0	4	14
6	1	0	0	1
7	0	0	0	0

FIGURE 36

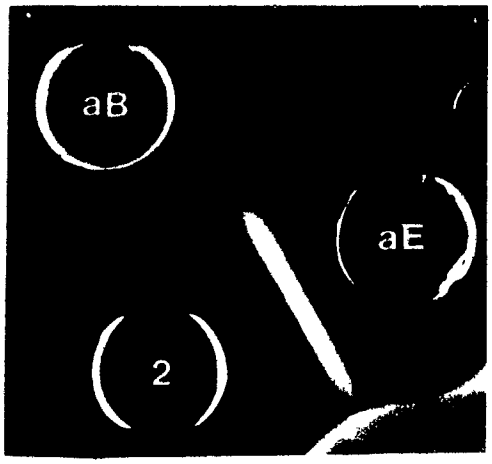
DOUBLE IMMUNODIFFUSION STUDIES OF COLUMN FRACTIONS 1 AND 2 NASCENT LIPOPROTEINS FROM INCUBATIONS OF NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RAT HEPATOCYTES

Immunodiffusion plates were prepared from 1% ion agar and 4.0mm wells were cut with a Bio-Rad gel punch. (Other conditions were as described in ref. 24. Fractions 1 and 2 were applied to wells marked 1 and 2 from normal (N1 and N2), hypothyroid (HT1 and HT2) and hypercholesterolemic (HC1 and HC2) rat hepatocyte incubations. Antibody (IgG) preparations raised against rat apo-B and apo-E were applied to wells marked $\bar{a}$B and $\bar{a}$E respectively. Photographs were taken of unstained gels.

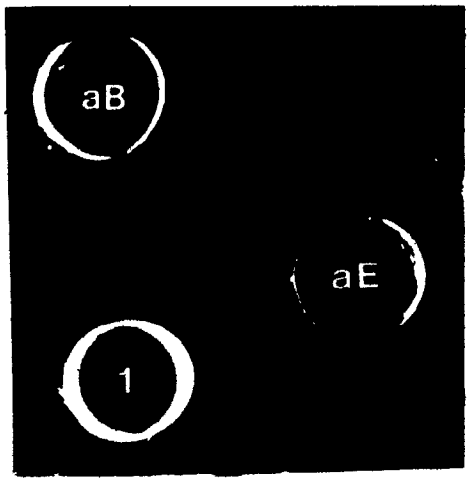
NI



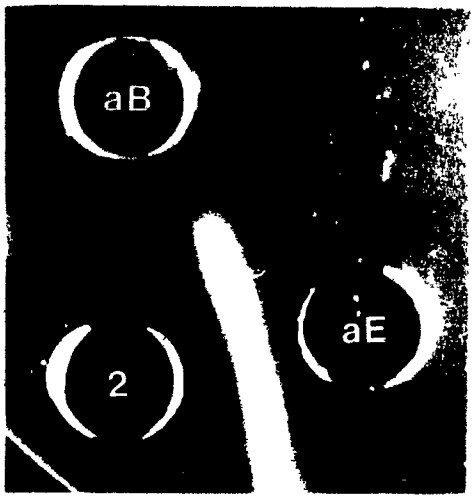
N2



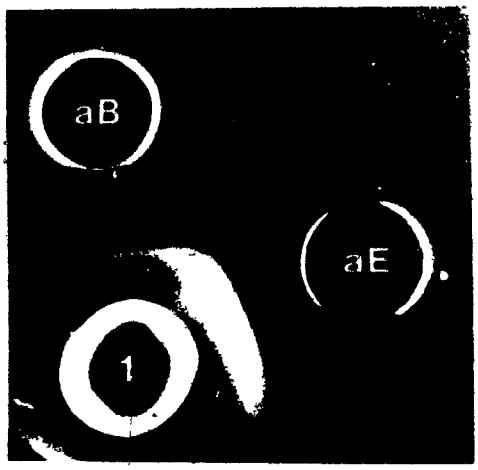
HTI



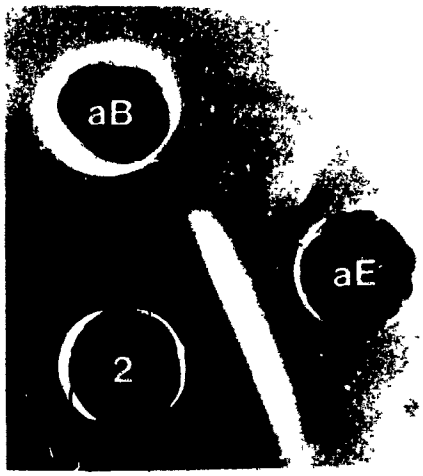
HT2



HCI



HC2



"reaction of identity" and "partial identity" usually refer to precipitin arcs bridging two antigenic wells. For the purposes of discussion here, these well established immunological terms will be used for simplicity).

Reactions of identity were observed between anti-apo-B and anti-apo-E for the lipoproteins secreted into fraction 1 by all three groups of hepatocytes. This immunodiffusion pattern suggests that both apolipoproteins reside on a single lipoprotein particle. In contrast, reactions of partial identity between the two antibody preparations were observed for nascent lipoproteins secreted into fraction 2 by hepatocytes from all groups of rats. The presence of a spur pointing towards the anti-apo-B well suggests that fraction 2 contains a species of lipoprotein having only apo-E immunoreactive sites on it. This particle then probably represents Lp-E (20).

Double immunodiffusion studies were performed on the other column fractions (3a to 5) and are shown in Fig. 37. Patterns were identical for all fractions from incubations of hepatocytes from all groups of rats studied. Fraction 3a gave a reaction of non- or partial identity between anti-rat-apo-E and anti-apo-C. The paucity of anti-apo-C reactive material present precludes a definite evaluation of this observation. The immunodiffusion patterns obtained from fractions 3b and 4 resemble each other closely, however, significantly more total apolipoprotein is present in normal fraction 4. Again the insensitivity of double immunodiffusion in detecting small quantities of apolipoprotein, fails to give a definite reaction of non - identity between anti-apo-E and anti-apo-AI, however if these two apolipoproteins did co-exist on a single particle their combined Stokes radius would be too great to elute in fraction 4. Only apo-E was detectable in Fraction 5.

