THE METABOLISM OF LIPOPROTEINS IN PRIMARY CULTURES OF RABBIT HEPATOCYTES

by

Patricia A. Soltys

A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon
Health Sciences Center
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
June 1980

APPROVED: [redacted]

. . . . . .

(Professor in Charge of Thesis)

[redacted]

. . . . . .

(Chairman, Graduate Council)

AN ABSTRACT OF THE THESIS OF

Patricia A. Soltys for the Ph.D. degree in Biochemistry

Date of receiving this degree: June 1980

Title: The Metabolism of Lipoproteins in Primary Cultures of Rabbit Hepatocytes

Approved . . . . . .

(Professor in Charge of Thesis)

The primary aim of this project was to examine lipoprotein catabolism in liver to determine if the liver has a significant role in low density lipoprotein (LDL) catabolism. A second aim was to examine the suitability of ^{125}I-labelled-apoproteins as a substrate for degradation studies in liver since significant amounts of deiodinase activity are present in that tissue.

Primary cultures of rabbit hepatocytes were developed to study lipoprotein catabolism at the cellular level. There are fewer uncontrolled variables in this system than in in vivo studies or in liver perfusion studies. Cultured cells also offer the advantage of allowing one to study several substrate concentrations at varying incubation times in cells from a single animal, thus eliminating interanimal variability and conserving animals. The technique of primary culture also allowed the cells to regenerate microvilli. These are removed by the cell isolation procedure. The ability of the cells to regenerate microvilli was another indication of cell viability in addition to trypan blue exclusion, adherence to the culture dish, and the ability to synthesize lipoproteins. The repair of surface damage is also an important prerequisite for studies involving binding to that surface.

After a 20-hour incubation in lipoprotein-deficient medium, the cultured hepatocytes bound, internalized, and degraded ^{125}I-LDL. The binding was examined and both high-affinity and low-affinity components were present. The high-affinity site was further characterized and found to have a K_m of 1.59×10^{-9} M at 37°C and a K_d of 3.97×10^{-9} M at 4°C. The K_d agreed with the value for LDL

binding in isolated porcine plasma membranes reported by Bachorik et al. (Biochem. Biophys. Res. Commun., 1979, 69, 927-935).

Comparison of two substrates, ^{3}H-leu-LDL and ^{125}I-LDL, showed no differences in binding to the specific high-affinity site and no difference in degradation rate as determined by appearance of either ^{3}H-leu or ^{125}I-tyrosine in the culture medium. Similarly, competition studies of ^{125}I-LDL with either native LDL or ^{127}I-LDL detected no difference between the native or nonradioactive iodinated LDL as competitor.

The putative LDL receptor was induced by preincubating the cells for 20 or 40 hours in lipoprotein-deficient medium before testing for high-affinity binding. Clearance studies revealed that hepatocytes, preincubated in lipoprotein-deficient medium, clear 40 times more (by volume) LDL than sucrose from the medium. It was also shown that, even after incubation in the presence of 40 μg of LDL/ml of medium, hepatocytes clear more LDL than sucrose. It was calculated from this data that receptor-depressed hepatocytes could account for as much as 56.9% of the LDL catabolism that occurs in vivo, thereby substantiating the view that the liver has an important role in LDL catabolism. A 50:1 molar excess of high density lipoprotein to LDL decreased binding, internalization, and degradation of LDL after 3 and 24 hours but not by a simple competition for binding sites since the binding after 0.2 hours was unaffected.

The addition of lipoproteins to the incubation medium increased hepatocyte cholesterol content, as measured by thin-layer chromatography, by a greater amount than could be accounted for by the amount of

cholesterol in the lipoproteins taken up by the cells. Further study of this phenomenon with lipoproteins labelled with both ^{125}I-apoprotein and ^{14}C-cholesterol clearly showed that a larger proportion of LDL free cholesterol than apoprotein was associated with the hepatocytes. It was not unequivocally established whether this cholesterol uptake represented only exchange or whether it also included net cholesterol uptake, but the increase in total cell free cholesterol strongly suggests that net cholesterol uptake can occur other than by uptake of intact lipoproteins.

Finally, in vivo studies showed that hyperlipemic LDL was catabolized faster than control LDL in both hyperlipemic and control rabbits. In vitro, 1.4 times more hyperlipemic LDL was internalized ($p < 0.001$) and degraded ($p < 0.05$) than control LDL thereby indicating that the liver may play a significant role in the development and control of hyperlipemia.

ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. Oscar W. Portman, for the many helpful and stimulating suggestions he has offered and the long and interesting discussions we have held during the course of this project. His support and understanding throughout a rather extended period of illness is particularly appreciated. I also want to thank the members of his laboratory--Manfred Alexander, Jeanne Brandt, Carol Jones, Hilda Justice, Jean O'Malley, Ofelia Svart--whose help and understanding have greatly contributed to the completion of this project.

Additionally, I want to acknowledge a few of the many people at the Oregon Regional Primate Research Center whose comments and advice have been very helpful. In particular, I thank Dr. Wolf Fahrenbach for his help with the scanning electron microscopic examinations of hepatocytes, Dr. Funan Hu for her advice and encouragement in developing liver cell cultures, and John Ochsner for help in acquiring the surgical skill to successfully remove and perfuse rabbit livers. There are many others who gave encouragement, advice, and help including Isabel McDonald, Kathy Kerr, Harry Wohlsein, and Janina Ely, to name only a few.

I am indebted to Margaret Shetler whose typing skill has made this dissertation a reality. She deciphered my handwriting without complaint.

Finally, I want to thank my entire family for the patient and understanding support they have given me through the many years that I have spent completing this work.

TABLE OF CONTENTS

	Page
Acknowledgments	ii
List of Tables	ix
List of Figures	xi
I. Statement of Problem	1
II. Background	2
A. Lipoproteins: General Considerations	2
1. Brief Historical Overview	2
2. Classification and Nomenclature	5
3. Chemical and Physical Properties	8
a. Composition	8
b. Apoprotein Structure and Function	11
c. Structural Models of Lipoprotein Particles	17
4. Metabolism of Lipoproteins	20
a. LpA	21
b. LpC	23
c. LpB	24
d. LpE	30
B. Cholesterol Metabolism and the Liver: Effects of Lipoproteins	32
1. Cholesterol Metabolism in the Liver: An Overview	32
2. The Liver and Lipoproteins	37

a. Synthesis 37

b. Degradation 39

c. Isolated Hepatocytes 44

1) Suspension Studies 45

2) Primary Culture Studies 48

3) Other Hepatocyte Preparations 53

C. The Low Density Lipoprotein Receptor 56

III. Materials and Methods 63

A. Animals 63

1. Lipoprotein and Serum Donors 63

2. Liver Donors 63

3. In Vivo Metabolic Studies 63

B. Preparation of Lipoproteins and Lipoprotein-deficient Serum 64

1. General Methods 64

2. Low Density Lipoprotein Isolation 65

3. High Density Lipoprotein Isolation 66

4. Lipoprotein-deficient Serum 67

5. Isotopic Labelling of Low Density Lipoprotein . 68

a. Iodination Method 68

b. Incorporation of ^{3}H-Leucine 70

C. Cell Cultures 70

1. Isolation of Hepatocytes 70

2. Establishment of Primary Cultures 73

3. Lipoprotein Uptake and Degradation 74

a. Degradation 74

b. Bound and Internalized 75

D. Analytical Procedures 77

1. General Protein Methods 77

2. General Lipid Methods 79

3. Determination of Radioactivity 80

4. Determination of Specific High-affinity and Low Density Lipoprotein Binding 81

5. Determination of the Apparent Equilibrium Constant for Low Density Lipoprotein Binding . 81

a. Scatchard Analysis 82

b. Determination of Rate Constants 83

6. Uptake of ^{14}C-Sucrose 84

7. Electron Microscopy 85

8. Immunologic Identification of Apolipoprotein B 85

9. Binding of Free $^{125}I^-$ to Serum and Hepatocyte Proteins 86

IV. Results 87

A. Characterization of Low Density Lipoprotein 87

1. Composition of Control and Hyperlipemic Low Density Lipoprotein 87

2. Characterization of ^{125}I and ^{3}H Label in Low Density Lipoprotein 88

a. ^{125}I-Low Density Lipoprotein 88

b. ^{3}H-leu-Low Density Lipoprotein 91

3. Proteolytic Enzyme Degradation of Low Density Lipoprotein 92

B. Characterization and Morphology of Hepatocyte Primary Cultures 98

1. Trypan Blue Exclusion 98

2. Cell Attachment 100

3. Lactic Dehydrogenase Release 102

4. Presence of Kupffer Cells 103

5. Retention of Liver Specific Functions 103

6. Surface Morphology 107

C. Characterization of ^{125}I-Low Density Lipoprotein Apoprotein Binding, Internalization, and Degradation in Primary Cultures of Hepatocytes 110

1. Effects of Substrate Concentration and Incubation Time 110

2. Determination of High-affinity Binding 113

3. Control Incubations 115

4. Determination of the Apparent Equilibrium Dissociation Constant 115

5. Investigation of the Effects of Iodination on Low Density Lipoprotein Metabolism in Hepatocytes 123

a. Dilution with Native Low Density Lipoprotein or ^{127}I-Low Density Lipoprotein . . . 123

b. Comparison of ^{3}H-Leu-Low Density Lipoprotein and ^{125}I-Low Density Lipoprotein . . 123

6. Additional Evidence for Specific High-affinity Low Density Lipoprotein Receptors in Hepatocytes 132

a. Demonstration of Specific High-affinity Binding 132

b. Comparison of the Rates of Internalization of Low Density Lipoprotein, Sucrose, and Dextran 133

7. Comparison of Homologous Low Density Lipoprotein Versus Heterologous Low Density Lipoprotein . . 137

D. Nutritional Effects on Low Density Lipoprotein Metabolism 138

1. In Vivo Studies 138

2. In Vitro Studies 145

E. Effects of High Density Lipoprotein on ^{125}I-Low Density Lipoprotein Catabolism in Hepatocytes . . . 147

F. Some Lipoprotein Effects on Hepatocyte Cholesterol Content 148

G. Fate of Bound Apoproteins: Pulse Studies 152

1. Experimental Design 152

2. Hepatocyte Uptake of Low Density Lipoprotein . 153

a. Bound 154

b. Internalized 154

3. Formation of Inorganic ^{125}I from ^{125}I Low Density Lipoprotein by Hepatocytes 155

4. Alteration of the Density of ^{125}I-Low Density Lipoprotein Released from Hepatocytes 158

V. Discussion 162

A. Choice of Hepatocytes 162

B. Suitability of ^{125}I-Low Density Lipoprotein as a Substrate 163

C. Specific High-affinity Binding of Low Density Lipoprotein by Hepatocytes 166

D. Low-affinity Binding of Low Density Lipoprotein by Hepatocytes 169

E. Compositional Differences in Hyperlipemic and Control Low Density Lipoprotein 173

F. Effect of Nutritional Differences on Low Density Lipoprotein Metabolism by Liver and Hepatocytes . . 173

G. Cholesterol Content of Hepatocytes 179

VI. Summary and Conclusions 182

References 183

List of Commercial Suppliers 217

Abbreviations 219

LIST OF TABLES

Page

1. Hyperlipoproteinemias 4
2. Relation between Nomenclature and Properties of Human Plasma Lipoproteins 8
3. Amino Acid Composition (in mole percent) of Human Low Density Lipoproteins 13
4. Molecular Weights of Apoprotein-B Subunits of Human Plasma Low Density Lipoprotein as Determined by Various Methods . 15
5. Percent Composition of Low Density Lipoprotein 87
6. Comparison of Control and Hyperlipemic Low Density Lipoprotein Apoproteins by 7.5% SDS-Polyacrylamide Gel Electrophoresis 89
7. Distribution of Radioactivity after ^{125}I-LDL Was Passed through a Sephadex Column in the Presence or Absence of Excess Iodide Ion 91
8. Effect of Whole Serum and Lipoprotein-deficient Serum on Cell Attachment 101
9. The Effect of Increasing Lipoprotein-deficient Serum Concentrations on Cell Attachment 102
10. Incorporation of ^{3}H-Leucine into Protein by Primary Cultures of Rabbit Hepatocytes 105
11. A Comparison of the Estimates of Endocytosis of ^{14}C-Sucrose, ^{14}C-Dextran, and ^{125}I-Low Density Lipoprotein 135
12. A Comparison of the Estimates of Endocytosis of ^{14}C-Sucrose and ^{125}I-LDL in Repressed and Derepressed Cells 137

13. Comparison of Human and Rabbit LDL as % Composition . . . 138

14. Comparison of Relative Amounts of Binding, Internalization, and Degradation of ^{125}I-Low Density Lipoprotein (^{125}I-LDL) Apoprotein when Hepatocytes Were Incubated with ^{125}I-LDL from Control or Hyperlipemic Donors 146

15. Effect of High Density Lipoprotein (HDL) on Low Density Lipoprotein (LDL) Binding and Internalization 147

16. Cholesterol Content of Hepatocytes 151

17. Selectivity of Cholesterol Uptake from Low Density Lipoproteins (LDL) or High Density Lipoproteins (HDL) by Isolated Hepatocytes 152

18. Changes in Distribution of Radioactivity with Time Following a 30-minute Pulse with ^{125}I-Low Density Lipoprotein . 155

19. Noniodide Trichloroacetic Acid-soluble (TCA-soluble) Radioactivity (amino acids and small peptides) and Total Trichloroacetic Acid-soluble Radioactivity Released into the Medium by Hepatocytes at 0.2, 3, and 24 Hours 157

20. The Quantity of ^{125}I Released from Hepatocytes and the Distribution of Radioactivity between Trichloroacetic Acid-Soluble Material and Protein That Was More or Less Dense Than 1.063 159

LIST OF FIGURES

Page

1. In Vivo Incorporation of ^{3}H-Leucine into Rabbit Lipoproteins 71
2. Degradation of ^{125}I-LDL by 0.1% Pronase 94
3. Average Rates of Degradation of ^{3}H and ^{125}I-LDL 95
4. Model Enzyme Degradation of LDL 97
5. Hepatocytes 99
6. ^{3}H-Leucine Incorporation into Lipoproteins 106
7. Hepatocyte Surface 108
8. Hepatocyte Surface 109
9. Bound, Internalized, and Degraded ^{125}I-LDL 112
10. LDL Specific Binding to High-affinity Receptors 114
11. Scatchard Plot (3 hours, 37°C) 116
12. Concentration-dependent Binding (4°C) 118
13. Scatchard Plot of LDL Binding 119
14. Time Course of ^{125}I-LDL High-affinity Binding 121
15. $t_{1/2_{(assoc)}}$ Versus ^{125}I-LDL Concentration 122
16. Binding, Internalization, and Degradation of ^{125}I-LDL Diluted with Either Native LDL or ^{127}I-LDL 124
17. Comparison of ^{125}I-LDL and ^{3}H-Leu-LDL Metabolism by Hepatocytes 126
18. Metabolism of ^{3}H-Leu-LDL and ^{125}I-LDL after 24 Hours . . . 129
19. High-affinity Binding of LDL 134
20. Competition of ^{125}I-LDL Binding (Homologous Versus Heterologous Competition) 139

21. Binding of Heterologous and Homologous LDL 140

22. In Vivo ^{3}H-LDL Protein in Rabbit Plasma 142

23. In Vivo ^{125}I-LDL Metabolism 143

24. The Effect of Cycloheximide on Reutilization of Radioactivity from Injected ^{3}H-LDL 144

25. The Effect of Lipoproteins on Hepatocyte Cholesterol . . . 149

I. STATEMENT OF PROBLEM

The aim of this study was to investigate the role of liver in low density lipoprotein (LDL) apoprotein uptake and degradation. Particular attention was directed to differences in hepatic catabolism of LDL from control and hyperlipemic donors.* The effect of high density lipoprotein (HDL) on hepatic catabolism of LDL was also investigated. Isolated rabbit hepatocytes, grown in primary monolayer culture, were used to investigate these liver-lipoprotein interactions at the cellular level. The importance of understanding the factors which affect the catabolism of LDL is illustrated by studies which link LDL to such serious human diseases as atherosclerosis and coronary heart disease.(315, 316, 352, 370)

*The terms control and hyperlipemic are used to differentiate the LDL obtained from animals fed Purina Rabbit Chow® (control) from the LDL obtained from animals fed chow supplemented with cholesterol and butter (hyperlipemic). Except where specifically noted, the LDL that was used came from control animals.

II. BACKGROUND

A. Lipoproteins: General Considerations

This section will provide a general overview of lipoprotein work as it stands at present. Although the entire field will be scanned, particular attention will be paid to low density lipoproteins as being of greater consequence to the studies undertaken and reported herein.

1. Brief Historical Overview

The existence of protein-lipid complexes in plasma was recognized by early investigators interested in lipid extraction and protein isolation. These complexes were discussed in the classical extraction studies of Schulz,[293] Nerking,[241] and Shimidzu.[300] Considerable data on protein-lipid complexes were accumulated by protein chemists such as Hardy,[152] Haslam,[153] Chick,[70] and Bang.[18] The first preparation comparable to lipoproteins as we currently recognize them was the work of Macheboeuf.[207] He isolated a well-defined plasma lipoprotein by adding ammonium sulfate to horse serum to 50% saturation and then acidifying the unprecipitated mixture to pH 3.9. He thereupon obtained a plasma lipoprotein precipitate containing cholesterol esters. We now know that the fraction isolated by Macheboeuf was HDL.

Lipoprotein research was stimulated during the 1940s by the development and general availability of three major methodological advances: electrophoresis, large scale fractionation procedures, and ultracentrifugation. In 1941, Blix et al. showed that

electrophoretic techniques separated the lipid-transport fractions of plasma into α and β-migrating globulins.(37) Using this technique Macheboeuf and Rebeyrotte showed that the acid-precipitable lipoprotein was an α-globulin.(208) About this same time Cohn and associates developed the first practical procedure for the large-scale preparation of the plasma lipoproteins for analysis.(150) They precipitated and separated the serum lipids into two fractions using ethanol-water mixtures; one fraction had α-mobility and the other β-mobility. The fractions differed in molecular size, shape, and lipid composition. Finally, and of particular importance, was the development of an ultracentrifugal flotation separation of plasma lipoproteins by Gofman and co-workers.(82, 137) These studies led to the subdivision of plasma lipoproteins into density classes.

Interest in the lipoprotein field increased dramatically during the 1950s with the realization that elevated plasma lipoprotein levels correlated with the appearance of cardiovascular disease.(136, 180, 181) Illustrative of this increased interest is the observation of Oncley and Harvie that between the years 1952 and 1962 the number of publications referring to lipoproteins increased from 40 to 300 per year.(252) During this period, much effort was directed toward measuring concentrations of plasma lipoproteins and their lipid components and relating these concentrations to clinical disease.

Dangerfield and Smith developed the use of paper electrophoresis for separating plasma lipoproteins,(77) and this technique was used in the early 1960s to categorize the clinical spectrum of plasma lipoprotein abnormalities.(197) This system was the basis of the

classification of hyperlipoproteinemia into five phenotypes as shown in Table 1.[126] In the past ten years it has become apparent that each of these phenotype patterns may result from a number of different biochemical defects in lipid metabolism.

TABLE 1

Hyperlipoproteinemias

	Observed Change in Lipoprotein Fraction			
Type	Chylomicron	LDL[a]	VLDL[a]	Floating β-Lipoprotein[b]
I	increased	--	--	--
IIa	--	increased	--	--
IIb	--	increased	increased	--
III	--	--	--	increased
IV	--	--	increased	--
V	increased	--	increased	--

[a]LDL = low density lipoprotein; VLDL = very low density lipoprotein.

[b]Floating β-lipoprotein is a lipoprotein with β electrical mobility that floats at VLDL densities.

In 1955, several authors independently described the precipitation of lipoproteins at neutral pH by sulfated polysaccharides.[30, 60, 253] The precipitate is an insoluble complex (lipoprotein-polyanion-cation) that is formed in the relative order of chylomicron > very low density lipoprotein (VLDL) > LDL > HDL. The ease of formation of complex is related to the protein:lipid ratio rather than the lipid composition of individual lipoproteins. (See Burstein and Scholnick for a good review of this area.[61])

This procedure permits the rapid isolation of lipoprotein fractions from a large volume of serum without contamination by other serum proteins and without the need for prolonged ultracentrifugation.

In the early 1970s, Rudel et al. developed the technique of separating individual lipoprotein classes on an agarose column after harvesting the entire lipoprotein fraction at a density of 1.225 g/ml.(283) Three major classes are obtained from the column. The chemical, immunological, electrophoretic, and electron microscopic characterizations of the properties of these column-isolated lipoproteins were compared with similar characterizations of ultracentrifugally prepared lipoproteins. By each criterion, peak-I lipoproteins were the same as VLDL, peak-II lipoproteins were the same as LDL, and peak-III lipoproteins were the same as HDL. In addition to eliminating several of the days required for the separation of lipoproteins by sequential ultracentrifugation, this method records the size distribution of each of the plasma lipoprotein fractions.

The term apoprotein was first used by Oncley in reference to a given lipoprotein-protein.(251) By the mid-1960s the apoproteins began to be the focus of serious chemical and physical characterization. As will be discussed in the following sections, it was discovered that the apoproteins were quite heterogeneous and that a single density class of lipoproteins contains multiple apoprotein components.

2. Classification and Nomenclature of Lipoproteins

Serum lipoproteins are complex particles of protein and lipid held together by noncovalent bonds. The apoprotein is defined by Osborne and Brewer as an homogeneous protein, composed of a single

polypeptide chain or several covalently bound polypeptide chains containing no detectable noncovalently bound lipid, that forms an integral part of a lipoprotein particle.[254] Several lipoprotein particles contain more than one type of apoprotein. Apoproteins combine noncovalently with lipid to form water-soluble particles (i.e., lipoproteins) whose major function is to transport lipid through the aqueous medium of plasma.

Three general classification systems have been employed to differentiate the serum lipoproteins. The original and simplest classification system is based on the electrophoretic separation of four major classes of lipoproteins. Paper and agarose are the most commonly employed support media for electrophoresis. Plasma lipoproteins separated by electrophoresis are designated as 1) those which stay at the origin, and those which migrate into 2) the pre-beta (α_2), 3) the beta, and 4) the α_1 zones.[197] Although a quantitative analysis of lipoproteins separated by agarose electrophoresis has been reported,[155] it is generally a qualitative technique.[245] This system is widely employed in clinical laboratories since it permits the rapid identification of altered plasma lipoprotein patterns which are characteristic of certain disease states.

The most widely employed classification system in research laboratories is based on the relatively low hydrated density of the plasma lipoproteins. This property was utilized by Gofman et al. to separate lipoproteins into four major density classes designated chylomicrons, VLDL, LDL, and HDL.[136, 137] These classifications have been further differentiated by defining narrower bands of

densities, and the subdivisions most commonly used today are chylomicron ($d < 0.95$ g/ml), VLDL ($d < 1.006$ g/ml), intermediate density lipoprotein (IDL; $d = 1.006$ to 1.019 g/ml), LDL ($d = 1.019$ to 1.063 g/ml), HDL_1 ($d = 1.063$ to 1.070 g/ml), HDL_2 ($d = 1.070$ to 1.125 g/ml), HDL_3 ($d = 1.125$ to 1.21 g/ml), and very high density lipoprotein (VHDL; $d = 1.21$ to 1.25 g/ml).[254]

The third classification system is based on the apoprotein constituents of the lipoprotein and was proposed by Alaupovic and co-workers in 1962.[2] Using double immunodiffusion, immunoelectrophoresis, and column and affinity chromatography, Alaupovic and co-workers have identified five major families of plasma lipoproteins.[1, 3, 76, 224, 225] These are LpA,* containing polypeptides A-I and A-II; LpB, containing the apolipoprotein B (apo-B) protein(s); LpC, containing polypeptides C-I, C-II, and C-III; LpD, containing apo-D, or the thin-line protein; and LpE, containing apo-E, or the arginine-rich apolipoprotein. While not yet universally adopted, this system of naming lipoprotein apoproteins is gaining wide acceptance. In terms of this concept, apoprotein A (apo-A) refers to the apoproteins that are primarily, but not exclusively, found in HDL. Apo-B is the major apoprotein of LDL, but also comprises about 35% of VLDL protein. Apoprotein C (apo-C) is a major component of VLDL but is also present in HDL. Apo-D and apo-E are found in both VLDL and HDL. Table 2 summarizes the interrelationships of these three classification systems.

In many disease states involving lipids, such as lecithin:cholesterol acyltransferase (LCAT) deficiency, obstructive liver disease,

*The designation "Lp" stands for lipoprotein family.

TABLE 2

Relation between Nomenclature and Properties of Human Plasma Lipoproteins

Properties	Nomenclature			
	Chylomicrons	VLDL	LDL	HDL
Density, g/ml	0.95	0.95-1.006	1.006-1.063	1.063-1.210
Electrophoretic mobility	Origin	Prebeta (α_2)	Beta (β)	Alpha (α_1)
Major apoprotein components	Apo-B Apo-C-I Apo-C-II Apo-C-III	Apo-B Apo-C-I Apo-C-II Apo-C-III Apo-E	Apo-B	Apo-A-I Apo-A-II

type III dyslipoproteinemia, and abetalipoproteinemia, these neatly interdigitating classifications deteriorate into confusion. In LCAT deficiency, for example, VLDL migrates electrophoretically as β-lipoprotein and HDL migrates as α_2 rather than α_1 bands.(133) There is also evidence to suggest that the nutritional status of rabbits affects the electrophoretic mobilities of lipoproteins isolated as VLDL, LDL, and HDL.(322)

3. Chemical and Physical Properties

a. Composition

Chylomicrons are the vehicles by which most dietary triglycerides are transported in the plasma. They are heterogeneous in size ranging in diameter from 500 to 5000 Å. They contain mainly triglyceride with a small fraction of phospholipid (8%), cholesterol

(5%), and protein (1-3%).[170] The protein content of chylomicrons is small and variable. Studies by Kostner and Holasek found the approximate protein composition of lymph chylomicrons to be 66% apo-C, 22% apo-B and 12% apo-A.[185] Schaefer et al. analyzed the apolipoproteins of human chylomicrons and reported the presence of apoproteins A-I, A-II, B, C-I, C-II, C-III, D, E, and albumin.[291] When intestinal chylomicrons are incubated in serum, however, they gain apo-C and lose apo-B. Even though apo-B accounts for less than 1% of total chylomicron mass, it appears to be essential for chylomicron formation since all of the apo-B-containing lipoproteins (chylomicrons, VLDL, and LDL) are absent from the plasma of individuals with abetalipoproteinemia in which apo-B synthesis is defective.[103]

The major transport vehicles for endogeneously synthesized triglyceride are the VLDL particles. They range in size from 280 to 750 Å and the size of the particle is directly related to the triglyceride content and inversely related to the phospholipid and protein content. By weight, VLDL is 8 to 10% protein with the rest of the particle being lipid: 56% triglyceride, 19 to 21% phospholipid, and 17% cholesterol. The ratio of esterified:unesterified cholesterol is approximately 1.[170] Apo-B and apo-C make up 90% of VLDL protein content. The rest of it is composed of apo-E and amounts of apo-A and apo-D comprising less than 1% of the protein content.

In primates, LDL are the principal vehicles by which plasma cholesterol is transported. They range in size from 170 to 260 Å.[125] Chemically, LDL are composed of lipid (75-78%), protein (20-22%), and carbohydrate (3-5%). The lipid portion of the particle

is approximately 50% cholesterol ester, 30% phospholipid, 10% unesterified cholesterol, and 10% triglyceride.[285] Phosphatidylcholine and sphingomyelin account for 65% and 25%, respectively, of the total phospholipids.[170] Traces of other lipids are also found, including much of the plasma α-tocopherol and β-carotene. The major fatty acid components of the lipids are oleic (18:1), linoleic (18:2), and palmitic (16:0).[285] The major apoprotein in LDL is apo-B. The apo-B content averages 98% of the total protein mass in LDL isolated in the 1.019 to 1.063 g/ml density range.[290] A small amount of apo-C (about 5% of the total protein) appears to be associated with lipoprotein that is isolated in the density range 1.006 to 1.019 g/ml (called IDL in current terminology).[196] This study indicates that apo-C associates with lipoproteins having densities of 1.006 to 1.019 g/ml but is not an integral part of the lipoproteins having densities of 1.019 to 1.063 g/ml.

The mass of B protein per LDL particle has been found to remain constant at about 510,000 daltons[232] although the molecular weight of monodisperse human LDL ranges from 2.4×10^6 to 3.9×10^6 daltons.[117] This range of molecular weights for the whole LDL particle is thought to be caused by the variable degree of lipidation of the apoprotein. This variable degree of lipidation is also responsible for the range of densities associated with LDL. A weight average molecular weight of 2.29×10^6 daltons has been reported for rhesus monkey LDL.[119]

The smallest lipoprotein particles are the HDL, having diameters of 90 to 120 Å. While the physiologic significance was unknown as

recently as 1976,[170] recent studies associate low HDL cholesterol levels with coronary artery disease;[67, 144, 230, 279] and Steinberg and co-workers have suggested that HDL may be important in removing cholesterol from tissue.[65, 229] Lipids account for about half of HDL by weight (55% of HDL_2 and 45% of HDL_3). Triglycerides account for 10% of the lipid, cholesterol for 32% (about three-fourths as cholesterol ester), and phospholipids for 42 to 51% of the lipid.[170] Linoleic is the predominant fatty acid of the cholesterol esters.[170] Apo-A-I and apo-A-II comprise 67% and 22%, respectively, of the total protein mass with apo-C peptides making up 5 to 11% of the remainder. Apo-D and apo-E have also been detected in HDL fractions.[290] Recent rat liver perfusion studies indicate that the liver produces a nascent discoidal HDL particle which has an apo-E: apo-A-I ratio of 10:1, while this ratio in the spherical HDL isolated from plasma is 1:7.[109]

b. Apoprotein Structure and Function

This section will briefly survey what is known about the isolated apoproteins. For more detailed information on the structural effects of interactions of these apoproteins with various combinations of lipids, the reader is referred to references 170, 233, 254, and 290.

The amino acid sequences of both of the A apoproteins have been determined although the sequence for apo-A-I as reported by Brewer et al.[47] differs in several positions from the sequence reported by Baker et al.[17] The reason for these differences remains unknown. Apo-A-I has 243 amino acids and is a single polypeptide chain. It

contains a large amount of helical structure, varying somewhat upon the conditions used and the amount of lipid present.[206] It has been reported to activate LCAT.[114, 320] Apo-A-I has been observed to have similar amino acid composition, molecular weight, and circular dichroic spectrum over a wide range of species. This similarity suggests a major structural and perhaps physiological role.[170]

Human apo-A-II consists of two identical polypeptide chains containing 77 residues each and linked through a single disulfide bond at residue 6.[48] The amino acid residues in both of the apo-A peptides are not segregated into polar and nonpolar regions; however, the helical portions of these peptides appear to be amphipathic (i.e., one side of the helix is polar and the other is nonpolar).[295] Such an arrangement would facilitate simultaneous interaction of the protein with nonpolar lipids and an aqueous environment. The dimeric structure of human apo-A-II has so far been found only in the chimpanzee. Edelstein et al. showed that rhesus monkey apo-A-II was a monomeric form without the disulfide linkage.[93] They also reported that preliminary studies of baboon, dog, rabbit, and cow showed similar monomeric structure for apo-A-II. The rat has also been shown to have monomeric apo-A-II.[160] The physiological function of apo-A-II has not yet been established but a structural role in the HDL particle has been suggested by Assmann and Brewer.[9, 10]

The amino acid composition of apo-B (apo-LDL) has been determined in several laboratories (see Table 3) over the last 12 years, and the agreement between these analyses is quite good. Studies to determine the terminal amino acids of the peptide chain(s) of apo-B

TABLE 3

Amino Acid Composition (in mole percent) of Human LDL[a]

Amino acid	Margolis & Langdon (219)	Granda & Scanu (147)	Levy et al. (198)	Chen & Aladjem (69)[b]	Aladjem (69)
Glycine	5.06	4.73	5.15	4.90	5.87
Alanine	6.42	6.68	6.50	6.10	6.73
Serine	8.56	9.45	9.07	8.60	8.39
Threonine	6.68	7.11	6.83	6.40	6.62
Valine	6.44	5.35	5.67	5.40	4.79
Isoleucine	6.42	5.76	5.99	5.70	5.87
Leucine	12.57	12.09	11.73	11.10	12.96
Phenylalanine	5.54	5.25	5.38	5.10	5.17
Tyrosine	3.11	3.46	3.51	3.30	3.55
Methionine	1.90	1.47	1.29	1.20	0.67
Aspartic acid	11.16	11.77	11.19	10.50	11.30
Glutamic acid	13.03	12.63	12.72	11.90	12.48
Arginine	3.34	3.16	3.65	3.50	3.88
Histidine	2.64	2.29	2.60	2.50	4.04
Lysine	7.06	8.71	8.66	8.10	8.13

[a]The content of proline, half-cystine, and trystophane were not determined by Chen and Aladjem so they were left out of this comparison.

[b]These values are from T. P. Bersot, Ph.D. Thesis, Vanderbilt University, p. 25 (1972) as cited in Chen and Aladjem.(69)

---

have demonstrated only 1 mole of glutamic acid per 500,000 molecular weight for the N-terminal.(147, 281, 303) Similarly Shore performed a C-terminal analysis and found only serine present in quantities equalling 1 mole per 500,000 molecular weight; alanine and glycine were present as 0.8 mole per 500,000 molecular weight.(303) In this

study it was not possible to determine if these latter two amino acids represented peptide subunits which do not give a quantitative yield or protein contaminants of the LDL preparation.

There is little agreement concerning the peptide subunit composition and properties of apo-B. One of the major hindrances to the characterization of apo-B has been its insolubility in aqueous solution in the lipid-free state. This has led to the use of various strategies, such as treatment with strong anionic detergents, alkaline pH, denaturants, and chemical modification, to render the apoprotein soluble in aqueous solution. As summarized in Table 4, molecular weight estimates for the apo-B monomeric subunit range from 8,000 to 275,000 daltons. Evidence for subunit heterogeneity shows a similar lack of agreement between investigators. Simons and Helenius separated maleylated delipidated apo-LDL into two fractions by gel filtration and showed them to be immunologically heterogeneous.(311) Shore and Shore separated two subunits on DEAE cellulose and found different amino acid contents.(304) However, they recovered only 30% of the protein initially put onto the column and neither peptide resembled the composition of whole apo-LDL. Kane et al. separated maleylated LDL on Sephadex and found two components, one very similar to whole apo-LDL in amino acid composition and the other different in many amino acids.(176) This component, however, was not similar to either of the peptides described by the Shores.(304) Chen and Aladjem separated LDL into at least seven bands on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and analyzed the amino acid content of each band.(69) They were all nearly identical

TABLE 4

Molecular Weights of Apoprotein-B Subunits
of Human Plasma LDL as Determined by Various Methods

Protein	Method	Molecular Weight	Reference
LDL	X-ray scattering	8,000	223
Apo-B	SDS-PAGE[a]	9,500 and 13,000	68; 69
Apo-B	SDS-PAGE	10,000	203
Apo-B	Cyanogen bromide fragmentation	25,000 to 35,000	43
LDL	Electron microscopy	27,000	265
Apo-B	Analytical ultracentrifugation, pH 11.5	27,500	265
Maleylated apo-B	Gel filtration	26,000 and 194,000	176
Succinylated apo-B	Analytical ultracentrifugation	36,000 to 38,000	287
Apo-B	Gel filtration	80,000	79
Apo-B	Analytical ultracentrifugation, pH 8.6	80,000 to 100,000	265
Apo-B	SDS-PAGE	230,000	312
Reduced, carboxymethylated apo-B	Analytical ultracentrifugation	250,000	317
Apo-B	SDS-PAGE	250,000 270,000	188
Apo-B	SDS-PAGE	255,000	317
Reduced, carboxymethylated apo-B	Gel filtration	275,000	317

[a] SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

to whole apo-LDL. Thus, depending on the methodology used to investigate the apo-LDL subunits, they are either homogeneous or heterogeneous and can be as small as 8,000 daltons or as large as 275,000 daltons. When the whole LDL particle is examined, optical studies suggest apo-B contains more β-structure than the other apoproteins. However, the quantity of this structure is decreased by delipidation.(170) Apo-B appears to have an essential function in transporting triglyceride out of the liver and intestine since when apo-B is absent from plasma, no triglyceride enters the blood stream despite the accumulation of vast amounts of intracellular triglyceride.(145)

There are three C apoproteins in the human being. They are low molecular weight proteins in the range of 6000 to 9000 daltons. The amino acid sequences of all three have been reported.(49, 168, 171, 307) These apoproteins appear to play an important role in the metabolism of triglyceride-rich lipoproteins. Ganesan and co-workers have shown that apo-C-I activates lipoprotein lipase purified from human postheparin plasma.(130, 131) It has also been reported to activate LCAT.(320) Apo-C-II has been shown to be a specific protein cofactor for the hydrolysis of triglyceride by lipoprotein lipases of extrahepatic origin.(157, 158, 195) Although the physiological functions of apo-C-III are uncertain, Brown and Baginsky have shown that it inhibits lipoprotein lipase at concentrations greater than 2% of the substrate.(57)

The arginine-rich protein or apo-E was originally isolated by Shore and Shore.(304) It has an approximate molecular weight of 33,000 daltons(297) and a great deal of α-helical structure.(305)

Similar arginine-rich proteins have also been identified in rabbit,[305] rat,[331] swine,[218] and dog.[216] The amino acid analysis of arginine-rich protein has been examined in a number of species and they are reportedly very similar.[357] The mole percent of tyrosine is very low (1.2 to 1.6%) and this probably accounts for the difficulties reported in iodinating this protein.[122]

Three additional apoproteins have been reported in human plasma: apo-D, apo-A-III, and apo-F. Apo-D has a molecular weight of 22,700 daltons and forms its own lipoprotein particle in the HDL density range.[225] Apo-A-III is also isolated in the HDL density range and differs from apo-D in that it lacks cystine. It was reported to activate LCAT by Kostner,[184] but Soutar et al. were unable to confirm this.[320] Apo-F has a mass of 26,000 to 30,000 daltons and is reported to form a distinct lipoprotein species within HDL.[250]

c. Structural Models of Lipoprotein Particles

The most current description of the lipid-core model for VLDL is found in the review of Morrisett et al.[233] This model describes VLDL as a sphere with a hydrophobic triglyceride core surrounded by a polar shell, 20 Å thick, consisting of B and C apoproteins, cholesterol, and phospholipids. The cholesterol ester of VLDL is thought to be randomly distributed throughout the hydrophobic core. Chylomicrons are generally assumed to have a similar micellar type structure.[369]

Osborne and Brewer describe HDL as a micellar lipoprotein particle with the proteins, phospholipids, and cholesterol located primarily at the surface and the nonpolar lipids (cholesterol esters

and triglycerides) forming a hydrophobic core.[254] There are currently two proposed models for the lipid-protein interaction in HDL. Segrest et al.[295] and Jackson et al.[169] have proposed an amphipathic helix model in which the proteins have only secondary (α-helical) structure. In the fluid mosaic model of HDL proposed by Assmann and Brewer, the apolipoproteins contain secondary, tertiary, and quaternary structure.[9] A fuller discussion of these features can be found in references 233 and 254.

Numerous physicochemical studies on intact LDL particles have led to general acceptance of the idea that LDL forms highly isotropic, sphere-like particles with a diameter of about 22 nm (for recent reviews, see references 125, 170, 190, and 288). Each particle contains protein in three structural forms (α-helix, antiparallel β-sheet, random coil), but the proportion of these structures in native LDL remains to be established since it appears to vary according to the source of the LDL, the exact chemical composition of the LDL, the ambient temperature, and the properties of the solvent used for the experimental determination.[288] An early model, proposed by Mateu et al. from low-angle x-ray scattering data, consisted of a small protein core enclosed by a lipid bilayer which contained the hydrocarbon chains of the phospholipids and cholesterol esters; this in turn was encased in a protein shell.[222] Although the nuclear magnetic resonance studies by Finer et al. were interpreted to support this model of a protein core,[116] other investigators, who used small-angle neutron scattering[328] and small-angle x-ray scattering,[334] did not agree with this interpretation. These studies suggested

that the internal structure of LDL was composed of a central core of neutral lipids surrounded by a shell of cholesterol, phospholipid, and protein. The cholesterol esters in the core display a reversible thermal transition between 20°C and 40°C (because of a transition from an ordered smectic-like liquid crystalline state at lower temperatures to a more disordered state at high temperatures.(81) This transition point varies depending upon the degree of saturation of the fatty acid esterified to the cholesterol.(81) The transition occurred above normal body temperature in cynomolgus monkeys fed an atherogenic diet, whereas LDL from monkeys fed a normal diet achieved the disordered core at temperatures lower than body temperature.(333) A detailed analysis of small-angle x-ray diffraction data by P. Laggner and his colleagues suggested that the cholesterol esters in the core of LDL formed two concentric rings when in the more ordered phase.(235)

Although uncertainty remains as tc the surface structure of the LDL particle, the nuclear magnetic resonance studies of Yeagle et al. established that about one-fifth of the phospholipid of LDL is immobilized by the B protein.(362, 363) Their evidence suggests that all of the mobile phospholipid head groups reside on the surface of the particle, and it also appears that most of the cholesterol is not associated with the mobile surface phospholipids. Tardieu et al. have proposed a deeply convoluted surface with spikes about 8 nm in length,(334) however Laggner and his colleagues collected data which suggest that the material of high electron density at the surface of the particle (i.e., the protein and phospholipid head groups) is

contained within a shell of 2-3 nm thickness.[191, 192, 235] Since this shell is only about 50% occupied, it would allow a surface structure to exist in which the constituents are arranged in clusters or patches. Similar ambiguities exist in the literature regarding electron microscopic studies of LDL structure.[125, 264, 265]

4. Metabolism of Lipoproteins

The metabolism of lipoproteins is a complicated subject because of the complex nature of the lipoproteins. The components of individual particles are not necessarily synthesized and secreted as a complete unit. The interrelationships governing synthesis and degradation of apoproteins, triglycerides, phospholipids, and cholesterol are numerous and difficult to isolate. The rapid exchange of phospholipid and free cholesterol between lipoproteins and between lipoproteins and plasma membranes, makes it difficult to use these compounds to study the metabolic functions of lipoproteins. With the advent of techniques allowing the apoprotein to be labelled with ^{125}I, this component of lipoproteins has been studied to provide information on lipoprotein metabolism.