The essentially lipid - free apo-E secreted into fractions 4 and 5 gave reactions of identity with rat serum apo-E (Fig. 38). Polyacrylamide gel electrophoretic patterns obtained after immunoaffinity chromatography of

FIGURE 37

DOUBLE IMMUNODIFFUSION STUDIES OF COLUMN FRACTIONS 3a, 4 AND 5 FROM INCUBATIONS OF NORMAL, HYPOTHYROID AND HYPERCHOLESTEROLEMIC RAT HEPATOCYTES

Immunodiffusion plates were prepared as in Fig. 36. Fractions 3a to 5 from an incubation of normal rat hepatocytes were applied to wells marked 3a to 5 respectively. Antibody (IgG) preparations raised against various rat apolipoproteins were applied to peripheral wells. Anti-apo-B, āB; anti-apo-E, āE; anti-apo-AI, āAI; anti-apo-C, āC (directed towards all apo-C proteins).

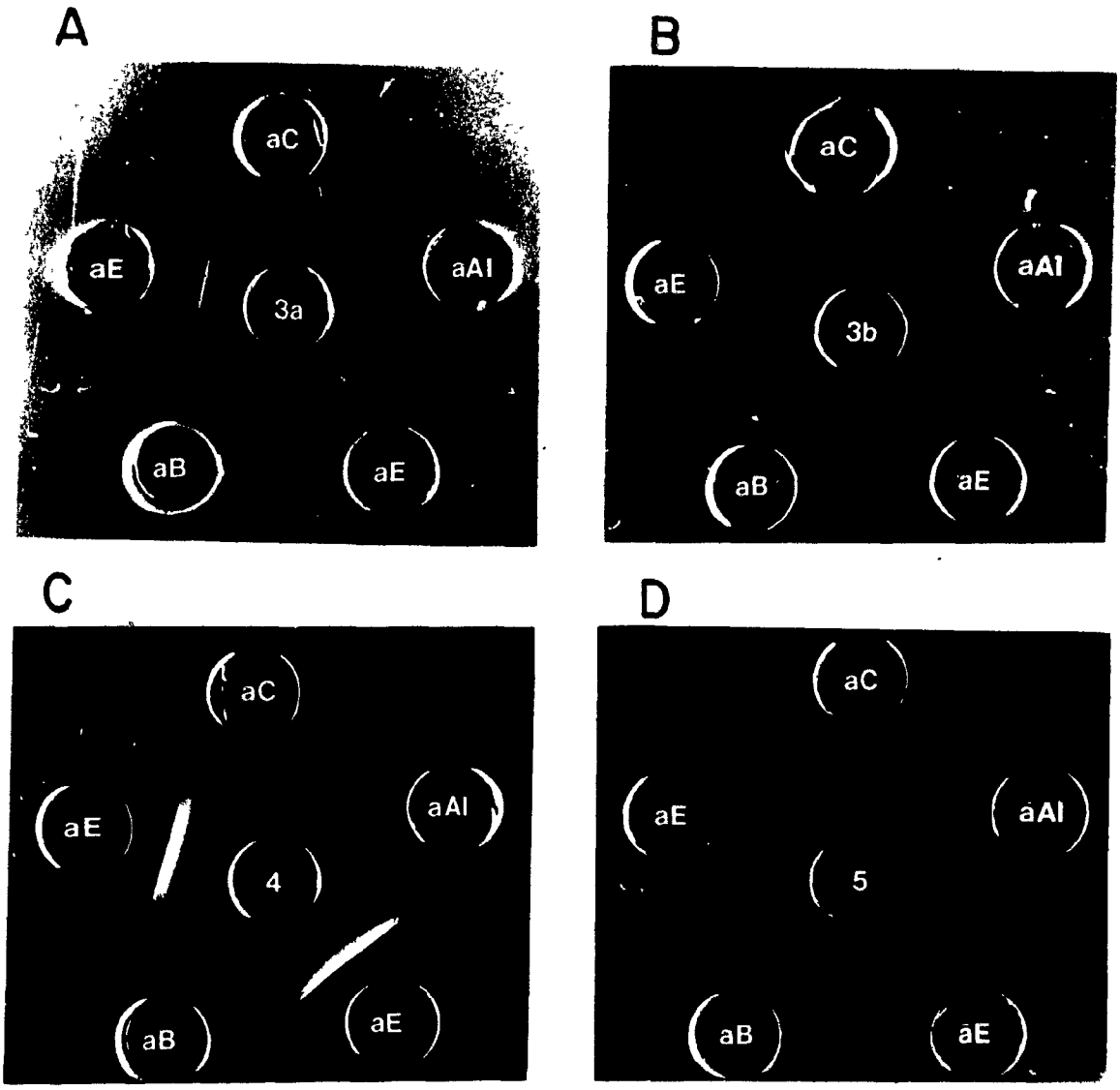
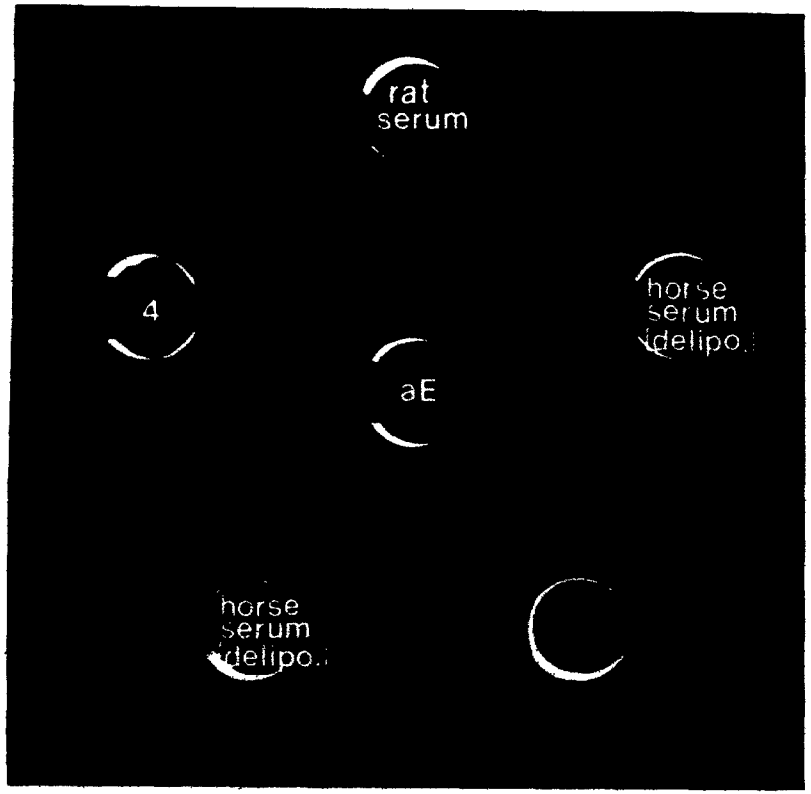
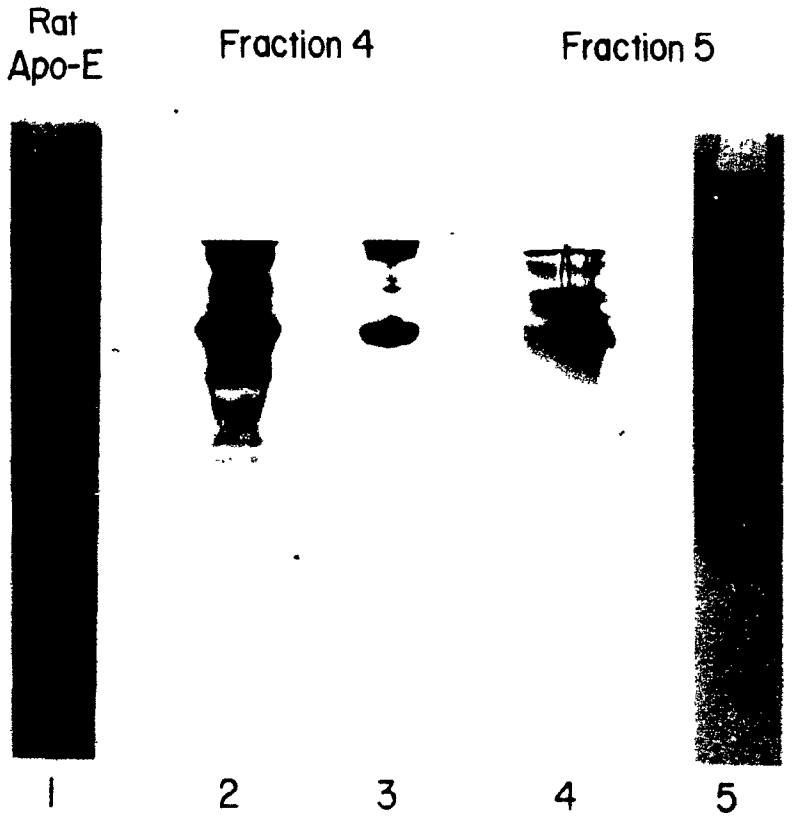

A
aC
aE
aAl
3a
aB
aE
B
aC
aE
aAl
3b
aB
aE
C
aC
aE
aAl
4
aB
aE
D
aC
aE
aAl
5
aB
aE

FIGURE 38

ANTI-RAT-APO-E IMMUNOAFFINITY CHROMATOGRAPHY OF COLUMN FRACTIONS 4 AND 5

Column fractions 4 and 5 from the medium of a 24 hour incubation of normal rat hepatocytes were applied to an anti-rat-apo-E immunoaffinity column as described in Materials and Methods. Urea polyacrylamide gel profiles of the fractions at various stages of the experiment are shown on the right. (1) Purified rat serum apo-E; (2) and (4), Column fractions 4 and 5 before application to the column; (3) and (5), Adsorbed protein eluted from column with 6M sodium thiocyanate, pH 7.2. Immunodiffusion plate on left prepared as in Fig. 36. Center well contained anti-apo-E (āE). Fraction 4 was applied to the well marked 4; Cab-O-Sil treated horse serum (used for incubations) was applied to the designated wells; fasted rat serum applied to topmost well.


rat
serum
4
horse
serum
delipo.
aE
horse
serum
delipo.
C


Rat
Apo-E
Fraction 4
Fraction 5
1
2
3
4
5

medium fractions 4 and 5 from incubations of normal rat hepatocytes are shown in Fig. 38. Radioactivity was associated with the adsorbed apo-E extracted from these fractions.

DISCUSSION

Isolated hepatocytes maintained in suspension for 24 hours proved to be a suitable system to measure lipoprotein secretion. The effects of dietary and hormonal status in the intact animal were reflected in the pattern of lipoprotein secretion by hepatocytes isolated from these rats. Metabolic distinctions have been shown to be retained in hepatocytes isolated from chow - fed, sucrose - fed, triglyceride - fed or starved rats (324, 378, 379, 380).

The hepatocytes isolated and incubated in the studies described herein showed a high percent (>60%) of viable cells (retaining LDH) even after 24 hours. Synthesis of cellular ATP, retention of cellular potassium concentrations and the incorporation of radioactively - labelled precursors into lipid and protein indicated that the cells were metabolically active throughout the incubation periods which confirmed the ultrastructural evidence for cellular integrity obtained by electron microscopy. The longer - term incubation techniques developed by Jeejeebhoy et al. (326) permitted studies to be carried out long after the "recovery" period for the hepatocytes. It is well known that hepatocytes, freshly isolated by collagenase enzyme usually demonstrate maximal hormone response (381), specific adhesion to sugars (382), specific receptor - mediated endocytosis of asialoglycoproteins (383) and chylomicron remnant uptake (384) only after 30 mins to 2 hours of incubation. It would appear that this period of time is required for the hepatocyte to "recover" from the injury induced by enzymatic disaggregation (385). Maintenance of a suspension culture in a large volume for our studies also permitted the isolation of synthesized and secreted material in a quantity sufficient to permit biochemical and immunological characterization by currently available techniques.

Our first objective was to investigate the nature of the lipoproteins secreted by hepatocytes from fasted, normal rats. Nascent VLDL secreted by

isolated normal rat hepatocytes and isolated ultracentrifugally at d < 1.063 $g.mL^{-1}$ exhibited several protein bands when subjected to urea polyacrylamide gel electrophoresis (see Table VII). However, ^{3}H-amino acids were only incorporated into apo-B and apo-E as Jeejeebhoy et al. previously noted (327). In contrast to the latter group, we observed proportionately more incorporation into apo-E. The nature of the darkly staining protein bands having little or no radioactivity associated with them was enigmatic. Unfortunately in the report by Jeejeebhoy et al. (327) whose system was essentially adopted for our studies, actual apo- VLDL gel profiles were not shown so that no comparison of the proteins could be made. Evidence presented here suggests that these protein bands are non - dialyzable horse - serum apolipoproteins which became dissociated from the lipoproteins during the ultracentrifugal method of delipidating the serum. When the serum was delipoproteinated using colloidal silicic acid (Cab-O-Sil), nascent apo-VLDL profiles on urea polyacrylamide electropherograms were identical to those obtained by Davis et al. (330) for apo-VLDL isolated from serum - free hepatocyte monolayer cultures. Colloidal silicic acid removes apolipoproteins from serum (341) and our results are consistent with this. Clearly, then, this method is superior to ultracentrifugation for delipoproteinating serum for lipoprotein metabolic studies. Although the nature of horse apolipoprotein binding to nascent VLDL was not investigated extensively, the phenomenon itself is interesting. Nestruck and Rubinstein (295) demonstrated that nascent VLDL isolated from hepatic Golgi acquired apolipoproteins from serum HDL when incubated together. They noted that whereas serum VLDL could acquire or exchange apo-E and apo-C fairly specifically from HDL, the acquisition of apolipoproteins by nascent VLDL was non - specific and included apo-AI, a protein not usually found in association with VLDL (295). Studies by Dolphin (334) also indicated that nascent Golgi VLDL acquired apo-E (in mass terms) from the serum d > 1.063 $g.mL^{-1}$ fraction