The study of lipoprotein apoprotein metabolism in terms of the major density classes is also difficult as it has been shown that some of the apoproteins are exchanged among the lipoproteins. This section will, therefore, discuss lipoprotein metabolism in terms of the major families of lipoproteins as defined by Alaupovic rather than in terms of density class.[1]

A recent study by Wu and Windmueller measured the incorporation of ^{3}H-leucine into lipoproteins in rats.[371] The small intestine

was resected and the liver removed from the circulation. They found no evidence of lipoprotein synthesis under these conditions. This study clearly identified the liver and the intestine as the only significant sites for apolipoprotein synthesis. Additionally, they determined the relative contributions of hepatic and intestinal synthesis to each of the apoproteins in the plasma of fat-fed rats. They used two different methods: a differential double-labelling approach and a relative organ contribution approach which compared synthesis in isolated organs with synthesis in the intact rat. They determined that the liver contributes 81% of the total apoprotein pool and the intestine synthesizes the remaining 19%. Their results showed greater than 50% of the plasma apo-A-I and apo-A-IV originated in the small intestine. Apo-A-II is a relatively insignificant apoprotein in rats and was present in insufficient quantity to determine its source. About 16% of the plasma apo-B was synthesized in the intestine but only 5% of the C apoproteins were derived from the intestine. Finally, little or no apo-E was synthesized by the intestine although it was the most actively synthesized of all the apoproteins in both the isolated liver and the whole animal (see also references 109 and 221).

a. LpA

The site of apoprotein A synthesis in man has not yet been identified; in the rat, however, both the intestine[151, 221] and the liver have been shown to synthesize apo-A-I.[109] No information is yet available on the synthesis of apo-A-II. Recent studies by Schaefer et al. with human ^{125}I-labelled lymph chylomicrons indicated

that chylomicron apo-A-I and apo-A-II served as precursors to plasma HDL apo-A-I and apo-A-II.[291] Over 90% of the apo-A radioactivity of the chylomicrons was recovered in the HDL fraction within one hour of injection. The nascent HDL particle secreted in rat liver perfusion studies has an apo-E to apo-A-1 ratio of 10:1, whereas this ratio in plasma HDL is 1:7.[109] Thus the A apoproteins appear to be redistributed in the plasma compartment after they are initially secreted. Additional studies are needed to gain complete understanding of the site(s) and control(s) of LpA synthesis.

Most of the knowledge about LpA catabolism has come from studies of HDL catabolism since the apo-A peptides are the major proteins in HDL. The metabolic fate of HDL in man has been investigated in several studies, and the reported HDL half-life values in normal subjects ranged from 3.3 to 5.8 days.[40, 128, 132, 286] The study by Blum et al. found that the specific activity of apo-A-I and apo-A-II decayed in parallel under both normal and perturbed conditions (i.e., high carbohydrate diet or nicotinic acid treatment).[40] This study suggested that apo-A-I and apo-A-II are metabolized as a unit. Recent observations of Tangier disease homozygotes and heterozygotes indicate an abnormality of LpA catabolism. In the homozygotes, HDL catabolism was very rapid and apo-A-I was catabolized at a significantly faster rate than apo-A-II.[290]

The factors that determine HDL catabolism are not yet clearly defined. Animal studies indicated an important role for kidney and liver lysosomes,[107, 239, 271] but recent tissue culture studies suggest peripheral tissue may be important.[65, 231] No explanation

has yet been offered for the enhanced catabolism found after carbohydrate feeding or in Tangier disease. The relative contributions of liver and intestine to apo-A-II synthesis in man have yet to be established. Current data are consistent with the concept that chylomicron apolipoproteins are precursors of a portion of the HDL A-apolipoproteins and that apo-A-I and apo-A-II are catabolized at similar rates.

b. LpC

The studies on LpC metabolism have not differentiated between the three known peptides so apo-C will be discussed as a single entity. The liver is the site of apo-C synthesis in the rat.(359) Only a small amount of apo-C synthesis has been detected in the intestine. When radioactive amino acids were added to a liver perfusion system, both the VLDL and the HDL isolated from the perfusate contained labelled apo-C proteins. It was not clear whether apo-C was secreted with the liver VLDL or if it was rapidly transferred to VLDL after secretion. Mahley et al., however, identified apo-C in VLDL particles isolated from rat liver Golgi cisternae.(211) Apo-C exchanged between VLDL and HDL in vitro as shown by the simultaneous decrease in specific activity of VLDL apo-C and increase in specific activity of HDL apo-C. A net transfer of apo-C from VLDL to HDL occurred in vitro when ^{125}I-labelled VLDL was incubated with lipoprotein lipase-rich plasma and the amount of apo-C transferred was proportional to the extent of triglyceride hydrolysis.(106) Further experiments showed that the dissociation of apo-C from VLDL in the presence of lipoprotein lipase is dependent on the degree of

lipolysis of VLDL rather than the presence of an apo-C acceptor.[134] Eisenberg calculated that the molar ratio of apo-B to apo-C at the surface of a VLDL particle may increase 15-fold or more during lipolysis.[100] This changing pattern may be one of the factors that determine subsequent catabolic pathways. The study of Schaefer et al. showed that when labelled chylomicrons were injected into a human subject, the apo-C radioactivity was initially lost from the chylomicrons at a somewhat slower rate than apo-A or apo-B were lost.[291] After 6 hours, however, an increase in chylomicron apo-C radioactivity was observed because of reassociation of apo-C with the triglyceride-rich particles. Most of the apo-C that was initially lost from the chylomicron fraction was recovered from either VLDL or HDL. The site(s) of apo-C catabolism are unknown but it has a short half-life in plasma (10 to 18 hours).[29] Since this is clearly much shorter than the half-lives of either apo-B (3 days) or apo-A (5 days), it has been suggested that either apo-C is preferentially associated with HDL of very short lifespans or that apo-C units are cleared from the circulation independently of either apo-A or apo-B. This is postulated to occur as the C-peptide is being transferred between triglyceride-rich particles and HDL.[290]

c. LpB

Apoprotein B is synthesized in both the liver[221] and intestine[359] of the rat and enters the circulation with both chylomicrons and VLDL. Apo-B is an integral part of three lipoprotein species: chylomicrons, VLDL, and LDL. It appears to be the structural skeleton that is essential for the transport of triglycerides out

of the liver and intestine since when no apo-B is present in plasma, as occurs in persons afflicted with abetalipoproteinemia, no triglyceride enters the circulation despite the presence of large amounts of intracellular triglyceride.(145)

Chylomicron catabolism occurs in two distinct steps. Initially it is depleted of much of its triglyceride by lipoprotein lipase,(28) and the resulting particle is referred to as a chylomicron remnant.(275) The remnant is then selectively removed by the liver and metabolized further.(74, 110) Sherrill and Dietschy demonstrated that the remnant is removed as a unit by a saturable high affinity process.(299) Florén and Nilsson investigated the degradation of ^{125}I-labelled apoproteins in these remnants by hepatocyte monolayers and were able to show that degradation of the protein moiety occurred.(122) Unfortunately, they were unable to distinguish degradation of the individual peptides present in the remnant since the protein moiety was composed of apo-E ($\cong$83%), apo-B ($\cong$4%), apo-A-I ($\cong$6%), and combined C-peptides ($\cong$8%); and the label was distributed as follows: 26% in apo-B, 50% in apo-A-I, 24% in C-peptide, and barely detectable in apo-E. Thus the liver appears to catabolize the apo-B that is secreted by the intestine in the form of chylomicrons.

In the late 1950s Gitlin et al. carried out studies that suggested that a protein moiety of VLDL could be the precursor of the LDL protein moiety.(132) The specific demonstration of this path was achieved 10 years later with the use of VLDL that was labelled with ^{125}I in its protein moiety.(102) It had been shown that during incubation of human plasma VLDL with either whole plasma or with isolated

lipoproteins, the apo-B of VLDL did not exchange with the apo-B in other lipoproteins.[101] Following the injection of ^{125}I-labelled VLDL, all apo-B was initially found in VLDL. With increasing time intervals, labelled apo-B began appearing in IDL and finally in LDL (human studies;[103] rat studies [104, 105]). Labelled apo-B did not appear in VLDL or IDL when ^{125}I-labelled LDL was injected.[194] These studies unequivocally established a one-way path in which VLDL was successively delipidated to form LDL. This conversion of VLDL to LDL was dependent upon lipoprotein lipases. The activation of the lipase system by heparin injection greatly accelerated the appearance of apo-B in LDL.[103]

Recent studies in the rat by Suri et al. suggest that there are normally two pathways for the degradation of VLDL in the intact animal.[330] Both paths are initiated by the removal of triacylglycerol from VLDL by lipoprotein lipase action in extrahepatic tissues. The path that predominates in the intact rat involves the removal or modification of triacylglycerol-depleted VLDL remnants by the liver leading to very low levels of plasma LDL since the remnants are metabolized before LDL is formed. The other path, which is probably more important in man, involves formation of LDL particles from VLDL. Suri et al. demonstrated this path in a supradiaphragmatic rat preparation in which VLDL remnants were degraded to LDL particles since the remnants could not be removed by the liver.[330] Thus in the rat, the liver appears to be a major site for catabolism of apo-B containing lipoprotein remnants.

In man the transformation of VLDL into LDL was demonstrated by the kinetic studies of Berman et al.[29] This study used the mathematical model developed by Phair et al. to analyze the kinetics of labelled apoproteins following the injection of ^{125}I-labelled VLDL.[258] According to the analysis of Berman et al., VLDL underwent a series of incremental density changes, probably caused by successive delipidation steps.[29] Apo-B remained with the particle until the IDL density range was achieved but apo-C was progressively lost along with the lipid. The apo-C lost from VLDL and IDL initially cycled to HDL and then recycled to newly synthesized VLDL. In normal subjects, some of the apo-B in IDL was degraded directly. A slowdown of the stepwise delipidation process was evident in the hyperlipemic subjects.[29]

A recent study by Bilheimer et al. compared LDL turnover in a group of normal persons and a group that was homozygous for familial hypercholesterolemia (FH).[35] The mean LDL apoprotein concentration in the FH patients was 7.5-fold greater than in the normals (362 $\pm$ 126 mg/dl vs. 48 $\pm$ 6 mg/dl). The fractional catabolic rate (FCR) in the FH group was 17% of the pool per day (compared with 45% in the normal group). However, when the FCR was multiplied by the total pool size, it showed that the absolute amount of LDL catabolized by the FH patients was actually threefold greater than in the normal group. Since all of the subjects were maintaining a stable metabolic steady state during the course of the study, the FH group was synthesizing three times more apo-B than the normal group. Interestingly, there was no clear-cut difference between the normal individuals and

the FH patients with respect to cholesterol synthesis in this study. Bilheimer et al. suggested that increased LDL synthesis without increased cholesterol synthesis indicated reutilization of the cholesterol released during LDL degradation.[35] There was no indication in this study as to where the threefold increase in LDL catabolism occurred. The authors, however, proposed that the decreased FCR indicated a decrease in the efficiency of LDL catabolism caused by the lack of functional LDL receptors.

Since it has been established that LDL apoprotein is normally 98% apo-B, the catabolism of LDL and that of apo-B are closely related. The site(s) of such catabolism is of particular interest since elevated LDL levels are highly correlated with atherosclerosis.[315, 316] Heterozygous FH (LDL apoprotein = 126 $\pm$ 21 mg/dl)[35] occurs with an estimated population frequency of about 1 in 500 and in about 5 of every 100 patients under 60 years who suffer myocardial infarction.[234] It should be remembered, however, that since this genetic problem is associated with greater rates of synthesis and absolute LDL catabolism, although the FCR is significantly reduced, the elevation of LDL levels may be caused, in part, by uncontrolled synthesis of apo-B as well as by faulty degradation.

The site(s) of apo-B catabolism in vivo have not yet been unequivocally identified. The role of the liver will be discussed in section II.B.2.b. In 1976, Brown and Goldstein hypothesized that one of the roles of LDL was to transport cholesterol to nonhepatic tissues where it would be utilized by the tissue in lieu of <u>de novo</u> cholesterol synthesis.[54] Studies utilizing a variety of nonhepatic cells

in culture have shown them to be capable of binding, internalizing, and degrading the apo-B contained in LDL. Tissues in which this has been demonstrated include fibroblasts,[141, 324] arterial smooth muscle cells,[33, 142] lymphoid cells,[162] endothelial cells,[350] and adrenocortical cells.[186] The comprehensive studies of Goldstein and Brown (reviewed in references 142 and 143) into the nature of LDL interaction with both normal and homozygous FH fibroblasts led to their delineation of the LDL pathway: a receptor-mediated mechanism for regulation of cholesterol metabolism. The main features of this path include: 1) the binding of LDL to a specific high-affinity receptor on the cell surface; 2) internalization of the receptor-LDL complex via endocytosis and fusion with lysosomes; 3) hydrolysis of the LDL protein and cholesterol esters in the lysosome which then allows the free cholesterol to suppress the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme of cholesterol synthesis; 4) the rate of intracellular cholesterol esterification is increased; and 5) the synthesis of high-affinity receptors is reduced. This pathway thereby provides a physiological system for regulating intracellular cholesterol levels. LDL that is internalized by other processes, such as nonspecific pinocytosis, does not perform these regulating functions even though it has been shown to be internalized and degraded in homozygous FH fibroblasts that lack the high-affinity receptor.[141] This raised the possibility that a prerequisite for the regulation of cholesterogenesis in normal fibroblasts is the initial binding of LDL to the

high-affinity receptor. The properties of this receptor will be discussed in section II.C.

d. LpE

HDL has been shown to accumulate in perfusates of both liver and intestine.(359) Recent studies of apoproteins of intestinal origin suggest that very little apo-E is synthesized in the gut.(164, 292, 361) Rat liver perfusion studies indicate that apo-E is secreted in nascent HDL particles and then is transferred to VLDL in the plasma.(109, 151, 221) The mechanism of transfer appears to involve the LCAT reaction since Ragland et al. showed that an HDL particle, which was abnormally rich in apo-E, accumulated in the plasma of patients in whom alcoholic hepatitis had caused a deficiency of plasma LCAT.(273, 274) With remission of the hepatitis, LCAT levels returned to normal and apo-E no longer accumulated in HDL but was found in VLDL. They also showed that the HDL isolated from the plasma of patients suffering from alcoholic hepatitis was a better substrate for LCAT than either normal HDL or HDL obtained from the same patients following recovery. This was consistent with the studies of Hamilton et al. which showed that nascent HDL from rats was a better substrate than plasma HDL for purified human LCAT.(151) Nascent HDL, containing high levels of apo-E, appeared as disc-shaped lipid bilayers whereas normal plasma HDL particles were spherical.(151, 273) These observations led Hamilton et al. to hypothesize that LCAT binds to the disc-like particle and forms 1) cholesterol esters which, by virtue of their insolubility in water, move into the hydrocarbon domain of the bilayer, and 2) polar lysolecithin which

transfers away to serum albumin.[151] This reaction consumes surface cholesterol and lecithin particles and generates an oily core which pushes apart the bilayer until a spherical pseudomicellar HDL is formed. Newly formed cholesterol esters transfer from HDL to LDL and VLDL,[243, 276] and unesterified cholesterol probably transfers to HDL from both cells and lipoproteins after HDL cholesterol esters have been formed by LCAT.[135, 237, 329] Ragland et al. suggested that a probable physiological role of apo-E is the transport of unesterified cholesterol and/or cholesterol esters in plasma.[273] Several observations are consistent with this idea. First, Ragland et al. always found a large quantity of apo-E associated with the presence of excess unesterified cholesterol.[273] Second, animal studies have demonstrated that feeding high cholesterol diets increases plasma levels of both apo-E and cholesterol. The species in which this has been demonstrated include swine,[218] dog,[216] monkey,[217], rat,[212] guinea pig,[149] and rabbit.[306]

Eisenberg and Rachmilewitz showed that the degradation of iodine-labelled VLDL in rats is accompanied by the transfer of apo-E activity to HDL; however, they were unable to assess the extent of this transfer.[104, 105] In a recent review, it was suggested that apo-E enters the circulation with nascent HDL particles and is transferred to VLDL during the process of cholesterol esterification.[290] Apo-E may then recycle back to HDL making its metabolism similar to that of apo-C.

Since 1975 when Assmann et al. showed that HDL_c* suppresses HMG CoA reductase activity in cultured swine aortic smooth muscle cells, much work has been done by Mahley and his co-workers with this lipoprotein.(11) They have shown that HDL_c is capable of interacting with the LDL receptor and that it is the apo-E component of the lipoprotein which binds to the receptor.(32, 213)

B. Cholesterol Metabolism and the Liver: Effects of Lipoproteins

1. Cholesterol Metabolism in the Liver: An Overview

The observation of Bloch and Rittenberg that labelled acetate is incorporated into both the aliphatic side chain and the tetracyclic moiety of cholesterol was the beginning of the systematic study of how two carbon units unite to form cholesterol.(39) In 1964 Feodor Lynen and Konrad Bloch received the Nobel Prize for having worked out the entire biosynthetic pathway of cholesterol. These studies are reviewed by Bloch.(38) During the decade of the 1950s, a great variety of environmental, dietary, and hormonal factors were shown to influence the rate of cholesterol synthesis both in the whole animal and in hepatic tissue specifically. Hormonal regulation of cholesterol synthesis is a complex and interesting subject that will not be discussed here. See reference 88 for a good review of this area.

In normally healthy animals the rate of cholesterol synthesis is primarily regulated by three physiologic variables: the amount of dietary cholesterol; the total caloric intake; and the functional

*HDL_c is a lipoprotein which is characteristic of experimentally induced hypercholesterolemia and which lacks apo-B but contains apo-E and apo-A-I.

integrity of the enterohepatic circulation of bile acids. The interrelationships between these variables and hormones is not within the scope of this discussion. The liver and the intestine appear to be the most important organs involved with cholesterol synthesis,[85] and while this discussion will emphasize the role of liver, the importance of the intestine should not be forgotten. The regulation of hepatic cholesterol synthesis by cholesterol feeding was first reported by Taylor and Gould.[335] No tissue other than liver showed such marked suppression of sterol synthetic activity.[83, 84, 146] The point of inhibition was shown, indirectly, to be localized at the point of reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonate.[59] In 1966, Siperstein and Fagan showed that the incorporation of ^{14}C-acetate into mevalonate was markedly inhibited by cholesterol feeding, but the incorporation of this labelled precursor into HMG CoA was virtually unaffected.[314] The next year Linn, using isolated hepatic microsomes and solubilized enzyme preparations, demonstrated that the activity of HMG CoA reductase was reduced in the livers of cholesterol-fed rats.[202]

Suppression of cholesterol synthesis also occurs during fasting. This was demonstrated in 1952 by Van Bruggen et al.[344] and Tompkins and Chaikoff.[338] The studies of Linn showed that hepatic HMG CoA reductase activity was reduced by fasting.[202]

Another dimension was added to the study of mechanisms that control cholesterol synthesis when separate studies revealed that there was a large diurnal fluctuation in the rate of cholesterol synthesis in both the rat[14] and mouse.[175] This rhythmic fluctuation was

shown to be cued to the feeding schedule of rats;[97] however, the rhythm also persisted in both fasted and cholesterol-fed animals.[296] The demonstration that the conversion of mevalonate to cholesterol did not exhibit rhythmical variation led to the proposal[14] and then documentation that the fluctuation in cholesterol synthesis is caused by a fluctuation in HMG CoA reductase activity.[89] Recently, Anderson and Dietschy disclosed that diurnal light cycling affects only hepatic cholesterogenesis; there was no evidence of diurnal variation in the 15 other rat tissues examined.[5]

The manner in which the activity of the rate-limiting enzyme in cholesterol synthesis is regulated has been the subject of a number of studies. It was originally thought that cholesterol provided a negative feedback regulation of the enzyme activity, but this was disproven when Linn showed that adding cholesterol to a solubilized enzyme preparation did not reduce the observed activity.[202] Edwards and Gould then showed that the hepatic enzyme turns over very rapidly with a half-life of about 4 hours.[97] The observed diurnal variation of enzyme activity was interpreted as being caused by changes in the rate of enzyme synthesis. However, Higgins and Rudney observed that there was an initial rapid decline in HMG CoA reductase activity in rats fed cholesterol in the absence of a concomitant decrease in immunoprecipitable reductase.[161] Thus a decrease in activity occurred which could not be adequately explained by a reduction in synthesis. Subsequently Beg et al. reported the in vitro inactivation of hepatic HMG CoA reductase when microsomes were incubated with cytosol in the presence of ATP and Mg^{2+}.[23] This has subsequently been confirmed

in several laboratories.[50, 72, 249] In 1978 two separate laboratories provided evidence that the rapid regulation of HMG CoA reductase occurs via a reversible phosphorylation-dephosphorylation reaction.[24, 165] In one of these studies, Beg et al. also reported that the addition of either coenzyme A, acetyl-CoA, acetoacetyl-CoA, or 3-hydroxy-3-methylglutaric acid to solubilized, highly purified HMG CoA reductase inhibited the activity via an allosteric (i.e., noncovalent) effect on the enzyme.[24] Brown, Goldstein, and Dietschy examined the active (dephosphorylated) and inactive (phosphorylated) forms of the enzyme in rat liver and concluded that the best interpretation of their data indicated that long-term alterations in cholesterol synthesis in rat liver are related to changes in the total amount of enzyme protein rather than a change in the state of phosphorylation.[56] However, they suggested that the phosphorylation-dephosphorylation mechanism could be used by the liver in the event of a sudden demand for a large amount of cholesterol since their data indicated a large reservoir of inactive enzyme was present in this organ. The question of how cholesterol directs the multifaceted control of HMG CoA reductase activity remains to be answered.

The role of the enterohepatic circulation of bile acids in the control of cholesterol synthesis first became apparent when Economou et al. showed that external diversion of the bile flow leads to a rapid increase in cholesterogenesis in the liver.[92] While early investigators were searching for a direct effect of bile acids in controlling cholesterol synthesis, Siperstein demonstrated that pure conjugated bile acids did not inhibit synthesis in liver slices.[313]

This together with the observation that obstruction of the common bile duct, which causes increased concentrations of bile acids in blood and liver, enhanced liver sterol synthesis[92, 127] led Weis and Dietschy to propose that the influence of bile acid on hepatic cholesterogenesis is secondary to its effect on the movement of cholesterol into lymph chylomicrons and lipoproteins.[355] In other words, bile acids cause the absorption of cholesterol from the gastrointestinal tract, and it is the cholesterol that controls hepatic synthesis. Experimental data supporting this hypothesis have been accumulating.[5, 25, 355] These studies showed that procedures, such as biliary fistula, biliary obstruction, or cholestyramine feeding, that caused the withdrawal of bile acids from the intestinal lumen and the subsequent failure of absorption of cholesterol into the intestinal lymph resulted in vastly increased hepatic synthesis of cholesterol. Additionally, feeding bile acid enhanced cholesterol absorption and decreased hepatic synthesis as did the infusion of chylomicrons in animals with biliary diversion. Only hepatic tissue showed suppressed cholesterol synthesis in response to chylomicron infusions.[5]

It has become clear that one of the major determinants of hepatic cholesterol synthesis is the rate of cholesterol uptake relative to the rate of cholesterol secretion.[242] This uptake, in turn, depends upon the interaction between the hepatocyte membrane and the cholesterol carriers (i.e., lipoproteins). Thus it is important to examine the roles of specific lipoproteins in the regulation of hepatic cholesterol synthesis as well as the effect of pathologic states on such regulation. In a recent paper, Anderson et al. showed that, in rats,

chylomicrons are the most important regulators of hepatic cholesterogenesis when rat chylomicrons, human LDL, and human HDL are compared.(6) They suggested that this was logical since the cholesterol delivered to the liver via chylomicrons was primarily derived from ingested cholesterol and de novo intestinal synthesis. It thus represented a net increase in total body cholesterol, and this results in the compensatory suppression of hepatic cholesterogenesis. They also suggested HDL and LDL had considerably less suppressive effect in the liver since most LDL, HDL, and VLDL cholesterol ultimately comes from liver synthesis, and thus hepatic uptake of these particles results in little net delivery of cholesterol to the liver and little net change in the rate of hepatic cholesterol synthesis. These in vivo studies did show that elevated levels of LDL suppress hepatic sterol synthesis although the suppression was not as dramatic as that produced by chylomicrons. The mechanism of the enhanced suppression of sterol synthesis by chylomicrons was not discussed.(6)

2. The Liver and Lipoproteins

a. Synthesis

Studies in isolated perfused rat livers have shown that labelled amino acids are incorporated into the protein moieties of both HDL and VLDL particles.(220, 247, 359) While the perfusion conditions (e.g., recirculating or nonrecirculating, presence or absence of serum, etc.) appeared to affect the relative amounts of label recovered in VLDL or HDL, the experiments all clearly showed synthesis and secretion of VLDL and HDL but were unable to determine whether the small amount of label recovered in the LDL fractions

represented VLDL catabolism, contamination from the HDL fraction, or true LDL secretion.

Evidence derived from turnover studies of isotopically labelled VLDL in man,[29, 102, 308] rat,[104, 105, 111] monkey,[163] and rabbit[189] indicate that the majority of plasma LDL comes from the catabolism of VLDL. In these studies VLDL degradation could not always account for all of the LDL formed. In the kinetic analysis worked out by Berman et al., for example, VLDL degradation could account for all of the LDL-apo-B in normal subjects; but in type III hyperlipidemic subjects, some LDL-apo-B had to come from a source other than VLDL.[29] Illingworth compared the simultaneous appearance of labelled LDL from injected ^{3}H-VLDL and ^{14}C-leucine in triton-treated, fasted squirrel monkeys and calculated that 10 to 19% of the B apoprotein entered the plasma directly in LDL rather than through the VLDL degradation pathway.[163] Similarly, Fidge and Poulis examined the apo-B kinetics of VLDL and LDL subfractions after injection of ^{125}I-VLDL in the rat and concluded that not all LDL apo-B is derived from VLDL apo-B.[113] Finally, Nakaya et al. perfused isolated pig livers with ^{14}C-leucine and found label incorporated into the LDL fraction.[240] Perfusing livers for a similar length of time with either ^{125}I-VLDL or ^{14}C-VLDL did not produce labelled LDL, thus indicating that VLDL was not catabolized to LDL during their liver perfusions. Taken altogether these experiments indicate that most LDL is derived from VLDL but a small amount enters the circulation from other sources, including the liver. The amount of LDL derived from sources other than VLDL degradation appears to vary from species to

species and also to depend in part on whether or not the animal was hyperlipemic.

b. Degradation

Perfused liver systems have been used to study liver degradation of chylomicrons and have repeatedly shown that intact chylomicrons are taken up to a limited extent whilst chylomicron remnants are rapidly removed from the perfusate.(74, 110, 246, 299) Felts et al. proposed that lipoprotein lipase, which is firmly bound to chylomicron remnants but not to intact chylomicrons, is the signal that allows the liver to "recognize" the remnants.(110) Cooper has shown that chylomicron remnants are removed as a unit by isolated liver and that the rate of catabolism of cholesterol ester from remnants is similar both in vivo and in perfused liver.(74) He also showed that the removal of remnants by the isolated liver suppresses cholesterol synthesis in that liver. A recent liver perfusion study raised the possibility that the degradation rate of chylomicron remnants is determined by the movement of the removed remnant to the site of hydrolysis since the cholesterol ester was hydrolyzed at a rate that was only about 0.5% of the remnant removal rate.(75) This hypothesis is consistent with the report of Florén and Nilsson that microtubule inhibitors blocked the hydrolysis of remnant cholesterol esters.(121)

As discussed earlier, most VLDL is degraded to LDL in normal human beings, but in untreated type III hyperlipemic patients, the kinetic analysis of Berman et al. indicates that 10 to 29% of the newly secreted VLDL apo-B disappears from the circulation without

being converted to either IDL or LDL.[29] The studies of Portman et al. in squirrel monkeys also indicated that some VLDL apo-B was cleared without forming LDL.[268] Liver participation in the removal of VLDL or VLDL remnants in the rat has been inferred from autoradiographic studies of ^{125}I-VLDL uptake by Stein et al.[323] Five minutes after the injection of ^{125}I-VLDL into the rat, 40% of the silver grains were seen over the hepatocyte cytoplasm. After 120 minutes, 80% of the label was intracellular and many grains were localized over secondary lysosomes. Finally, studies by Suri et al. demonstrated that LDL accumulated in a supradiaphragmatic rat preparation.[330] In the intact animal, the liver rapidly removed VLDL remnants leading to very low LDL levels.

A number of studies in the early seventies, in which labelled HDL was injected and the tissue radioactivity analyzed at various intervals, indicated that the greatest amount of radioactivity accumulated in the liver.[107, 271, 282] This was interpreted to mean that the liver was a major site of HDL catabolism. Studies with isolated hepatocytes also indicated that HDL was taken up and degraded.[87, 238] Recent quantitative studies by Sigurdsson et al. in the isolated perfused rat liver indicate, however, that only about 7% of the HDL apoprotein mass is degraded directly by the liver.[310] These data are compatible with the partial hepatectomy study of van Tol et al. which found that neither the initial rapid decay of ^{125}I-HDL from the serum nor the slow phase of disappearance were influenced by the removal of two-thirds of the liver.[346] Consequently the fractional catabolic rate remained unchanged. The serum level of HDL was

decreased by the partial hepatectomy but the chemical composition of HDL was unchanged. They concluded that the in vivo degradation rate of HDL-apoproteins was not influenced by the removal of two-thirds of the liver and that the decreased HDL level in serum was caused by impaired hepatic synthesis. They suggest these results can be explained by the possibility of extrahepatic HDL-apoprotein catabolism or by a stimulation of HDL-apoprotein degradation in the remaining liver lobes. The second explanation was not supported by finding an increased level of hepatic HDL radioactivity in the remaining liver lobes.

In 1975 when this study was begun, the role of the liver in the catabolism of low density lipoproteins had been considered for many years without the emergence of definitive experiments to determine its importance. In vivo studies in several species had consistently shown that after injection of ^{125}I-LDL, the greatest concentrations of total radioactivity and of protein-associated radioactivity were found in the liver.(62, 107, 159, 294) This was recently verified in the rabbit by Portman, Alexander, and Kannan in studies that showed that 15 minutes after the injection of ^{125}I-labelled LDL, the liver accounted for 56% of the control LDL and 72% of the hyperlipemic LDL that had disappeared from the plasma.(267) The high level of ^{125}I in the liver was not a function of alterations in LDL or its parts during iodination, since experiments using LDL labelled in vivo with ^{3}H-leucine gave the same results.(270) These data showed that the liver was a part of the extravascular pool of LDL, but they did not indicate whether LDL was degraded there.

Hay et al. showed that perfused rat livers altered ^{125}I-LDL apoprotein, since the appearance of trichloroacetic acid-soluble (TCA-soluble) radioactivity was coupled with the loss of TCA-precipitable radioactivity from the medium.(159) Thus the liver was actively removing the LDL and either degrading the LDL apoprotein or cleaving its ^{125}I label from the unaltered apoprotein. Because the liver system removed a greater quantity of ^{125}I-LDL from the perfusion medium than was cleared from the blood of an intact rat over a similar time period, they concluded that the liver accounted for a major part of the in vivo catabolism of ^{125}I-LDL. This conclusion was recently challenged when Sigurdsson et al. performed liver perfusions under somewhat different conditions and found only about 7% of the LDL apoprotein catabolism that was observed in intact animals.(309)

Sniderman et al. and Steinberg et al. observed that ^{125}I-LDL disappeared more rapidly from the plasma of pigs after total hepatectomy than from the plasma of intact animals.(318, 325) This observation tended to minimize the role of the liver in the catabolism of LDL apoprotein. Their experiments showed that the disappearance of labelled LDL apoprotein after hepatectomy was monoexponential; thus they confirmed that the liver contains the major part of the extravascular LDL pool that is exchanged readily with plasma LDL and that accounts for the first rapid exponential phase of the disappearance of ^{125}I-LDL from the blood of intact animals. Conversely, the in vivo studies of van Tol et al. showed that when rats were subjected to a partial hepatectomy in which two-thirds of the liver was removed, the FCR of LDL was decreased by 62%, the serum LDL level was somewhat

increased after 8 hours, and the triglyceride content of LDL was decreased.[345, 347] They concluded that the liver determines the degradation rate of serum LDL in vivo.

A definitive experiment to assess the cumulative lysosomal degradation of proteins was proposed by Pittman and Steinberg in 1976.[262] This involved coupling the protein to a small molecule, like sucrose, that is cleaved from the protein only after entry into the lysosome and that is not metabolized further in lysosomes but remains "trapped" there. Thus the small molecule is accumulated in lysosomes in direct proportion to the amount of protein degraded. Sucrose is an excellent choice for such a marker as it is taken up by fluid pinocytosis and accumulates in lysosomes which contain little or no sucrose activity.[73] A method for covalently linking sucrose to LDL, which did not alter the metabolism of the LDL by cultured fibroblasts, was described by Pittman et al. in the summer of 1979.[261] The application of this method to the in vivo study of the fate of LDL in swine was subsequently reported in October, 1979.[260] The in vivo FCR of ^{14}C-sucrose-LDL was the same as that of ^{125}I-LDL. ^{14}C-Labelled degradation products (i.e., nonprotein-bound) were determined 24 hours after injection of ^{14}C-sucrose-LDL. In three studies 37.9, 39.6, and 37.8% of the degraded ^{14}C-sucrose-LDL was recovered in the liver. After 48 hours, 38.7 and 39.9% of the total LDL degradation products were recovered in the liver. After 24 hours, less than 6% of the degraded LDL had appeared in the urine and less than 13% had appeared in urine after 48 hours. This is a sharp contrast with ^{125}I-LDL degradation products which are rapidly and almost quantitatively recovered in the

urine. In two 24-hour experiments, parenchymal and nonparenchymal cells were separated and over 90% of the ^{14}C-sucrose activity in the liver was shown to be associated with the parenchymal cell fraction.

c. Isolated Hepatocytes

The description of a method for isolating viable hepatocytes in high yield from rat liver provided a new and better technique to examine lipid metabolism in the liver.(31) For the first time it was possible to separate the contributions of parenchymal and nonparenchymal cells. Within a few years, several laboratories had developed the methodology necessary to keep the isolated hepatocytes in primary culture for up to five days.(36, 41) Both techniques (i.e., freshly isolated suspensions of cells and primary cultures) have been utilized to examine various aspects of hepatic lipid and lipoprotein metabolism. The ability to control the cellular environment is a major advantage of studies in isolated cell systems as compared with in vivo studies. Additionally, comparative studies in hepatocytes which are derived from a single liver eliminate the differences which occur between animals. These methods also permit a greater number of variables to be examined than is feasible when designing liver perfusion studies. Cell suspension studies have two disadvantages not encountered in primary culture studies. First, any damage to the cell surface(244) and receptors(236) that is incurred during the isolation procedure will not be repaired before the short-term suspension experiment. Surface damage can be repaired during the 20-hour incubation that establishes the viable primary culture. Second, the primary culture can be incubated for 20 or more hours in

lipoprotein-deficient medium in order to maximize LDL-receptor synthesis. One disadvantage of cell culture is that dedifferentiation of the cell line may occur and thus give misleading results. Several studies suggest that this does not occur in hepatocytes earlier than four to five days.[36, 41, 129] The following sections will review the studies in which the relationships between serum lipoproteins, hepatocytes, and hepatic cholesterol metabolism have been examined.

1) Suspension Studies

Edwards and co-workers investigated the activity of HMG CoA reductase in freshly isolated rat hepatocytes.[94-96, 98, 99] They reported that a 3-hour incubation of the hepatocytes in Swims S-77 medium resulted in a twofold increase in enzyme activity, whereas inclusion of 10% serum in the medium caused a sevenfold increase in activity. The addition of HDL or lecithin dispersions also enhanced enzyme activity above control values, but LDL or cholesterol-lecithin dispersions reduced the enzyme activity.[94] Further work showed that a sterol loss by the hepatocytes preceded the increase in enzyme activity. This supported the hypothesis that one mode of regulation of HMG CoA reductase is dependent upon the relative rates of movement of cholesterol into and out of the cells.[96]

A completely separate study by Nordby et al. confirmed the finding that serum, added to the medium, enhanced the secretion of cholesterol by freshly isolated hepatocytes.[248] This study showed that LCAT was secreted by the isolated hepatocytes. Concomitantly there was secretion of unesterified cholesterol and triglyceride. They showed that all of these are optimally secreted in the presence of

25% serum in the medium. One experiment specifically showed that cholesterol secretion was enhanced in the presence of increased serum concentrations up to 25% of the total medium. Thus, serum enhanced the outflux of cellular unesterified cholesterol, and the Edward's study implied that HDL was the serum component involved in this outflux.(94)

Other workers have specifically investigated the uptake and degradation of HDL by suspensions of hepatocytes. Nakai et al. showed that hepatocytes bind, internalize, and degrade ^{125}I-HDL$_3$.(238) Their observations indicated the presence of both a saturable (specific) component and a nonsaturable (nonspecific) component for binding and uptake of HDL$_3$. The specific binding component was largely temperature-dependent being very small at 4°C. Competition studies showed HDL$_3$ inhibited the binding and uptake of ^{125}I-HDL$_3$ much more effectively than either LDL or VLDL. They calculated the apparent dissociation constant of the specific receptor to be 60×10^{-8} M. This calculation used a molecular weight of 28,000 daltons for apo-A-I, the predominant protein moiety of rat HDL$_3$. The presence of 5 mM chloroquine inhibited the proteolysis of ^{125}I-HDL$_3$. This supported previous studies which revealed the importance of liver lysosomes in the degradation of HDL$_3$.(238, 239) Berg et al. and Drevon et al. investigated the uptake of ^{3}H-cholesterol ester-labelled HDL in preparations of rat hepatocytes and also in nonparenchymal cells.(27, 87) While both types of cells showed uptake and hydrolysis of the cholesterol ester portion of HDL, only the hepatocytes showed a distinct saturation of uptake. No saturation was observed in the nonparenchymal cells.

Concanavalin A reduced the uptake of lipoprotein by hepatocytes but not by nonparenchymal cells, thus suggesting that carbohydrate sites on the hepatocyte surface were involved in HDL uptake.

Lakshmanan et al. examined the effect of chylomicrons, chylomicron remnants, VLDL, LDL, and HDL on fatty acid synthesis and cholesterol synthesis in hepatocytes isolated from meal-fed rats.(193) While chylomicrons, VLDL, and remnants caused significant inhibition of fatty acid synthesis within one hour, none of the fractions inhibited cholesterol synthesis in the same time period. They suggested this lack of inhibition was due to the short incubation time since Brown et al. had shown that four to six hours were required for lipoproteins to suppress HMG CoA reductase activity in fibroblasts.(51)

The catabolism of ^{125}I-LDL and ^{125}I-VLDL by isolated rat and pigeon hepatocytes was reported.(63) A recent study by Capuzzi, Sparks, and DeHoff suggested that most of the degradation of VLDL observed in hepatocyte suspensions was caused by residual enzymes adsorbed from the dispersion medium.(64)

Takeuchi et al. studied labelled cholesterol uptake from serum and cholesterol-phospholipid dispersions by isolated rat hepatocytes.(332) Cells incubated with increasing concentrations of serum cholesterol (0.25 to 2.0 μmol/ml) showed no increase in total cholesterol content over a 2-hour period. However, incubation with serum containing labelled free and esterified cholesterol showed that labelled cholesterol accumulated within the cells thus indicating exchange was occurring. When the uptake of label from the various lipoprotein components of the serum was investigated over a range of cholesterol

concentrations (0.5 to 10 μmol/ml), the uptake (exchange) of label from LDL was twice as great as it was from HDL at any given cholesterol concentration.

2) Primary Culture Studies

Floren and Nilsson have published a series of papers reporting their investigations of the uptake and degradation of labelled chylomicron remnants in primary cultures of rat hepatocytes. In the presence of chylomicrons or chylomicron remnants containing equal amounts of cholesterol ester, hepatocytes take up and hydrolyze greater amounts of cholesterol ester from the chylomicron remnants. (120, 121) The presence of 15% fetal calf serum in the incubation medium reduced the uptake and degradation of cholesterol ester from both native chyle lipoproteins and chylomicron remnants. Chloroquine and colchicine inhibited the hydrolysis of remnant cholesterol ester but increased the cell-associated label.(121) This was shown to be an increase of interiorized rather than surface-bound cholesterol ester.(120) VLDL and chylomicrons inhibited both the uptake and hydrolysis of remnant cholesterol ester, while HDL inhibited hydrolysis of remnant cholesterol ester but had little effect on the amount of surface-bound label. They suggested that chylomicrons, VLDL, and remnants compete for the same surface receptor whereas HDL interferes with remnant cholesterol ester hydrolysis after both particles are interiorized. The pretreatment of cells with mild pronase reduced remnant binding at 4°C by 63%, but the binding at 37°C (after a 4-hour incubation) was reduced by only 17%. If cycloheximide was added to the 37°C incubation, the remnant binding was reduced by 52%.(123)

The receptor that bound the remnants thus appeared to be a protein that can be about 66% replaced during a 4-hour incubation. Since remnant uptake was not inhibited by EDTA, asialofetuin, or neuraminidase treatment, the remnant receptor appeared to be different from the protein receptors described for desialylated glycoproteins and for LDL.[123]

In order to determine if remnants are taken up and catabolized as whole particles, Florén and Nilsson labelled chylomicrons with ^{125}I by the iodine monochloride method of McFarlane.[122, 226] Remnants were prepared by treating the labelled chylomicrons with postheparin plasma. Since saturation of uptake is observed with both cholesterol ester-labelled and ^{125}I-labelled remnants, the V_{max} of total uptake (i.e., bound + interiorized + degraded) and the apparent K_m could be calculated. The V_{max} of ^{125}I-remnant uptake was 300 ng of remnant apoprotein/hr/mg cell protein and the apparent K_m was 7.7 μg of remnant apoprotein/mg cell protein. Since the added remnants contained 35.8 μg of cholesterol/53 μg of remnant apoproteins, the V_{max} for the cholesterol ester uptake was calculated to be 200 μg/hr/mg cell protein and the apparent K_m was 5.1 μg cholesterol ester/mg cell protein. These values are within the range of values previously determined for cholesterol ester-labelled remnant preparations having varying compositions. These values ranged between 80 and 320 μg cholesterol ester/hr/mg cell protein for V_{max} and between 1.4 and 5.9 μg cholesterol ester/mg cell protein for the apparent K_m.[120, 122] This study also compared ^{125}I-labelled remnant uptake with ^{14}C-sucrose and determined that the remnant uptake was 77 times greater

than labelled sucrose uptake.[122] This was evidence that a process other than bulk fluid pinocytosis was involved in the uptake of remnants by rat hepatocytes.