whereas serum VLDL did not. These observations suggest that nascent VLDL obtained from Golgi or isolated hepatocytes represents a transient form of lipoprotein which can readily be modified following its secretion. As apo-C acquisition would appear to be the major modification of nascent VLDL (295, 327, 328, 330) it is reasonable to assume that the acquisition of apo-C is dependent on the availability of apo-C "donors". In situations of increased chylomicron and/or VLDL production, the reservoir of apo-C in HDL would be relatively depleted as apo-C exhibits a net transfer to these triglyceride - rich lipoproteins upon their entry into the serum compartment (386). Apo-CII, which functions as the activator of lipoprotein lipase, therefore would be less available to newly secreted VLDL. During lipolysis, net transfer of apo-C back to HDL occurs (109) and this would serve to replete the "donor" supply. Taskinen et al. (387) noted that the distribution of lipid and apo-C within the various HDL subfractions was dependent on the ratio of VLDL - triglyceride and HDL_3 protein present in in vitro incubations. It is interesting to speculate that the apo-C distribution amongst lipoproteins functions as a mode of regulating lipolytic action. Such a mechanism may moderate lipolysis to ensure that lipoprotein receptors are not saturated and that lipoprotein remnants do not accumulate in the plasma.

The lack of visually or immunologically detectable apo-C on PAGE gels or by immunodiffusion of nascent VLDL secreted by the isolated hepatocytes confirms an earlier observation (327). However, some investigators have reported small amounts of visually detectable and radioactively labelled material on PAGE gels in the region corresponding to apo-C (224, 330, 332) for VLDL secreted by hepatocytes. Apo-C is virtually absent from Golgi VLDL (295) however, when secretory functions are impaired by the administration of colchicine, apo-C is found to be associated with the isolated Golgi VLDL (295, 301). These findings can be partly explained by the observation that apo-C

is acquired by the VLDL just prior to secretion by the Golgi secretory vesicles as was shown by Dolphin et al. (301). Apo-C immunoreactive material, however, was detectable in nascent lipoproteins isolated in the high density range in the studies described here. However, the small quantities of material available precluded any further study by the methods employed for the other apolipoproteins. As suggested by Hamilton (314), this is probably due to the low hepatic secretion rate of HDL.

Ultracentrifugation to isolate the nascent lipoproteins was a time - consuming procedure and for the purposes of studying nascent lipoprotein secretory products did not appear to be suitable. Apolipoprotein E was consistently found in the $d > 1.21\ g.mL^{-1}$ fraction of the incubation medium, a finding that had been reported by Dashti et al. (331) and was conceivably due to the ultracentrifugal procedures (36). Therefore gel filtration on Sephacryl - 300 columns was used in all subsequent studies to separate the nascent lipoprotein species. This system provided the added advantage that the entire spectrum of secretory products could be examined for apolipoproteins in a single step. Fractionation of normal rat serum by this method resulted in a failure to detect significant amounts of apo-E or apo-AI as lipid - free components, confirming the earlier work of Fainaru et al. (36) which indicated that gel filtration was a gentler procedure to separate the lipoprotein classes and did not give rise, as ultracentrifugation did, to lipid - free apolipoproteins. Also, the horse serum used in the incubations for all subsequent studies was routinely delipidated by colloidal silicic acid to eliminate any possible modifications of the secreted nascent lipoproteins by horse apolipoproteins.

Our second aim was to distinguish between the effects of chow feeding, hypothyroidism and hypothyroidism with cholesterol feeding on hepatic lipoprotein biosynthesis and secretion. Previous studies in this laboratory had

provided evidence for the direct secretion of abnormal lipoproteins by hypothyroid, cholesterol - fed (hypercholesterolemic) rats (120).

A fundamental determinant of nascent VLDL size appears to be the quantity of triglyceride to be transported out of the hepatocyte (388). In a lipid - deficient medium, hepatocytes are capable of de novo synthesis of fatty acids, phospholipids and cholesterol (221). A close relationship between the rate of hepatic fatty acid synthesis and the production and release of triglycerides has been demonstrated (221). Fatty acid synthesis rates are quite variable between animals (221), which, in part could explain the large standard deviations in the absolute amounts of lipid secreted by the normal rat hepatocytes in this study (Table XII). The lipoproteins isolated from fractions 1 and 2 of the incubation medium secreted by normal rat hepatocytes were cholesterol ester poor when compared with the serum VLDL (126), an observation also noted by Kempen (332) and Davis et al. (330). The higher cholesteryl esters in serum VLDL may be a result of the contamination of this fraction with chylomicron remnants. However, the higher proportion of cholesterol - ester in serum versus nascent VLDL may arise from the net transfer of this lipid component from HDL in the serum compartment (389). Although the cholesterol ester transfer activity in rats is reported to be low relative to the activity in human serum (51), one cannot preclude a possible physiological role . Since measurements of the rates of cholesterol ester transfer have been conducted on incubations of isolated serum HDL and VLDL, it is conceivable that the measurable cholesterol ester transfer from HDL to VLDL would be low if any transfer did occur, in vivo, upon secretion of the nascent VLDL. In any case, however, the net transfer of cholesteryl ester does not compare to that of humans, as rat HDL is rich in cholesteryl arachidonate and very little of this species is found in rat serum VLDL.

Hepatocytes from hypothyroid, chow - fed rats exhibited an elevated lipid

secretion rate compared to normal rat hepatocytes which was solely accounted for by an increase in triglyceride and phospholipid secretion. This is consistent with a generalized reduction in calorigenesis observed in hypothyroid animals (390). It has been observed in perfused livers that thyroid dysfunction causes a marked diminution of fatty acid oxidation through the tricarboxylic acid cycle resulting in increased VLDL triglyceride secretion (336). Triglyceride synthesis is increased in hypothryoid rat hepatocytes and as a result they synthesize and secrete larger lipoproteins rather than an increased number of particles of the same size as secreted by normal rat hepatocytes. The secretion of apo-B into the medium by hypothyroid rat hepatocytes, however, was the same if not lower, than that of normal rat hepatocytes. As this apolipoprotein is known to be essential for lipoprotein assembly (109), it would appear that each molecule of apo-B can accomodate a larger triglyceride load when concentrations of this lipid are elevated within the cell. The assembly and secretion of larger lipoproteins at the same apolipoprotein secretory rate would provide a rapid mechanism whereby hepatocytes could prevent the intracellular accumulation of excess lipid.

In vivo studies indicate that triglyceride secretion by the liver in hypothyroid rats is decreased relative to euthyroid rats (391). These findings are apparently at variance with the results obtained here and those of Keyes and Heimberg (392) who also noted increased triglyceride secretion by isolated perfused livers from hypothyroid rats. A possible explanation for the observed differences is suggested here. The production of VLDL is related to the balance between fatty acid oxidation and esterification (223, 393, 394). Thyroid dysfunction causes a reduction in fatty acid oxidation (336) whereas fasting (217) results in an increase. Thyroxine raises the activity of outer mitochondrial carnitine palmitoylacyltransferase in isolated rat hepatocytes, the committed step in fatty acid oxidation (395). Hepatocytes from euthyroid,

fasted rats exhibit increased ketone body production (380) relative to fed rats, and in a fatty - acid deficient medium such as that utilized in these studies would rely on endogenously synthesized fatty acids for oxidation. The increased triglyceride synthesis observed for the hypothryoid rat hepatocytes would indicate that thyroid dysfunction arrests oxidation to an extent that endogenously synthesized fatty acids are directed towards net lipogenesis to a larger degree than in the normal rat hepatocytes. However, in vivo, the normal rat liver is exposed to higher insulin levels (396) and probably higher concentrations of plasma fatty acids (397) than in hypothyroid rats which could account for the relatively higher rate of triglyceride secretion observed in the intact normal animal. It is also pertinent to note that, in vivo, thyroxine enhances the activities of insulin (triglyceride synthesis) and epinephrine (free fatty acid release by adipose tissue) (398).

The lipoproteins that were secreted by hypothyroid, chow -fed rat hepatocytes are evidently suitable substrates for lipolytic activity as the total serum triglyceride levels in the intact animals were lower than that observed in normal animals (Table VIII). Walton et al. (399) noted that in human hypothyroid patients the mechanism of disposal of triglycerides in the serum compartment was not impaired although a prolonged half - life for LDL was observed. This is consistent with the hypothesis that decreased removal of lipoproteins occurs in the hypothyroid state as suggested by studies in humans (399, 400) and rats (335, 391, 401). The lower triglyceride levels in the serum, may be accounted for by increased lipolysis of the lipoproteins in the serum compartment as a result of the impaired removal. These factors could give rise to the β- migrating lipoproteins which accumulate in the sera of these animals.

It is of interest to note that the uptake of normal chylomicron remnants by isolated hepatocytes from hypothyroid rats was not impaired relative to

euthyroid rats (374). However, normal chylomicron remnant uptake by livers from hypercholesterolemic, hypothyroid rats was delayed (374, 402). In these animals, in vivo, only chylomicron remnant cholesteryl ester clearance was impaired whereas chylomicron triglyceride is cleared efficiently (403). Cholesterol feeding alone did not cause any diminution of uptake (404).

These findings suggest that elevated cholesterol levels can act synergistically with hypothyroidism to induce decreased lipoprotein uptake. It appears that either condition alone does not elicit decreased lipoprotein uptake which raises some interesting points worthy of consideration.

Drastic differences in fasting serum cholesterol levels have been reported for various strains of rat (405). These differences are abolished upon hypophysectomy (405), suggesting strain - specific modulating hormonal levels or target tissue response. In the study quoted previously, showing normal chylomicron remnant uptake by hypothyroid rat hepatocytes, the donor albino strain had cholesterol levels that were less than one - half (374) those of Long - Evans rats utilized in the present study. Also, serum triglyceride levels in the hypothyroid albino rats were similar to the untreated rats (374) indicating a clear difference to the results obtained in the present study.

In view of the observation that removal of the thyroid in rat causes a marked rise in plasma cholesterol (406), it is surprising that the relatively high serum cholesterol level in Long - Evans rats is accompanied by a high normal thyroxine level (7.4 µg/dl) compared to albino rats (approx. 4.0 µg/dl) (335, 374, 391). It is also interesting to note that hypophysectomy causes an increase in cholesterol levels in rats, the increase being more dramatic (190%) for strains having initially low levels than for those with high levels (60%) (405). The net result is that the final serum cholesterol concentrations are equivalent in both strains. The exact nature of the genetic

differences between strains that gives rise to variant cholesterol levels remains to be determined, however, it is interesting to speculate that the higher cholesterol levels in chow - fed Long - Evans rats with concomitant hypothyroidism would parallel the studies demonstrating decreased chylomicron remnant uptake by hypothyroid, hypercholesterolemic albino rats (374, 402). In fact, a recent report demonstrated, in vivo, the decreased removal of chylomicron remnant cholesterol ester by normal chow - fed Long - Evans rats versus albino rats (407). The relative deficiency in chylomicron remnant removal by Long-Evans rats correlated with an increased cholesterolemic response compared to albino rats when 1% cholesterol was added to the diet (407).

Increased proportions of cholesterol within the liver plasma membrane are observed in conditions of elevated serum cholesterol levels in the rat (408). The decreased fluidity of such membranes could account for the relative deficiency of chylomicron remnant uptake via specific membrane - bound receptors.

In this study, hepatocytes from hypothyroid, cholesterol - fed (hypercholesterolemic) rats secreted cholesterol-rich and triglyceride - poor lipoproteins even after 24 hours of incubation in a lipid - deficient medium, in contrast to the triglyceride - rich, cholesterol - poor lipoproteins secreted by normal or hypothyroid rat hepatocytes. The highly increased secretion rate for all lipid components, particularly cholesterol, by hypercholesterolemic rat hepatocytes most probably reflects hepatocyte secretion of dietary - derived lipids taken up as chylomicron remnants and retained by the liver (95, 374). The lipoproteins were similar with respect to particle size as the lipoproteins secreted by hypothyroid rat hepatocytes and were similarily more heterogeneous than normal nascent lipoproteins. The significantly different lipid composition of hypercholesterolemic versus simply hypothyroid nascent lipoproteins suggests that cholesterol esters can successfully substitute for

triglyceride in the hydrophobic core of the nascent lipoproteins (95). In a recent report, Davis et al. (95) demonstrated that the neutral lipid composition within the hepatocyte (which is proportionately higher in cholesteryl ester during cholesterol feeding) largely dictates the core lipid composition of de novo synthesized VLDL.