Analysis of both the apoprotein content and the distribution of ^{125}I label in the chylomicron remnants gave the following results. The protein content, as estimated by gel scanning, indicated a protein composition of $4 \pm 1\%$ apo-B, $83 \pm 4\%$ apo-E, $6 \pm 2\%$ apo-A-I and $8 \pm 4\%$ apo-C-peptides. The distribution of iodine label in the protein moiety was $50 \pm 4\%$ in apo-A-I, $26 \pm 3\%$ in apo-B, and $24 \pm 6\%$ in the C-peptides.[122]

Two separate groups have described the synthesis and secretion of VLDL by isolated rat hepatocytes. Jeejeebhoy et al. described the synthesis and secretion of VLDL in isolated hepatocyte suspensions in which all of the hepatocytes isolated from a rat liver were incubated for 24 to 48 hours.[173] The medium was examined for the presence of immunologically identifiable VLDL and for the incorporation of ^{3}H-valine into VLDL-protein. This study was included in this section because the length of the incubation suggests that at least some of the cells are actually in the primary culture phase. Davis et al. also showed that hepatocytes cultured in an arginine-free medium synthesize and secrete ^{3}H-leucine-labelled, immunologically identifiable VLDL apoprotein. Additionally, they showed that orotic acid acted directly on cultured hepatocytes to inhibit VLDL secretion.[78]

Lin and Snodgrass showed that the initial difference in HMG CoA reductase activity observed in hepatocytes isolated from rats during the peaks of the light or dark cycles decreases during the first 48

hours in culture.[200] From 48 to 96 hours of culture, cells from both types of animals maintained a similar steady-state activity of HMG CoA reductase in serum-free medium.

Breslow and his colleagues studied the effect of lipoproteins on the regulation of HMG CoA reductase in primary cultures of rat hepatocytes[44-46] and saw an increase of enzyme activity in the presence of rat or human HDL[44, 46] similar to that observed by Edwards in suspensions.[94] Additionally, VLDL promoted a small increase in activity, but LDL had no effect. The cells were incubated in serum-free medium for 48 hours and then the lipoproteins were added for an additional 24 hours before HMG CoA reductase activity was assessed. Lipoproteins of $d < 1.063$ g/ml from hypercholesterolemic rats were potent inhibitors of HMG CoA reductase activity,[46] and the $d < 1.006$ g/ml and $1.006 \text{ g/ml} < d < 1.1019$ g/ml fractions reduced the activity twice as far as the $1.019 \text{ g/ml} < d < 1.063$ g/ml fraction.[44] The inhibition was maximum at a concentration of 25 μg of lipoprotein/ml with no further decrease in activity being achieved in the presence of 100 μg of lipoprotein/ml. The inhibitory lipoproteins contained 1.5- to 2-fold more apoprotein with a molecular weight of 35,000 d* and 3- to 9-fold more cholesterol than the noninhibitory proteins. Since the cells were examined 24 hours after the addition of the lipoproteins, the inhibition of enzyme activity was probably caused by suppression of enzyme synthesis. This was consistent with the report of Gaertner that after 48 hours in culture, the second 24 hours in the

---

*Shelburne and Quarfordt reported a molecular weight of 33,000 d for apo-E.[297]

absence of lipoproteins, LDL added to the medium did not suppress HMG CoA reductase activity.[129] When this experiment was carried out in cultures that are 120 hours (5 days) old, LDL did suppress the enzyme activity by approximately 66% after a 6-hour incubation and by 100% after 12 hours. At this point in the culture, he claimed the cells are beginning to dedifferentiate and suggested they are becoming more fibroblast-like.

In 1978 Pangburn and Weinstein reported that primary cultures of rat hepatocytes degraded 4 times as much HDL as LDL when the lipoproteins were present at equimolar concentrations ranging from 10 to 250 pmol/ml. HDL degradation at 200 pmol/ml (22 μg/ml) was reported to be 2.4 μg/mg cell protein/day.[256] Since uptake of labelled sucrose has been described as a convenient measure of bulk fluid endocytosis,[22, 327, 351] they compared total lipoprotein uptake (i.e., internalized + degraded) with sucrose uptake. At lipoprotein concentrations of 10 μg/ml, the observed uptakes of LDL and HDL were 10 times and 43 times greater, respectively, than the uptake of ^{3}H-sucrose by bulk fluid endocytosis. At a lipoprotein concentration of 50 μg/ml, the observed uptake of HDL fell to only 15 times greater than the fluid endocytosis of ^{3}H-sucrose whereas the uptake of LDL was still 10 times greater.

Finally, the catabolism of labelled desialylated LDL (70% of sialic acid residues removed) was compared with the catabolism of labelled native LDL in rat hepatocytes.[12] Both lipoproteins were observed to be degraded at similar rates over a range of concentrations ranging from 20 to 100 μg/ml. Their value of 0.8 μg of LDL degraded/mg cell protein/day agreed fairly well with the estimated

value of 0.6 μg of LDL degraded/mg cell protein/day obtained from the Pangburn and Weinstein abstract.(256) The cells did differentiate between asialo-fetuin and native fetuin, binding and degrading the former much more rapidly. This was a clear indication that the carbohydrate moiety of LDL is not involved in hepatic recognition and uptake.

3. Other Hepatocyte Preparations

Van Berkel et al. examined the catabolism of LDL and HDL in homogenates of isolated parenchymal or nonparenchymal cells.(340, 343) They found that at pH 4.2, the homogenates degraded 5- to 6-fold more HDL than LDL. Furthermore, although the nonparenchymal cells hydrolyzed 7.5 times more LDL and 5.5 times more HDL per milligram of homogenate protein than did parenchymal cells, the nonparenchymal cells comprise only 10% of the total liver protein(353) and therefore accounted for only 45% and 38%, respectively, of the LDL and HDL degradation that occurred in whole liver homogenates.(343) These studies indicated that nonparenchymal cells contribute significantly to liver degradation of lipoproteins. This group then investigated the contribution of nonparenchymal cells to the in vivo uptake of lipoproteins. They injected either ^{125}I-labelled or ^{3}H-cholesterol ester-labelled LDL or HDL intravenously into rats.(341, 342) After 6 hours, they perfused the liver with collagenase, separated the parenchymal from the nonparenchymal cells, and examined each cell type for accumulated label. In all cases. the nonparenchymal cells contained 3 to 4 times more radioactivity per milligram of cell protein than the parenchymal cells. Thus Van Berkel and his colleagues have shown that

nonparenchymal cells take up approximately 30% of the total activity recovered in the liver and that these cells account for approximately 40% of the lipoprotein degradation observed in liver homogenates.[341, 343]

Bachorik et al. isolated a plasma membrane fraction from porcine liver that binds LDL.[15, 16] He defined high-affinity binding as the difference between the amount of labelled LDL associated with the membranes in the presence or absence of excess unlabelled lipoprotein. Studies with the crude plasma membrane fraction showed that 40% of the total ^{125}I-LDL binding was inhibited by adding bovine serum albumin (5 mg/ml) to the incubation system. Further decreases in binding did not occur with increasing amounts of albumin. After excluding this component of nonspecific binding by including albumin in the medium, they showed that concentrations of LDL from 0 to 100 μg/ml depressed labelled LDL binding by 80%. A Scatchard plot of the concentration dependence of ^{125}I-LDL binding to crude plasma membrane preparations revealed a concave slope. This was interpreted to mean that at least two separate binding sites were present, a high-affinity saturable site with an apparent dissociation constant (K_d) of 11 μg/ml and a low-affinity nonsaturable site. The nonspecific component of total LDL binding was maximal by 10 minutes, the shortest time measured. The high-affinity binding was markedly temperature sensitive and the nondisplaceable binding was somewhat less affected by temperature. The percent of total binding due to the displaceable component fell from 76% at 35°C to 64% at 24°C and finally to 53% at 4°C. The absolute amount of displaceable LDL bound to membrane protein fell from

140 ng/mg/30 min to 25 ng/mg/30 min. They also isolated two subfractions of plasma membranes, light and heavy. Only the heavy membrane fraction was enriched in LDL-displaceable binding (about 20-fold) compared with the unfractionated microsomal fraction. When this fraction was characterized, it was found that the presence or absence of bovine serum albumin did not influence LDL binding as in the crude plasma membrane fraction. Additionally, treatment of the heavy membrane with trypsin or pronase had no effect on high-affinity binding. They also showed that HDL (without detectable apo-E) and phosphatidylcholine inhibited ^{125}I-LDL binding to heavy membranes. Divalent cations stimulated but were not absolutely required for binding. These effectors of binding were not examined in the crude plasma membrane fraction. The finding that LDL binding was not destroyed by treatment of the membrane with proteolytic enzymes is puzzling since Goldstein and Brown showed that in fibroblasts brief treatment with pronase, papain, or trypsin destroyed the LDL receptors and that cycloheximide prevented the reappearance of the receptors.(143) Also Carrella and Cooper showed that high affinity binding of chylomicron remnants to rat liver plasma membranes decreased when the membranes were treated with trypsin.(66) The LDL receptor characterized by Goldstein and Brown also had an absolute requirement for divalent cations.(143) Thus while the investigations of Bachorik et al. into the binding of LDL to a crude preparation of plasma membranes yielded data which were compatible with what is known about LDL binding to the fibroblast receptor, the binding which they described in the "purified" fraction indicated that binding to a nonprotein site was

occurring.[15, 16] Was this perhaps an artifact of the preparation?

Recently Carrella and Cooper reported the high-affinity binding of chylomicron remnants to rat liver plasma membranes.[66] They showed that the remnant particle appeared to bind to the membrane as a unit and was not displaced by albumin, VLDL, LDL, or cholesterol-lecithin vesicles. Chylomicrons, remnants, and HDL displaced the labelled remnants to varying extents. Binding was decreased after the membranes were treated with trypsin.

C. The Low Density Lipoprotein Receptor

The LDL receptor was discovered and described in 1973 by Michael Brown and Joseph Goldstein through studies in cultured human fibroblasts.[51, 52, 140] They compared LDL catabolism in normal fibroblasts with that in fibroblasts from patients homozygous for familial hypercholesterolemia. They demonstrated that a specific high-affinity receptor bound LDL in normal fibroblasts but that this receptor was absent in the genetically defective fibroblasts. The work of Brown, Goldstein, and others on the LDL receptor has been comprehensively reviewed and will only be summarized here.[138, 143]

Biochemical evidence has indicated the receptor is a protein.[143] Subjecting fibroblasts to a brief treatment with the proteolytic enzymes pronase, trypsin, or papain destroyed the receptor, and cycloheximide blocked its reappearance. A variety of other enzymes, including various glycosidases, phospholipases, aryl sulfatases, and acid and alkaline phosphatases, did not influence receptor activity.

The fibroblast LDL receptor has an absolute requirement for divalent cations.[141] Calcium or other divalent cations were also

required for receptor activity in preparations of isolated fibroblast membranes.[20] Bachoric et al. found that calcium stimulated receptor binding of LDL two- to threefold in plasma membranes isolated from porcine hepatocytes.[15]

Competitive binding studies showed that LDL from several species actively competes with human LDL for binding to the receptor.[143] All apo-B-containing lipoproteins compete with LDL for binding to the receptor. Innerarity and Mahley used competitive binding assays to show that canine HDL_c (a lipoprotein containing apo-E as its only detectable protein) possessed an enhanced binding activity, which ranged from 10- to 100-fold greater than was observed with human or canine LDL.[166] No other proteins or lipoproteins have been found that compete for the receptor binding site.

These observations led to speculation that apo-E and apo-B have a common structural sequence which is responsible for the binding to the high-affinity receptor. Recently, Mahley and his colleagues showed that the modification of either lysine or arginine residues on LDL or HDL_c abolished their ability to react with the receptors.[214, 356] These procedures specifically modified either lysine or arginine without causing irreversible alteration of the chemical or physical properties of either LDL or HDL_c.

In addition, Weisgraber et al. showed that it was the modification of the lysine residue, not the neutralization of the positive charge on lysine, that abolished the receptor binding activity of apo-B and apo-E.[357] Two of the chemical modifications, acetoacetylation and carbamylation, modified the lysine chemically and

neutralized the positive charge of the ϵ-amino group. However, the third method, reductive methylation, modified the lysine chemically but preserved the positive charge. This modification destroyed the binding activity of the apoprotein as effectively as had the other two.

Another study showed that partial delipidation, which removed more than 75% of the lipid, did not alter the reactivity of HDL_c or LDL with the high-affinity receptor sites.(166) Total delipidation of apo-E abolished its ability to bind to the high-affinity receptor, but binding was restored when phospholipid was added back to the apo-E.(167) It was suggested that the increased α-helical structure caused by the presence of the phospholipid material oriented the molecule into the proper configuration for recognition and binding by the receptor. Complete delipidation of apo-B and the subsequent formation of an apo-B-albumin complex to solubilize the apo-B, however, caused greatly increased binding to the high-affinity receptor.(302) These opposite effects on receptor binding caused by the total delipidation of apo-E and apo-B are very interesting. Further investigation is needed in order to provide an explanation of these differences.

Another contrast was observed in the high-affinity binding of HDL_c and LDL to the fibroblast receptor. Goldstein et al. showed that heparin and other sulfated glycosaminoglycans cause the release of LDL from its cell surface receptor.(139) Release of LDL by heparin has subsequently been used in many laboratories to measure specific binding. However, Pitas et al. found that receptor-bound HDL_c was only minimally released from heparin-treated fibroblasts.(259) They suggested that the resistance of HDL_c to release by heparin could be

explained by the increased affinity HDL_c displays for the receptor. They also noted that there was a weaker interaction of HDL_c with heparin affinity columns than was observed with LDL. It can therefore be surmised that although the same amino acids, lysine and arginine, are involved in the binding of both apo-E and apo-B to the high-affinity receptor on fibroblasts, the binding also includes other elements which are not identical and which give rise to the observed differences in binding affinity of apo-B and apo-E.

Visualization of LDL receptors on the plasma membrane of normal fibroblasts was first described by Anderson et al.[7] Normal fibroblasts were incubated with ferritin-labelled LDL, and then thin sections were viewed with an electron microscope. Seventy percent of the surface-bound ferritin cores were localized in short segments of the membrane where it appeared to be indented and coated on both sides by a fuzzy material. These "coated pits" had been previously described in other cell types, but this was the first evidence that they were involved with LDL binding.[7] Although the same number of indented, coated membrane regions per millimeter of cell surface were present in fibroblasts from patients homozygous for familial hypercholesterolemia, no LDL-ferritin was observed to bind to the cell membrane.[7] These studies were recently confirmed for unmodified LDL when Vermeer et al. visualized the bound LDL by an indirect immunoperoxidase technique combined with the use of an antiserum against apo-B.[348] Immunoreactive regions representing bound apo-B were found on the plasma membrane in indented regions that had a fuzzy coat on the cytoplasmic side (i.e., coated pits). Fibroblasts from

a patient homozygous for familial hypercholesterolemia showed no immunoreactive material in the indented regions.

Anderson, Brown, and Goldstein have described a mutant fibroblast that binds LDL but does not internalize it.[8, 55, 138] Investigation by electron microscopy and LDL-ferritin disclosed that the LDL receptors were not localized in the coated pits but were scattered along noncoated segments of the membrane.[8] On the basis of these studies they constructed a model in which the LDL receptor is a transmembrane protein that has two active sites. One is the binding site for LDL and is located on the external surface of the membrane. The other is the internalization site, which causes the receptor to be recognized as a component of the coated pits, and is located on the cytoplasmic surface of the membrane. Receptors that contain this site migrate laterally in the plane of the membrane and cluster together in regions containing coated pits. Brown and Goldstein hypothesize that the clustering occurs on the cytoplasmic surface of the membrane as a result of an interaction of the internalization site with either the coat protein, clathrin, or some other protein that is bound to clathrin.[55] The binding of LDL to the receptor does not appear to induce the clustering since the clustering phenomenon was also observed in cells that had been partially fixed prior to exposure to LDL-ferritin.[7]

The two-site receptor model is consistent with the finding of Ostlund et al. that microtubules mediate the process of intracellular degradation of LDL.[255] Colchicine, which causes the depolymerization of microtubules, did not influence LDL binding but reduced the

endocytosis of bound LDL to 58% of that occurring in nontreated cells and also reduced the release of LDL degradation products to only 34% of that in the control cells.

Interpretation of kinetic experiments indicated that LDL receptors escape degradation in the lysosomes after internalization of the LDL-receptor complex and return to the plasma membrane surface where they again cluster in coated pits.(8, 139) The recycling of receptors would account for the observation that fibroblasts ingested saturating levels of LDL at a uniform rate for more than 6 hours after the synthesis of new receptors had been blocked by cycloheximide.(8) Goldstein et al. contend that if recycling did not occur, all of the receptors would be consumed within 10 minutes after exposure of the cells to LDL.(138) To date, however, there has been no direct evidence that such recycling occurs although other investigators have proposed models for membrane recycling.(80, 91)

There is currently some disagreement in the literature concerning the role of the carbohydrate moiety in the binding of LDL to its receptor. Filipovic et al. reported that fibroblasts and smooth muscle cells showed enhanced binding and internalization of ^{125}I-LDL that had been desialylated with neuraminidase from Vibrio comma cholerae (Behringwerke).(115) On the other hand, Shireman and Fisher reported the absence of a role for the carbohydrate moiety in the binding of LDL to the high-affinity receptor of fibroblasts.(301) They desialylated LDL with an enzyme preparation containing neuraminidase, β galactosidase, endo-β-galactosidase, β-N-acetyl glycosaminidase, and endo-N-acetyl-glucosaminidase which had been prepared from

Diplococcus penumonie by Dr. Gilbert Ashwell. Finally, Attie et al. desialylated LDL by incubation with agarose-bound proteinase-free neuraminidase from Clostridium perfringens (Sigma).[12] Since liver parenchymal cells have been identified in rats and rabbits as the site of uptake of asialo-glycoproteins,[336] Attie et al. examined the degradation of native and desialylated LDL and native- and asialo-fetuin in primary cultures of rat hepatocytes and also in vivo in the pig.[12] In the hepatocyte cultures, the degradation rates of native and desialylated LDL were indistinguishable whereas asialo-fetuin was degraded at six to ten times the rate of native fetuin. In the in vivo studies, the kinetics of decay of simultaneously injected ^{125}I-labelled LDL and ^{131}I-labelled desialylated LDL were not significantly different. On the other hand, asialo-fetuin was cleared very rapidly with a $t_{1/2}$ of 90 seconds whereas intact fetuin was cleared much more slowly with a $t_{1/2}$ of 42 hours. Since all three studies utilized different enzyme preparations to desialylate the LDL, it is possible that the inconsistent results of the study reported by Filipovic et al. were caused by an unidentified proteinase contaminant.[115] They did not screen the neuraminidase they used for proteinase activity as did Attie et al.[12]

Thus much has been speculated and learned about the LDL receptor and how it functions but relatively little solid information yet exists. The studies reviewed in this section, however, provide numerous insights and intimations from which future attempts at finding the complete solution will arise.

III. MATERIALS AND METHODS*

A. Animals

1. Lipoprotein and Serum Donors

Control LDL donor rabbits were bred and raised to 8 to 9 weeks of age by the Grand Ronde Rabbitry. They were 4- to 5-pound New Zealand white rabbits of both sexes and had been maintained on a nonmedicated diet of commercial alfalfa pellets. These animals were also the source of the serum used in the hepatocyte incubations.

Hyperlipemic LDL donors were also New Zealand white rabbits. These were male, 8 to 10 pounds, and 20 to 40 weeks of age. They were fed Purina® Rabbit Chow coated with 0.5% cholesterol (dissolved in 1.4% butter) for a period of one to three months before donation of hyperlipemic blood.

2. Liver Donors

Four- to 5-pound male New Zealand white rabbits, fed Purina® Rabbit Chow, were the best liver donors. Larger, older animals were more apt to have tougher connective tissue.

3. In Vivo Metabolic Studies

Hyperlipemic rabbits maintained on diets containing 0.5% cholesterol and 1.4% butter for one month had livers that were neither enlarged nor grossly fatty; those maintained on the same regimen for six months did have enlarged and fatty livers. The hyperlipemic

*Abbreviations used in this section: EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDH, lactic dehydrogenase; LDL, low density lipoprotein; leu, leucine; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TMU, tetramethylurea; Tris, tris(hydroxymethyl)aminomethane; VLDL, very low density lipoprotein.

rabbits and control rabbits were given injections of low density lipoproteins isolated from either control or hyperlipemic donors and labelled with ^{3}H-leucine (^{3}H-leu) or ^{125}I as described in section III.B.5.a,b. The lipoproteins were injected into the marginal ear vein, and blood samples were withdrawn from the central artery of the ear. The animals were killed by exsanguination 1, 3, 6, or 24 hours later under ether anesthesia and then were perfused with 3 liters of isotonic saline to remove blood from the tissues. Samples of plasma, plasma lipoprotein, and tissues were analyzed for the radioactivity in the protein, lipid, and water-soluble fractions as described in sections III.D.1,2,3 and Portman et al.(270)

B. Preparation of Lipoproteins and Lipoprotein-deficient Serum

1. General Methods

The original description of lipoprotein fractionation appeared in 1954.(82) This was subsequently modified; the version described by Havel et al., in which density adjustments are made up to d = 1.063 g/ml by addition of KBr solutions of various densities, is probably the most commonly used method for separating lipoproteins.(156) Radding and Steinberg reported a calculation that adjusts the density through the direct addition of solid KBr to serum.(272) In the formula used for this calculation

$$X = \frac{Vi\ (d_f - d_i)}{1 - 0.298\ d_f}$$

"X" is the grams of solid KBr to be added for adjustment; "Vi" is the initial volume of the solution to be adjusted; "d_f" is the final

density desired; "d_i" is the initial density; and "0.298" is the partial specific volume of KRb calculated by Hatch and Lees[154] from the data of Baxter and Wallace.[21] The practical details of lipoprotein separation and analysis have been clearly described by Hatch and Lees.[154]

All centrifuge runs were performed at 5°C in a model L2-75B ultracentrifuge (Beckman) with polycarbonate tubes. Density adjustments were made by the method of Radding and Steinberg.[272] Lipoprotein fractions were removed by gentle aspiration of the floating layer from the tube. The rim of the tube was then wiped free of residual lipid, and the infranate was removed with a pipette. When sequential lipoprotein fractions were removed, the infranate was returned to the original volume by the addition of a KBr solution of the same density before the density was raised to the next level by the addition of crystalline KBr. These KBr solutions were made by addition of the appropriate amount of crystalline KBr to a sodium chloride solution containing 22.8 g of NaCl, 0.2 g of ethylenediaminetetraacetic acid (EDTA), and 2.0 ml of 1 N NaOH (made up to 2 liters with water) plus 6.0 ml of water (d = 1.006 g/ml). Dialysis of all lipoprotein fractions and of lipoprotein-deficient serum was carried out in a 4°C cold room. After isolation, lipoprotein samples were stored in ice. Lipoprotein-deficient serum was stored at -15°C.

2. Low Density Lipoprotein Isolation

Control rabbit blood was purchased by the quart from the Grand Ronde Rabbitry. Each quart contained the pooled blood from approximately four to six rabbits. The blood was allowed to stand at

room temperature for 30 minutes and then was refrigerated overnight before the serum was harvested. The serum was adjusted to a density of 1.019 g/ml with crystalline KBr and centrifuged for 20 hours at 40,000 rpm in either a 42.1 or a 60 Ti rotor (Beckman). The lipoprotein fraction was gently removed with a Pasteur pipette. The sides of the tubes were wiped free of lipid and then the infranate was removed and placed in clean centrifuge tubes. The density of the serum was readjusted to 1.063 g/ml with KBr. The LDL fraction ($1.019 < d < 1.063$) was removed by gentle aspiration after 20 hours at 40,000 rpm, and it was concentrated and purified by recentrifugation at $d = 1.063$ g/ml for 20 hours at 45,000 rpm in a 50 Ti rotor (Beckman). This LDL was then exhaustively dialyzed against 0.9% NaCl containing 0.05 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.4) and 0.001 M disodium EDTA and stored in ice. Before use in cell experiments, the LDL was dialyzed against unbuffered saline to remove the Tris.

Hyperlipemic LDL was obtained in similar fashion from the serum of rabbits given food containing 0.5% cholesterol for a period of one to three months.

3. High Density Lipoprotein Isolation

After removal of lipoproteins of $d = 1.019$ to 1.063 g/ml, the tubes were wiped free of lipid. The infranate was then removed and adjusted to a density of 1.21 g/ml with KBr and spun for 40 hours at 40,000 rpm in a 42.1 or a 60 Ti rotor. The high density lipoprotein (HDL) fraction was concentrated by spinning 40 hours at 45,000 rpm in the 50 Ti rotor, exhaustively dialyzed against buffered saline, and stored in ice. Before use in cell experiments, the HDL was

dialyzed against unbuffered saline.

4. Lipoprotein-deficient Serum

Fresh rabbit serum was adjusted to a density of 1.25 g/ml with KBr and centrifuged at 40,000 rpm for 40 hours in a 42.1 rotor or at 55,000 rpm for 24 hours in a 60 Ti rotor, and the lipoprotein fraction was removed. Several lots of serum had lipoproteins of increasing density removed sequentially, and other lots had all the lipoproteins removed in a single step as described above. After exhaustive dialysis against isotonic saline, two 3-ml aliquots of serum were removed for analysis and the remaining serum was frozen until a pool of approximately two liters had been collected. The pooled serum was thawed, thoroughly mixed, and refrozen in 100-ml aliquots until needed for cell experiments. This method provided a pool of lipoprotein-deficient serum from approximately 20 to 25 rabbits.

The aliquots for analysis were mixed with 4 ml of d = 1.006 saline, and the final density was adjusted to 1.063 g/ml or 1.21 g/ml with KBr. The samples were centrifuged for 20 hours and 40 hours, respectively, at 45,000 rpm in a 50 Ti rotor. The top 1 ml was removed from each tube and dialyzed overnight against saline. The protein value was determined by the method of Lowry and an aliquot was subjected to electrophoresis in a 7.5% sodium dodecyl sulfate (SDS) (Schwarz Mann) gel containing polyacrylamide as described in section III.C.1.[204] The $d < 1.063$ sample contained no detectable protein, and there were no Coomassie-blue-stained bands on the gels. The $d < 1.21$ sample contained apoprotein A and C bands, earmarks of

HDL. The lipoprotein-deficient serum was free of LDL apoprotein and contained only 42 μg of HDL protein per ml (whole serum contains 800 to 1000 μg of HDL protein/ml). Thus, only 4 to 5% of the original HDL and no detectable LDL remained in the lipoprotein-deficient serum.

5. Isotopic Labelling of Low Density Lipoprotein

a. Iodination Method

Isolated control or hyperlipemic LDL was labelled with carrier-free Na-^{125}I obtained from Amersham/Searle (pH 10.0) by the iodine monochloride method of McFarlane[226] as modified by Fidge and Poulis.[112] The LDL to be iodinated was dialyzed against 0.02 M glycine buffer (pH 9.0) for 30 minutes and then placed in tube A. Tube B contained 0.2 ml of 0.02 M glycine buffer (pH 10.5) and 0.5 mCi of Na-^{125}I/mg of LDL protein. Eight microliters of 3.3 mM ICl (prepared by the method of McFarlane) per milligram of LDL protein were added to tube B and the entire contents were immediately mixed with the LDL in tube A.[227] The reaction was allowed to continue for two minutes, and then the pH was lowered to 7.4 with 0.1 M Tris. The unbound iodide was removed by passage of the ^{125}I-LDL preparation through a 9 x 100 mm Sephadex G-50 fine column (Pharmacia). The iodinated preparation was washed through the column with 10 ml of buffer containing 0.05 M Tris and 0.001 M EDTA (pH 7.4). The void volume of the column was 2.0 ml. The iodinated protein was collected in the 1.5-ml fraction immediately after the void volume. The next 0.5-ml fraction was relatively free of protein or iodide. Most of the iodide was removed in the next 2.5 ml of buffer. The iodinated protein was dialyzed (4°C) against three changes of buffer comprised

of 0.05 M Tris (pH 7.4), 0.001 M Na_2EDTA, 0.85% NaCl, and 0.05% NaI. Finally, the ^{125}I-LDL was dialyzed against 0.9% NaCl with Tris (0.05 M) and EDTA (0.001 M). Some preparations were adjusted to d = 1.063 g/ml with KBr and reisolated by ultracentrifugation. The LDL was then dialyzed against saline to remove the KBr.

Some samples of control LDL were also iodinated in this fashion with the stable Na-^{127}I isotope rather than the radioactive Na-^{125}I isotope.

Several LDL preparations were subjected to additional manipulations in an attempt to further reduce the amount of free iodide present. One preparation of iodinated LDL was passed through two additional Sephadex columns. A 5-μl aliquot of the LDL fraction from each column was precipitated with 10% TCA, and the ^{125}I in the precipitate and the soluble fraction was counted. A second preparation was divided equally into part A and part B. Part A was brought to a final concentration of 2.6% NaI. Part A and part B were then passed through separate Sephadex columns, and the LDL fraction of each was collected. Twenty-microliter aliquots were precipitated with 10% TCA or 2:1 chloroform-methanol. The ^{125}I in the precipitates and the soluble fractions was counted. A third preparation of LDL was subjected to the conditions used for iodination but without the addition of ICl, and then Na-^{125}I was added two minutes before the preparation was passed through the Sephadex column. The LDL and iodide fractions were collected, and 5-μl aliquots were analyzed for free $^{125}I^-$ by scintillation counting. The LDL and iodide fractions were again passed through separate Sephadex columns, and aliquots of the LDL and

iodide fractions were anlayzed for free iodine (for results, see section IV.A.2.).

b. Incorporation of ^{3}H-leucine

A small dose (0.8 mCi) of L-[4,5-^{3}H]leu (Amersham/Searle) was injected into a 4-kg male New Zealand white rabbit. Blood samples were withdrawn at intervals of 30 minutes for 4 hours and then again at hours 6 and 24 in order to determine the time at which maximum incorporation of ^{3}H-leu occurred in the LDL. Very low density lipoprotein (VLDL), IDL, LDL, HDL_2, and HDL_3 were isolated from each sample by sequential flotation in an ultracentrifuge. The lipoprotein fractions were analyzed for protein content and radioactivity. The maximum specific activity in LDL was reached after 3 hours, and it then gradually diminished to half of the maximum value during the succeeding 2 hours (Figure 1). It was decided that ^{3}H-leu LDL specific activity would be maximized by injection of 15 mCi of ^{3}H-leu in three 5-mCi doses at minutes 0, 30, and 60. The label was injected into the marginal ear vein of the rabbit, and 3 hours after the final injection, the animal was exsanguinated via a catheter placed in the femoral artery. The ^{3}H-leu LDL was isolated from the serum as described in section III.B.1.

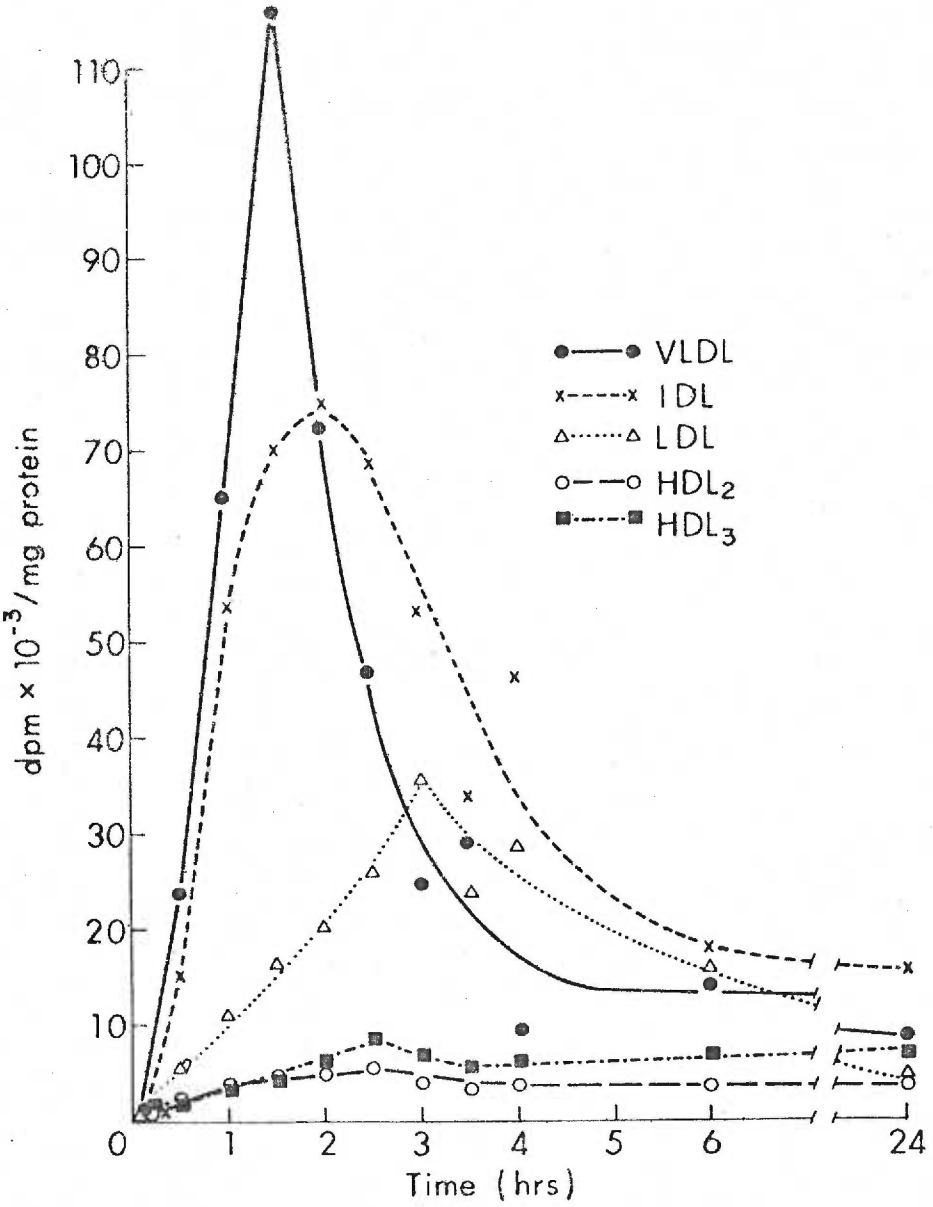
C. Cell Cultures

1. Isolation of Hepatocytes

Rabbit hepatocytes were isolated by a slightly modified version of the method described by Bonney for rat hepatocytes,[41] which had been based on the liver perfusion technique originally developed by Berry and Friend.[31] Each liver was cannulated via the

Figure 1. In vivo incorporation of ^{3}H-leucine into rabbit lipoproteins. Plasma samples were withdrawn at various times after the injection of ^{3}H-leucine and dialyzed overnight to remove free ^{3}H-leucine. The samples were then subjected to sequential ultracentrifugation and the isolated lipoproteins analyzed for radioactivity and protein. These results are illustrated in the figure.

In vivo Incorporation of ^{3}H-Leucine into Rabbit Lipoproteins



the portal vein and flushed in situ with 500 to 700 ml of Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (Gibco) (37°C) containing 0.5% bovine serum albumin (Sigma), 0.55 μg of insulin/ml (Sigma, bovine pancreas, 25.3 IU/mg), 70 μg of penicillin/ml, and 110 μg of streptomycin/ml. The liver was then removed from the animal, the peripheral edges of the lobes were trimmed away, and the gallbladder was clamped off. The liver was then placed in a closed recirculating perfusion system (37°C) containing 100 ml of the supplemented Hank's balanced salt solution, to which had been added 80 mg of hyaluronidase (Sigma, type 1), 50 mg of collagenase (Sigma, type II), and enough saturated $NaHCO_3$ solution to bring the perfusion medium to pH 7.4. This medium was continuously gassed with a mixture of 5% CO_2 and 95% O_2, and the perfusion was continued until the liver was easily penetrated with a blunt instrument (35 to 60 minutes). The liver was then removed to a steel tray containing 50 ml of oxygenated Hamm's F-12 medium (Microbiological Associates) (37°C). The cells were combed away from the remaining connective tissue, filtered through 253-μm-mesh nylon gauze (Tetko) and then through 64-μm-mesh nylon gauze, and centrifuged at a low speed (300 rpm) to separate the cells from the medium. The cells were resuspended twice in 37°C oxygenated medium and gently centrifuged. This process removed any residual enzyme and the majority of the Kupffer cells.[187] Cell viability was determined with a trypan blue exclusion test and preparations in which fewer than 90% of the cells excluded the dye were discarded.[364]

2. Establishment of Primary Cultures

The primary culture technique was chosen because it offered a way of examining several hundred replicate cultures while providing a system in which cells could reverse damage incurred to microvilli during isolation. The separation of viable cells from irreversibly damaged cells and cell debris was readily accomplished when the medium was changed after 20 hours. Primary culture also permitted the assessment of changes occurring in the cells over a period of time up to 72 hours. This was an important consideration since data from experiments utilizing both fibroblasts[53] and lymphocytes[162] indicated that cells had to be exposed to LDL-free medium for 20 hours before specific high-affinity binding could occur.

The isolated cells were diluted with an equal volume of Hamm's F-12 medium supplemented with 0.55 μg of insulin/ml, 70 μg of penicillin/ml, 110 μg of streptomycin/ml, and 20% lipoprotein-deficient serum. The cells were counted in a hemacytometer, and 4 to 12 x 10^6 cells were plated in 60 x 15 mm tissue culture dishes (Falcon) containing 3 ml of the supplemented Hamm's F-12 medium. After 20 hours, the medium was changed and unattached cells were discarded.

Viability of the cultured cells was routinely assessed by the dye-exclusion test with 0.21% trypan blue (Dr. G. Grübler & Co.) as excluded dye.[42] This technique was fully described in 1917 by Pappenheimer[257] and had been used even earlier to differentiate vital cells that excluded dye from nonviable cells that absorbed it.[148] Two drops of trypan blue solution (0.21%) were added to the culture dish after removal of the medium. A glass coverslip was

placed gently over the stain, and the plate was examined with a microscope at a magnification of 40X.

Lactic dehydrogenase (LDH) in the medium was assayed with a Sigma LDH diagnostic kit. This determination, based on the method developed by Wroblewski and LaDue, gave an index of enzyme leakage from the cells.(360)

3. Lipoprotein Uptake and Degradation

Experiments were carried out after 2 or 3 ml of fresh, lipoprotein-deficient medium had been added to the cell cultures. The contents of each dish were analyzed for bound, internalized, and degraded LDL apoprotein according to the following procedures.

a. Degradation

The medium was removed from each dish, an aliquot was removed for assay of the total radioactivity, and the remainder of the medium was brought to a final concentration of 10% trichloroacetic acid (TCA) by the addition of an appropriate amount of 50% TCA. After this mixture had been stored overnight in ice, it was heated to 100°C for 10 minutes and the precipitate was removed by centrifugation. In some cases the precipitate was dissolved with 0.5 ml of 5 N NaOH at 60°C, was neutralized with concentrated HCl to a phenophthalein end point, and was transferred with 10 ml of Formula 950-A scintillation fluid (New England Nuclear) and 1 ml of methanol to a scintillation vial for determination of radioactivity. One aliquot of the TCA-soluble fraction was taken for direct radioassay, and a second 2-ml aliquot was treated with 10 μl of KI and 50 μl of H_2O_2 for 10 minutes. This procedure converted inorganic iodide, but not tyrosyl iodide, to

iodine.[34] The iodine was removed by two extractions with 5 ml of $CHCl_3$, and the iodide-free, TCA-soluble material remaining was assumed to be degraded LDL apoprotein.

Because I wanted to confirm the validity of the claim of Bierman et al. that the iodine moieties of tyrosyl iodide and other small iodinated peptides of tyrosine resist oxidation with H_2O_2 and extraction with $CHCl_3$,[34] ^{125}I-L-tyrosine, ^{125}I-L-tyrosylglycine, ^{125}I-L-tyrosyl-L-leucine, and ^{125}I-L-tyrosyl-L-phenylalanine were synthesized and tested. The L-tyrosine and the dipeptides were all obtained from Sigma and iodinated as described in section III.B.5.a. The iodinated product was added to an equal volume of 20% TCA. The TCA-soluble material was treated with 10 μl of KI and 50 μl of H_2O_2 and extracted two times with 5 ml of $CHCl_3$. The radioactivity recovery rates were 92.1% for ^{125}I-L-tyrosine, 99.4% for ^{125}I-L-tyrosylglycine, 94.4% for ^{125}I-L-tyrosyl-L-leucine, and 89.9% for ^{125}I-L-tyrosyl-L-phenylalanine. The material was treated with H_2O_2 and extracted with $CHCl_3$ a second time without the recovery of the iodinated peptides or tyrosine being compromised. The results of this experiment showed that the iodide moiety on ^{125}I-tyrosine resists oxidation with H_2O_2.

b. Bound and Internalized

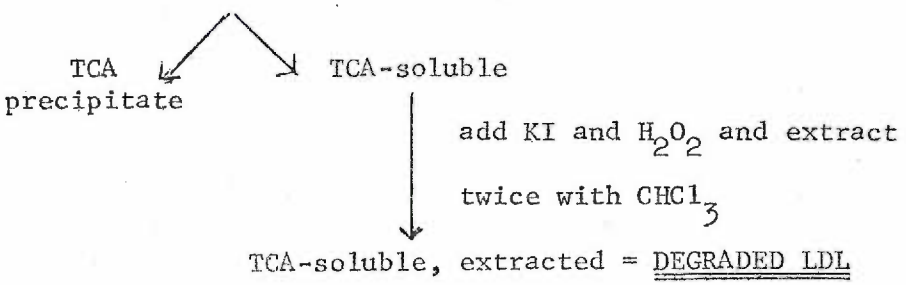
The cells were washed three times with phosphate-buffered saline (pH 7.4) and then were trypsinized for 5 minutes (37°C) with 0.5 ml of 0.05% trypsin solution; the reaction was stopped with an equal volume of medium.[34] A 100-ml portion of trypsin solution contained 0.05 g of trypsin (trypsin 1:250, Gibco), 1 ml of 2 M Tris buffer (pH 7.4), and 10 ml of 10X Versine (0.2 g of EDTA,

8.0 g NaCl, and 0.4 g of KCl/100 ml). The cells were centrifuged gently (400 rpm for 5 minutes) to remove the trypsin and were washed again with phosphate-buffered saline. The radioactivity in the trypsin fraction was defined as LDL bound to the outer surface of the cell. The cell pellet was brought to a volume of 0.5 ml with distilled water and frozen. After the pellet had been thawed and thoroughly mixed, duplicate aliquots were removed for protein determination; the remainder was added to a scintillation vial with scintillation fluid. The radioactivity associated with the cell pellet was considered to be internalized LDL. An abbreviated protocol is given below.

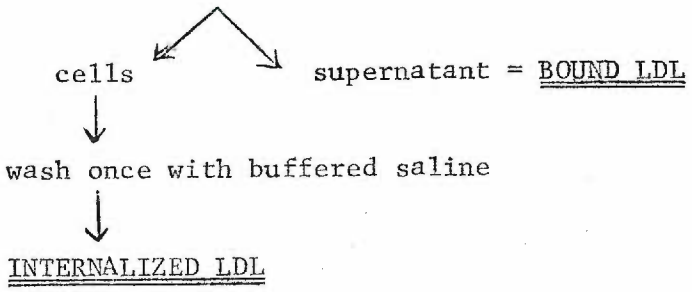
PROTOCOL FOR HANDLING CELLS

1. Plate 4 to 12 x 10^6 cells in 3 ml of Hamm's F-12 medium (20% lipoprotein-deficient serum) in 60 x 15 mm culture dishes
2. After 20 hours, remove medium and unattached cells, wash gently, and add 3 ml of fresh medium
3. Add lipoprotein according to the experiment being conducted and incubate 0 to 24 hours
4. Remove medium and add TCA to the medium

   [final concentration of TCA = 10%]



5. Wash cells three times with buffered saline and then trypsinize with 0.05% trypsin for 5 minutes at 37°C; stop with an equal volume of medium; remove to a centrifuge tube and spin for 5 minutes at 400 rpm



D. Analytical Procedures

1. General Protein Methods

Total protein concentrations of lipoprotein fractions and cell preparations were routinely determined by the Lowry method with human serum albumin as the standard.(204) Some lipoprotein preparations were examined for tetramethylurea-soluble (TMU-soluble) protein as described by Kane et al.(177) The apo-B content of the lipoprotein was determined as the difference between the soluble protein content of a lipoprotein solution treated with TMU (Sigma) and an untreated sample. The lipoprotein solution containing EDTA (1 mM) was diluted so that it contained approximately 600 μg of protein/ml. It was important to keep the ionic stength $\geq$ 0.05 and the pH between 6.0 and 9.0. The lipoprotein solution and the glass-distilled TMU were separately warmed to 37°C; 250 μl of the sample was placed in a small glass tube, and an equal volume of TMU was added with immediate mixing on a mechanical vortex mixer. The final molarity of the TMU in this

mixture was 4.2. The mixture was held at 37°C for 30 minutes and then filtered through 5.75-inch Pasteur pipettes packed with glass wool which had been washed with $CHCl_3$. One hundred-microliter aliquots were diluted with 400 μl of 0.15 M NaCl for determination of the protein content by the Lowry technique.(204) The protein standards for this analysis contained 10% TMU.

The identities of apoproteins other than apoprotein B were assessed by SDS-polyacrylamide gel electrophoresis of samples containing 10 to 20 μg of protein. The samples were diluted 1:1 with buffer* and then were boiled for 5 minutes; afterwards, 5 μl of a 1:10 dilution of pyronin Y was added. The protein preparation was then layered on top of precast Bio-Phore 7.5% gels (BioRad), to which the electrophoretic buffer had been introduced by 12 hours of electrophoresis on a model 51515 Gelcell column electrophoresis unit (Gelman), driven by a model 2103 power supply (LKB). The electrophoretic buffer consisted of 2.0 g of SDS, 44.65 g of Tris base, and 23.4 ml of acetic acid/2 liters. The gels were run at 2 mA/tube until samples entered the gels, and then the current was increased to 3 mA/tube. The electrophoresis was contined until the pyronin Y dye reached the base of the gel. The gels were stained with Coomassie blue.(108) These gels were scanned with a model 250 spectrophotometer (Gilford), and the amount of protein in the band identified as apoprotein E was estimated with human serum albumin as the standard. Some gels were cut up, and the

*The buffer contained 2.4 g of urea, recrystallized; 1.16 ml of 0.5 M Tris (pH 6.8); 1.34 ml of a 10% SDS solution (the SDS was recrystallized from ethanol before use); 0.7 ml of H_2O; and 12 mg of dithiothreitol.

radioactivity of each protein band was estimated by scintillation counting after the band had been dissolved and bleached by addition of 1 ml of H_2O_2 and heating to 70°C for 12 hours.

Several lipoprotein samples were also examined by agarose gel electrophoresis. I used Pol-E-Film prepared agarose gels (Pfizer) and followed their standard method (based on the work of Noble)[245] for determining lipoprotein phenotypes.

2. General Lipid Methods

Lipid analyses of the individual lipoprotein fractions and the cell preparations were carried out after the lipid had been extracted in 2:1 chloroform-methanol and washed by the procedure of Folch et al.[124] The $CHCl_3$ extract was taken to dryness under N_2. The total lipid residue was then dissolved in a measured volume of $CHCl_3$, from which aliquots were taken for analysis. Phospholipid phosphorus was determined by the method of Bartlett.[19] Individual neutral lipids (i.e., unesterified fatty acids, free cholesterol, esterified cholesterol, and triglycerides) were separated by thin-layer chromatography on 0.3-mm layers of silica gel H (E M Laboratories) in the solvent system n-heptane-diethyl ether-methanol-acetic acid (85:15:3:2 by volume) developed by Belfrage et al.[26] Five aliquots of a standard mixture of known lipid content were run on each plate. The mass of the separated lipids was determined by charring of the plates and measurement of the intensity of the char with a VIS-UV chromatogram analyzer (Farrand) as described by Portman and Alexander.[266] This technique accurately quantified 1 to 3 μg of each of the major lipid constituents.

3. Determination of Radioactivity

Radioactivity was assayed in a Tri-Carb model 526 liquid scintillation counter (Packard). The gain and window settings that resulted in the greatest efficiency were determined for each isotope. Lipid samples dissolved in chloroform were dried in the counting vials, and then 1 ml of water and 10 ml of Formula 950A scintillation fluid were added. Aqueous samples were prepared for counting by addition of enough water to bring the sample volume to 1 ml. Ten milliliters of scintillation fluid were then added. Protein precipitates were dissolved in 0.5 ml of 5 N NaOH and then neutralized with concentrated HCl to a phenophthalein end point. The neutralized precipitates were then transferred to the counting vial with 1 ml of methanol and 10 ml of Formula 950A.

Since ^{125}I has a half-life of 60 days, an aliquot of each ^{125}I-protein preparation was counted at the beginning of the experiment and then used as the standard to correct for isotope decay during the course of analysis. Quenching was not detected when an internal ^{125}I standard was added to several representative samples, so quench corrections were unnecessary over the energy spectrum examined. ^{125}I was counted with an efficiency of 45% in the Packard liquid scintillation counter.*

*Some gamma emitters can be counted at equal or greater efficiency by liquid scintillation counting than by conventional gamma counting because these radioisotopes emit beta particles or Auger electrons, as well as gamma-ray photons, during their decay.(182) Additionally, Compton electrons are generated by the interaction of gamma photons with the counting solution and the vial and they simulate beta particles in the liquid scintillation medium. Consequently, gamma emitters are used as the external standard in many liquid scintillation counters. Those gamma emitters, including ^{131}I, ^{125}I, ^{60}Co, ^{59}Fe, and

Because tritium is much more sensitive to quenching, the channels ratio method was used to calculate the efficiency of counting.[58] Unquenched samples were counted with an efficiency of 18.6%.

Carbon-14 samples were also corrected for quenching with the channels ratio method.[58] Unquenched samples were counted with an efficiency of 76%.

4. Determination of Specific High-affinity Low Density Lipoprotein Binding

In several studies it was necessary to separate specific high-affinity LDL binding from nonspecific LDL binding. This distinction was made in binding studies performed at 4°C in which LDL was bound to the cell but internalization and degradation did not take place. Iodinated LDL was added to the cultures in concentrations ranging from 1 to 10 μg/ml to measure total LDL binding (specific and nonspecific). The nonspecific component was determined by addition of labelled LDL in the presence of a 35-fold excess of unlabelled LDL. Specific high-affinity binding was defined as the difference between these two values.[162] All determinations were performed in duplicate.

5. Determination of the Apparent Equilibrium Constant for Low Density Lipoprotein Binding

Mathematical methods derived for the study of binding of small ligands to macromolecules have been used in a variety of systems to study hormone-receptor interaction. The application of these methods involve several important assumptions which are fully

---

^{57}Cr, that also emit beta particles or Auger electrons should be classed as beta emitters together with ^{14}C and tritium.[182]

described by Kahn.[174] When these assumptions are made, it is possible to derive a series of equations which describe both the kinetics and equilibrium of a specific binding reaction.

Goldstein and Brown and Pitas et al. applied these methods to lipoprotein-receptor interactions by making the additional assumption that the interaction of lipoproteins with the high-affinity cell surface receptor is analogous to hormone-receptor interactions.[141, 259] In order to better compare my studies on hepatocytes with those on fibroblasts, I used these assumptions to calculate an apparent equilibrium dissociation constant for LDL binding by two separate methods of calculation.

If these assumptions are made, the following equations describe both the kinetics and equilibrium of the specific binding reaction:

$$[Lp] + [R] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Lp \ldots R] \qquad (1)$$

$$\text{and } \frac{k_{-1}}{k_1} = \frac{[Lp]\,[R]}{[Lp \ldots R]} = K_d \qquad (2)$$

where [Lp], [R], and [Lp ... R] represent the concentration of lipoprotein, receptor, and receptor-bound lipoprotein, respectively; k_1 and k_{-1} are the association and dissociation rate constants; and K_d is the equilibrium dissociation constant.

a. Scatchard Analysis

I determined the equilibrium dissociation constant by plotting the ratio of receptor-bound lipoprotein to free lipoprotein against receptor-bound lipoprotein, as described by Scatchard.[289]

The slope(s) of the straight segment(s) is equal to $-(\frac{1}{K_d})$. When the Scatchard plot was concave upward, this nonlinearity was attributed to heterogeneity of the receptor sites, with the presence of several orders of dissociation constants.(280) The X intercept(s) of the straight segment(s) of the curve represented the concentration at which the receptor was saturated, and a segment parallel to the X-axis represented a receptor with "zero" affinity but "infinite" capacity. A molecular weight of 3×10^6 d, of which 20% is protein, was used to convert nanograms of LDL apoprotein to moles.(118)

b. Determination of Rate Constants

Since K_d is equal to $\frac{k_{-1}}{k_1}$, it is possible to obtain an independent estimation of K_d by determining these rate constants. The rate constants (k_1, k_{-1}) for the interaction of LDL with specific high-affinity cell surface receptors were determined from the time course of binding at four lipoprotein concentrations. Assuming that the simple reversible bimolecular equilibrium model expressed in equation (1) correctly represented the lipoprotein-receptor interactions, one obtains the rate of formation of the lipoprotein-receptor complex ([Lp ... R]) with the equation below.

$$\frac{d\,[Lp \ldots R]}{dt} = k_1\,[Lp]\,[R] - k_{-1}\,[Lp \ldots R] \qquad (3)$$

Solution of this equation (Described by Kahn(174) for hormone-receptor interactions) led to equation (4):

$$\frac{\ln 2}{t_{1/2(assoc)}} = k_1\,[Lp] + k_{-1} \qquad (4)$$

where $t_{1/2_{(assoc)}}$ is the time required for [Lp ... R] to reach 50% of its equilibrium value. The time course of binding at 4°C was ascertained at four concentrations of ^{125}I-LDL and plotted. The $t_{1/2_{(assoc)}}$ at each concentration of lipoprotein was obtained by use of a double-reciprocal plot of nanograms bound ($\frac{1}{B}$, ordinate) versus time ($\frac{1}{t}$, abscissa). The maximum bound LDL apoprotein at equilibrium was determined from the Y intercept. Half of this value gave the half maximum LDL bound, and $t_{1/2_{(assoc)}}$ was then obtained from the previously plotted time course of binding. A plot of $\frac{\ln 2}{t_{1/2_{(assoc)}}}$ versus the LDL concentration gave a straight line with slope k_1 and intercept k_{-1}. These values were used to calculate K_d (see section IV.C.4.).

6. Uptake of ^{14}C-Sucrose

The uptake of radiolabelled sucrose by cultured fibroblasts has been shown to be a suitable marker for the quantification of fluid uptake by pinocytosis.[22, 327] Experiments designed to examine fluid endocytosis by hepatocytes utilized either ^{14}C-sucrose (Amersham/Searle) or the polymer ^{14}C-dextran (mol wt 70,000; New England Nuclear). After the initial 20-hour incubation in lipoprotein-deficient medium, fresh medium containing 2.3 μmoles of ^{14}C-sucrose, 1 nmole of ^{14}C-dextran or 25 μg of ^{125}I-LDL protein/ml was added, and the incubation was continued for 24 hours at 37°C. The medium was removed, an aliquot was taken to determine the radioactivity, and the remainder was adjusted to 10% with TCA. The cells were washed three times with phosphate-buffered saline (pH 7.4), trypsinized to remove them from the dish, washed again, and then brought to 0.5 ml with water and

frozen. After thawing, duplicate aliquots were removed for protein determination and the remaining cells were added to a scintillation vial with 10 ml of Formula 950A scintillation fluid. Uptake of dextran or sucrose was calculated on the basis of internalized radioactivity. The LDL uptake was calculated as the summation of internalized plus degraded (TCA-soluble, extracted) radioactivity. Clearance of ^{14}C-sucrose, ^{14}C-dextran, or ^{125}I-LDL (microliters of medium per milligram of cell protein) was calculated by division of the uptake of radioactivity (counts per minute per milligram of cell protein) by the concentration of radioactivity in the medium (counts per minute per microliter of medium).

7. Electron Microscopy

Cells were prepared and examined with a scanning electron microscope by Dr. Wolf Fahrenbach. The cultures were fixed by addition of a phosphate-buffered 2.5% glutaraldehyde, 5% formalin solution at 37°C. They were dehydrated through critical-point drying from Freon and then were spattered with gold-palladium. A model 1000 scanning electrom microscope (AMR) was used for viewing.

8. Immunologic Identification of Apolipoprotein B

The chase medium of pulse experiments was examined for the presence of immunologically identifiable LDL by a modified version of the double-antibody radioimmunoassay of Albers et al.[4] Sheep anti-rabbit-LDL serum was obtained from Holly Hill Laboratory. Fresh, nonfrozen rabbit antisheep-IgG serum was obtained from Gibco. The lipoproteins were removed from the antibody-containing serum by ultracentrifugation at a density of 1.21 g/ml. The lipoprotein-deficient

serum was then exhaustively dialyzed before it was frozen for storage. To assay for immunologically identifiable LDL, I used the following method. An excess of sheep antirabbit-LDL antibody containing serum (200 μl) was added to the chase medium and the mixture was allowed to sit in ice overnight. Then 2.2 ml of rabbit serum containing anti-sheep-IgG antibody was added to precipitate the ^{125}I-LDL-antibody complex and again was kept in ice overnight. The precipitated LDL-antibody complex was separated by centrifugation, dissolved in 5 N NaOH, and counted as described in section III.D.3. An aliquot of the supernatant was counted before 50% TCA was added to make a final concentration of 10% TCA. The precipitate was separated by centrifugation, dissolved in 5 N NaOH, and counted.

9. Binding of Free $^{125}I^-$ to Serum and Hepatocyte Proteins

I checked for binding and coprecipitation of free $^{125}I^-$ with the serum proteins in the medium by adding a small amount of Na-^{125}I (11,000 cpm) to 3 ml of medium and incubating for 3 hours at 37°C; 50% TCA was added to make a final concentration of 10% TCA. The precipitate was collected by centrifugation, dissolved in 5 N NaOH, and counted.

Binding and coprecipitation of free $^{125}I^-$ with hepatocyte proteins was determined as follows. Hepatocytes that were suspended in medium (0.6 ml) were homogenized in 2.4 ml of saline and incubated with Na-^{125}I (38,213 cpm) at 37°C for 30 minutes. Medium controls (0.6 ml) without hepatocytes were also incubated in 2.6 ml of saline. The proteins were precipitated with TCA and counted as described above.

IV. RESULTS

A. Characterization of Low Density Lipoprotein

1. Composition of Control and Hyperlipemic Low Density Lipoprotein

The lipid and apoprotein composition of LDL isolated from hyperlipemic rabbits differed from the composition of LDL from control rabbits. The mean values for seven samples of hyperlipemic LDL and seven samples of control LDL are given in Table 5. Examination of the protein moieties showed that 36.2% of the hyperlipemic LDL protein and 11.9% of the control LDL protein were soluble in 50% TMU. Since TMU selectively precipitates apo-B, the hyperlipemic LDL contained considerably greater amounts of nonapo-B protein.(177)

TABLE 5

Percent Composition of Low Density Lipoprotein

	Hyperlipemic LDL	Control LDL
Phospholipid	20.9 ± 1.0	23.0 ± 1.2
Cholesterol ester	38.9 ± 1.2	21.3 ± 3.7
Unesterified cholesterol	13.7 ± 0.4	9.1 ± 0.7
Triglyceride	5.4 ± 1.1	19.5 ± 3.0
Protein	21.1 ± 0.4	27.2 ± 1.4

One preparation was further characterized by isolating light (d = 1.019 to 1.040 g/ml) and heavy (d = 1.040 to 1.063 g/ml) fractions from control and hyperlipemic sera; 85% of the total control

LDL protein and 78% of the total hyperlipemic LDL protein were recovered in the 1.019 to 1.040 g/ml fraction. These light and heavy LDL fractions were examined by agarose gel and SDS-polyacrylamide gel electrophoresis for evidences of HDL_c as described by Mahley (i.e., α_2 mobility, high concentrations of apo-A and apo-E, and low concentrations of apo-B).[210] The results of the SDS-polyacrylamide gel analysis are shown in Table 6. Fourteen percent of the d = 1.019 to 1.040 g/ml hyperlipemic LDL and 12% of the d = 1.040 to 1.063 g/ml hyperlipemic LDL had α mobility on agarose gel electrophoresis while less than 2% of either control LDL preparation had α mobility.

2. Characterization of ^{125}I and ^{3}H Label in Low Density Lipoprotein

a. ^{125}I-Low Density Lipoprotein

Aliquots of ^{125}I-labelled control and hyperlipemic LDL were treated with chloroform-methanol (2:1) to extract the lipid. The protein precipitate was removed by centrifugation and washed with additional chloroform-methanol that was added to the chloroform-methanol extract. This extract was washed by adding one volume of water to four volumes of extract and removing the aqueous phase. The chloroform-soluble component (i.e., lipid-bound ^{125}I) contained 5.5 to 7.6% of the radioactivity in control LDL and 4.0 to 30.0% of the radioactivity in hyperlipemic LDL. Trichloroacetic acid (TCA) precipitated 77.8% (SE = 3.2; n = 15) of the radioactivity of the control LDL and 77.5% (SE = 3.83; n = 9) of the activity of hyperlipemic LDL. Ninety-eight percent of the radioactivity of the TCA-soluble fraction was estimated to be unreacted $^{125}I^-$ (see section III.C.3.a.). One

TABLE 6

Comparison of Control and Hyperlipemic LDL Apoproteins by 7.5% SDS-Polyacrylamide Gel Electrophoresis[a]

Apoprotein	B	E	A-I	C-II	C-III	Total
Hyperlipemic LDL						
light fraction[b]	14.38 μg (71.9%)	5.24 μg (26.2%)	0.09 μg (0.5%)	0.14 μg (0.7%)	0.15 μg (0.8%)	20 μg (100.1%)
heavy fraction[c]	18.55 μg (92.8%)	1.05 μg (5.3%)	0.30 μg (1.5%)	-- --	0.10 μg (0.5%)	20 μg (100.1%)
Control LDL						
light fraction[b]	19.75 μg (98.8%)	0.21 μg (1.1%)	-- --	0.04 μg (0.2%)	-- --	20.25 μg (100.1%)
heavy fraction[c]	19.72 μg (98.6%)	0.25 μg (1.3%)	0.03 μg (0.2%)	-- --	-- --	20.0 μg (100.1%)

[a]The μg were determined by scanning the gels with a Gilford gel scanner using human serum albumin as the standard protein. 20 μg of LDL protein were applied to each gel.

[b]The light fraction was between 1.019 and 1.040 g/ml and contained 78% of the hyperlipemic LDL protein and 85% of the control LDL protein.

[c]The heavy fraction was between 1.040 and 1.063 g/ml and contained 22% of the hyperlipemic LDL protein and 15% of the control LDL protein.

preparation was subjected to more extensive analysis. Agarose gel electrophoresis showed that 95% of the protein-bound radioactivity in hyperlipemic LDL had β mobility and less than 2% had α mobility. Corresponding values for control LDL were 98% and 1%. SDS-polyacrylamide gel electrophoresis revealed that 95% of the radioactivity in control LDL and 84% of the radioactivity in hyperlipemic LDL behaved like B protein. Radioactivity was barely detectable in the apoprotein E locus accounting for only 1% and 2% of the total radioactivity, respectively, in control LDL and hyperlipemic LDL apoproteins.

Passing the ^{125}I-LDL preparation through two additional Sephadex columns did not change the ratio of counts recovered as TCA-soluble material to counts recovered as TCA-precipitable material in the LDL fraction. The total radioactivity recovered in the LDL fraction decreased after each column fractionation.

One ^{125}I-LDL preparation was divided into two parts, hereafter referred to as (A) and (B). NaI was added to (A) to give a final concentration of 2.6%. Preparations (A) and (B) were then passed through separate Sephadex columns and aliquots of the LDL fraction were examined for TCA-precipitable and chloroform-methanol-precipitable radioactivity. As shown in Table 7, 90.1% of the LDL counts of (A) were TCA-precipitable whereas only 81.8% of the LDL counts of (B) were TCA-precipitable. This experiment clearly indicated that excess, unlabelled iodide added to the LDL preparation before putting it through Sephadex reduced the amount of free ^{125}I$^-$ associated with the LDL particle.

TABLE 7

Distribution of Radioactivity after ^{125}I-LDL Was Passed through a Sephadex Column in the Presence (A) or Absence (B) of Excess Iodide Ion

Preparation		TCA[a] Precipitate	TCA-soluble		CM[c] Precipitate	$CHCl_3$	MeOH-H_2O
			Total	Noniodide[b]			
(A)	cpm[d]	208,572	22,922	1,956	118,014	38,367	17,099
	%	90.1	9.9	0.8	68.0	22.1	9.9
(B)	cpm	281,553	62,827	5,210	158,869	73,565	40,615
	%	81.8	18.2	1.5	58.2	26.9	14.9

[a]TCA = trichloroacetic acid

[b]This fraction was comprised of free amino acids and peptides after the free $^{125}I^-$ was extracted (section III.C.3.a.).

[c]CM = chloroform-methanol (2:1)

[d]cpm = counts per minute. Each value is a mean of duplicate 20 μl aliquots of the LDL fractions from the Sephadex columns that were precipitated with TCA or CM.

In a final experiment, $^{125}I^-$ was added to LDL in the absence of ICl and the mixture was separated by passage through a Sephadex column. Only a small amount (0.05%) of the $^{125}I^-$ associated with the LDL particle, but one-half of the associated $^{125}I^-$ coprecipitated with the protein after the addition of TCA or chlorform-methanol. Passing this LDL through a second Sephadex column removed 59.4% of the label in the free I^- fraction and the rest was still associated with the LDL fraction (20.8%) or remained on the column.

b. ^{3}H-Leu-Low Density Lipoprotein

Chloroform-methanol precipitation of an aliquot of biosynthesized ^{3}H-leu-LDL indicated $< 1\%$ of the label was in the lipid

fraction. TCA precipitation showed > 99% of the label in the protein precipitate.

3. Proteolytic Enzyme Degradation of Low Density Lipoprotein

The two kinds of labelled LDL were compared in an in vitro system with several proteolytic enzymes to examine the effect of incorporating iodine into the LDL structure. This is an important consideration since an iodine atom is roughly the same size as a benzene ring,(205) and this may well modify the stereochemistry and physical chemistry of the particle and influence its affinity towards proteolytic enzymes and membrane receptors. Iodination of the tyrosyl residue has the consequence of increasing the hydrophobicity of the phenoxy ring while decreasing the pK_a of the hydroxyl group of the tyrosine,(199) and this too might alter the reactivity of the protein.

Three proteolytic enzyme preparations were examined: 0.1% trypsin (Sigma, bovine pancreas, Type XI), 0.1% pronase (B grade, Calbiochem), and 0.05% collagenase plus 0.1% hyaluronidase (the enzymes used to isolate hepatocytes). Proteolysis was examined in five separate preparations of ^{125}I-hyperlipemic LDL, ^{125}I-control LDL, and ^{3}H-leu-control LDL. The concentrations were chosen to approximate the conditions which were used in the cell studies. Nonsaturating conditions and the presence of albumin in some incubations were an attempt to imitate conditions in the actual liver system. The reaction was initiated by adding the lipoprotein to the enzyme solution (dissolved in Tris buffer, pH 7.4) and stopped by adding 50% TCA to make a final concentration of 10% TCA. Degradation was estimated

by examining the TCA-soluble products as described in section III.C.3.a.

As shown in Figure 2, degradation of ^{125}I-LDL in the pronase system was linear over the concentration range of LDL examined in these experiments. The addition of 12.5 mg of albumin/ml did not saturate the enzyme although the rate at which the labelled LDL was degraded was reduced. From this figure it can be seen that the rate of LDL degradation decreases with increased incubation times. At an LDL concentration of 20 μg/ml, the average rates of degradation were only 2104 ng/ml/min after 1 minute, 244 ng/ml/min after 10 minutes, and only 130 ng/ml/min after 60 minutes. In the presence of 12.5 mg of unlabelled protein/ml the average rates were 358 ng/ml/min, 229 ng/ml/min, and 76 ng/ml/min, respectively. It can thus be seen that after one hour the rate of degradation had decreased considerably.

Figure 3 shows the results of an experiment in which differences in pronase degradation of ^{125}I-LDL and ^{3}H-leu-LDL were examined in the presence of 12.5 mg of albumin/ml. After 60 minutes of incubation, equal amounts of both proteins were degraded at all concentrations. As can be seen from Figure 3, the average rate of degradation of ^{3}H-leu-LDL during the first 10 minutes far exceeded the average rate of degradation of ^{125}I-LDL during that period. The average rate of lipoprotein degradation during the next 50 minutes of incubation was then calculated and is also shown in Figure 3. This figure shows that ^{125}I-LDL continued to be degraded during the time from 10 minutes to 60 minutes although at much slower rates than during the initial 10 minutes of incubation. It is also clear that pronase degradation

Figure 2. Degradation of ^{125}I-LDL by 0.1% pronase. Degradation was examined in the presence (---) or absence (——) of 12.5 mg/ml of albumin at 1 (■), 10 (●), or 60 (▲) minutes. The reaction was initiated by adding the protein and stopped by adding TCA to a final concentration of 10%. Values were the average of duplicate samples.

Pronase Degradation of Control ^{125}I-LDL

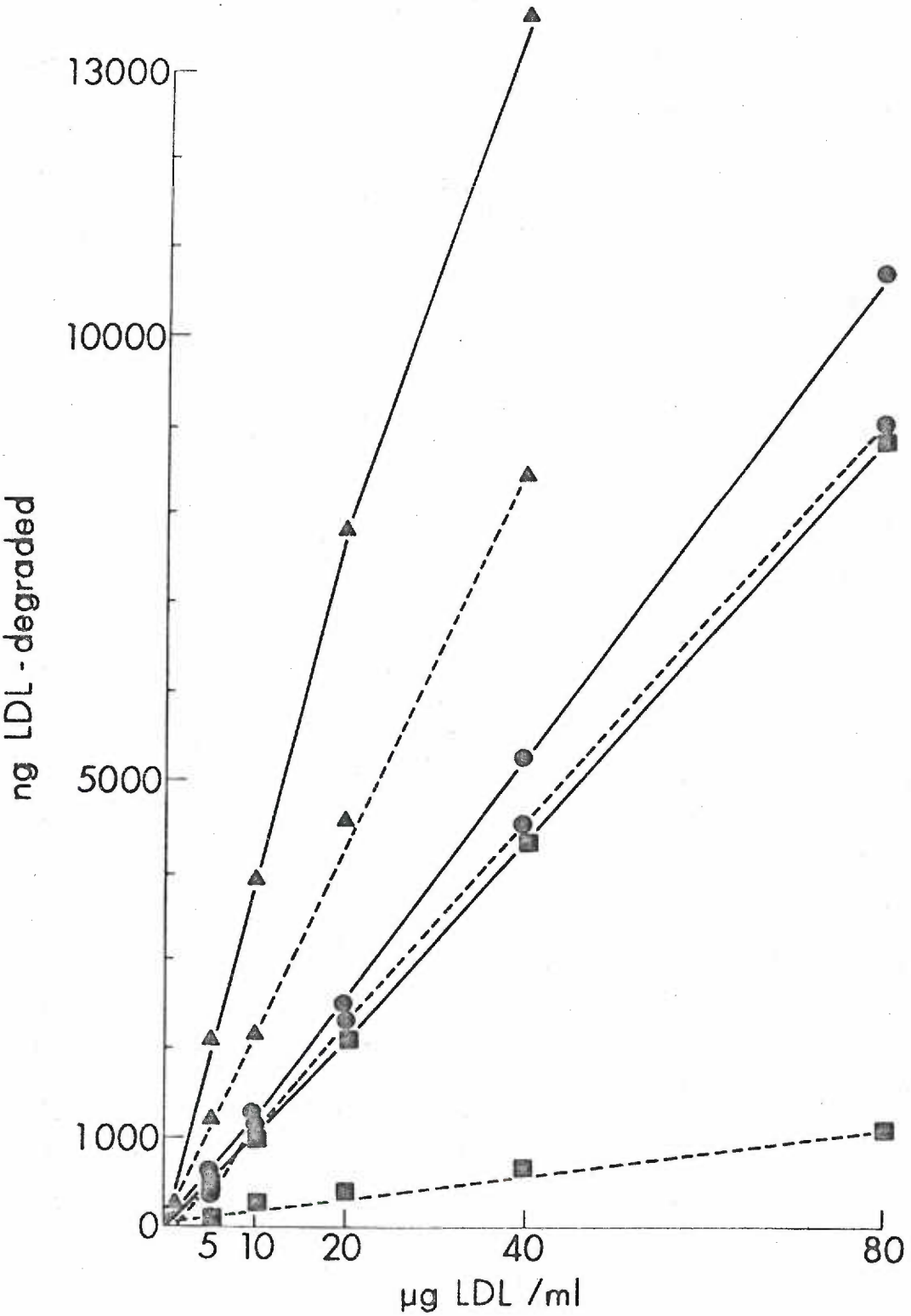
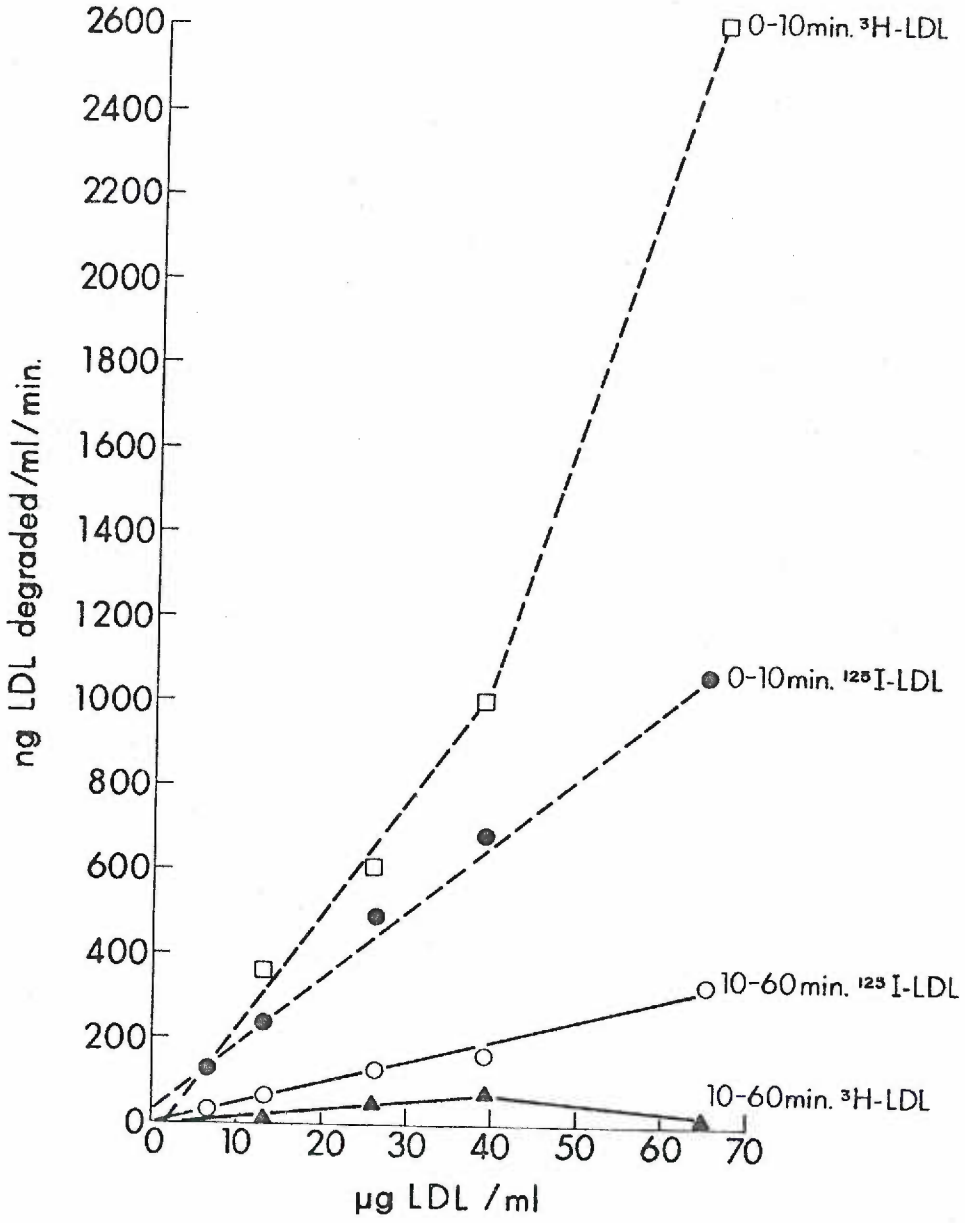


Figure 3. Comparison of the average rates of degradation of ^{3}H-leu-LDL and ^{125}I-LDL by 0.1% pronase. The average rates during the first 10 minutes of incubation (i.e., $\frac{\text{total ng degraded/ml}}{\text{10 min}}$) are shown by the --- lines. The average rate for the subsequent 50 minutes was calculated (i.e., total ng LDL/ml degraded after 60 minutes minus total ng LDL/ml degraded after 10 minutes divided by 50 minutes) and is shown by the —— lines. Duplicate determinations were performed for each point.

Average Rates of Degradation of ^{3}H & ^{125}I-LDL



of ^{3}H-leu-LDL was essentially complete after the initial 10 minutes of incubation.

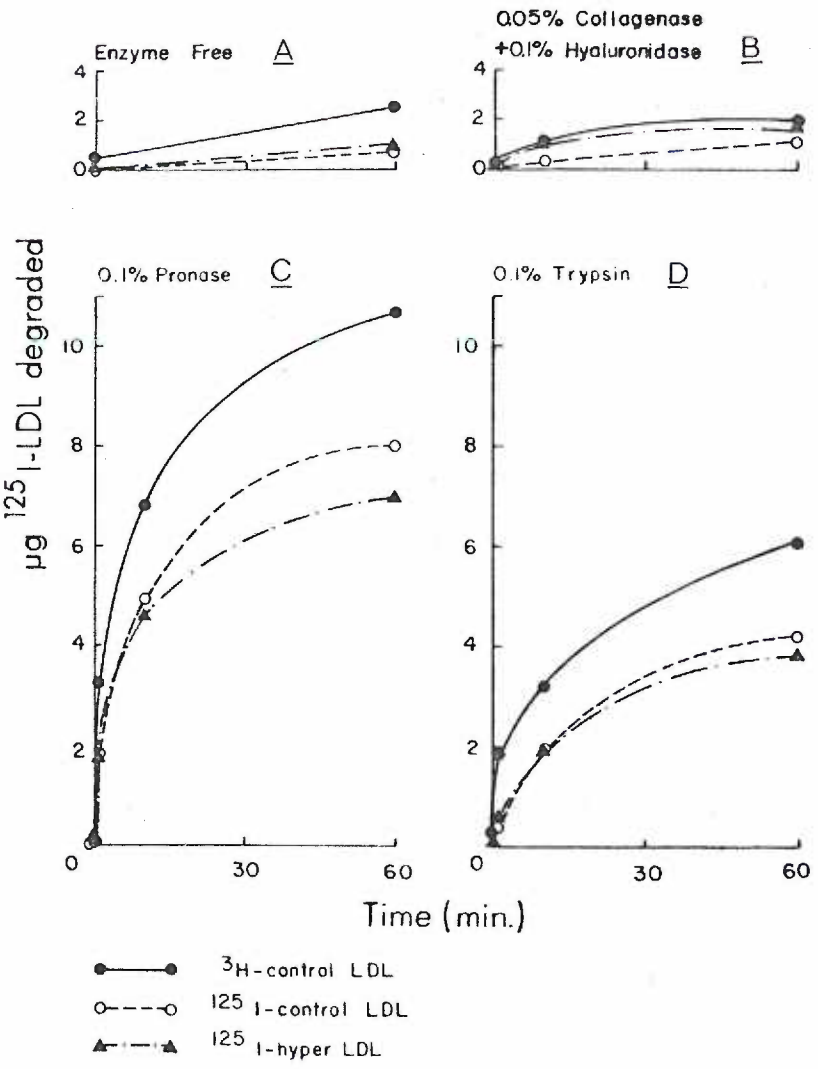
This study revealed that the presence of an iodinated tyrosine on the lipoprotein slowed the proteolytic degradation of ^{125}I-LDL. While the reaction was slower with ^{125}I-LDL, eventually equal amounts of ^{125}I-LDL and ^{3}H-leu-LDL were degraded in the isolated enzyme systems. The decreased degradation rate may be caused by the iodine sterically hindering the approach of the protein to the active site of the enzyme or perhaps there is a decreased affinity for the active site caused by the increased tendency for the iodotyrosyl phenoxy group to ionize as the pH of the solution approaches 8.2 (which is the value for the pK_a of the iodotyrosyl phenoxy group.(199)

Figure 4 shows the results of the experiment which compared the degradation of ^{3}H-leu-LDL and ^{125}I-LDL in four systems in the absence of other serum proteins. In this experiment 14.2 μg protein/ml hyperlipemic ^{125}I-LDL was compared with the same concentration of control ^{125}I-LDL and ^{3}H-leu-LDL. As can be seen in Figure 4, C and D, the ^{3}H-leu-LDL was degraded more rapidly than either of the iodinated lipoproteins by both pronase and trypsin. There was little difference observed in the rate or amount of degradation of the two iodinated lipoproteins. It thus appears that nutritionally induced changes in lipid and apoprotein composition of the lipoprotein have less effect on proteolytic enzymes than does the iodine moiety.

Since it had been shown that isolated proteolytic enzymes did not degrade ^{3}H-leu-LDL and ^{125}I-LDL at the same rate, it was deemed necessary to compare these two types of labelled lipoproteins in the

Figure 4. Comparison of degradation of 14.2 μg of protein/ml of control and hyperlipemic LDL labelled with either ^{125}I or ^{3}H-leu by several isolated enzyme systems. A. Spontaneous appearance of degradation products in the absence of any added enzyme. B. Degradation of LDL by the enzyme mixture used to isolate the hepatocytes. C. Degradation in the presence of 0.1% pronase. D. Degradation in the presence of 0.1% trypsin. The values plotted are the average of duplicate determinations. The blank values shown in a. were not subtracted from the degradation values shown here. ● —— ● = ^{3}H-leu-control LDL; o --- o = ^{125}I-control LDL; and ▲ --.-- ▲ = ^{125}I-hyperlipemic LDL.

MODEL ENZYME DEGRADATION OF LDL



hepatocyte system that was used to study LDL catabolism. These results will be presented in section IV.C.5.b.

The collagenase-hyaluronidase system was examined because many collagenase preparations are contaminated with a protease that actively degrades LDL. As can be seen from Figure 4, A and B, no degradation of ^{3}H-leu-LDL (above the blank) had occurred after 60 minutes and less than 0.3 μg (2%) of the ^{125}I-LDL was degraded after 60 minutes. Since the hepatocytes were well washed before adding LDL, collagenase degradation of LDL did not appear to be a significant problem.