Discoidal lipoproteins observed in incubations of hypercholesterolemic rat hepatocytes no doubt arise as a result of the excess cholesterol present within these cells. These abnormal lipoproteins were not detected in incubations of hypothyroid or normal rat hepatocytes. The dimensions of these particles (227 X 54 Å) (Table XIII) closely resemble those for the discoidal HDL present in the plasma of hypercholesterolemic guinea pigs (252 X 51 Å) (203). Compared to normal plasma HDL, the discoidal lipoproteins secreted by the hypercholesterolemic hepatocytes are relatively deficient in core lipids and are apo-E - rich rather than being spherical and having apo-AI as their major apolipoprotein (Table XIII and Fig. 35). The plasma of patients with LCAT deficiency contain similar apo-E - rich discoidal HDL (5, 409). Such particles are also secreted by isolated rat livers in the presence of an LCAT inhibitor (205). The LCAT enzyme is believed to be responsible for the conversion of nascent discoidal HDL to spherical HDL, and this enzyme has been shown to catalyze this conversion in vitro (5). These studies conducted on discoidal HDL from LCAT deficient patients demonstrated that LCAT action not only gave rise to cholesteryl esters but elicited the acquisition of apo-AI by the HDL (probably from the $d > 1.25$ $g.mL^{-1}$ protein fraction) and a concomitant loss of apo-E (5).

As the secretion of LCAT by suspended rat hepatocytes has been shown (410), it is reasonable to assume that incubations of normal and hypothyroid rat hepatocytes secrete nascent HDL which can be efficiently modified by this enzyme. Felker et al. (323) had shown that normal isolated perfused livers

secreted a significant proportion of the total apo-AI as a lipid- free particle, much as was observed in this study (Fig. 26). Addition of the LCAT inhibitor (DTNB) in the former study resulted in the secretion of discoidal apo-E - rich HDL with a significantly larger proportion of the apo-AI present as a lipid - free particle in the $d > 1.21$ $g.mL^{-1}$ fraction of the liver perfusate (323). These findings are consistent with the concept that apolipoprotein AI is acquired by normal nascent HDL from the interaction of nascent HDL with either LCAT and apo-AI or a complex of both (205, 411).

As the incubation medium contains no LCAT activity per se, the occurrence of discoidal particles can result from one of the following possibilities. Firstly, a lower than normal LCAT output by hypercholesterolemic rat hepatocytes could account for the observations, however, Bosisio et al. (412) failed to observe any marked changes in the serum cholesterol - esterifying capacity in the intact hypercholesterolemic animal. Therefore it is possible that in hypercholesterolemic rat hepatocyte suspensions, the capacity of the LCAT enzyme is exceeded as a result of the exorbitant cholesterol load. As discoidal particles were not observed in the sera of intact fasted hypercholesterolemic rats, the plasma LCAT levels may be sufficiently high (as a result of the additional contribution to LCAT levels by other tissues such as intestinal mucosa and adrenal) (4) to permit the conversion of these particles to spherical HDL.

Secondly, the excess of free cholesterol secreted by these hepatocytes may evoke a functional deficiency of LCAT by virtue of the higher cholesterol : phospholipid molar ratios in the discoidal HDL (413). The molar ratios of unesterified cholesterol to phospholipid of the spherical lipoproteins in fractions 1 and 2 from 24 hour incubations of normal and hypothyroid rat hepatocytes are 0.6 and 0.5 respectively whereas the corresponding ratio in nascent hypercholesterolemic lipoproteins is 1.4 (Table XIV). A molar ratio of

free cholesterol to phospholipid of 1 : 1 or greater has been shown to completely inhibit LCAT activity in vitro (413). As a result, a functional deficiency of LCAT activity would be expected for the particles having a high ratio and such a mechanism is thought to give rise to the discoidal HDL (cholesterol to phospholipid 2 : 1) that accumulate in the sera of hypercholesterolemic guinea pigs (203). These observations are consistent with the concept that the disc - to - sphere conversion of HDL is retarded in incubations of hypercholesterolemic rat hepatocytes by virtue of the substrate composition. However, nascent hypercholesterolemic lipoproteins secreted into fraction 1 are spherical despite their high free cholesterol to phospholipid molar ratios. These lipoproteins have significantly more core lipids than the discoidal particles of fraction 2 (Table XIII) and thus it would appear that the amount of non- polar lipid present is an important factor in determining particle morphology.

Thirdly, it is possible that a deficiency of fatty acids for cholesterol - esterification occurs within hypercholesterolemic cells incubated in the lipid - deficient medium. Intracellular ACAT activity in hepatocytes is increased during cholesterol feeding (273, 414) which therefore places a demand for cellular fatty acids. As hepatocytes incubated under the conditions described here are dependent on endogenously synthesized fatty acids for substrate, the rate of synthesis could limit cholesterol esterification by ACAT. Therefore despite the increased cholesterol load the amount of cholesteryl ester synthesized as VLDL would be less than optimal. This possibility is indicated in our studies by the lower proportion of cholesterol ester with respect to total cholesterol secreted as VLDL-like particles by the hypercholesterolemic hepatocytes compared to the particles secreted by the isolated liver (120). Similarily, in the guinea pig the inherently low liver ACAT activity may be partly responsible for the high proportion of

unesterified cholesterol secreted as discoidal HDL (415).

The difference in agarose gel electrophoretic profiles of nascent versus serum lipoproteins (Fig. 30) is probably related to the difference in apolipoprotein composition, as the latter largely confers the net charge on the particle. However, there was no significant qualitative difference between the nascent lipoproteins secreted by the three groups of hepatocytes which suggests that a similar proportion of the apolipoproteins contribute to the lipoproteins' overall charge, despite differences in lipid composition. Hypothyroid and hypercholesterolemic nascent lipoproteins secreted into fraction 1 had less charge heterogeneity than the normal lipoproteins. The former lipoproteins exhibited heavy lipid stainable bands migrating as fast pre-β.