No active deiodination was observed in any of these systems.

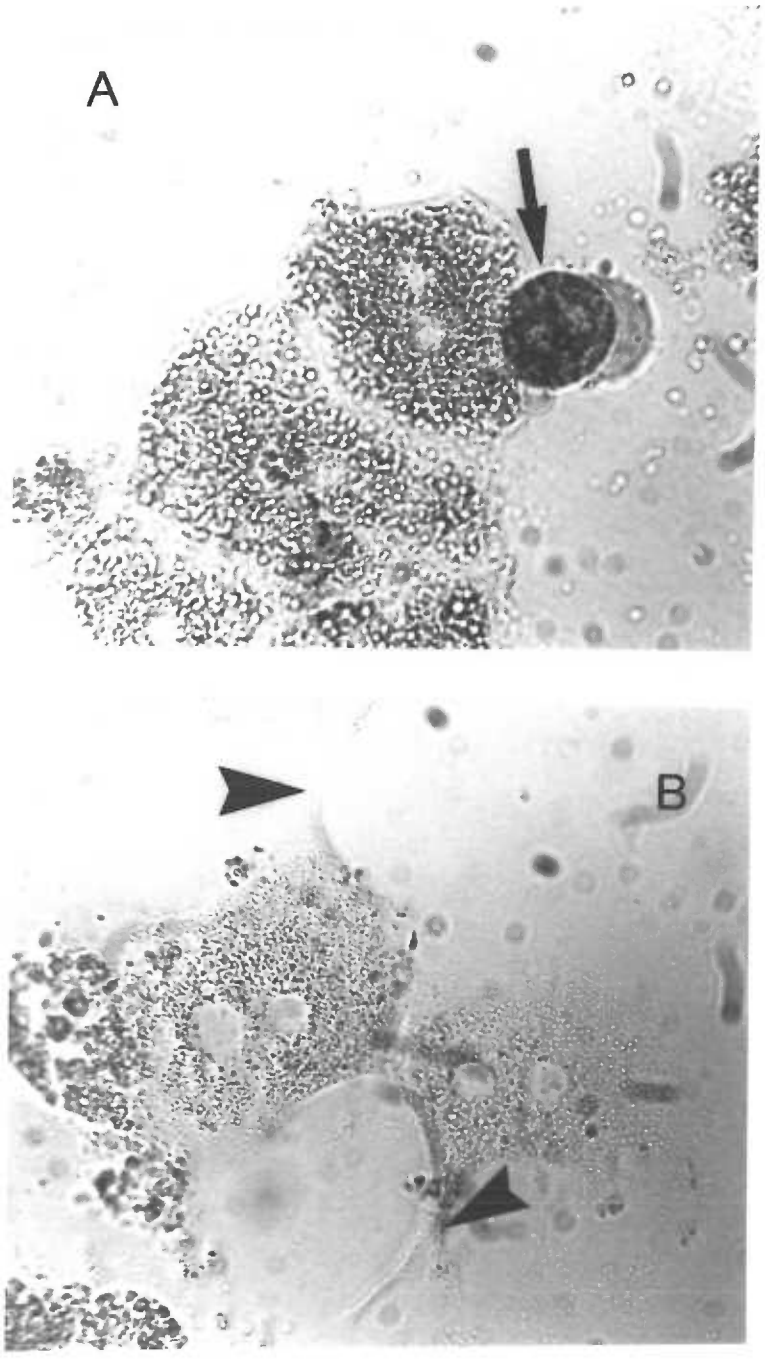
B. Characterization and Morphology of Hepatocyte Primary Cultures

1. Trypan Blue Exclusion

Trypan blue was routinely used to assess hepatocyte viability. Figure 5 shows that nonviable cells are clearly distinguishable from the viable ones. Cell preparations in which less than 90% of the cells excluded trypan blue were discarded. When the original medium was changed after 20 hours, all of the attached cells excluded the dye. After 48 hours in lipoprotein-deficient medium, the cells began to show visible degeneration; counting 13 fields of cells on a single plate gave an estimate of 62.5% viable cells. After trypsinization to release cells from the dish, estimates from three separate dishes averaged 58.4% viable cells after 48 hours. Thus the trypsinization procedure did not lyse significant numbers of hepatocytes.

Figure 5. Light micrographs (40X+ photoenlargement) of discontinuous monolayer of rabbit hepatocytes. In the presence of trypan blue (A), viable cells appear flattened and are frequently binucleate. Arrow points out nonviable cell. Note darker color caused by dye uptake; also, it is spherical rather than flat. (B) Hepatocytes with well-defined extensions (arrowheads) which give the appearance of seeking contact with other cells. Hepatocytes, which initially are mostly individual cells, frequently form "clusters" when they attach to the culture dish.

Hepatocytes



2. Cell Attachment

Another criterion of viability was the ability of cells to attach to the culture dish. None of the cells which were visibly attached to the dish took up trypan blue. When the medium was changed after the initial 20-hour incubation, 95% of the nonattached cells stained with trypan blue. If the nonattached cells were transferred to a second culture dish with fresh medium, no further attachment was observed. One dish from each liver preparation was examined after the initial 20-hour incubation for trypan blue uptake. If the cells had attached, no dye uptake was observed.

Several liver preparations yielded apparently viable cells after the initial isolation; that is, greater than 90% of the cells excluded trypan blue, but the cells did not attach to the culture dish. These preparations were discarded. The reason for the nonattachment is unknown, however this phenomenon has also been reported for primary cultures of rat hepatocytes.(78)

The number of cells which attached to the culture dish was considerably greater if the initial plating medium contained 20% whole serum rather than 20% lipoprotein-deficient serum. These differences from two cell preparations are shown in Table 8. Despite the better plating efficiency exhibited in the presence of whole serum, cells were routinely plated in lipoprotein-deficient serum for three reasons. In the first place, plating the cells in lipoprotein-deficient serum initially permitted experiments to be done between 24 and 48 hours after isolation, which is well before the observed dedifferentiation of specific liver function begins to occur.(129) Second, when cells

TABLE 8

Effect of Whole Serum and Lipoprotein-deficient Serum on Cell Attachment[a]

Medium[b]	Experiment	n	Average Cell Protein Per Dish (mg) ± S.D.[c]
Medium 1	1	4	1.405 ± 0.225
	2	12	1.208 ± 0.230
Medium 2	1	8	0.266 ± 0.072
	2	12	0.806 ± 0.059
Medium 3	1	4	0.235 ± 0.031
	2	--	--
Medium 1 for 20 hr, then Medium 2 for 24 hr	1	--	--
	2	12	0.852 ± 0.127

[a]The cells were plated in 3 ml of medium and incubated for 20 hours at 37°C. Fresh medium was added for 24 hours and then removed. The cells were removed from the dish by trypsin and washed.

[b]Medium 1 was Hamm's F-12 medium supplemented with 20% whole serum. Medium 2 was Hamm's F-12 medium supplemented with 20% lipoprotein-deficient serum. Medium 3 was Hamm's F-12 medium supplemented with 20% lipoprotein-deficient serum plus 20 μg LDL protein/ml.

[c]Protein determinations were performed as described in section III.D.1.

were plated in medium with whole serum and then changed to medium with lipoprotein-deficient serum, the increased numbers of cells which had attached in whole serum sloughed off in the lipoprotein-deficient serum as shown in Table 8. Third, this condition is necessary to induce LDL receptors.(143)

The amount of lipoprotein-deficient serum in the plating medium was examined in another experiment in order to determine the optimum concentration to use in the plating medium. These data are shown in

TABLE 9

The Effect of Increasing Lipoprotein-deficient Serum Concentrations on Cell Attachment

% serum in Hamm's F-12 medium	10%	15%	20%	25%
mg attached hepatocyte protein	0.775	0.703	1.319	1.114

Protein values were determined 20 hours after plating. Attached cells were washed with fresh medium and then were removed with trypsin. Duplicate dishes were examined at each serum concentration.

Table 9 and clearly show that almost twice as many cells attached in the presence of medium containing 20% lipoprotein-deficient serum than attached at the lower serum concentrations. Increasing the amount of serum to 25%, however, did not further increase cell attachment.

3. Lactic Dehydrogenase Release

Lactic dehydrogenase (LDH) concentration in cell media has been shown to correlate with cell membrane disruption and is thus regarded as a good marker of cell damage and/or death.[90, 337] Cells were grown for 48 hours in lipoprotein-deficient medium (28 hours after medium change) and LDH in the medium was compared with LDH released to the medium when the cells were homogenized. By this criterion, 25.4% of the cells had released LDH (i.e., were nonviable) after 48 hours in culture.

Several dishes of cells from this preparation were subjected to an additional 2-hour incubation at 4°C and then tested for LDH release. The analysis showed that 58.2% of these cells had released LDH. The proportion of dead cells increased from 25.4% to 58.2% during a 2-hour

exposure to 4°C. Thus exposure of hepatocytes to 4°C for two hours resulted in the loss of 44% of the cells that were viable prior to the exposure.

4. Presence of "Kupffer Cells

"Kupffer cells were easily distinguished by scanning electron microscopy. A count of one cell preparation showed 90.8% of the attached cells were hepatocytes. Since hepatocytes comprise 66% of the liver by cell number but 92% by mass,(342) each hepatocyte has 5.924 times the mass of each "Kupffer cell. In this preparation, then, "Kupffer cells comprised only 1.68% of the total cell mass.

5. Retention of Liver Specific Functions

It has been demonstrated by several investigators that primary cultures of rat hepatocytes retain many of the functions of normal liver. Bissell et al. showed that such cultures 1) synthesized and secreted albumin (the liver is the major source of plasma albumin (228)), 2) synthesized glucose from pyruvate and lactate, 3) catabolized glycogen in response to 2×10^{-10} M glucagon, 4) increased glycogen stores in response to 1 or 4 milliunits of insulin, 5) induced the detoxification enzyme p-nitroanisole O-demethylase in response to benzo[α]pyrene, and finally 6) restored intracellular ATP levels to normal with 24 hours of plating.(36) Savage and Bonney demonstrated the induction of two important liver enzymes: tyrosine aminotransferase and phosphoenol pyruvate carboxykinase.(284) Recently Katz et al. reported the induction of the liver-specific key enzyme of glucose metabolism, glucokinase, in the presence of dexamethasone and insulin.(179) The capacity to induce this enzyme is lost when hepatocyte

cultures are carried past the primary stage.[354] Breslow et al. mentioned that primary cultures of rat hepatocytes were able to conjugate the bile acids, cholic and chenodeoxycholic, with both taurine and glycine but did not present any data.[44]

A recent study demonstrated the ability of rat hepatocytes to synthesize and secrete VLDL.[78] This process was shown to be inhibited by colchicine and cycloheximide and enhanced if the liver donor had been maintained on a sucrose-containing high carbohydrate diet for several days prior to sacrifice.

Similarly, investigations carried out in collaboration with Jeanne Brandt and Oscar Portman in this laboratory examined the ability of primary cultures of rabbit hepatocytes to incorporate ^{3}H-leucine into lipoproteins. In these experiments ^{3}H-leucine was incubated with the cells for 0, 3, or 24 hours. After the incubation, the medium was dialyzed to remove free ^{3}H-leucine. An aliquot of the dialyzed medium was added to 2:1 chloroform-methanol to precipitate the protein in the medium. The rest of the medium was subjected to sequential ultracentrifugation to harvest any lipoproteins secreted by the cells. These fractions were also precipitated with chloroform-methanol. The cells were washed and harvested by scraping with a rubber policeman. An aliquot was taken for protein determination and the remainder precipitated with chloroform-methanol. This precipitation step helped remove the small amount of free ^{3}H-leucine not removed by dialysis and also removed any leucine that had been converted into lipid material. Table 10 shows that leucine was actively incorporated into both cellular and secreted (medium) protein, and

TABLE 10

Incorporation of ^{3}H-Leucine into Proteins by Primary Cultures of Rabbit Hepatocytes

	pmol of Leucine Incorporated/mg Cell Protein		
Hour	Cells	Total Medium	Medium of $1.006 < d < 1.210$
0	1.2	4.1	--
3	649.1	188.3	19.4
24	1669.2	699.7	46.9

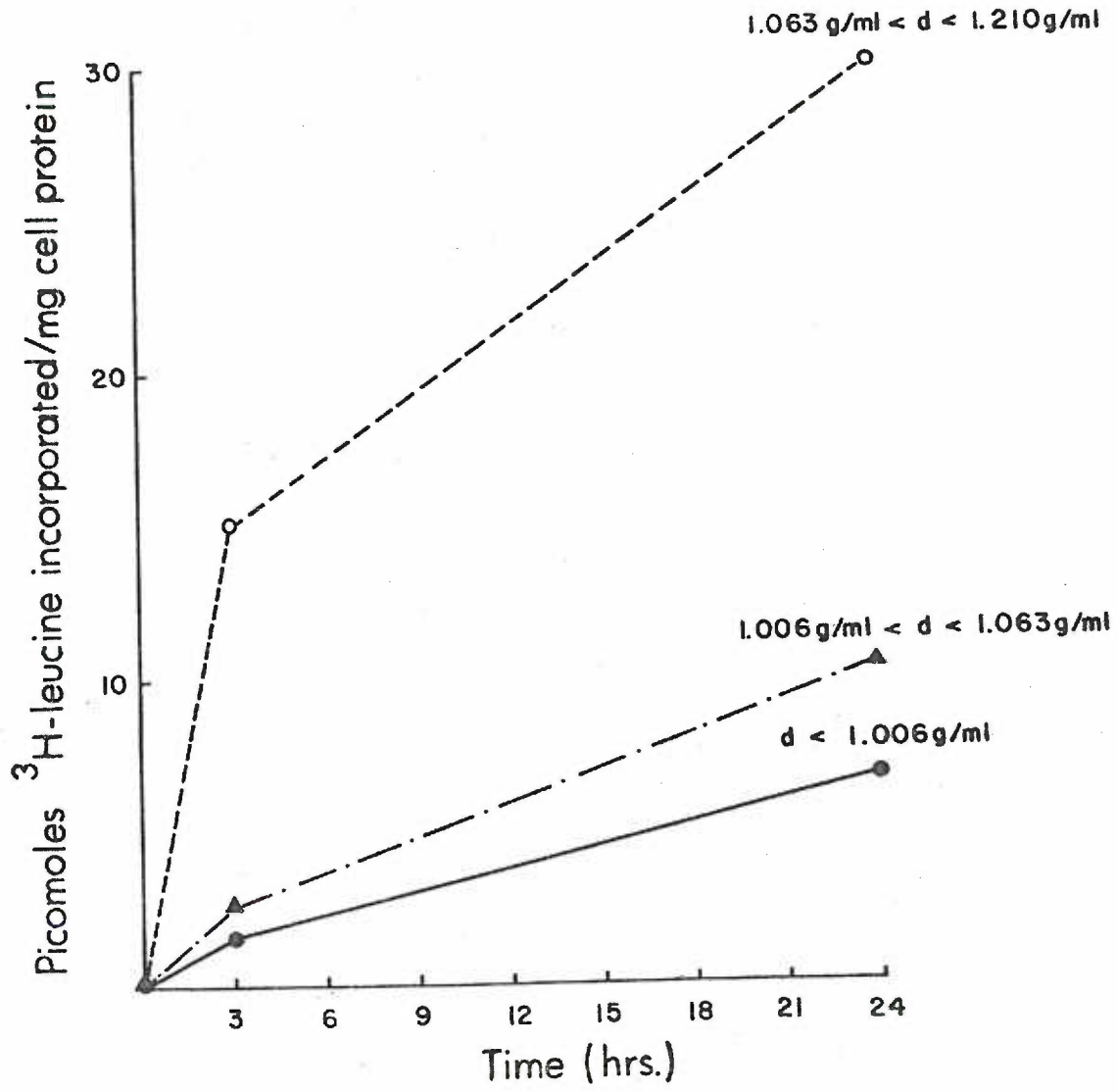
Cells were incubated with medium containing 13.1 μg of ^{3}H-leucine per ml. The picomoles of leucine incorporated into precipitable protein in each fraction were calculated on the basis of the specific activity of the leucine. Incorporation into medium protein that was less dense than 1.210 g/ml was considered to be caused by de novo synthesis of lipoprotein apoproteins by the cells.

that between 6.7 and 10.3% of the secreted protein could be recovered at densities less than or equal to 1.21 g/ml. The recovery of labelled protein in individual density cuts with time is shown in Figure 6.

Other studies indicated that the cells actively incorporated ^{14}C-choline into both cellular and secreted phospholipids and ^{14}C-palmitate into both cellular and secreted triglycerides. In one experiment after a 24-hour incubation with ^{14}C-palmitate, the medium was subjected to ultracentrifugation and ^{14}C-triglyceride was identified in all three density cuts (i.e., 1.006 g/ml, 1.063 g/ml, and 1.21 g/ml) by using thin-layer chromatography. These studies indicate that primary cultures of hepatocytes retain differentiated liver function and can be utilized to study liver metabolism at the cellular level.

Figure 6. Incorporation of ^{3}H-leucine into lipoproteins of various densities by primary cultures of rabbit hepatocytes. The medium from duplicate sets of dishes was subjected to sequential ultracentrifugation at increasing densities to isolate labelled lipoproteins. The data are presented as picomoles of ^{3}H-leucine incorporated per mg cell protein into the chloroform-methanol (2:1) precipitable protein of the lipoprotein fractions recovered at $d < 1.006$ g/ml (● — ●), at $1.006 \text{ g/ml} < d < 1.063$ g/ml (▲ — . — ▲), and at $1.063 \text{ g/ml} < d < 1.210$ g/ml (o --- o).

INCORPORATION OF ^{3}H-leucine INTO LIPOPROTEINS ISOLATED AT VARIOUS DENSITIES BY HEPATOCYTE PRIMARY CULTURES



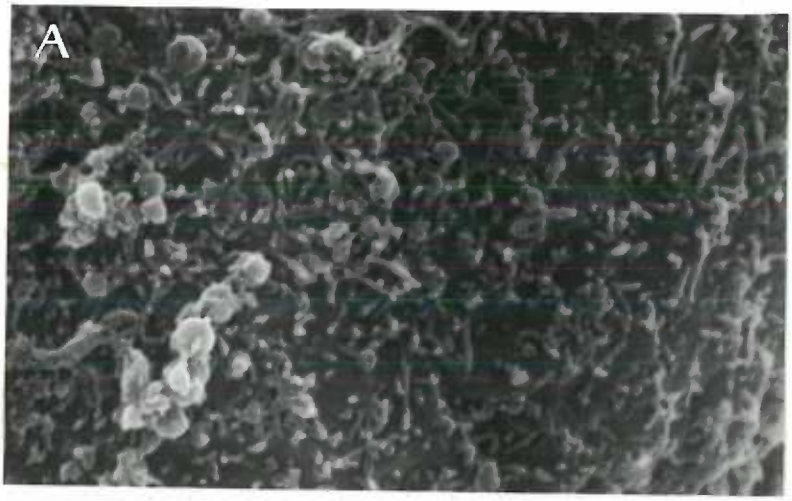
6. Surface Morphology

The surface of isolated hepatocytes was examined by scanning electron microscopy. Immediately after isolation the cells were not attached to a surface, thus it was very difficult to prepare the cells for examination. Therefore, the cells were cultured for several days and then exposed to the collagenase-hyaluronidase mixture used to isolate the cells. Figure 7 shows the surface of the cells after 24 (A) and 72 (B) hours in culture and after exposure to the enzyme mixture for one hour (C). It can readily be seen that the surface underwent a dramatic change upon exposure to the enzyme mix. The microvilli were almost totally removed by the collagenase treatment. This was also seen in cells immediately after isolation, but the lack of attachment to the surface caused the cells to become very spherical and it was not possible to take a photograph in which the surface was in focus. The reappearance of the microvilli was one more indication that the cells recovered during the initial 20-hour incubation after isolation.

Figure 8 shows scanning electron micrographs of the surface of hepatocytes after several different treatments. Since some of the binding studies subjected hepatocytes to 4°C for several hours, Figure 8(A) shows the surface after a 2-hour incubation at 4°C. The microvilli were fewer in number than was seen after 37°C incubations (Figure 7, A and B). Several areas of the cell surface were completely denuded of microvilli. These denuded patches were not observed in cells maintained at 37°C. In addition, the microvilli which were present give the appearance of resorbing into the surface. The

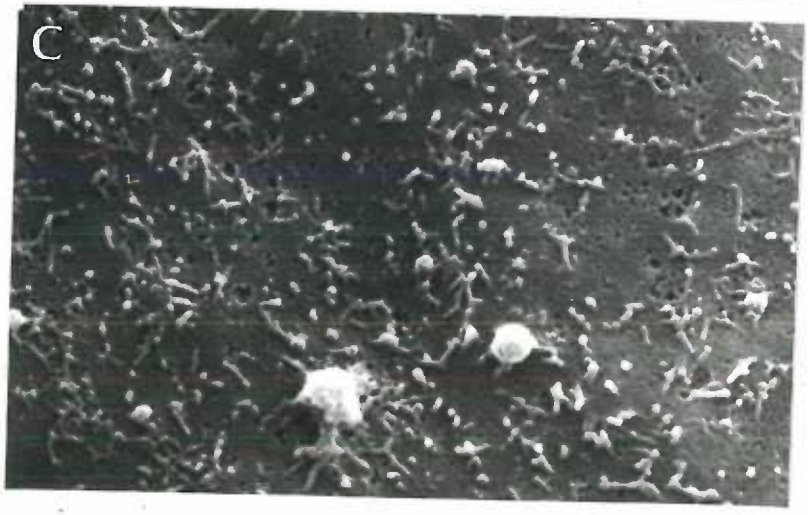
Figure 7. Scanning electron micrographs of hepatocyte surface (10,000 X). (A) Incubation at 37°C for 24 hours after initial isolation in Hamm's F-12 medium plus 20% lipoprotein-deficient serum. (B) Incubation at 37°C for 72 hours after initial isolation. (C) Incubation as (B) followed by removal of medium and addition of medium containing 0.1% hyaluronidase plus 0.05% collagenase (as used to isolate the hepatocytes initially). The cells were incubated at 37°C for one hour in this mixture.

Hepatocyte Surface



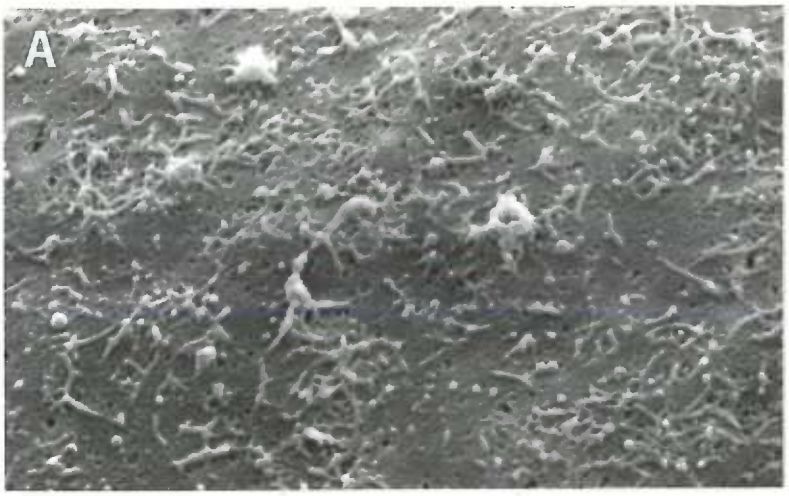
24hr.

72 hr.



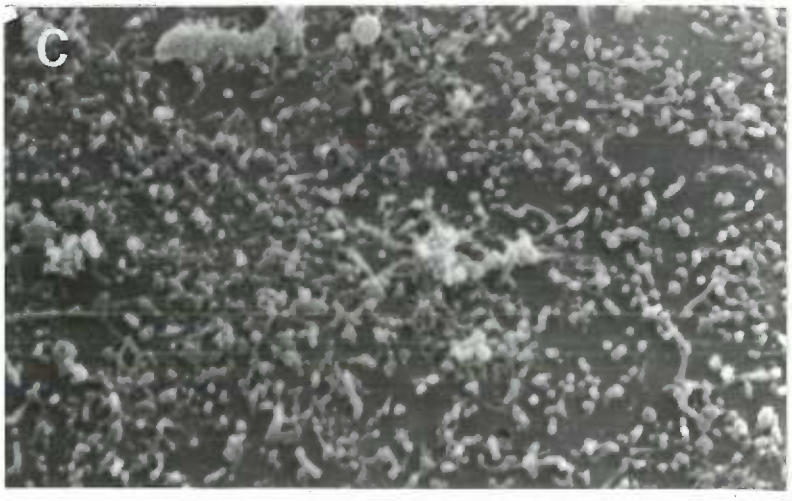
72hr.
plus collagenase

Figure 8. Scanning electron micrographs of hepatocyte surface (10,000 X). (A) Incubation at 37°C for 24 hours after initial isolation in Hamm's F-12 medium plus 20% lipoprotein-deficient medium followed by a 1-hour incubation at 4°C. (B) Same as (A) except that 10 mg/ml of heparin was added to the medium during the 4°C incubation. (C) Incubation at 37°C for 24 hours after the initial isolation in Hamm's F-12 medium plus 20% lipoprotein-deficient serum. This medium was removed and 2 ml of trypsin solution (section III.C.3.b.) was added and incubated for 5 minutes at 37°C.



cold

heparin



trypsin

surface was also riddled with pits which appear to be similar to the pits seen after the collagenase-hyaluronidase treatment (Figure 7C).

The surface of heparin (sodium salt, Sigma, grade 1) treated hepatocytes could not be distinguished from that of the cold-treated cells (Figure 8, A and B). This was probably because the heparin treatment was conducted at 4°C. This particular treatment was examined because incubation of cells with heparin at 4°C is an alternate method of detecting specific LDL binding.(143)

Figure 8C shows the cell surface after trypsin treatment as used to detect total LDL binding. The microvilli still cover the entire surface and no pits are evident in the underlying surface. More of the microvilli appear as knobs or balls than is seen in Figure 7A and B where the microvilli appear somewhat more tubular. Since both the surface appearance and LDH release indicate that two-hour incubations in the cold are deleterious to the well being of hepatocytes, trypsinization rather than heparin treatment in the cold was the method chosen to examine LDL binding to the cells.

C. Characterization of ^{125}I-Low Density Lipoprotein Apoprotein Binding, Internalization, and Degradation in Primary Cultures of Hepatocytes

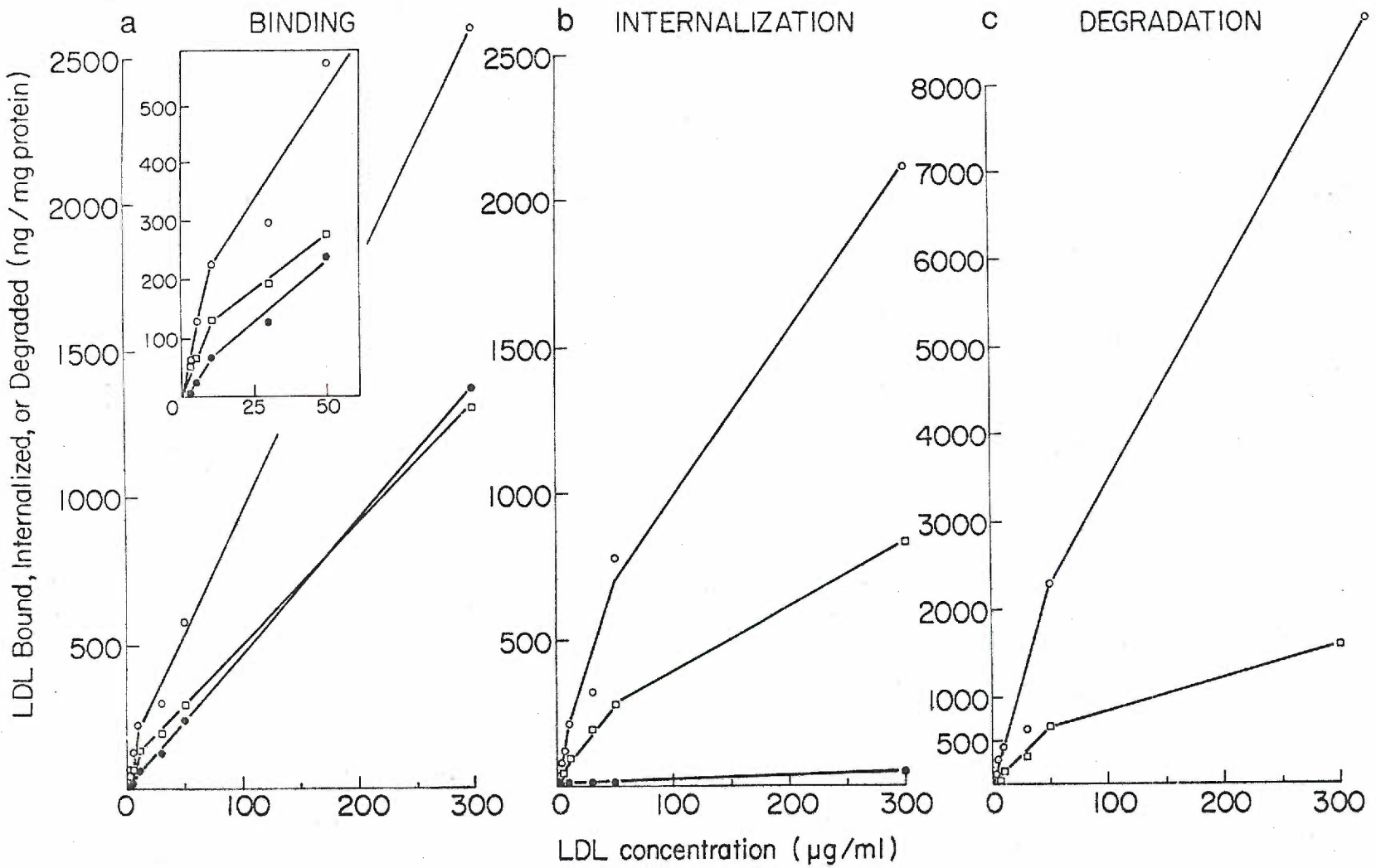
1. Effects of Substrate Concentration and Incubation Time

Primary cultures of rabbit hepatocytes were used to study the binding, internalization, and degradation of LDL. The initial 20-hour incubation of the newly isolated hepatocytes in medium containing 20% lipoprotein-deficient rabbit serum allowed the viable cells to become firmly attached to the culture dish. Since preincubation in

lipoprotein-deficient medium has been shown to be a necessary condition for fibroblasts[142] and lymphocytes[162] to achieve maximal LDL binding and degradation, this initial incubation period also allowed the hepatocytes to respond to the LDL-free medium before binding and degradation studies were carried out.

Experiments designed to examine the ^{125}I-LDL concentrations at 0, 3, and 24 hours were conducted in three different hepatocyte preparations. Figure 9 illustrates the results of one of these experiments. In all three experiments, approximately 50% of the binding seen at 24 hours had occurred within 5 minutes after the addition of LDL to the cells; as seen in Figure 9, the remaining 35 to 53% of binding occurred between 3 and 24 hours. A change in the slope of the binding curve occurred between LDL-apoprotein concentrations of 10 and 30 μg/ml. This was an indication that two classes of binding (i.e., high- and low-affinity) were present in hepatocytes similar to those reported for fibroblasts.[142] No indication of saturation of the low-affinity component was observed over an increasing range of concentrations up to 300 μg of LDL protein/ml. The quantity of LDL internalized by hepatocytes increased with both time and increasing LDL concentrations, but at each time and substrate concentration there was always less LDL internalized than bound. Low density lipoprotein degradation also increased with time and substrate concentration, but the absolute values showed greater variability than the data for bound and internalized LDL. Some of this variability can be attributed to the fact that some cells die and become unattached during the incubation. Although before death they have contributed to the total

Figure 9. Variation with substrate concentration in the quantities of low density lipoprotein (LDL) bound (A), internalized (B), and degraded (C) by isolated rabbit hepatocytes after 0 (●), 3 (□), and 24 (o) hours of incubation. Hepatocytes that formed a discontinuous monolayer had previously been incubated for 20 hours in a medium free of lipoproteins. (From reference 319, used with permission)


a
BINDING
b
INTERNALIZATION
c
DEGRADATION
LDL Bound, Internalized, or Degraded (ng / mg protein)
LDL concentration (μg/ml)
2500
2000
1500
1000
500
0
100
200
300
500
400
300
200
100
0
25
50
8000
7000
6000
5000
4000
3000
2000
1000
500

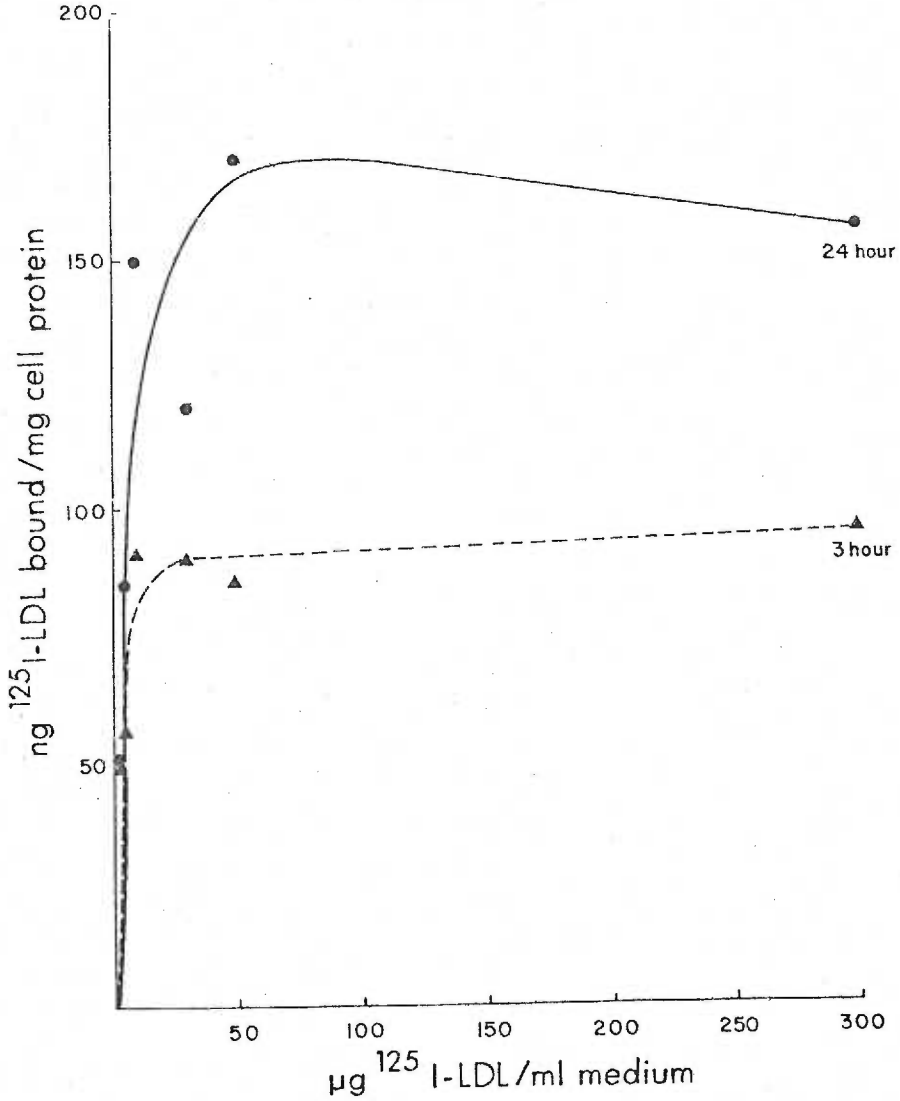
degradation products of LDL, these dead cells are eliminated when the incubation medium is removed, and so only the viable cells are used to determine the internalized and bound LDL and milligrams of cell protein. Therefore, when degradation products are calculated as nanograms per milligram of cell protein using the value for milligrams of cell protein that was determined from the viable cells, the contribution of the dead cells yields variation between samples. Determining the milligrams of cell protein in the nonvital cells and adding this to the value for the vital cells to calculate degradation per milligram of cell protein does not necessarily decrease the variability since there is no way to determine when cell death occurred or how much each cell contributed before death. Interanimal differences and the presence of an active deiodination system (section IV.G.3.) also may contribute to variability between hepatocyte preparations.

2. Determination of High-affinity Binding

The high-affinity process for binding at 3 and 24 hours was examined by replotting the data shown in Figure 9 using the "slope peeling" technique described by Goldstein and Brown to subtract the contribution of low-affinity binding from the total amount bound.(141) The contribution of low-affinity binding was derived by extrapolating the slope of the terminal linear portion of the binding curve (Figure 9A) to zero on the Y axis. A line with this slope is then drawn through the origin to show the contribution to total binding caused by low-affinity binding. The contribution of high-affinity binding, as determined by the slope peeling method, is shown in Figure 10. When

Figure 10. Specific binding of LDL to high-affinity hepatocyte receptors. This was determined by removing the contribution of low-affinity or nonspecific binding by the technique of slope peeling as described in the text (section IV.C.1.) from the binding data shown in Figure 9. △ --- △ shows receptors at 3 hours and ● —— ● shows receptors at 24 hours.

LDL SPECIFIC BINDING
TO HIGH AFFINITY RECEPTORS



this figure is examined, it is apparent that the high-affinity binding process is saturable.

A Lineweaver-Burk plot of the data in Figure 10 disclosed an apparent K_m of 1.75×10^{-9} M and B_{max} (i.e., maximum amount bound) of 169 ng of LDL protein/mg cell protein after 24 hours.[201] After 3 hours the K_m and B_{max} were 1.57×10^{-9} M and 105 ng of LDL protein/mg cell protein, respectively. Thus the LDL concentration at which half-maximal binding to the high-affinity receptor occurs remained constant during the 24-hour incubation. The maximum amount bound to the high affinity receptors, however, increased more than 40% between 3 and 24 hours.

3. Control Incubations

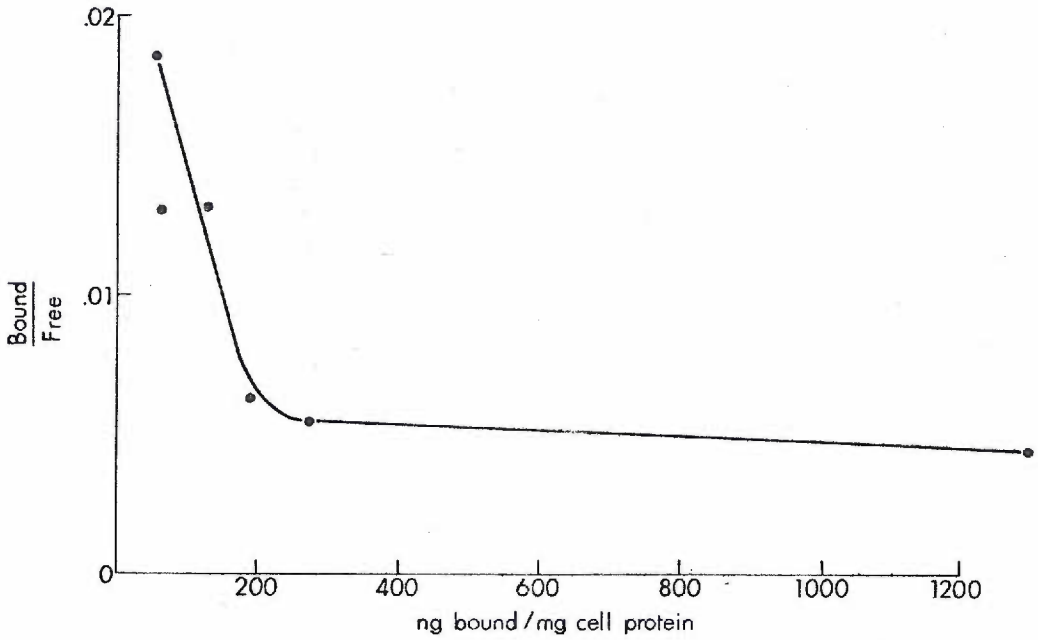
Control incubations of LDL without cells showed that a negligible amount of LDL binds to the dish (i.e., LDL that was trypsin releasable). There was no increase in noniodide TCA-soluble material during a 24-hour incubation at 37°C; however, 2.9% of the original counts were converted to free iodide. A 35-fold excess of unlabelled LDL added to the control incubations had no effect on either of these variables.

4. Determination of the Apparent Equilibrium Dissociation Constant

The apparent equilibrium dissociation constant (K_d) was calculated from binding studies carried out at both 4°C and 37°C. A Scatchard plot of the 3-hour binding data of Figure 9 is shown in Figure 11.[289] A similar figure was obtained from the 24-hour data. The presence of two distinct components in the plot suggests the

Figure 11. Scatchard plot of the data on LDL binding by hepatocytes after 3 hours at 37°C (taken from Figure 9). An apparent dissociation constant of 11.9 μg/ml was calculated from the first leg of the curve. Slope = $\frac{-1}{K_d}$.

Scatchard Plot (3 hours, 37°C)



presence of two types of binding sites: one of high-affinity and finite capacity and the other of low-affinity and infinite capacity.(280) At both 3 and 24 hours, the K_d, calculated from Scatchard plots, for the high-affinity binding site was 3.95×10^{-9} M (11.86 μg/ml). This value for K_d is in good agreement with the value of 10.7 μg/ml (3.57×10^{-9} M) reported by Bachoric et al. for the binding of LDL to an isolated fraction from porcine liver plasma membranes.(15) Brown and Goldstein examined LDL binding to fibroblasts at 37°C and calculated a K_d of 15 μg/ml (2.5×10^{-8} M).(52) Thus at 37°C fibroblasts appear to have a lesser affinity for LDL than do hepatocytes.

Since one of the important assumptions in equilibrium studies is that the substrate-receptor interaction behaves as a simple bimolecular reversible reaction, these binding studies were repeated at 4°C. At this temperature there is binding but no internalization or degradation, so that the total reaction being considered is

$$[Lp] + [R] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Lp \ldots R]$$

rather than $[Lp_{free}] + [R] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Lp \ldots R] \xrightarrow{k_2} [Lp_{internal}] + [R]$,

which is the total reaction occurring at 37°C. The high-affinity binding of ^{125}I-LDL at 4°C as a function of lipoprotein concentration in the medium is shown in Figure 12. The Scatchard analysis of these data (Figure 13) gave a nonlinear plot with an upward concavity. This type of nonlinear plot has generally been attributed to

Figure 12. Concentration-dependent binding of ^{125}I-LDL to specific high-affinity receptor on hepatocytes at 4°C. After a 20-hour preincubation in lipoprotein-deficient medium, 22 ml of iced medium was added and the cells were kept at 4°C for 20 minutes. ^{125}I-LDL of the indicated concentration was then added in the absence or presence of a 27-fold excess of unlabelled LDL, and the cells were incubated at 4°C for 4 hours. The cells were scraped from the dish and sedimented by centrifuging at 1000 rpm for 3 minutes at 4°C. The medium was removed and the cells were washed once with phosphate-buffered saline containing 2 mg/ml of albumin and a second time with phosphate-buffered saline alone. Since no label is internalized at 4°C, all of the label associated with the cells was considered to be bound.(143) High-affinity binding was determined as described in section III.D.4. Each point represents the average of duplicate determinations. The mean cellular protein was 279 μg/dish.

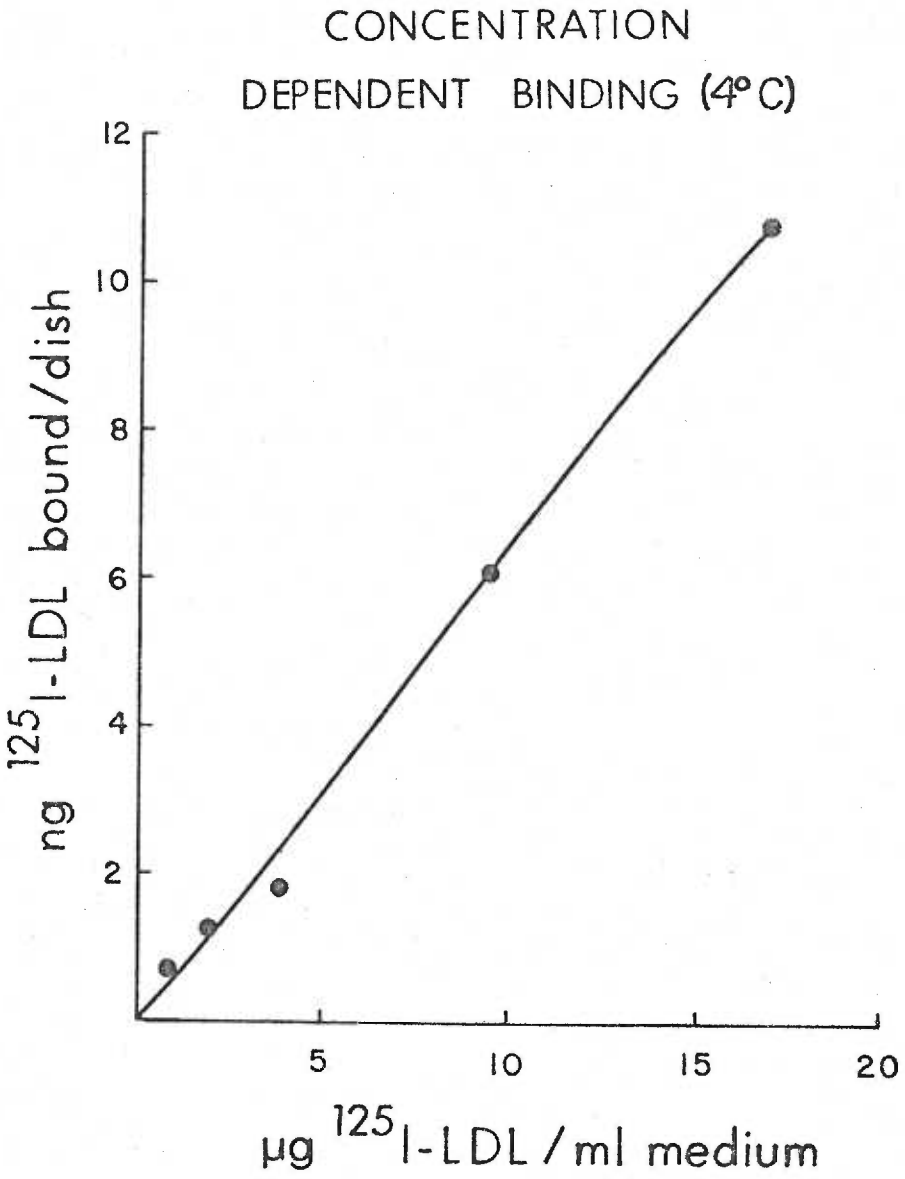
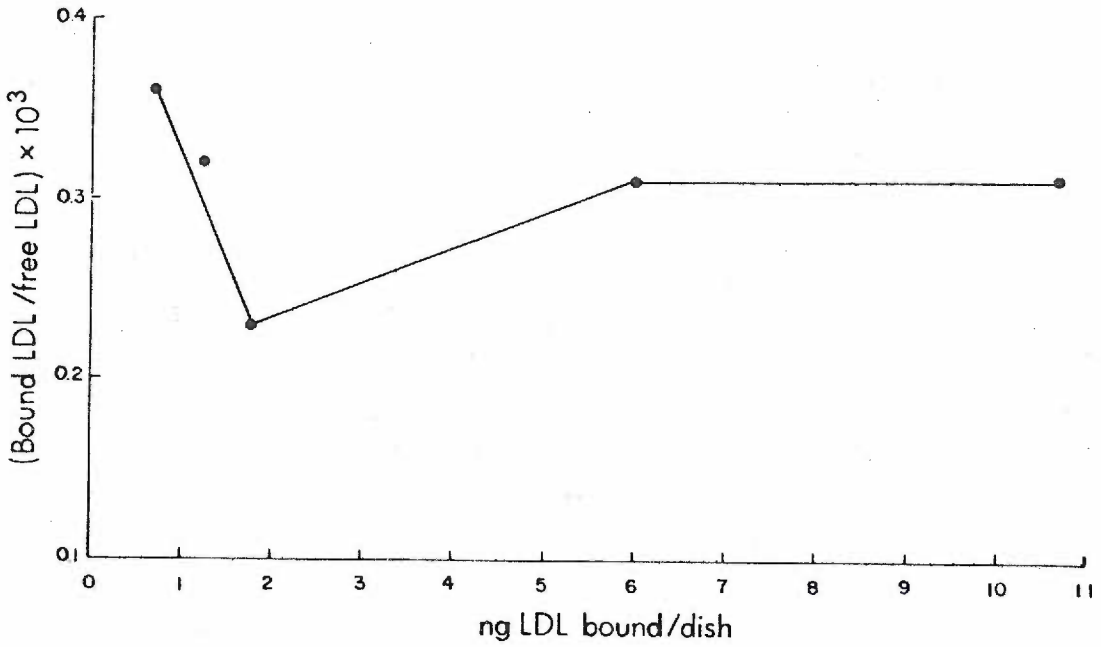

CONCENTRATION
DEPENDENT BINDING (4° C)
ng 125I-LDL bound/dish
12
10
8
6
4
2
5
10
15
20
µg 125I-LDL / ml medium

Figure 13. The Scatchard plot of the data on LDL binding by hepatocytes after 4 hours at 4°C taken from Figure 12. The apparent dissociation constant from the first leg of the curve is 3.97×10^{-9} M. Slope = $\frac{-1}{K_d}$.

SCATCHARD PLOT OF LDL BINDING



heterogeneity of the receptor sites with two or more classes or "orders" which differ in binding affinity.[174, 280] The K_d of the high-affinity portion of the 4°C plot was calculated to be 3.97×10^{-9} M which is not different from the K_d of 3.95×10^{-9} M seen at 37°C.

The Scatchard method of analysis involves several important assumptions (see reference 174). Perhaps the most questionable of these assumptions for the LDL receptor model is that the lipoprotein-receptor interaction is the simple bimolecular reversible reaction expressed in the preceding paragraph. A kinetic analysis was therefore undertaken to evaluate the validity of this model by obtaining an independent estimate for K_d.

The rate constants for LDL association (k_1) and dissociation (k_{-1}) were determined from the time course of specific binding of the lipoprotein at several different concentrations (Figure 14). Plotting the data as described in Equation 4 (section III.D.5.b.) yielded a straight line (Figure 15) in which the slope (k_1) = 1.08×10^{4} M^{-1} sec^{-1} and the Y intercept (k_{-1}) = 0.48×10^{-4} sec^{-1}. The K_d was calculated from these rate constants by $K_d = \frac{k_{-1}}{k_1}$, and the value obtained was 4.44×10^{-9} M for LDL. These results were in reasonable agreement with the values obtained from the equilibrium binding studies, and this agreement provided added confidence in the methods.

Figure 14. Time course of ^{125}I-LDL high-affinity binding to cultured rabbit hepatocytes as a function of concentration. Cells were incubated at 4°C for the indicated times with: 1) 0.96 (o -- o), 2) 1.91 (▲ --- ▲); 3) 3.82 (△ -.-△); or 4) 9.56 (● — ●) μg of ^{125}I-LDL protein/ml. Receptor-bound ^{125}I-LDL was determined as the difference in amount of ^{125}I-LDL bound in the presence or absence of a 27-fold excess of unlabelled LDL. Each point represents the average of duplicate determinations. The mean cellular protein was 279 μg/dish.

TIME COURSE OF LDL BINDING AS A FUNCTION OF CONCENTRATION

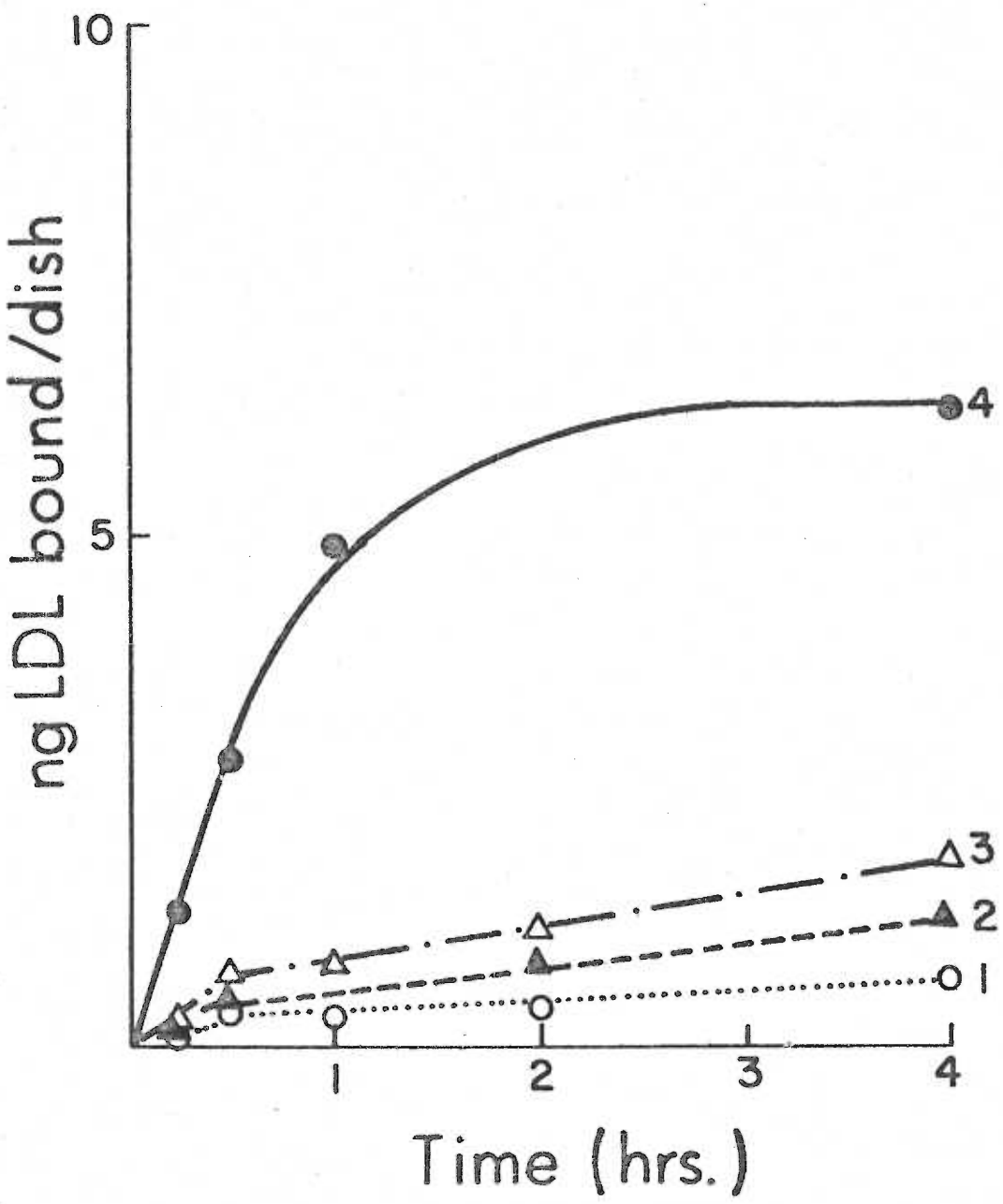
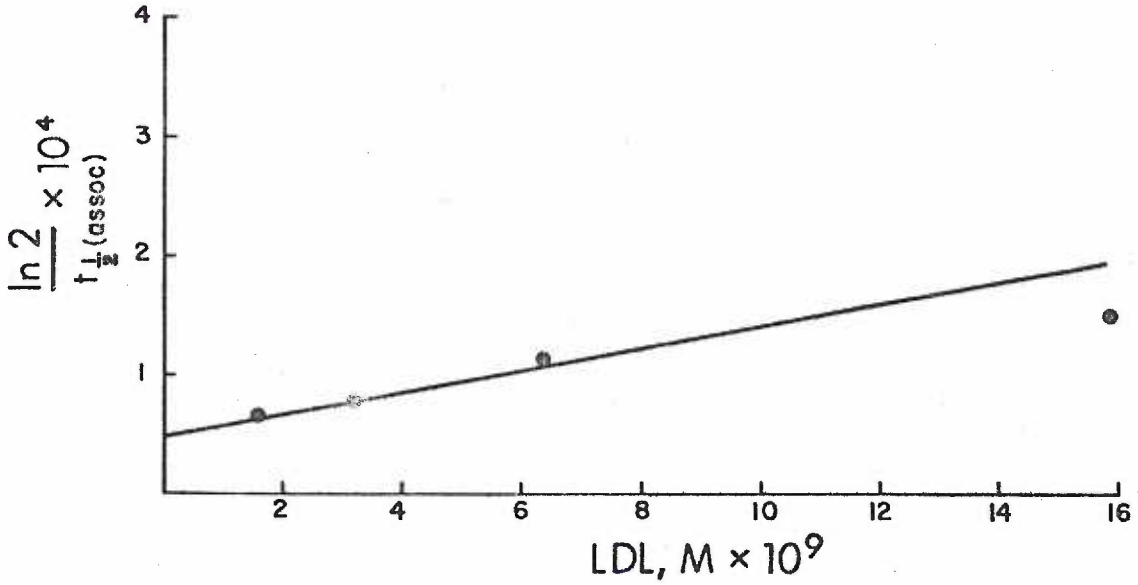


Figure 15. Plot of $t_{1/2}$ (association) against the ^{125}I-LDL concentration in the tissue culture medium. See section III.D.5. for details. Slope (k_1) = 1.08×10^{-4} M^{-1} sec^{-1}; Y intercept (k_{-1}) = 0.48×10^{-4} sec^{-1}. The mean cellular protein was 279 μg/dish.

$t_{1/2}$ (ASSOCIATION) vs [LDL] IN THE MEDIUM



5. Investigation of the Effects of Iodination on Low Density Lipoprotein Metabolism in Hepatocytes

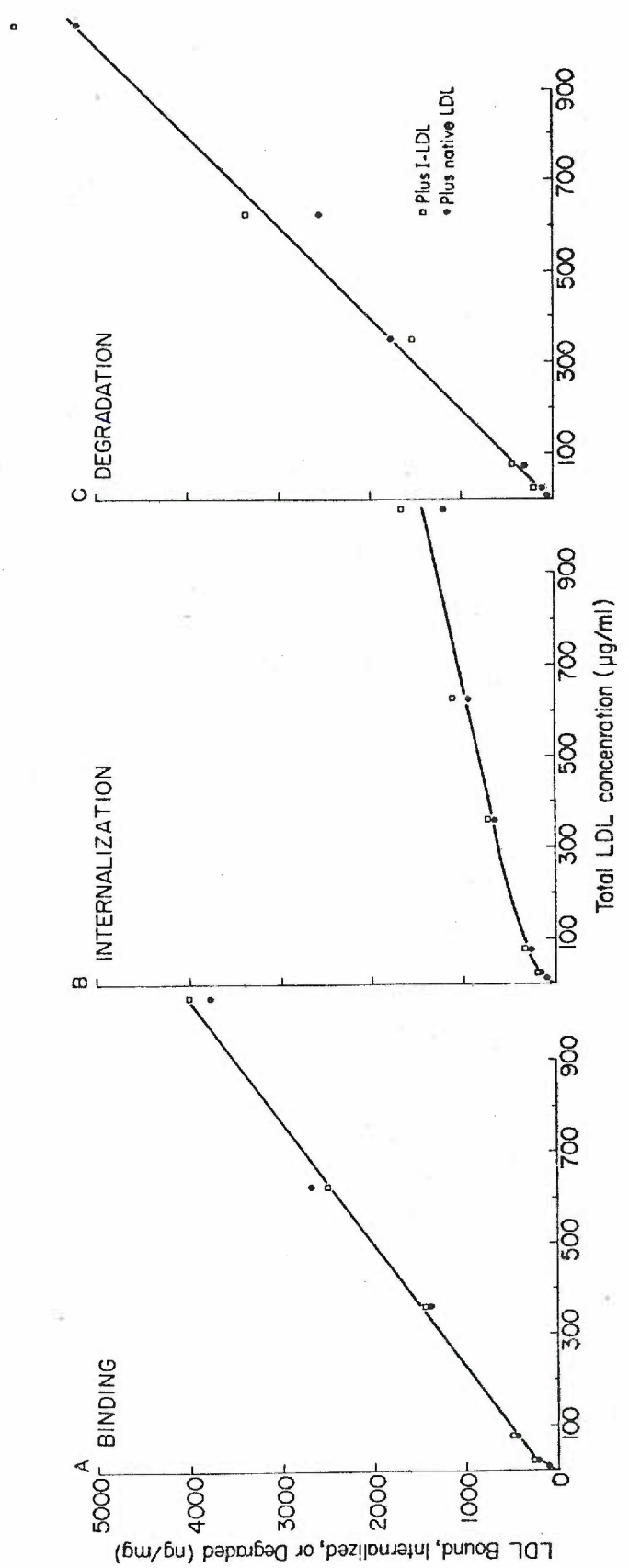
a. Dilution with Native Low Density Lipoprotein or ^{127}I-Low Density Lipoprotein

The effect of the iodine moiety in ^{125}I-labelled LDL on the patterns of metabolism was examined in a set of dilution experiments in which 10 μg of ^{125}I-labelled LDL was diluted with increasing concentrations of native LDL or ^{127}I-labelled LDL and incubated for 24 hours. These data were used to calculate the total amount of labelled plus unlabelled LDL that was bound, internalized, and degraded by the hepatocytes. If the native LDL was bound, internalized, and/or degraded more effectively than the iodinated LDL, the dilution of ^{125}I-LDL with native LDL would suppress binding, internalizing, and/or degradation to a greater extent than would dilution with nonradioactive iodinated LDL. This was not the case as is illustrated in Figure 16. There was no apparent difference between the two diluents. This experiment also showed that nonspecific, low-affinity binding increased with increasing LDL concentrations.

b. Comparison of ^{3}H-Leu-Low Density Lipoprotein and ^{125}I-Low Density Lipoprotein

Since it has been shown that there is some difference in the way proteolytic enzymes degrade LDL labelled with ^{3}H-leu or ^{125}I (section IV.A.3.), these two components were compared in relation to hepatic degradation of LDL. Comparison of these compounds would also ascertain if the active deiodination system of liver (section IV.G.3.) had skewed the degradation data obtained using ^{125}I-labelled

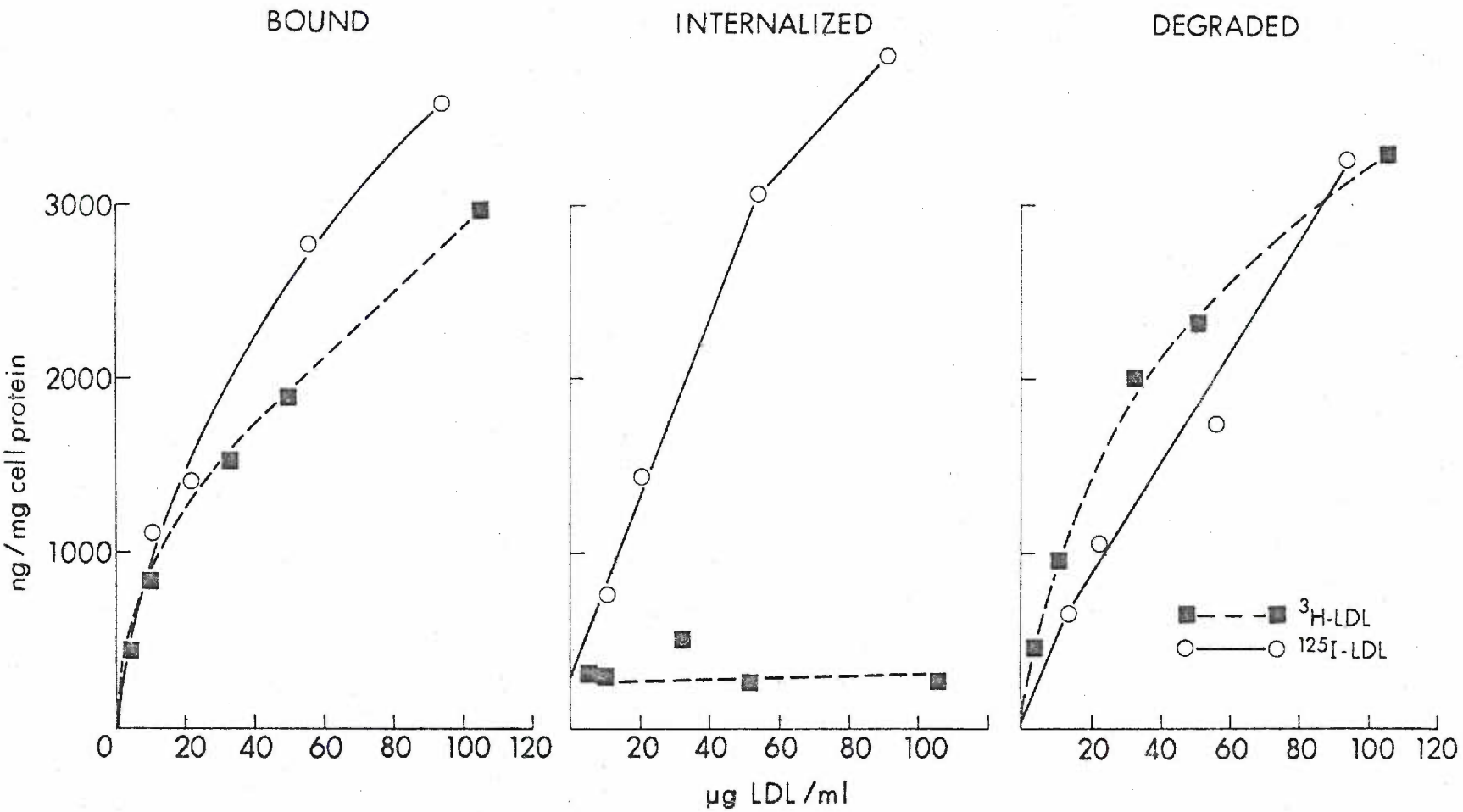
Figure 16. Comparison of native low density lipoprotein (LDL; ●) and ^{127}I-LDL (□) as the diluent of ^{125}I-LDL studies of lipoprotein binding (A), internalization (B), and degradation (C) by primary cultures of rabbit hepatocytes after 24 hours. The quantities of lipoproteins in the three reactions were derived from the products of the fractions of radioactivity involved and the combined amounts of labelled and unlabelled LDL in the medium. From reference 319; used with permission.


A BINDING
B INTERNALIZATION
C DEGRADATION
LDL Bound, Internalized, or Degraded (ng/mg)
5000
4000
3000
2000
1000
0
100
300
500
700
900
Total LDL concentration (µg/ml)
Plus I-LDL
Plus native LDL

LDL. Inasmuch as the criterion for proteolysis of LDL apoproteins is the appearance of ^{125}I-tyrosine or labelled small peptides, removal of ^{125}I-iodide from ^{125}I-tyrosine would result in an underestimation of the true degradative activity.

This study utilized a 24-hour incubation period because it was not possible to obtain ^{3}H-leu-LDL with a high enough specific activity to be able to accurately assess the radioactive degradation products after a 3-hour incubation. As shown in Figure 9, there was a substantial increase in the accumulation of degradation products between 3 and 24 hours. The longer incubation would, therefore, allow more accurate measurement of the ^{3}H-leu-LDL degradation products and would also maximize any differences in the accumulation of degradation products as measured by the appearance of ^{125}I-tyrosine or ^{3}H-leucine in the medium. Figure 17 shows the results of this study and indicates similarities in the binding and degradation of these two types of LDL at 24 hours. The similar degradation data were a good indication that ^{125}I-LDL can be used to study LDL degradation in hepatocytes even in the presence of the cellular deiodination system.(71, 178, 321) ^{125}I-LDL was bound in slightly greater amounts than ^{3}H-LDL, particularly in the range where nonspecific low-affinity binding predominates. This increased nonspecific binding could be caused by an interaction of the iodinated tyrosine moiety of the apoprotein with a receptor similar to or identical with that for thyroxine (T_4) or triiodothyronine (T_3). When the slope peeling method was used to subtract the contribution of nonspecifically bound material, the amount of specific high-affinity binding was the same for both ^{125}I-LDL and ^{3}H-leu-LDL.(141)

Figure 17. Comparison of ^{125}I-LDL (o —— o) and ^{3}H-leu-LDL (▣ --- ▣) metabolism by rabbit hepatocytes. The cells were preincubated for 20 hours in lipoprotein-deficient medium. Then fresh medium containing the indicated concentration of either ^{125}I- or ^{3}H-leu LDL was added and the incubation continued for 24 hours.


BOUND
INTERNALIZED
DEGRADED
ng / mg cell protein
3000
2000
1000
0
20
40
60
80
100
120
μg LDL / ml
^{3}H-LDL
^{125}I-LDL

As indicated by Figure 17, considerably more ^{125}I label was internalized by the cells after a 24-hour incubation than was ^{3}H-leucine label. In order to account for this difference, the internalized label was examined after a 24-hour incubation with 25 μg/ml of either ^{125}I-LDL or ^{3}H-leu-LDL. Ten percent TCA was added to the cells and the TCA-soluble material was examined before and after oxidation with H_2O_2 and extraction with chloroform in order to estimate free $^{125}I^-$ and ^{125}I-tyrosine. The TCA precipitate was washed once with 10% TCA and a second time with 2:1 chloroform-methanol. The cells incubated with ^{125}I-LDL contained ^{125}I-label distributed as follows: 25 to 35% was free iodide, 8% was ^{125}I-tyrosine, and 57 to 67% precipitated with TCA. After the TCA precipitate was washed with chloroform-methanol, only 27 to 37% of the total internalized counts remained with the precipitate. The remainder was soluble in chloroform-methanol. When this was separated into chloroform-soluble (i.e., lipid) and methanol-water-soluble radioactivity, 23% of the total internalized label could be accounted for in the lipid fraction. This material was not analyzed further. The identity of the material in the methanol-water fraction is not known, but it is suspected to be free iodide that was not removed from the internalized lipoprotein by the TCA precipitation of the cells.

The presence of free $^{125}I^-$ inside the cell is not caused by the direct uptake of free $^{125}I^-$ from the surrounding milieu since a control experiment clearly showed that the hepatocyte plasma membrane is impermeable to inorganic iodide which is present in the culture medium. Na-^{125}I was added to the culture medium in three

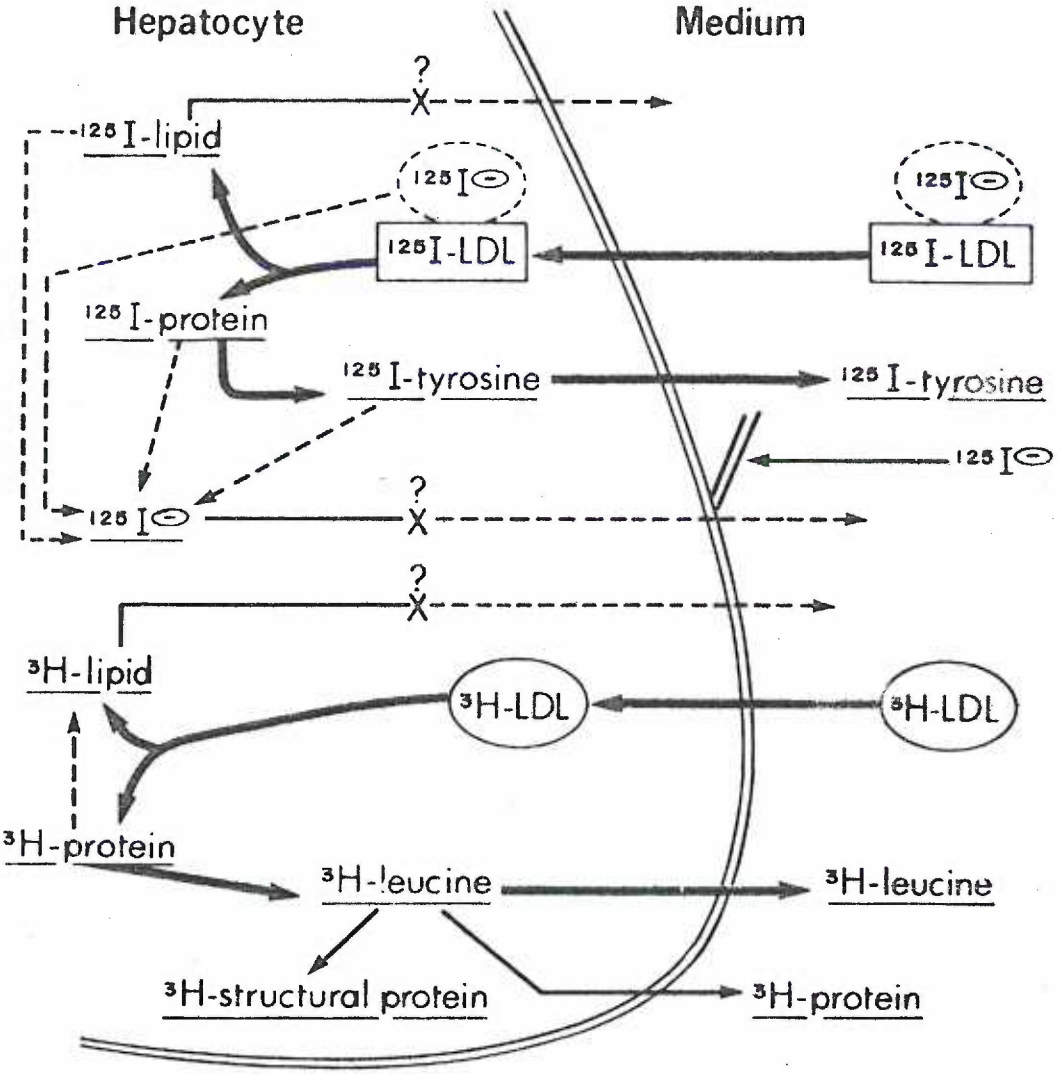
concentrations and incubated for 0, 3, or 24 hours. Analysis of the cells showed that free $^{125}I^-$ was neither internalized by the hepatocytes nor bound to the cell surface.

When internalized ^{3}H-leu-LDL was similarly examined, 82% of the label was TCA-precipitable, less than 1% was TCA-soluble (i.e., free ^{3}H-leucine), and the remainder was recovered from the chloroform-methanol wash of the TCA precipitate. Analysis showed that one third of the label in the chloroform-methanol was in the lipid fraction. This suggested that the tiny portion of ^{3}H-leu-LDL that is lipid (i.e., 1%) had accumulated within the cell or that some of the ^{3}H-leucine freed during the lipoprotein degradation had been incorporated into cell lipid since leucine can be a precursor for lipid and steroid synthesis or that a combination of these possibilities had occurred. (358) The material in the methanol-water portion was not identified.

Figure 18 shows a diagrammatic representation of the metabolism of labelled lipoprotein inside the hepatocyte after a 24-hour incubation with ^{125}I-LDL or ^{3}H-leu-LDL and tabulates the distribution of internalized label. Iodinated lipid, which accounted for 10% of the label in the substrate LDL, accounted for 23% of the label within the hepatocyte. Whether this was a reflection of natural lipid accumulation subsequent to lipoprotein ingestion or was an indication that the iodine moiety affected the ability of the cell to utilize and/or dispose of the lipid is not known. The finding that 6% of the internalized ^{3}H-leu-LDL was recovered as lipid when only 1% of the substrate label was lipid also suggested that lipid accumulation has occurred

Figure 18. Distribution of internalized label in hepatocytes after 24 hours in culture with 25 μg protein/ml of either ^{3}H-leu-LDL or ^{125}I-LDL. ═══ represents major pathway. ——— represents reactions known to occur but their importance in LDL catabolism may be small. - - - represents suspected but as yet unproven pathways. —$\overset{?}{X}$--→ represents unknown amount of secretion by the cell.

Metabolism of ^{125}I-LDL and ^{3}H-LDL at 24 hours



% Distribution of Internalized Label After 24 Hours		
	^{125}I	^{3}H
Protein	27-37	82
Lipid	23	6
Free amino acid	8	1
Free iodide	25-25	0
Unidentified	7	11

in these cells although hepatocytes are able to synthesize lipid from ^{3}H-leucine.

The distribution data in Figure 18 and the internalization data in Figure 17 were used to make the following calculations. In the presence of 50 μg of LDL protein/ml, 27% of the internalized ^{125}I-LDL label (810 ng) is protein associated, and 82% of the internalized ^{3}H-leu-LDL label (287 ng) is protein associated. Since the evidence obtained from the model proteolytic enzyme studies (section IV.A.3.) revealed that the initial rate of degradation for ^{125}I-LDL is slower than for ^{3}H-leu-LDL, it can be surmised that similar rate differences occur within the hepatocyte proteolytic system. Inasmuch as the amount of nonspecifically bound ^{125}I-LDL was somewhat greater than that of ^{3}H-leu-LDL at the concentration used to examine internalized label, more ^{125}I-protein would be internalized by simple bulk pinocytosis. Over the 24-hour period studied, this could conceivably lead to the accumulation of ^{125}I-protein, especially if the initial rates of degradation in the cell are similar to those observed in the model enzyme system.

An alternate explanation for the presence of more ^{125}I-protein than ^{3}H-leu-protein in the cell would be that the free ^{125}I^{-} forms a complex with one or more of the hepatic proteins and coprecipitates with the proteins in the presence of TCA. This hypothesis was examined by another experiment. Hepatocytes were homogenized in a small volume of medium plus saline. This homogenate was incubated with free ^{125}I^{-} for 30 minutes in order to expose internal cell proteins to ^{125}I^{-}. A control containing only medium and saline was similarly

incubated. The incubation was terminated by the addition of TCA to a final concentration of 10%. The results showed that 4.5% of the $^{125}I^-$ in the incubation coprecipitated with the protein in the presence of liver homogenate whereas only 2.8% of the $^{125}I^-$ coprecipitated with protein in the control. This experiment was not directly comparable to the conditions that occur in intact hepatocytes during a 24-hour incubation, but it did indicate that some free $^{125}I^-$ coprecipitates with hepatocyte protein and this could account for some, if not all, of the excess ^{125}I-protein found in the cells after 24 hours.

The remainder of the excess ^{125}I-label within the hepatocyte can be attributed to the greater accumulation of label in lipid form caused by the greater amount of labelled lipid in the ^{125}I-LDL substrate and to the accumulation of free $^{125}I^-$. There are several possible sources of this free $^{125}I^-$. One is the $^{125}I^-$ which can only be dissociated from the substrate LDL by either TCA or chloroform-methanol precipitation. This free $^{125}I^-$ can account for up to 20% of the substrate label (section IV.A.2.a.). Since the rate and manner of egress of iodide ion from hepatocytes is not known, it is possible that uptake of free $^{125}I^-$ with the LDL particle could lead to some accumulation. Another source of internalized free iodide would be through the action of the hepatic microsomal deiodinase system on the labelled lipid, protein, and/or tyrosine moieties.[13]

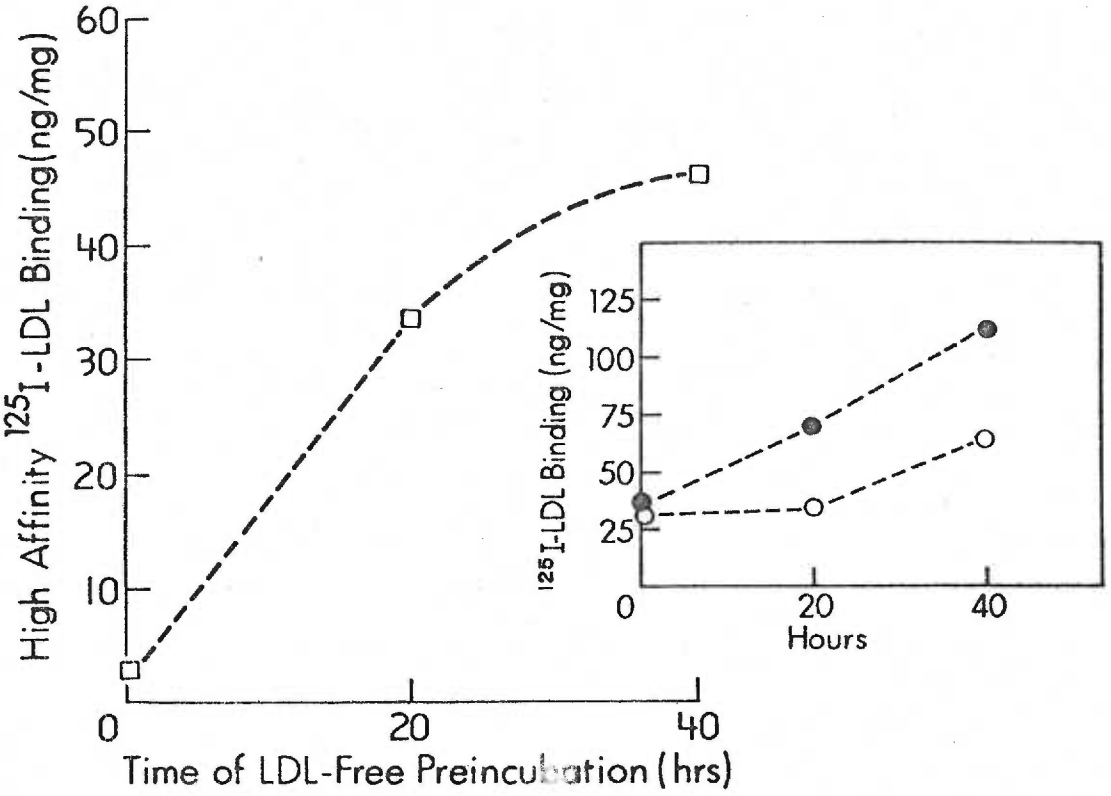
6. Additional Evidence for Specific High-affinity Low Density Lipoprotein Receptors in Hepatocytes

a. Demonstration of Specific High-affinity Binding

After showing that hepatocytes incubated in a primary culture medium without LDL for 24 hours were able to bind, internalize, and degrade LDL, I did an experiment to determine whether LDL binding would be repressed by exposing the cells to LDL prior to addition of the test medium containing ^{125}I-LDL. The cells were divided into 3 groups for the initial incubation conditions. Group I (derepressed cells) was incubated in LDL-free medium for 40 hours. Group II was incubated in medium supplemented with 40 μg of LDL protein/ml for 20 hours and then changed to LDL-free medium for an additional 20 hours. Group III (repressed cells) was incubated for 40 hours in medium supplemented with 40 μg of LDL protein/ml. The binding experiment was fashioned after that of Ho et al. to detect whether the development of specific high-affinity LDL binding and the enhancement of LDL binding can be induced by exposure to LDL-free medium.(162) Ho et al. defined specific high-affinity LDL binding as "the amount of ^{125}I-LDL that is prevented from binding to the cell membrane by an excess of unlabelled LDL."(162) After the cells had been incubated in the absence of LDL for 0, 20, or 40 hours, the medium was changed to LDL-free medium to which had been added either 10 μg of ^{125}I-LDL protein/ml or 10 μg of ^{125}I-LDL plus 350 μg of native LDL protein/ml. I determined binding after 2 hours at 4°C to ensure that the LDL was not metabolized after binding. Three different hepatocyte preparations were tested in this fashion and representative results

Figure 19. The effect of incubation time in medium free of low density lipoprotein on the binding of ^{125}I-LDL by isolated hepatocytes. The cells were incubated for 2 hours at 4°C with 10 μg of ^{125}I-LDL protein/ml alone (o) or with 10 μg of ^{125}I-LDL protein/ml plus 350 μg of unlabelled LDL protein/ml (•). The inset graph at the right is the plot from which the difference in ^{125}I-LDL binding in the presence or absence of unlabelled LDL was calculated. The difference curve (□) is an index of high-affinity binding sites. From reference 319; used with permission.