Lipoproteins secreted into fraction 2 of the incubation medium by all three groups of hepatocytes exhibited β-mobility on agarose gel electrophoresis, despite significantly different lipid compositions between the nascent hypercholesterolemic and hypothyroid or normal lipoproteins. The β-migration of the discoidal lipoproteins secreted into fraction 2 by hypercholesterolemic hepatocytes has also been observed for similar particles in human cholestasis (190) and the less intense staining of the observed band on agarose gel is typical of lipoproteins containing predominantly polar lipids (205).

Apolipoprotein secretion and distribution amongst nascent lipoproteins can be altered by dietary or hormonal manipulation (95, 120, 334, 416, 417). As apolipoproteins essentially function as metabolic programmers for the lipoprotein particles, their distribution amongst nascent lipoproteins is clearly significant with respect to the subsequent metabolism of these particles.

In the present study, apo-E and apo-AI were secreted by normal rat hepatocytes at a rate that closely resembled the secretion rates observed by

Dashti et al. (331) for hepatocyte monolayers incubated for similar periods of time. The latter group employed ultracentrifugation to isolate the nascent lipoproteins and a significant proportion of apo-E (65%) was found to be secreted as a lipid - free particle. In the present study, gel filtration of the nascent lipoprotein species revealed that only 45% of the total apo-E was secreted as a particle of low lipid content (fractions 3b,4 and 5). Therefore, up to 20% of the total apo-E found in the $d > 1.21$ g.mL^{-1} fraction in the study by Dashti et al. (331) could have resulted from ultracentrifugation, an observation previously reported by this laboratory (126).

Both apo-B and E secretion rates were significantly increased in incubations of hypercholesterolemic rat hepatocytes, paralleling the increased secretion of lipid (Table X, Table XII). After 24 hours of incubation the secretion rate for these apolipoproteins decreased. It is possible that the decreased secretion rate after a longer period of incubation simply reflects the hepatocytes' reduced need to secrete lipoproteins at such an accelerated rate once removed from the high serum lipid levels.

Apo-AI secretion by hypercholesterolemic rat hepatocytes was slightly but not significantly increased, yet the serum levels of this apolipoprotein were elevated in the intact animals. As the liver only contributes approximately 44% of the total serum apo-AI (308), it would appear that either the intestinal secretion rate of apo-AI is increased in PTU - cholesterol feeding or that decreased removal of apo-AI - containing lipoproteins occurs to account for the elevated serum levels. Riley et al. (418) did not observe any increase in the total amount of apo-AI secreted by the intestine in cholesterol - fed rats. However, unlike the studies described here, Riley et al. (418) did not induce hypothyroidism, a condition which is known to increase the cholesterol absorption by the intestine (265).

The secretion rates of apolipoproteins B, E and AI by hypothyroid rat

hepatocytes was not significantly different from normal rat hepatocytes. Serum levels of apo-AI in the intact animals were identical to normal, whereas apo-B and E levels were increased. Since the liver is the primary source of serum apo-B and E (308) and the observed secretion rates by hypothyroid rat hepatocytes are normal, it follows that there is a defective removal of apo-B and E - containing lipoproteins from the circulation of hypothyroid animals.

Serum levels of apo-AI in hypothyroid, chow - fed rats may be subject to differences in strain response to the degree and duration of hypothyroidism. This is suggested by the observation that no change in apo-AI levels in our animals occurred whereas Dory et al. (335) reported increased apo-AI levels in their albino rats maintained on the same regimen. However, the Long - Evans rats used in the present study demonstrated elevated levels of apo-AI after being administered PTU for an extended period of time (419). On the other hand, Wilcox et al. (420) noted that mild hypothyroidism had no effect on rat plasma apo-AI levels.

The distribution of the apolipoproteins amongst the nascent lipoproteins secreted by the hepatocytes was clearly affected by the dietary and hormonal manipulations. Hypercholesterolemic rat hepatocytes secreted proportionately more of the total apo-B and E associated with larger lipoprotein particles than normal rat hepatocytes. A similar observation was made by Patsch et al. (416) who noted a redistribution of apo-B and E into larger lipid - rich lipoproteins after incubating hepatocytes in the presence of added fatty acid. Conversely, when triglyceride and phospholipid secretion was acutely inhibited by insulin treatment, it was noted that proportionately more apo-B and E was secreted either associated with smaller particles or essentially lipid - free (417). This apparently lipid - dependent redistribution of the apolipoproteins was demonstrated in the present study. Significantly less of the total apo-E was secreted into the medium as a lipid

- free particle (26% in fractions 3b,4 and 5) by hypercholesterolemic rat hepatocytes than by normal cells (44%), consistent with earlier studies of nascent lipoprotein secretion by isolated perfused livers (120). Similarily, the increased secretion and redistribution of apo-B towards larger lipoprotein particles in these hepatocyte incubations is in agreement with the increase in the absolute amount of triglyceride secreted when compared to normals and supports the concept that apo-B is essential for the transport of triglyceride out of the liver (109). There was only a slight increase in triglyceride secretion by hypothyroid rat hepatocytes with no significant increase in apo-B secretion, although a redistribution of the apolipoprotein towards larger lipoprotein particles was observed. This suggests that sufficient apo-B is secreted by hypothyroid, chow - fed rat hepatocytes to accomodate the relatively small increase in triglyceride secretion with the result that larger lipoproteins are secreted. The distribution of apo-E, in contrast, appeared to be more sensitive to hormonal alterations. The hypothyroid rat hepatocytes secreted proportionately more apo-E in association with lipoprotein complexes and less as a lipid - poor particle (Fig. 26). The absolute amount of apo-E secreted by these cells was not significantly different to that secreted by normal hepatocytes. This is of interest as there is evidence to indicate that total liver protein synthesis is reduced in hypothyroidism (421). As only the secretion of apolipoproteins into the medium was measured in this study, it is not possible to ascertain whether or not the normal output of apo-E by hypothyroid rat hepatocytes resulted from active synthesis or simply additional secretion from a large intracellular pool. Lin-Lee et al. (290) observed an increase in total liver cell apo-E mRNA in cholesterol fed rats despite concomitant hypothyroidism. In hypercholesterolemia much of the newly synthesized apo-E was not actively secreted into the medium but expanded the hepatocyte intracellular pool of this

apolipoprotein (290). The higher proportion of radioactive amino acid incorporation into apo-E versus apo-B in lipoproteins secreted by hypercholesterolemic hepatocytes compared to normal (Fig. 35) supports the concept of a more active synthesis of the former protein. The data presented here suggests that the highly elevated apo-E secretion rates in hypercholesterolemic rat hepatocyte incubations probably reflect an increase in apo-E biosynthesis whereas in hypothyroid rat hepatocytes no significant increase in apo-E biosynthesis occurs.

Hepatocytes from all three groups of rats studied secreted apo-AI at similar rates and primarily as a particle with a Stokes radius smaller than albumin. Less than 30% of the total apo-AI secreted was associated with larger lipoprotein particles. It is possible that the characteristics of the large nascent lipoproteins are not suitable for apo-AI acquisition. Tall et al. (422) demonstrated that multilamellar liposomes incorporated far less human apo-AI with increasing liposomal molar ratios of cholesterol to phospholipid. The relatively higher free cholesterol content of nascent serum lipoproteins versus serum lipoproteins leads us to suggest that similar principles may govern apolipoprotein acquisition in the rat. This possibility is supported by the observation that a significantly lesser proportion of the total apo-AI secreted by hypercholesterolemic hepatocytes was found to be associated with the large cholesterol-rich lipoprotens when compared to normal or hypothyroid nascent lipoproteins (Fig. 26).

An interesting observation was the similarity in immunodiffusion patterns exhibited by the lipoproteins secreted by the hepatocytes of the three groups of rats (Figs. 36 and 37). Reactions of identity between apo-B and apo-E for lipoproteins in fraction 1 would indicate that both these apolipoproteins reside on a single particle. (The levels of apo-AI in these fractions were too low to permit similar studies.) In contrast, the reaction of partial identity

for lipoproteins in fraction 2 with a spur extending towards apo-B infers that at least a portion of the apo-E is secreted as Lp-E, i.e. with apo-E as the sole apolipoprotein present on these particles. To our knowledge the secretion of an Lp-E particle has not previously been reported. The observation that the patterns for nascent lipoproteins from the three groups of rats are the same, would indicate that the basic mechanisms of lipoprotein assembly and secretion are identical despite the variant amounts of lipid secreted per particle.

A faint reaction indicating the presence of apo-C in fraction 3a from incubations of rat hepatocytes (Fig. 37) is probably analogous to the single reaction of partial identity observed for ultracentrifugally isolated HDL (Fig. 21). The secretion of apo-C in association with nascent HDL had been proposed by Hamilton et al. (328) and is present in HDL secreted by perfused normal rat livers (320). This may provide a source of apo-C for nascent VLDL within the space of Disse. The relative deficiency of apo-C observed in VLDL secreted by hypercholesterolemic rat livers versus normal rats, then, (120) could be the result of a defective acquisition of HDL- apo-C by the nascent VLDL.

In summary, under conditions of hypothyroidism and cholesterol feeding, the liver cell secretes increased amounts of apo-B, E and lipid (especially cholesterol) as large abnormal lipoprotein complexes. In conditions of mildly elevated lipid secretion (hypothyroidism) more apolipoprotein (apo-E) may be required for the assembly of larger particles having relatively normal composition. This would cause an increased proportion of apolipoprotein (especially apo-E) to be associated with these lipoproteins, suggesting that the lipid - poor apolipoproteins secreted by the hepatocytes represents an "excess" pool of apolipoprotein. Such a mechanism of apolipoprotein acquisition by nascent lipoproteins during their intracellular assembly would not necessitate the increased biosynthesis and secretion of the apolipo-

proteins per se. The lipid - free apolipoprotein secreted by the hepatocytes may also serve as a readily available pool of serum apolipoproteins that can associate with enzymes (e.g. LCAT) and lipoproteins (e.g. chylomicrons) to mediate the degradation and cellular uptake of the latter. As both apo-B and E have been implicated in receptor - mediated uptake of lipoproteins by hepatic and extrahepatic tissues (107, 423), it is likely that higher proportions of these apolipoproteins would be required for nascent lipoproteins when the hepatocytes are secreting higher amounts of lipid. Thus, the apo-B and apo-E would elicit the removal of the lipoprotein remnants from the circulation or would confer stability to the more lipid - rich particles.

The presence of cholesterol - rich, β - migrating lipoproteins in simple hypothyroidism does not arise from the direct hepatic secretion of these particles but may be derived either from VLDL or chylomicron metabolism. Their accumulation may result from impaired removal, possibly as a consequence of decreased lipoprotein - receptor number. In dietary - induced hypercholesterolemia with hypothyroidism, in contrast, the liver responds to the increased cholesterol load by secreting cholesterol - rich particles which contribute directly to the accumulating serum pool of these lipoproteins, a condition which is only exacerbated by the concomitant thyroid deficiency.

SUMMARY

1) Separation of the lipoprotein secretory products of isolated hepatocytes by gel filtration indicates that the liver cell secretes apolipoproteins in association with large lipoprotein complexes as well as in essentially lipid - free forms. This is one of the first demonstrations that essentially lipid - free apolipoproteins not arising from ultracentrifugation are secreted by isolated hepatocytes.

2) The secretion rates of apo-B, E and AI by hepatocytes from euthyroid, hypothyroid and hypercholesterolemic rats were determined. All three apolipoproteins were secreted at higher rates by hypercholesterolemic hepatocytes. No significant difference was observed for hypothyroid hepatocytes compared to normal.

3) Total lipid secretion by the three groups of hepatocytes was determined and was found to be markedly elevated in the hypercholesterolemic hepatocytes, whereas only a slight increase in triglyceride and phospholipid secretion was observed for hypothyroid hepatocytes compared to normal.

4) Lipid compositional analysis of the large nascent lipoprotein complexes secreted by the hepatocytes indicated that the hypercholesterolemic hepatocytes secreted abnormal, cholesterol - rich particles whereas the composition of the hypothyroid and normal nascent lipoproteins was similar.

5) The hypercholesterolemic hepatocytes secreted a discoidal, apo-E - rich lipoprotein which was visualized by electron microscopy. Such particles were not detected in electron micrographs of nascent lipoproteins isolated from incubations of normal or hypothyroid hepatocytes.

6) Fractionation of the nascent lipoprotein products by gel filtration

revealed the redistribution of apo-B and E from hypercholesterolemic hepatocytes and apo-E from hypothyroid hepatocytes to larger lipoproteins compared to normal apolipoprotein distributions.

7) Evidence is provided to suggest that apo-C is secreted by the hepatocytes as an HDL - like particle and that apo-E can be secreted as an LP-E lipoprotein.

CONTRIBUTION TO KNOWLEDGE

The author considers all of the findings outlined in the summary as original contributions to the understanding of lipoprotein metabolism.

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