Induction of High Affinty Binding



are shown in Figure 19. The amounts of ^{125}I-LDL that were bound in the presence of an excess of unlabelled LDL indicated that there was a relatively high level of nonspecific binding of LDL by hepatocytes compared with lymphocytes.(162) This may be caused by the marked sensitivity of hepatocytes to incubation at 4°C (see section IV.B.3. and 6.), or it could represent a real difference in nonspecific binding by the two cell types. There was a 300% enhancement of labelled LDL binding in the absence of excess native LDL after the hepatocytes had been kept free of LDL in the pretest medium for 40 hours. The induction of specific high-affinity binding over a 40-hour period in LDL-free medium is illustrated by the curve in Figure 19 which shows the difference between the amount of ^{125}I-LDL that is bound in the absence of native LDL and the amount of ^{125}I-LDL that is bound in the presence of a 35-fold excess of LDL. No high-affinity binding was seen when the cells had continually been exposed to a physiological level of LDL before the experiment was carried out. This study clearly demonstrated that exposure to LDL-free medium induced specific high-affinity binding in hepatocytes as it had in lymphocytes.(162)

b. Comparison of the Rates of Internalization of Low Density Lipoprotein, Sucrose, and Dextran

To further characterize the uptake of LDL by hepatocytes, I compared the internalization of ^{125}I-labelled LDL, ^{14}C-sucrose, and ^{14}C-dextran after suitable incubation. The studies of Wagner et al. demonstrated that ^{3}H-sucrose provides a marker for quantitation of nonadsorptive or bulk fluid encodytosis and that it

neither induces nor stimulates endocytosis in Chang liver cells.[351] Additionally, the impermeability of cell membranes to sucrose as well as the indigestability of sucrose by liver have been reported.[172, 351] In the first experiment, cells were incubated for 24 hours (37°C) in the presence of 2.3 μmol of ^{14}C-sucrose, 1 nmol of ^{14}C-dextran, or 25 μg of ^{125}I-LDL protein/ml of medium, and the uptake of each compound was measured. Table 11 shows the uptakes calculated as microliters of medium internalized per milligram of cell protein per 24 hours. LDL uptake was estimated according to the method of Miller et al. in which the total LDL uptake was considered to be the sum of the LDL degraded during the incubation plus the internalized LDL.[231]

TABLE 11

A Comparison of the Estimates of Endocytosis of ^{14}C-Sucrose, ^{14}C-Dextran, and ^{125}I-Low Density Lipoprotein (^{125}I-LDL)[a,b]

Material Tested	Volume of Media Internalized (μl/mg cell protein/24 hr)
^{14}C-Sucrose	1.09 μl ± 0.37
^{14}C-Dextran	0.44 μl ± 0.05
^{125}I-LDL	39.05 μl ± 4.35

[a]The cells were incubated for 24 hours at 37°C in the presence of 2.3 μmol of ^{14}C-sucrose, 1 nmol of ^{14}C-dextran, or 25 μg of ^{125}I-LDL per milliliter.

[b]Values represent mean values from quaduplicate dishes ± S.E.

Sucrose and dextran uptakes were taken to be the total amount internalized. After 24 hours, the uptake of LDL was almost 40-fold greater

than that of sucrose or dextran. This is another indication that a more specific process than bulk fluid pinocytosis is responsible for LDL uptake by hepatocytes.

A second experiment was designed to test the process of nonadsorptive pinocytosis under conditions which previously had been shown to cause either the repression or the derepression of the putative LDL receptor in hepatocytes. Repressed cells were preincubated for 20 hours in medium containing 20% whole serum plus 20 μg of LDL protein/ml medium. Derepressed cells were preincubated for 20 hours in medium containing 20% lipoprotein-deficient serum. During the 5-hour experimental incubation, the medium contained either 6 μg of ^{125}I-LDL protein/ml or 0.77 μmol of ^{14}C-sucrose/ml. In order to ensure that the hepatocytes remained repressed or derepressed throughout the experiment, the test media were supplemented with the same serum constituents that were added to the preincubation media.

The results of this experiment are presented in Table 12. The presence or absence of lipoproteins in the medium clearly had no effect on the nonadsorptive endocytotic uptake of ^{14}C-sucrose. It was also evident that under repressed conditions, when specific high-affinity LDL binding is either absent or inoperative, a greater volume of medium was cleared of LDL than could be accounted for by nonadsorptive endocytosis alone. This may be a function of the large amount of nonspecific LDL binding which appears to be characteristic of hepatocytes. It has not been shown that the "nonspecific" binding site is nonspecific in a biological sense; and, indeed, this experiment implies that nonspecific binding contributes substantially to

TABLE 12

A Comparison of the Estimates of Endocytosis of ^{14}C-Sucrose and ^{125}I-LDL in Repressed and Derepressed Cells[a,b]

Material Tested	Volume of Media Internalized (μl/dish/5 hr)	
	Repressed Cells	Derepressed Cells
^{14}C-Sucrose	1.68	1.64
^{125}I-LDL[c]	10.07	18.06

[a]The cells were incubated in the presence of 2.3 μmol of sucrose or 18.8 μg of ^{125}I-LDL in 3 ml of media for 5 hours at 37°C.

[b]The values represent mean values from triplicate dishes. Average protein per dish was 0.73 mg.

[c]The clearance values for ^{125}I-LDL in repressed and derepressed cells are not directly comparable since the total LDL protein/ml medium was 46 μg for the repressed cells but only 6.3 μg for the derepressed cells.

hepatocyte ingestion of LDL under conditions which have been shown to inhibit LDL binding by the putative specific high-affinity LDL receptors.

7. Comparison of Homologous Low Density Lipoprotein Versus Heterologous Low Density Lipoprotein

A competition study was used to examine the ability of rabbit hepatocytes to bind heterologous as well as homologous LDL. The heterologous LDL was freshly prepared human LDL. Table 13 shows the comparative analysis of these two lipoproteins.

Figure 20 shows how increasing the concentration of either human or rabbit LDL affects the binding of ^{125}I-LDL (2.6 μg protein/ml) at 4°C. Figure 21 shows these data redrawn in terms of the total

TABLE 13

Comparison of Human and Rabbit LDL as % Composition

	Human LDL	Rabbit LDL
Phospholipid	26.1	26.2
Cholesterol ester	34.1	21.8
Unesterified cholesterol	11.1	8.8
Triglyceride	3.1	11.8
Protein	25.6	31.7

nanograms of LDL bound: that is, labelled plus unlabelled lipoprotein. Very little difference was observed at concentrations of LDL below 50 μg of LDL protein/ml, thus indicating that rabbit hepatocytes do not distinguish between rabbit and human LDL.

D. Nutritional Effects on Low Density Lipoprotein Metabolism

1. In Vivo Studies

The in vivo studies presented in this section were a joint effort with other investigators from Dr. Portman's laboratory and have been published in detail elsewhere.[268-270] The data shown here provided the basis for the in vitro studies comparing hepatocyte catabolism of control and hyperlipemic LDL which was one of the major objectives of this thesis. This series of studies examined the effects of nutritionally induced hyperlipemia on the catabolism of LDL in vivo. LDL isolated from either hyperlipemic or control donor rabbits was labelled with either ^{3}H-leucine or ^{125}I as described in Methods (III.B.5.). The labelled LDL was then injected into recipient rabbits that were maintained on either hyperlipemic or control

Figure 20. Competition of homologous or heterologous LDL with ^{125}I-LDL for binding sites at 4°C. Rabbit hepatocytes, preincubated for 20 hours in LDL-free medium, were chilled to 4°C; and then iced medium containing 2.6 μg of rabbit ^{125}I-LDL protein/ml plus the indicated concentration of native rabbit or human LDL was added, and the incubation was continued for 2 hours at 4°C. The cells were washed three times with chilled phosphate-buffered saline containing 2 mg of albumin/ml and once with plain phosphate-buffered saline. They were then scraped from the dish and all cell-associated radioactivity was considered to be bound since no internalization occurs at 4°C.(143)

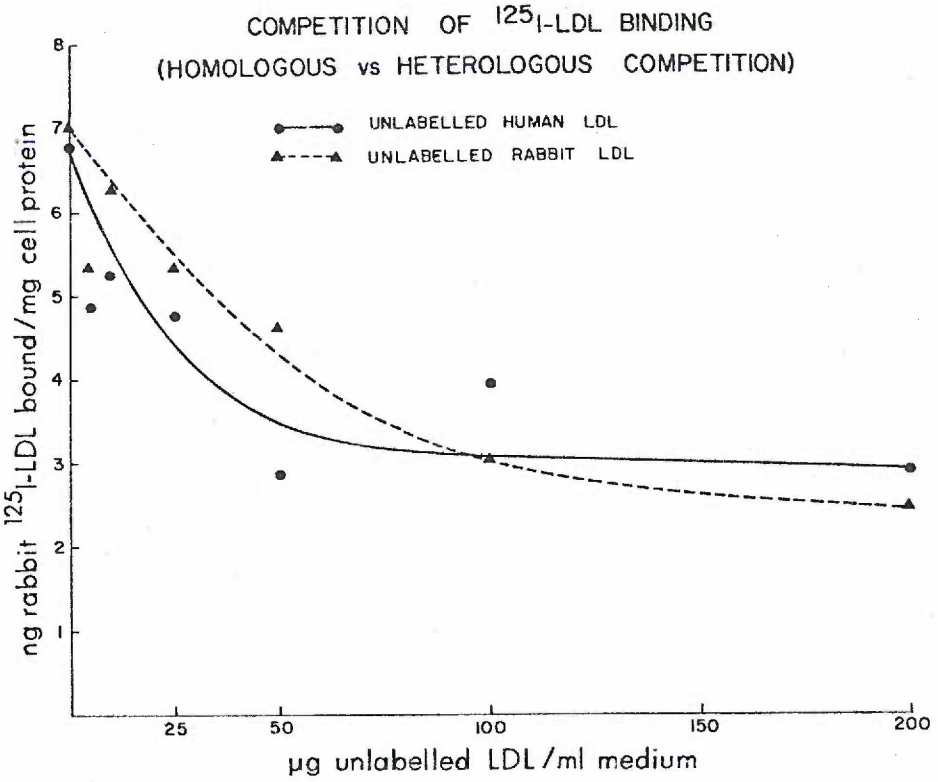
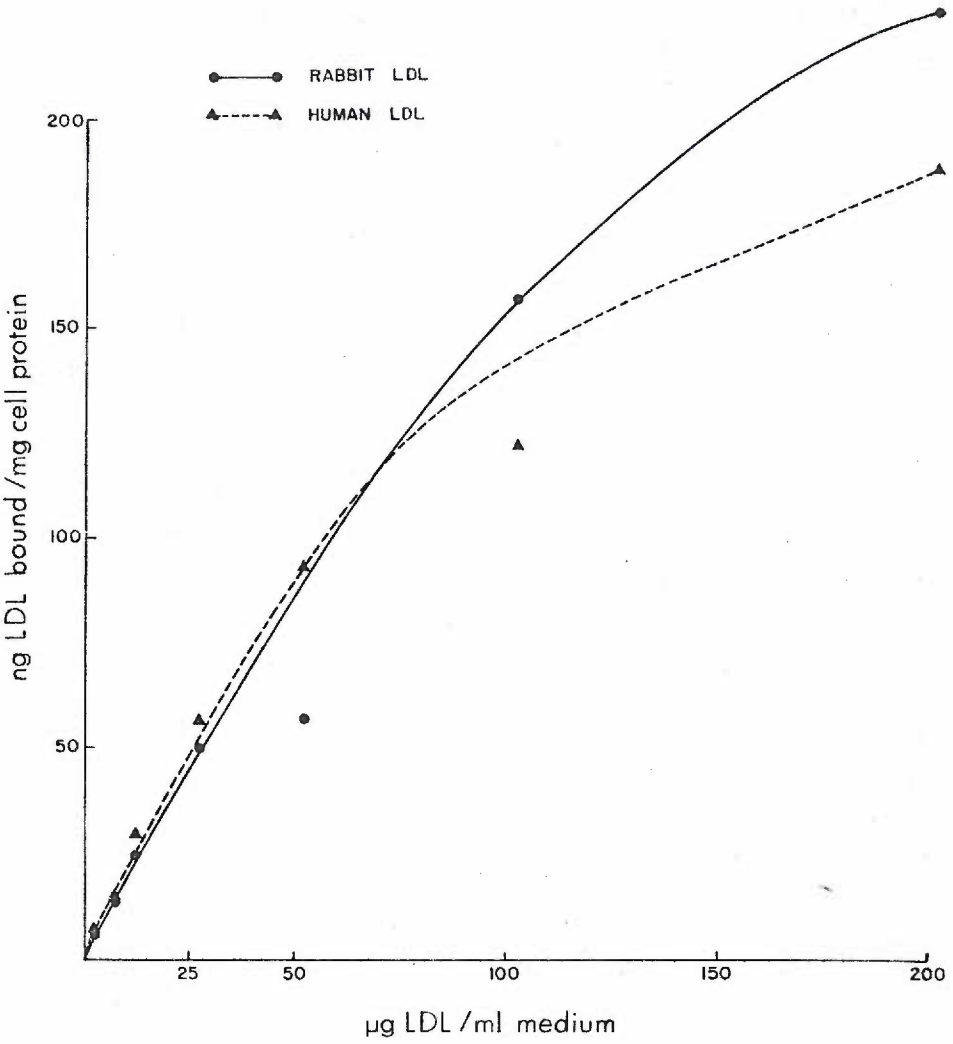

COMPETITION OF 125I-LDL BINDING
(HOMOLOGOUS vs HETEROLOGOUS COMPETITION)
UNLABELLED HUMAN LDL
UNLABELLED RABBIT LDL
ng rabbit 125I-LDL bound/mg cell protein
µg unlabelled LDL/ml medium
1
2
3
4
5
6
7
8
25
50
100
150
200

Figure 21. Comparison of homologous (rabbit) and heterologous (human) LDL as the diluent of rabbit ^{125}I-LDL in a study of specific high-affinity binding at 4°C by rabbit hepatocytes in primary culture. The absolute quantity of lipoprotein bound was derived from the product of the proportion of radioactive LDL bound times the total amount of LDL (labelled plus unlabelled) in the medium. Each point represents the average of duplicate determinations.

BINDING OF HETEROLOGOUS AND HOMOLOGOUS LDL



diets. Figure 22 shows the die-away curves of ^{3}H-LDL from the plasma of control and hyperlipemic rabbits.(270) It can be seen that while the diet regimen of the recipient produced the larger effect, the source of the donor lipoprotein, especially in control animals, also strongly influenced the die-away curves. LDL of both kinds was cleared much more rapidly from the control animals than from the hyperlipemic animals. Hyperlipemic LDL, however, was cleared more rapidly from the plasma than was control LDL. Very similar results were obtained when the injected lipoproteins were labelled with ^{125}I rather than ^{3}H-leucine. These results are shown in Figure 23.(268)

The extent to which the free ^{3}H-leucine liberated during the catabolism of ^{3}H-LDL was reutilized for total plasma protein synthesis and for LDL protein synthesis was determined by blocking protein synthesis in the recipient by injecting cycloheximide (3 mg/kg body weight) 2 hours before the injection of the lipoproteins. This concentration of cycloheximide had been shown (in other experiments) to reduce protein synthesis in the liver several-fold for a few hours. It is shown in Figure 24 that the inhibitor blocks the reutilization of ^{3}H-leucine from ^{3}H-LDL for the synthesis of total plasma proteins, particularly albumin, but the recycling of released ^{3}H-leucine into nascent LDL was not significantly different in normal rabbits and in those with cycloheximide-inhibited synthesis.(270)

These studies showed unequivocally that the metabolism of hyperlipemic LDL in a given group of recipients is more rapid than is the metabolism of control LDL. A possible role for the liver in LDL catabolism was strongly indicated since the protein radioactivity in the

Figure 22. The effect of the dietary group of rabbits in which labelled low density lipoproteins were biosynthesized and of the group of lipoprotein recipients on the disappearance of radioactivity from plasma low density lipoprotein protein. Control low density lipoprotein hyperlipemic indicates that lipoproteins were synthesized in control rabbits, isolated and purified by ultracentrifugation and dialysis, and injected into hyperlipemic rabbits. From reference 270; used with permission.

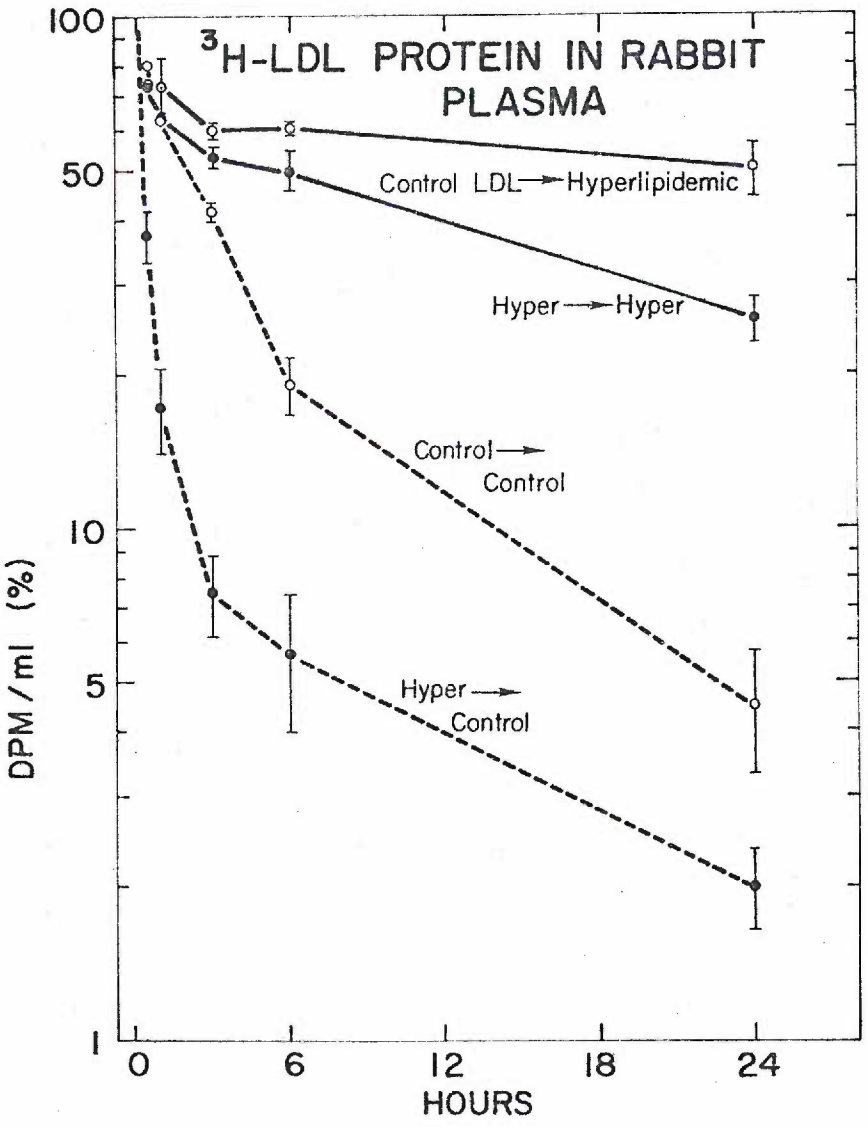

^{3}H-LDL PROTEIN IN RABBIT PLASMA
Control LDL → Hyperlipidemic
Hyper → Hyper
Control → Control
Hyper → Control
DPM / ml (%)
100
50
10
5
1
0
6
12
18
24
HOURS

Figure 23. The effect of the diet of the LDL donor and of the LDL recipient on the die-away curve of ^{125}I-LDL protein from the blood of rabbits. Control ⟶ hyper indicates that LDL from control rabbits was labelled and injected into hyperlipemic rabbits. From reference 268; used with permission.

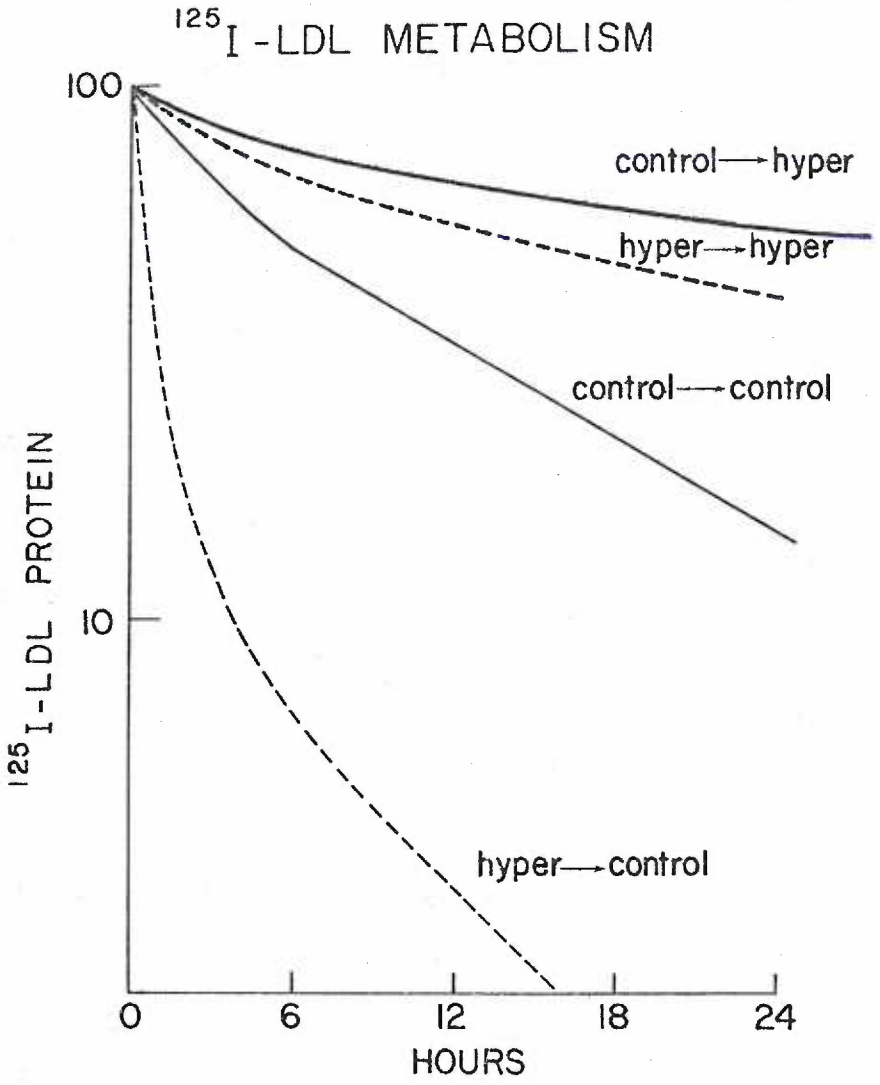
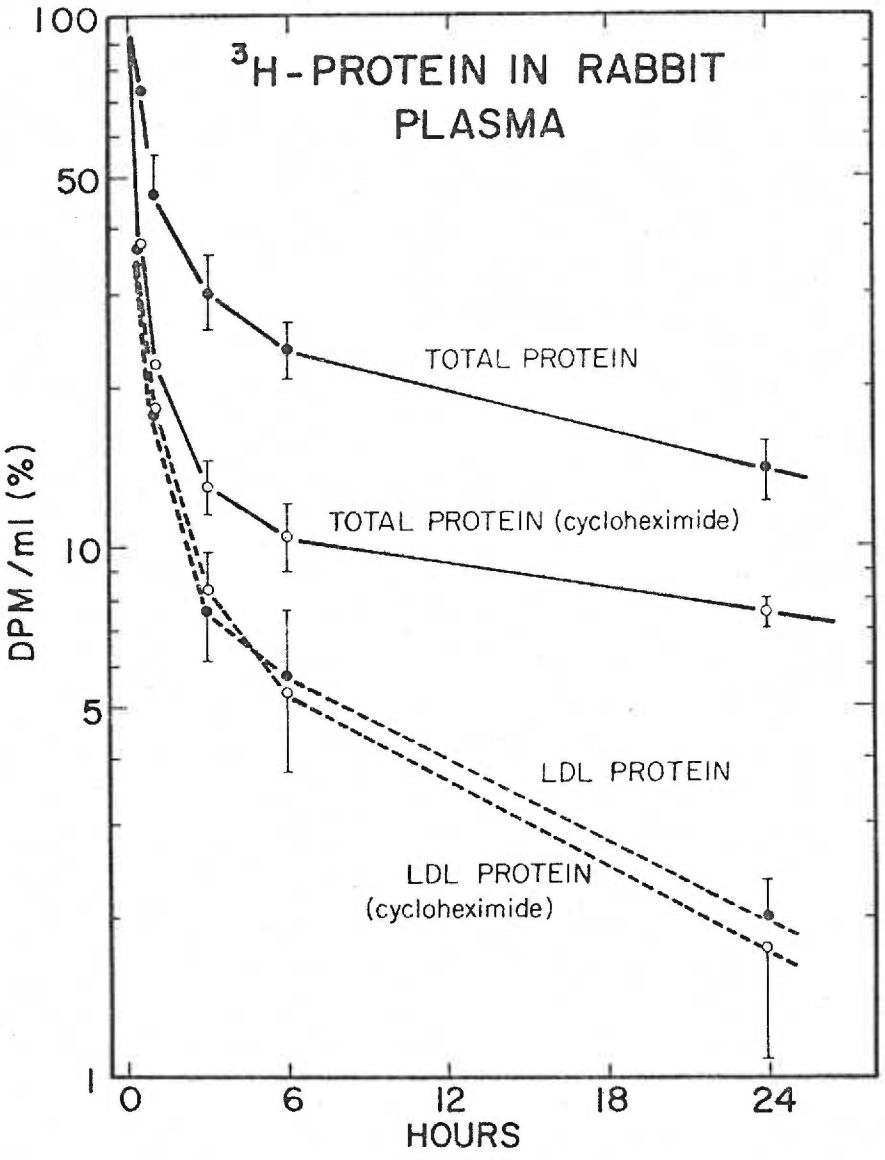

^{125}I-LDL METABOLISM
100
10
^{125}I-LDL PROTEIN
control→hyper
hyper→hyper
control→control
hyper→control
0
6
12
18
24
HOURS

Figure 24. The effect of intravenous injections of cycloheximide (3 mg/kg) 2 hours before the injection of labelled low density lipoproteins into control rabbits on the disappearance of radioactivity from the total and low density lipoprotein of plasma. The use of the protein synthesis inhibitor showed that there was some reutilization of ^{3}H for total protein but not for low density lipoprotein synthesis. From reference 270; used with permission.


^{3}H-PROTEIN IN RABBIT PLASMA
TOTAL PROTEIN
TOTAL PROTEIN (cycloheximide)
LDL PROTEIN
LDL PROTEIN (cycloheximide)
DPM/ml (%)
100
50
10
5
1
0
6
12
18
24
HOURS

liver (with either ^{125}I or ^{3}H label) was much greater than that recovered in any other tissue at all times after the injection of labelled LDL.

Another in vivo study in squirrel monkeys compared the die-away curves of LDL labelled by three different methods: in vitro with ^{125}I by 1) iodine monochloride or 2) the Bolton-Hunter method or 3) in vivo by injection of ^{3}H-leucine into a donor animal.(269) The method of labelling did not significantly affect the observed metabolism of LDL in squirrel monkeys.

2. In Vitro Studies

The binding, internalization, and degradation of iodinated hyperlipemic LDL was compared with that of iodinated control LDL at several different times and concentrations. The results, presented in Table 14, show that the amount of hyperlipemic LDL bound to hepatocytes was not different from the amount of control LDL; however, hyperlipemic LDL was internalized and degraded 1.4-fold better than control LDL. This is a strong indication that the liver is responsible for at least a portion of the more rapid catabolism of hyperlipemic LDL in vivo. These studies were performed with cells from control animals since it was difficult to isolate cells under identical conditions from control and hyperlipemic rabbits and to get consistent animal-to-animal results. However it was possible to get consistent results within a given primary culture in which variations in the media were tested.

There were special problems in isolating cells from the livers of hyperlipemic rabbits since preliminary studies showed that their

TABLE 14

Comparison of Relative Amounts of Binding, Internalization, and Degradation of ^{125}I-Low Density Lipoprotein (^{125}I-LDL) Apoprotein when Hepatocytes Were Incubated with ^{125}I-LDL from Control or Hyperlipemic Donors

Fate of LDL	n	Hyperlipemic	Control[a]	Difference	P
Bound	24	1.09	1.00	0.09 ± 0.10[b]	NS[c]
Internalized	25	1.38	1.00	0.38 ± 0.08	< 0.001
Degraded	25	1.40	1.00	0.40 ± 0.17	< 0.05

[a]All values were normalized so that control LDL determinations were equal to 1.0. Thus, the two lipoprotein preparations were compared over a range of substrate concentrations (5 to 100 μg of protein/ml) and in experiments with 5 different livers. Each experiment used freshly prepared lipoproteins.

[b]Mean ± S.E.M.

[c]NS = not significant

---

livers contain a great deal more connective tissue than control livers. This meant that the cells had to be exposed to proteolytic enzymes for longer periods during the perfusion in order to degrade the additional connective tissue. The cells also contained more lipid, which made them less dense, and, therefore greater centrifugal force was required to sediment the cells when the enzymes were removed. Both the prolonged exposure to enzymes and the greater lipid content of the cells appeared to increase their fragility during the isolation procedure. Because of these difficulties, I decided that the isolated hepatocytes were not representative of hepatocytes in the intact, hyperlipemic animal so attempts to compare liver cells from control and hyperlipemic animals were discontinued.

E. Effects of High Density Lipoprotein on ^{125}I-Low Density Lipoprotein Catabolism in Hepatocytes

The effect of a 50:1 molar excess of control HDL on both hyperlipemic and control LDL binding, internalization, and degradation was examined after incubations of increasing duration: 0.1, 3, 24, and 42 hours. As shown in Table 15, the effect is complex. After 3-hour incubations, there was no discernible effect of HDL on the binding of either control or hyperlipemic LDL; however the amounts of these

TABLE 15

Effect of High Density Lipoprotein (HDL) on Low Density Lipoprotein (LDL) Binding and Internalization

	Hours of Incubation			
	0.1	3	24	42
Bound:				
Control LDL	110	127	319	234
Control LDL + HDL		139	93	90
Hyperlipemic LDL	110	147	359	379
Hyperlipemic LDL + HDL		142	92	139
Internalized:				
Control LDL	5	49	189	196
Control LDL + HDL		29	70	64
Hyperlipemic LDL	3	99	536	640
Hyperlipemic LDL + HDL		73	171	239

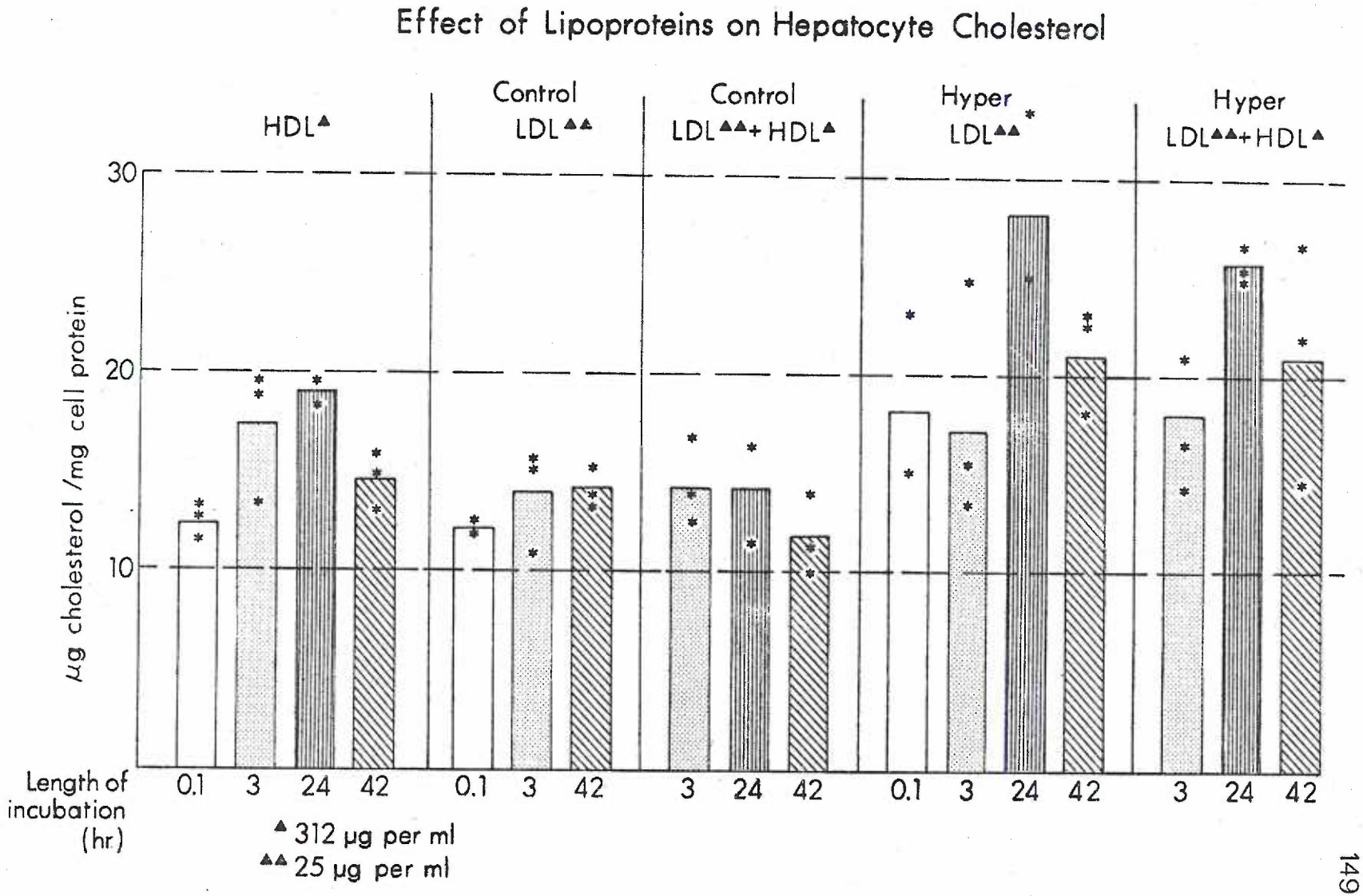
Values are expressed as ng of LDL/mg cell protein in the presence or absence of a 50:1 molar excess of HDL; i.e., 25 μg of LDL protein/ml + 312 μg of HDL protein/ml. These values are the means for triplicate incubations.

lipoproteins that were internalized were reduced by 40 and 25%, respectively. After 24- and 42-hour incubations, a progressive increase in the binding of control or hyperlipemic LDL was observed in the absence of HDL, but the presence of HDL markedly suppressed the binding of both kinds of LDL to levels less than observed after the 3-hour incubation. Internalization was reduced to about 35% of that observed in the absence of HDL although some increase did occur in the presence of HDL in contrast with the absolute decrease seen in binding. The degradation data were much more variable as stated earlier, but degradation was also depressed in the presence of HDL during the 24- and 42-hour incubations. These observations suggested that HDL did not directly compete for LDL binding sites but influenced the number of sites by some other means. The effect of HDL on hepatocyte cholesterol content will be examined in the next section.

F. Some Lipoprotein Effects on Hepatocyte Cholesterol Content

In an attempt to explain how HDL influenced LDL binding and internalization (section IV.E.), the cholesterol content of the hepatocytes in that experiment was determined. Figure 25 shows the results of that analysis. Since the cells were trypsinized before being analyzed, cholesterol contained in surface-bound lipoproteins would have been removed. Between 0.1 and 3 hours, the cell cholesterol content was increased by 2 μg of cholesterol/mg cell protein when the cells were incubated with 25 μg of control LDL protein/ml medium. The total amount of LDL protein bound, internalized, and degraded by these cells was calculated to be 0.416 μg of LDL protein/mg cell protein. Calculations based on the composition data

Figure 25. The effect of lipoproteins on hepatocyte cholesterol. The cellular content of cholesterol was measured at several times after the addition of selected lipoproteins to lipoprotein-deficient medium. The cells were maintained for 20 hours in lipoprotein-deficient medium before adding the lipoproteins. The concentrations of free and total cholesterol per milliliter of medium were estimated to be 10 and 25 μg for control LDL, 13 and 34 μg for hyperlipemic LDL, and 28 and 79 for HDL. The bars are the average values from duplicate or triplicate dishes and (*) represents the individual value per dish. The cholesterol content was estimated by thin-layer chromatography of the chloroform extract of trypsinized hepatocytes.


Effect of Lipoproteins on Hepatocyte Cholesterol
HDL▲
Control LDL▲▲
Control LDL▲▲+ HDL▲
Hyper LDL▲▲*
Hyper LDL▲▲+HDL▲
μg cholesterol /mg cell protein
30
20
10
Length of incubation (hr)
0.1 3 24 42 0.1 3 42 3 24 42 0.1 3 24 42 3 24 42
▲ 312 μg per ml
▲▲ 25 μg per ml

in Table 5 show that this would be equivalent to taking up 0.332 μg of total cholesterol/mg cell protein. Thus uptake of whole LDL particles can account for only 16.6% of the increased cellular cholesterol. And, indeed, this figure is an overestimation since the bound lipoproteins were removed before the cell cholesterol content was determined.

Delipidation was observed in the pulse studies (section IV.G.4.), and this suggested that a net transfer of lipid from lipoproteins to cells had occurred. The increase in cholesterol shown in Figure 25 was greater than could be accounted for by uptake of intact LDL particles and was another indication that cholesterol can move into the cell without the accompanying apoprotein moiety. Cells that were incubated with hyperlipemic LDL showed even larger increases in cholesterol content, probably because of the fact that hyperlipemic LDL contains twice as much cholesterol per milligram protein as does control LDL. The story was complicated by the observation that adding LDL and HDL together did not produce an additive effect on the cholesterol content of the cell. The results of two additional composition studies are shown in Table 16. These also show far greater increases in cholesterol content than can be accounted for by intact lipoprotein uptake as determined in the earlier experiments. The total cholesterol uptake that can be attributed to the LDL protein bound, internalized, and degraded after 3 hours (from Figure 9; 25 μg of LDL protein/ml) is only 0.476 μg/mg cell protein. This is much less than the increase of 5 to 6 μg cholesterol/mg cell protein shown in Table 16.

Two cell preparations were incubated for 2, 30, or 180 minutes with HDL or LDL doubly labelled with ^{125}I-apoprotein and unesterified

TABLE 16

Cholesterol Content of Hepatocytes (μg/mg cell protein)[a]

Experiment	20-hr Incubation in LDM[b]	20 Hours in LDM followed by 3 Hours with LDL[c]
I	25.5[d]	32.0
II	15.0 $\pm$ 1.5[e]	20.0 $\pm$ 2.1

[a]Cholesterol content was estimated by thin-layer chromatography of the chloroform extract of trypsinized hepatocytes as described in section III.D.2.

[b]LDM = lipoprotein-deficient medium.

[c]Concentration of LDL was 25 μg of protein/ml.

[d]S.D. not calculated for mean value from duplicate samples.

[e]S.D. for mean value from triplicate samples.

---

^{14}C-cholesterol. As can be seen from Table 17, the ratio of cholesterol radioactivity to protein radioactivity increases with time. These values were normalized so that a ratio of one is equivalent to the proportion of labelled cholesterol to labelled protein in the lipoprotein that was added to the medium. Thus, as the values in Table 17 become greater than one, the amount of labelled cholesterol associated with the cell has increased more than has the amount of labelled apoprotein. Although this experiment was unable to differentiate between the exchange of labelled free cholesterol among lipoproteins and cells and the net transfer of labelled cholesterol from lipoproteins to cells, it clearly showed that free cholesterol

TABLE 17

Selectivity of Cholesterol Uptake from Low Density Lipoproteins (LDL) or High Density Lipoproteins (HDL) by Isolated Hepatocytes

	Experiment I		Experiment II	
Incubation Time	LDL_I[a]	HDL_I	LDL_{II}	HDL_{II}
2 minutes	1.05[b]	1.23	0.68	0.57
	1.05	0.69	0.72	0.84
30 minutes	0.96	1.88	--	1.77
	0.96	2.13	--	2.11
180 minutes	3.45	3.46	2.19	7.50
	3.50	4.87	2.13	8.96

[a]The concentration of lipoprotein in the medium was as follows: LDL_I contained 51.6 μg of LDL apoprotein/ml; LDL_{II} contained 34.6 μg of LDL apoprotein/ml; HDL_I contained 53.3 μg of HDL apoprotein/ml; and HDL_{II} contained 47.6 μg of HDL apoprotein/ml.

[b]The selectivity of cholesterol uptake is determined by the following equation:

$$\text{Selectivity}_i = \frac{\dfrac{\text{amount of labelled cholesterol taken up by the cell}}{\text{amount of labelled protein taken up by the cell}}}{\dfrac{\text{amount of labelled cholesterol in the lipoprotein}}{\text{amount of labelled protein in the lipoprotein}}}$$

movement from lipoprotein to hepatocyte was not dependent upon concomitant apoprotein uptake.

G. Fate of Bound Apoproteins: Pulse Studies

1. Experimental Design

These studies were designed to examine additional aspects of LDL metabolism including the process of deiodination of ^{125}I-LDL and the reversibility of LDL binding. The experimental design for three

preparations was as follows. Hepatocytes were incubated for 15 or 30 minutes at 37°C with ^{125}I-LDL of high specific activity (>98 cpm/ng LDL protein) at a concentration of 15 μg of LDL protein/ml medium. The pulse medium was removed and the cells were washed five times with 3 ml of lipoprotein-free medium. Then 3 ml of unlabelled medium containing 0, 30, or 300 μg of native LDL protein/ml was added to the cells for incubations lasting from 0.2 to 24 hours. The cells were examined for bound and internalized radioactivity as previously described (section III.C.3.b.). The medium from some of the dishes was examined for degraded LDL (section III.C.3.a.). In addition, the amount of free iodide in the medium was estimated as the amount of radioactivity that was removed from the TCA-soluble fraction by oxidation with H_2O_2 followed by extraction with $CHCl_3$. The medium from some dishes was adjusted to a density of 1.063 g/ml with crystalline KBr and centrifuged for 20 hours at 40,000 rpm in a 50 Ti rotor. The lipoprotein fraction was removed and the distribution of radioactivity in both the LDL and the LDL infranate was determined. Some of the medium was examined for immunologically identifiable LDL (section III.D.8.).

2. Hepatocyte Uptake of Low Density Lipoprotein

At the earliest time examined after the removal of the ^{125}I-LDL pulse, 83.2% of the label was cell associated. A little more than half of this cell-associated label (54%) was internalized. The presence or absence of unlabelled LDL in the medium during the second incubation did not affect the percent distribution of label at 0.2, 3.0, or 24 hours.

a. Bound

Table 18 shows the changes in distribution of radioactivity with time. The amount of bound radioactivity fell sharply between 0.2 and 3.0 hours from 37.2% to 6.2%. Approximately half of this decrease could be accounted for by the increased amount of label recovered as TCA-precipitable material (i.e., protein or large peptides) in the medium. These data suggest that intact LDL may have become detached from the cells during the 3-hour incubation. It was not possible to determine from this experiment, however, if the remainder of the decrease was due to internalization and degradation of the LDL or if the LDL was deiodinated at the cell surface. Maciel et al. have recently presented evidence that the two main sites of hepatic deiodination of thyroxine and reverse-triiodothyronine are found on the plasma membrane and in the microsomes.(209) Thus it is possible that ^{125}I-LDL could be deiodinated without being internalized if the plasma membrane deiodinase mistakes the iodinated tyrosine of the LDL for the iodinated tyrosine of thyroxine or triiodothyronine. Deiodination of LDL by hepatocytes will be discussed in section IV.G.3.

b. Internalized

As shown in Table 18, the amount of internalized radioactivity decreased more slowly than the bound radioactivity during the interval from 0.2 to 3.0 hours. The percentage of radioactivity internalized continued to decrease up to 24 hours. The decrease observed between 3 and 24 hours could be accounted for by the increased radioactivity in the medium recovered as iodide (i.e., the

TABLE 18

Changes in Distribution of Radioactivity with Time Following a 30-minute Pulse with ^{125}I-Low Density Lipoprotein

Second Incubation (hours)	Cells		Medium		
	% Bound	% Intracellular	% TCA-precipitable[a]	% TCA-soluble[a] Total	% TCA-soluble[a] Iodide-free
0.2	37.2 ± 1.2[b]	46.0 ± 1.7	10.5 ± 0.6	6.5 ± 0.4	0
3	6.2 ± 0.4	24.3 ± 1.5	26.2 ± 0.7	44.0 ± 1.3	2.5 ± 0.2
24	3.3 ± 0.2	7.0 ± 0.6	26.4 ± 1.2	63.5 ± 1.0	2.7 ± 0.2

The cells were pulsed with 15μg of ^{125}I-LDL/ml medium (SpA = 131 cpm/ng) for 30 minutes at 37°C. They were washed 5 times with lipoprotein-free medium and then incubated for the indicated time in fresh lipoprotein-free medium. The total recovery of labelled material from each dish averaged 11,070 cpm (n = 18; S.E. = ± 266), and the mean amount of protein per dish was 441.1 μg (n = 18; S.E. = ± 31.09).

[a]TCA = trichloroacetic acid.

[b]The values are the mean of 6 dishes ± S.E.

total TCA-soluble radioactivity minus TCA-soluble radioactivity after extraction). These data suggest that internal deiodination of LDL may occur with the subsequent slow excretion of the liberated iodide.

3. Formation of Inorganic ^{125}I from ^{125}I-Low Density Lipoprotein by Hepatocytes

The liver is active in the deiodination of thyroxine and its derivatives.(71, 178, 321) Because the iodinated tyrosine moiety of ^{125}I-LDL resembles the iodinated outer ring of thyroxine, the pulse study was examined to determine whether hepatocytes actively

deiodinate ^{125}I-LDL. This method was chosen since it avoids the problems involved in quantifying the newly formed free ^{125}I$^-$ in the presence of a large background of free ^{125}I$^-$ since LDL preparations may contain as much as 23% of the total label as free iodide, which is not easily displaceable (section IV.A.2.a.). Several levels of unlabelled LDL were used in the second incubation to see if it would displace the ^{125}I-labelled LDL that bound to the cell during the initial pulse or affect the subsequent deiodination and proteolytic reactions. Table 19 shows that the amount of LDL in the second incubation had no effect on either degradation or deiodination. The level of iodinated tyrosine in the medium did not increase after the first 3 hours, but the amount of free ^{125}I-iodide increased from 6.5% at 0.2 hour to 41.5% after 3 hours and finally to 60.8% of the total radioactivity after 24 hours (Tables 18 and 19). This was clear evidence of the presence of deiodinase activity in hepatocytes since in control incubations without cells there was only a 2.9% increase in free iodide. Protein degradation calculated from the appearance of noniodide TCA-soluble material therefore is the minimum value since in this experiment it could not be determined if the observed deiodination occurs before or after ^{125}I-labelled LDL has been degraded to ^{125}I-tyrosine or other small ^{125}I-tyrosine-containing peptides.

A separate experiment showed that hepatocytes can deiodinate ^{125}I-tyrosine. Two levels of ^{125}I-tyrosine were incubated with hepatocytes for 0 or 3 hours. After 3 hours, between 54 and 58% of the label in ^{125}I-tyrosine was lost and could be recovered as free iodide. In the control incubations without cells, no ^{125}I-tyrosine was

TABLE 19

Noniodide Trichloroacetic Acid-soluble (TCA-soluble) Radioactivity (amino acids and small peptides) and Total Trichloroacetic Acid-soluble Radioactivity Released into the Medium by Hepatocytes at 0.2, 3, and 24 Hours[a]

Time (hours)	Noniodide TCA-soluble Radioactivity			Total TCA-soluble Radioactivity		
	LDL in Second Incubation (μg protein/ml)			LDL in Second Incubation (μg protein/ml		
	0	30	300	0	30	300
0.2	29[b]	26	29	781	615	550
3	312	305	283	5019	4700	5800
24	305	310	266	7200	7000	7150

[a]The cells were pulsed with medium containing ^{125}I-low density lipoprotein for 30 minutes and then were transferred to a radioactivity-free medium. The second medium contained 0, 30, or 300 μg of LDL protein/ml.

[b]All values are cpm recovered/mg cell protein.

converted to free iodide plus tyrosine. After the 3-hour incubation, less than 0.3% of the radioactivity in the incubation was cell associated (i.e., bound and internalized).

The presence of deiodinase activity in both the microsomal and plasma membrane fractions of the liver indicates that deiodination can occur at the surface of the cell as well as internally.(209) Since the deiodination of ^{125}I-tyrosine occurred without much accumulation of labelled material inside the hepatocytes, it is probable that this reaction occurred on the cell membrane. While the possibility of rapid excretion of the free ^{125}I$^-$ cannot be excluded by this

experiment, this occurrence would be inconsistent with the prior experiments which showed that considerable amounts of $^{125}I^-$ accumulate within the cell.

When the internalized label was examined after the addition of 10% TCA to trypsinized cells (as described in section IV.C.5.b.), approximately 35% of the radioactivity was estimated to be free iodide. Since control experiments, in which $Na^{125}I$ was incubated with hepatocytes, showed that the free iodide did not enter the cells to any significant extent during a 24-hour period, the recovery of 35% of the total internalized label as free iodide indicated that some, if not all, of the observed deiodination occurred intercellularly. These experiments were not designed to examine the rate at which free iodide leaves hepatocytes.

4. Alteration of the Density of ^{125}I-Low Density Lipoprotein Released from Hepatocytes

In the experiments described above, the quantity of label that was recovered as TCA-precipitable material from the medium after the second incubation increased when the length of the second incubation was increased from 0.2 to 3 hours, but the amount of LDL bound to the cells decreased. This relationship suggested that intact LDL particles were being detached from the cells. In order to examine the nature of the labelled TCA-precipitable particles, which were detached from the hepatocytes during the second incubation, the experiment was repeated with 3- and 10-hour incubations in LDL-free medium following the 30-minute pulse with ^{125}I-LDL. The medium from duplicate dishes was then either added to TCA in order to precipitate

the proteins (treatment A) or adjusted to a density of 1.063 g/ml with KBr and centrifuged at 40,000 rpm for 20 hours in a 50 Ti rotor in order to isolate lipoproteins (treatment B). Table 20 shows the quantity of label released from the cells and the distribution of radioactivity between TCA-soluble and TCA-precipitable (i.e., protein) label that was more or less dense than 1.063 g/ml. The amount of radioactivity in protein material in the infranate of treatment B

TABLE 20

The Quantity of ^{125}I Released from Hepatocytes and the Distribution of Radioactivity between Trichloroacetic Acid-Soluble Material and Protein That Was More or Less Dense Than 1.063[a]

Incubation period (hr)	Total ^{125}I released (cpm)[b]	Percentage of Released Counts[b]		
		Trichloroacetic Acid-soluble	Protein	
			$d < 1.063$	$d > 1.063$
3	4701	72.3	3.7	23.5
10	6098	73.0	4.5	22.5

[a]Cells were pulsed for 30 minutes with ^{125}I-low density lipoprotein and then transferred to a medium free of radioactivity for a 3- or 10-hour incubation.

[b]All values are the average of duplicate determinations.

($d > 1.063$ g/ml) was estimated by subtracting the total number of TCA-soluble counts in the medium (determined by treatment A) from the total number of counts in the infranate of treatment B. When this estimate of the radioactivity in proteins having a density greater

than 1.063 g/ml is added to the radioactivity recovered in the lipoprotein fraction of treatment B, one gets an estimate of the total radioactivity in protein material in the medium. This estimate agreed very well with the amount of radioactivity recovered in the total protein fraction that was precipitated by TCA (treatment A). Only about 18% of the TCA-precipitable material released from the cells still had the density of LDL, and the transformation occurred within the first 3 hours of incubation.

In a similar experiment, a specific anti-rabbit-LDL antibody was added to the second medium in order to precipitate antibody-recognizable protein. After this precipitate was removed, TCA was added in order to precipitate any remaining protein material. When the total protein counts precipitated by these two methods were compared, 53.9% of the labelled protein material was precipitated by the anti-rabbit-LDL antibody. This was an indication that some LDL was being delipidated by the hepatocytes without concomitant degradation of the protein moiety since approximately 54% of the labelled protein material in the second medium could be precipitated by anti-rabbit-LDL antibody but only 18% of the labelled protein material in the medium still had the density of LDL.

In order to rule out the possibility that the labelled protein material of density $>$ 1.063 g/ml was not the result of free $^{125}I^-$ coprecipitating with serum proteins in the medium, a control experiment was carried out to determine how much free $^{125}I^-$ will coprecipitate with the proteins in the medium. Eleven thousand counts of free $^{125}I^-$ (equivalent to the total counts bound after a 30-minute pulse)

were added to 3 ml of medium containing 20% lipoprotein-deficient serum and incubated for 3 hours. TCA was added to a final concentration of 10% and the amount of ^{125}I associated with the precipitate was found to be 5.5% of the total label. Table 18 shows that 26.2% of the total counts are recovered in the TCA precipitate of the medium after a 3-hour incubation subsequent to the ^{125}I-LDL pulse. Since 94% of the TCA-soluble counts recovered in the medium are free $^{125}I^-$ after 3 hours and the total TCA-soluble fraction accounts for 44% of the label (Table 18), the amount of label as free $^{125}I^-$ would be 4550 cpm. If 5.5% of 4550 cpm coprecipitates with serum proteins, that would mean that 2.3% of the original 11,000 cpm initially bound to the cells had coprecipitated with serum proteins when TCA is added to the medium. Hence only 2.3/26.2 or 8.8% of the total amount of TCA-precipitable label can be accounted for by coprecipitation of free iodide with serum proteins.

V. DISCUSSION

A. Choice of Hepatocytes

Since primary cultures of rat hepatocytes were first described in the early 1970s, they have become an increasingly useful tool for studying hepatic function and the factors that influence it.[36, 41, 42] I developed primary cultures of rabbit hepatocytes to study the role of hepatocytes in LDL degradation. I chose rabbit hepatocytes over those of rats for several reasons. First, and perhaps most important, was the fact that normal rat plasma contains very low levels of LDL; therefore, this species may not have developed the same mechanisms to catabolize the LDL particle that have been developed in species that normally have greater concentrations of plasma LDL. Second, it has been observed that hyperlipemia and subsequently atherosclerosis rapidly develop in rabbits maintained on diets containing 0.5% cholesterol.[270] Since differences in the catabolism of hyperlipemic and control LDL might be important in the development of atherosclerosis, it was reasonable to study this question in a species that is readily susceptible to the disease. Finally, a great deal of work on the in vivo catabolism of labelled lipoproteins in rabbits had been done in this laboratory.[268-270] The fact that the greatest concentration of label in any tissue appeared in the liver suggested that the liver might play an important role in the different rates of disappearance of hyperlipemic and control LDL from the plasma of rabbits. Primary cultures of hepatocytes thus appeared to be a useful system in which to examine this problem in greater detail.

Rabbit hepatocytes have not been used as extensively as rat hepatocytes. Zaleski and Bryła used suspensions of rabbit hepatocytes to study gluconeogenesis.[366-368] Continuous culturing of normal adult rabbit hepatocytes was reported by Vickrey et al.[349] After the 30th passage, these cells still displayed the specific liver parenchymal functions of albumin synthesis and tyrosine aminotransferase activity. A literature search revealed only one report on the use of primary cultures of rabbit hepatocytes.[319]

The cultured hepatocytes were viable for at least 3 days. They had the morphological appearance of hepatocytes, that is, they contained numerous microvilli and were generally binucleate. Retention of liver-specific functions by these cultured cells was illustrated by their ability to synthesize identifiable lipoproteins from amino acids.

B. Suitability of ^{125}I-Low Density Lipoprotein as a Substrate

A primary objective of this project was to show that ^{125}I-LDL labelled by the iodine monochloride method of McFarlane[226] is a suitable substrate for the investigation of hepatic catabolism of LDL. Currently this is the most widely used method for iodinating lipoproteins because of its simplicity, mildness, and relatively low cost.

Low density lipoprotein labelled with ^{125}I by the method of McFarlane[226] was compared with ^{3}H-leu-LDL prepared by in vivo synthesis. Comparison of the degradation of these two substrates by isolated proteases revealed that the ^{3}H-leu-LDL was degraded somewhat faster at nonsaturating concentrations. On the other hand, in vivo

studies had shown that ^{3}H-leu-LDL and ^{125}I-LDL disappear from the plasma compartment and appear in various tissues, especially liver, at very similar rates. In order to resolve the question, further studies were undertaken.

The uptake and degradation of these labelled LDL substrates were examined in primary cultures of hepatocytes. There was an increase in nonspecific binding of ^{125}I-LDL at elevated levels of substrate, possibly caused by the binding of the iodinated tyrosines to thyroid hormone binding sites on the membrane.(263) When the nonspecific component of binding was subtracted by the slope-peeling technique,(141) it was found that very similar amounts of ^{125}I-LDL and ^{3}H-leu-LDL were bound to the high-affinity receptors at all concentrations. This was evidence that the iodine moiety does not hinder this process and that intact tyrosine residues are not crucial for binding to the receptor, as are arginine and lysine.(214, 356)

The major difference observed was the amount of internalized ^{3}H-leu or ^{125}I. While most of the difference could be attributed to accumulation of both free ^{125}I^{-} and ^{125}I-lipid, there was also a greater accumulation of ^{125}I-protein. Several alternative explanations can be proposed to account for the greater amount of internalized ^{125}I-protein. Since the difference in internalized label increased with increasing substrate concentration, as did the observed small increase in ^{125}I-LDL binding over ^{3}H-leu-LDL, in all probability greater amounts of ^{125}I-LDL were internalized. If the rates of degradation of these two substrates were the same, ^{125}I-LDL would accumulate within the cell. Equal internalization rates but

slower degradation of ^{125}I would also account for the increased ^{125}I-protein. This possibility, however, seems highly improbable since the observed amounts of degradation products in the medium were very similar for the two substrates at all concentrations. The most attractive explanation is that some of the free $^{125}I^-$ inside the cell formed complexes with cell proteins and was coprecipitated with those proteins in the presence of TCA.

It can therefore be concluded that ^{125}I-LDL is a satisfactory substrate to use for the study of certain aspects of LDL catabolism in hepatocytes, namely: 1) binding in the range of physiological concentrations (i.e., 50 μg of LDL protein/ml or less); 2) binding to the specific high-affinity receptor; and 3) degradation as measured by the appearance of TCA-soluble products in the culture medium. It does not appear to be the most satisfactory substrate to use for the elucidation of the processes accompanying internalization. I suggest the ^{14}C-sucrose-LDL described by Pittman et al. would be a more suitable substrate to use for this purpose.[260, 261] The dilution experiments with native LDL and ^{127}I-LDL were examined to determine if the increased amount of internalized iodide that results from the presence of ^{127}I-LDL as the diluent has an effect on the observed uptake or degradation of ^{125}I-LDL. They indicated that the additional accumulation of iodide inside hepatocytes, which occurs in the presence of ^{127}I-LDL, does not interfere with the process of LDL uptake or degradation. Since, after 24 hours, similar amounts of both ^{125}I-LDL and ^{3}H-leu-LDL were degraded, protein degradation does not appear to be the rate-limiting process. If it were rate limiting, larger

amounts of TCA-soluble ^{3}H-leucine than TCA-soluble ^{125}I-tyrosine would have been evident. Since they were not, it is probable that degradation is limited by the rate of internalization. Neither does deiodination of ^{125}I-tyrosine appear to occur to a significant extent since this too would have resulted in the appearance of more ^{3}H-leucine than ^{125}I-tyrosine in the medium. The deiodination which is observed thus seems to be separate from the degradation pathway. The studies described in section IV.G.3. suggested that the binding of ^{125}I-LDL to the plasma membrane of the hepatocyte was necessary for deiodination to occur. However, these studies were unable to discern whether internalization of the ^{125}I-LDL particle was also necessary. I suggest that this question could be answered by binding ^{125}I-LDL to Sepharose beads as described by Steinberg et al.(326) This prevents internalization of the ^{125}I-LDL particle. The medium could then be examined for free ^{125}I^{-} after incubations of increasing duration.

C. Specific High-affinity Binding of Low Density Lipoprotein by Hepatocytes

The initial objective of this project was to determine if primary cultures of hepatocytes like fibroblasts, smooth muscle cells, and lymphocytes, could be induced to bind and degrade LDL. As described in section IV.C.1., I have shown that hepatocytes preincubated for 20 hours in lipoprotein-deficient medium do bind, internalize, and degrade LDL. The characteristics of this binding were examined over a range of LDL concentrations and several time periods at both 37°C and 4°C.

The existence of specific high-affinity binding* was indicated by several indirect approaches. The slope-peeling technique of Goldstein and Brown showed the presence of two separable components of binding.(141) One of these predominated at small concentrations of LDL and was saturated at concentrations greater than 25 μg of LDL protein/ml. The K_m of this component was calculated to be 1.59×10^{-9} M at 3 hours and 1.75×10^{-9} M at 24 hours. The maximum amount bound, however, increased from 105 μg of LDL protein/mg of cell protein at 3 hours to 165 μg of LDL protein/mg of cell protein at 24 hours. Thus, the concentration at which half-maximal binding occurs does not change with increased time in culture even though the continuing adaptation of the hepatocytes causes an increase in the maximum amount of bound LDL. This could occur through either of two mechanisms: increases in the amount of LDL bound to each high-affinity receptor or increases in the total number of receptors. The latter is the more probable explanation.

The second indication that hepatocytes can bind LDL by a specific high-affinity process came from the Scatchard plots of the binding data. As shown in Figures 11 and 13, the Scatchard plots for binding at both 4°C and 37°C were nonlinear and had an upward concavity. This type of nonlinear plot is generally interpreted as showing the heterogeneity of receptor sites with two or more classes or orders that differ in binding affinity.(174, 280) It must be kept in mind, however, that this is not absolute evidence of heterogeneous receptors

*High-affinity binding is defined as "the amount of ^{125}I-LDL that is prevented from binding by an excess of unlabelled LDL."(162)

since, as Rodbard has pointed out, nonlinear Scatchard plots with upward concavity can also be produced 1) by errors in separation of bound and free substrates, 2) by differences in affinity of labelled and unlabelled substrate, or 3) by the occurrence of site-site interactions of the type called "negative cooperativity."[280] I avoided errors of the first type by carefully washing the cells with phosphate-buffered saline containing 2 mg of albumin/ml after the test medium had been removed. There was no difference in the binding affinities of labelled and unlabelled substrate, as was demonstrated by competition studies utilizing ^{127}I-LDL or native LDL to compete with ^{125}I-LDL (section IV.C.5.a.). The occurrence of site-site interactions leading to "negative cooperativity," however, could not be ruled out.

I evaluated the validity of assuming that the LDL-receptor interaction behaved as a simple reversible bimolecular reaction at 4°C by undertaking a kinetic analysis to obtain an independent estimate for K_d. By this method, a K_d of 4.44×10^{-9} M was obtained and this agreed reasonably well with the value of 3.97×10^{-9} M obtained from the Scatchard plot at 4°C.

The apparent dissociation constants obtained from the Scatchard analysis of the 4°C and 37°C data for hepatocytes were very similar, i.e., 3.95×10^{-9} M and 3.97×10^{-9} M, respectively. This finding differs somewhat from that reported by Brown and Goldstein who studied fibroblasts and found a somewhat greater affinity at 4°C (4.5×10^{-9} M) than at 37°C (2.5×10^{-8} M).[52] Pitas et al. reported a similar value of 2.8×10^{-9} M for the apparent dissociation constant of

fibroblasts at 4°C.[259] The difference in binding affinity seen in fibroblasts but not hepatocytes at 4°C and 37°C indicates that there may be differences between the hepatocyte receptor and the fibroblast receptor. This would not be surprising since the milieu surrounding hepatocytes in vivo contains a relatively large concentration of LDL. This concentration has been estimated to be about 42% of the plasma concentration in rabbit hepatic lymph.[365] The LDL concentration in human peripheral lymph, on the other hand, has been found to be only about 10% of the concentration found in plasma.[277, 278]

Finally, specific high-affinity LDL receptors were demonstrated directly in a study (section IV.C.6.a.; Figure 19) that showed incubation in LDL-free medium induces high-affinity binding. There is a possibility that some of the observed binding to specific receptors after the 20-hour preincubation in LDL-free medium was the result of the filling of vacated receptors since it was shown (section IV.G.2.a.) that about 50% of the bound LDL becomes detached during a 3-hour incubation. Whether or not LDL was present in the incubation medium did not influence this detaching of bound LDL. Filling of vacated receptors, however, would not account for the additional 39% increase in binding to specific high-affinity receptors that resulted when the LDL-free preincubation was lengthened from 20 to 40 hours. Cultured hepatocytes, as well as fibroblasts and lymphocytes,[143] therefore respond to lipoprotein-deficient medium by synthesizing LDL receptors.

D. Low-affinity Binding of Low Density Lipoproteins by Hepatocytes

Although there is good evidence that hepatocytes, after preincubation in lipoprotein-deficient medium, can induce specific

high-affinity LDL receptors that behave in a fashion similar to that described for fibroblasts[143] and lymphocytes,[162] there is less reason to believe that this mechanism operates in vivo under normal circumstances. As stated earlier, the LDL content in lymph that bathes rabbit hepatocytes in vivo contains about 40% of the plasma concentration of LDL, or about 40 μg/ml for a control rabbit.[365] On the basis of the evidence presented in section IV.C.6.a., this level of LDL in the medium prior to the binding test eliminates observable binding to specific high-affinity receptors, a finding consistent with the finding of Brown and Goldstein that in the steady-state, body cells manifest only a small fraction of their maximal number of LDL receptors.[53, 54]

The study in which the clearance of LDL was compared with the clearance of sucrose provided additional information on the means by which LDL is taken into the cell, even in the absence of induced LDL receptors. In the first experiment, derepressed cells were incubated with either sucrose or LDL for a 24-hour test period and the volume (microliters) of medium cleared of substrate during that time was calculated. Since clearance of sucrose measured simple bulk pinocytosis, any clearance of substrate greater than the sucrose clearance indicated the presence of a more specific mode of uptake. In this experiment, derepressed cells cleared 40 times more (by volume) LDL than sucrose from the medium. Because hepatocytes in vivo exist under repressed conditions, the experiment was repeated in order to examine clearance in both repressed and derepressed cells. As shown in Table 12, repression or derepression did not affect the bulk fluid

pinocytosis of sucrose. In both conditions, however, the volume of medium cleared of LDL greatly exceeded that cleared of sucrose. Unfortunately, this type of procedure cannot be used to compare LDL uptake in repressed and derepressed hepatocytes since at 37°C, continuous metabolism occurs and the cells continue to respond to changes in the medium. Adding medium containing 40 μg of LDL protein/ml to derepressed cells would begin to repress the cells, and after 5 hours, the observed clearance would be a combination of repressed and derepressed responses.* The addition of inhibitors of protein synthesis could conceivably overcome a part of this problem, but since these substances cause increased cell detachment, i.e., cause cell deterioration, such an approach would not be comparable to cells without inhibitors; however, protein synthesis inhibitors would not interfere with recycled receptors.(8)

If one assumes that the total amount of LDL in the volume of medium cleared by the repressed cells is taken up and degraded, the following calculation is possible:

$$\frac{10.07\ \mu l\ \text{medium cleared}}{0.73\ \text{mg protein}} \times \frac{0.046\ \text{mg LDL}}{1000\ \mu l\ \text{medium}} \times \frac{1}{5\ \text{hours}} \times \frac{24\ \text{hours}}{1\ \text{day}} \times \frac{194\ \text{mg protein}}{1\ \text{g liver}}^{**} \times \frac{34.4\ \text{g liver}}{1\ \text{kg of rabbit}}^{**x} \times 0.9^{*xx} =$$

18.27 mg LDL of protein/kg of rabbit/day

---

*Five hours is the minimum time necessary to accumulate sufficient amounts of labelled sucrose for a dependable radioassay.

**Unpublished data.

***x*Unpublished data: 34.4 ± 1.2 (SE) g of liver/kg of rabbit; n = 19.

*xxHepatocytes make up 90% of the liver mass.(353)

Since the total in vivo catabolic rate of control LDL in control rabbits is 0.73 ± 0.1 mg of LDL protein/day/ml of plasma,[270] and since rabbit plasma volume is approximately 4.4% of the body weight,[86] the total amount of LDL protein catabolized is 32.1 mg/kg/day. Thus, the observed uptake and degradation of LDL by repressed hepatocytes in primary culture can account for 57 % of the total LDL catabolism observed in vivo. This value is greater than the figure obtained by Pittman et al. when they injected LDL labelled with ^{14}C-sucrose in vivo and recovered 40% of the ^{14}C-sucrose from degraded LDL in the liver lysosomes.[260] The larger amount of catabolism detected in culture could have been caused by a greater proportion of the cell surface being bathed with lipoprotein-rich medium than would be bathed in intact liver where the cells are found in a three-dimensional matrix and it is probable that considerably less surface area per cell would be bathed with lipoprotein-rich lymph.

Recently Shepard et al. modified the arginyl residues on LDL and showed that modification significantly reduces the fractional clearance rate from plasma and permits calculation of receptor-independent catabolism of LDL in vivo since arginine is necessary for receptor recognition.[298] They demonstrated that this receptor-independent catabolism accounts for 66% of the in vivo LDL catabolism in normal human beings. This observation is consistent with the demonstration that the liver accounts for 40% of the in vivo LDL catabolism[260] and with the hypothesis developed in these studies that under normal circumstances, the LDL receptors in hepatocytes are repressed and the

hepatic catabolism that is observed can be accounted for by receptor-independent uptake.

E. Compositional Differences in Hyperlipemic and Control Low Density Lipoproteins

The composition of hyperlipemic LDL as prepared and isolated for these studies was markedly different from that of control LDL. The percentage of cholesterol ester in hyperlipemic LDL was almost twice that of control LDL, whereas the percentage of triglyceride was reduced from 19.5% in control LDL to 5.4% in hyperlipemic LDL. There was also a reduction in the relative amount of protein, from 27.2% to 21.1%.

Examination of the protein moiety of hyperlipemic and control LDL revealed that the greatest differences were in the light fraction (d = 1.019 to 1.040 g/ml) of the LDL density range of 1.019 to 1.063 g/ml. The light fraction also contained approximately 80% of the total LDL protein. As shown in Table 6, 26% of the hyperlipemic light fraction protein was apo-E; only 1% of the control light fraction was apo-E.

F. Effect of Nutritional Differences on Low Density Lipoprotein Metabolism by Liver and Hepatocytes

The in vivo studies described in section IV.D.1. showed unequivocally that catabolism of hyperlipemic LDL in a given group of recipients is more rapid than the catabolism of control LDL. A possible role for the liver in this catabolism is indicated by the fact that the greatest concentration of radioactivity in any tissue other than

plasma (for both ^{125}I- and ^{3}H-labelled LDL) was found in the liver at all times after injection of labelled LDL.

Catabolism of ^{125}I-hyperlipemic and catabolism of ^{125}I-control LDL were compared in five separate hepatocyte preparations. Although there was not a significant difference in the amounts of these two types of lipoproteins that were bound to the surfaces of the hepatocytes, 1.4 times more hyperlipemic LDL was internalized and degraded. These differences were significant at the $p < 0.001$ and $p < 0.05$ levels, respectively. The more rapid degradation of hyperlipemic LDL by cultured hepatocytes indicated that the liver is an important organ in determining the results of the in vivo experiments, in which labelled hyperlipemic LDL was cleared from the plasma of both control and hyperlipemic recipients more rapidly than was labelled control LDL (Figures 22 and 23). The fractional catabolic rates (FCRs) in control recipients (0.835 per hour for hyperlipemic LDL and 0.244 per hour for control LDL) were vastly greater than the FCRs in hyperlipemic recipients (0.047 per hour for hyperlipemic LDL and 0.018 per hour for control LDL).[270] The fact that hyperlipemic LDL was cleared more rapidly under both homologous (i.e., hyperlipemic LDL into hyperlipemic recipient) and heterologous (i.e., hyperlipemic LDL into control recipient) conditions indicated that this was not an artifact of the heterologous system. In fact, when control LDL was examined in an heterologous system (i.e., control LDL into hyperlipemic recipient), it was cleared more slowly than the homologous lipoprotein (i.e., hyperlipemic LDL). These differences in LDL metabolism in control and hyperlipemic animals clearly need to be examined at the

cellular level in the liver. Unfortunately, as explained in section IV.D.2., the problems encountered in isolating representative hepatocytes from hyperlipemic rabbits have not yet been solved.

There are several plausible reasons for differences between the observed catabolic rates in the in vivo and the in vitro studies. First, in the in vivo experiments, LDL is catabolized by several tissues in addition to the liver; and as Van Berkel et al. have shown, the nonparenchymal cells of the liver also play an important role in lipoprotein catabolism.[341, 343] In my studies, the function of nonparenchymal cells in the primary cultures was much less than in whole liver. Second, the cultured hepatocytes were preincubated in lipoprotein-deficient medium in order to maximize the number of LDL receptors (section IV.C.4.). This condition does not occur in vivo and the use of these conditions may select an atypical population of hepatocytes, which can adhere under these conditions. Third, these cells were incubated in the absence of HDL. In subsequent experiments a 50:1 molar excess of HDL to LDL in the incubation medium had the long-term effect of decreasing the amounts of both hyperlipemic and control LDL bound and internalized (Table 14) and thence degraded by the hepatocytes. This 50:1 molar ratio of HDL to LDL approximated the ratio in normal rabbit hepatic lymph.[365] It was shown, however, that even in the presence of HDL, hepatocytes metabolize more hyperlipemic LDL than control LDL. These data are consistent with the hypothesis that the liver has a significant role in the more rapid disappearance of hyperlipemic LDL that occurs in vivo in both control and hyperlipemic animals.[268, 270]

I would like to propose that apolipoprotein E, which is present in increased amounts in hypercholesterolemic plasma, may be an important factor in achieving a new steady-state level of serum cholesterol when nutritionally induced hyperlipemia develops. Apolipoprotein E has two properties which would be important to this role. First, an increase in apo-E appears concomitantly with the increase in plasma cholesterol ester,[149, 212, 218, 306] and it has been suggested that apo-E plays an important role as a cholesterol-carrying protein.[273] Second, Innerarity and Mahley have shown that fibroblast "LDL receptors" have a greater affinity for apo-E-containing lipoproteins than for apo-β-containing lipoproteins.[166] Both the increased internalization and degradation of hyperlipemic LDL by hepatocytes and the increased degradation of hyperlipemic LDL in vivo could be explained by the greater affinity that "LDL receptors" have for apo-E-rich lipoproteins. I did not see increased binding of hyperlipemic LDL, but an increase in binding to specific receptors could have been masked by the large amount of nonspecific LDL binding that occurred in hepatocytes. The fact that internalization of hyperlipemic LDL was 1.4 times greater than for control LDL suggested that such an increase did occur. Thus, an increase in the amount of lipoproteins containing apo-E and cholesterol, coupled with the greater affinity of apo-E for "LDL receptors" and the subsequent increase in hepatic uptake of the cholesterol-rich lipoprotein, would act to shut down hepatic cholesterol synthesis as nutritionally induced hyperlipemia developed. If the cholesterol derived from the diet and incorporated into plasma lipoproteins was greater than that provided by the liver

before synthesis was reduced or eliminated, a new, albeit higher, steady-state level of plasma cholesterol would result. Since the increase in plasma cholesterol of rabbits that are fed cholesterol occurs very rapidly, there is only a relatively short period in which the rates of addition of cholesterol to plasma and subtraction from plasma are not in equilibrium.

The studies of Breslow et al. are in accord with this proposal.(44) They showed that when hyperlipemic LDL, but not control LDL, was incubated with primary cultures of rat hepatocytes, the activity of HMG CoA-reductase is reduced. Unfortunately, their studies did not examine binding of the lipoproteins.

Kahn has suggested that the presence of both high-affinity, low-capacity receptors and low-affinity, high-capacity receptors may serve as a buffer against transient large substrate concentrations while maintaining sensitivity during periods of small concentrations.(174) Although Kahn was describing the actions of hormone receptors, the same two types of receptors appear to be involved in LDL metabolism, particularly in the liver, where the low-affinity, high-capacity binding of LDL might effectively serve as the hepatic extravascular LDL pool. If the current concept of a recycling LDL receptor is valid, it is possible that the marker on the cytoplasmic side that directs the receptor to the coated pit area in fibroblasts(8, 138) also serves in hepatocytes to direct the receptor to the specific area necessary for the internalized LDL to reduce HMG CoA-reductase activity. Low density lipoprotein internalized by the low-affinity process would not be specifically channeled to this area, and

therefore, would be ineffective in reducing enzyme activity. Breslow et al.[44] did not determine whether the enzyme activity was reduced by an acute mechanism, e.g., phosphorylation of the enzyme,[56] or by a slower means, e.g., reduced synthesis of the enzyme,[97] or perhaps by both processes. If the lipoprotein needs to be bound to the high-affinity receptor in order to regulate the HMG CoA-reductase enzyme, this requirement would explain the finding of Breslow et al. that hyperlipemic LDL reduces enzyme activity better than does control LDL since hyperlipemic LDL contains twice as much apo-E and thus binds to the LDL receptor with a much greater affinity.[44]

The recent findings of Mahley et al. are also compatible with the proposal.[215] Mahley and his colleagues compared the in vivo metabolism of canine apo-E-HDL_c that contained acetoacetylated lysine residues with unmodified canine apo-E-HDL_c. After 20 minutes, 64% of the control HDL_c had left the plasma and 61% of that (i.e., 39% of the injected material) appeared in the liver; in a parallel experiment only 45% of the acetoacetylated apo-E-HDL_c was cleared from the plasma and less than 25% of that (i.e., less than 10% of the injected material) appeared in the liver. The rapid clearance of native apo-E-HDL_c from the plasma of both hypercholesterolemic and normal dogs suggested to them that the liver might be the normal site for removal of cholesterol ester-rich apo-E-HDL_c. The retarded removal of modified apo-E-HDL_c suggested to them that the uptake process is mediated by a lysine-dependent recognition system. Their results with apo-E-rich HDL_c are consistent with my hypothesis that the liver has a special role in the removal of all apo-E-rich hyperlipemic lipoproteins.

It appears from many lines of evidence, those just discussed as well as the experiments that showed apo-E-rich chylomicron remnants regulate hepatic cholesterol synthesis,[74] that apo-E is the messenger that signals the hepatocytes that the plasma cholesterol has increased. To carry this idea one step further, I conjecture that the increased concentration of apo-E-containing lipoproteins that develop during cholesterol feeding[149, 306] are associated with increased rates of apo-E synthesis and degradation. The increased synthesis could be caused by the same chain of events, as yet not fully elucidated, that turns off the synthesis of HMG CoA-reductase. As cholesterol levels decline, on the other hand, HMG CoA-reductase synthesis is turned on and apo-E synthesis would decrease. This model describes a sensitive system (HMG CoA-reductase in liver) for maintaining cholesterol homeostasis during relatively small variations in dietary cholesterol and also a very efficient carrier, apo-E-rich lipoproteins, for the transport of excess loads of cholesterol in plasma and their removal by the liver. In this model, liver cholesterol synthesis would not be turned off by the presence of apo-B as occurs in peripheral cells[143] but only by apo-E.[44] Apolipoprotein-B would have the functional role of providing structure for triglyceride carrying lipoproteins[145] and would also be the primary carrier of cholesterol to peripheral cells as described by Goldstein and Brown.[142, 143]

G. Cholesterol Content of Hepatocytes

The interaction of lipoproteins and hepatocytes is very complex and can clearly involve lipid movement by processes other than uptake of intact lipoproteins. One study described here (section IV.F.)

attempted to determine the influence of lipoproteins in the medium on the cholesterol content of the hepatocyte. These experiments were difficult to interpret and raised more questions than they answered. The level of hepatocyte cholesterol clearly rose in the presence of hyperlipemic LDL. The effect of HDL alone appeared to be different from the effect of HDL added with either control or hyperlipemic LDL (Figure 25). When control LDL or HDL labelled in both the protein and free cholesterol moieties was incubated with the cells, the cholesterol label was taken up to a greater extent than the protein label (Table 17). This finding was consistent with calculations showing that the increase in cellular cholesterol content was greater than could be accounted for by the amount of lipoprotein uptake, as calculated by apo-B uptake.

The increased cholesterol content of the cells was an indication that net uptake of cholesterol from the lipoproteins had taken place. It was not absolute proof since measurable synthesis could have occurred, and the greater increase in labelled cholesterol than in labelled protein could have been due to a simple exchange of cholesterol, which is known to occur rapidly between lipoprotein and erythrocytes. The presence of immunologically identifiable, delipidated apo-B in the incubation medium after a pulse of labelled LDL (section IV.G.4.) strongly supported the suggested occurrence of net transport of cholesterol from LDL to hepatocytes. Studies on the transfer and exchange of cholesterol and cholesterol ester among lipoproteins and hepatocytes constitute an ongoing effort to differentiate these

processes and to learn how the process of cell culture affects the need of hepatocytes for cholesterol.

VI. SUMMARY AND CONCLUSIONS

In summary, I have shown that ^{125}I-LDL can be used to study lipoprotein binding and degradation by hepatocytes even though hepatocytes have an active deiodination system. The agreement of the binding and degradation data for ^{125}I-LDL and ^{3}H-leu-LDL indicated that deiodination of ^{125}I-LDL did not alter the results obtained with the iodinated lipoprotein.

I demonstrated that a 20-hour incubation in lipoprotein-deficient medium results in increased specific high-affinity binding to the putative LDL receptors which are induced in the absence of lipoproteins. The high-affinity binding was characterized at 37°C by a K_d of 3.95×10^{-9} M and a K_m of 1.57×10^{-9} M after 3 hours. The large amount of nonspecific low-affinity binding that was observed may actually serve as the extravascular hepatic LDL pool.

Additional evidence that LDL is not simply taken up by bulk fluid pinocytosis was provided by the study that compared the clearance of ^{14}C-sucrose and ^{125}I-LDL in receptor-repressed and receptor-derepressed hepatocytes. Derepressed cells cleared 40 times more (by volume) ^{125}I-LDL than ^{14}C-sucrose from the medium. While repression or derepression of receptors did not change the observed clearance of ^{14}C-sucrose, receptor-repressed cells cleared less ^{125}I-LDL than did receptor-derepressed cells. It was calculated that the observed uptake and degradation of LDL by repressed hepatocytes could account for 56.9% of the total LDL catabolism observed in vivo. This supports the view that the liver is an important site of LDL catabolism.

I also showed that after preincubation in lipoprotein-deficient medium, nutritionally induced hyperlipemic LDL is taken up and degraded 1.4-fold better than control LDL. A model that has been proposed suggests that the increased apo-E content of hyperlipemic LDL is related to the increased uptake of hyperlipemic LDL and the decreased HMG CoA-reductase activity, which has been described in hepatocytes incubated with hyperlipemic LDL.[44] It is suggested that these changes give rise to the new, albeit higher, steady-state level of cholesterol, which is observed in rabbits maintained on a diet regimen which includes 0.5% cholesterol and butter. Thus, although hepatocytes have been shown to be important in the overall catabolism of LDL-apo-B, it has been suggested by several lines of evidence[44, 74] that apo-E is more important in the regulation of cholesterol synthesis in liver.

Finally, primary cultures of hepatocytes have been shown to be a useful model for the study of hepatic metabolism of lipoproteins. Additionally, the successful binding of heterologous LDL to the high-affinity receptor suggests that this model would be useful for examining the binding characteristics of the polymorphic forms of apo-E described by Uterman et al.[339] Hepatocyte cultures are also proving useful in the on-going effort to differentiate between the processes of transfer and exchange of cholesterol and cholesterol ester among lipoproteins and hepatocytes.

REFERENCES

1. Alaupovic, P. Apolipoproteins and lipoproteins. Atherosclerosis, 1971. 13, 141-146.

2. Alaupovic, P., Gustafson, A., Sanbar, S.S., & Furman, R.H. Protein moieties of human serum lipoproteins: a basis for classification. Circulation, 1964. 30, Suppl. III, 1. (Abstract).

3. Alaupovic, P., Kostner, G., Lee, D.M., McConathy, W.J., & Magnani, H. Peptide composition of human plasma apolipoproteins A, B, and C. Expos. Annu. Biochim. Med., 1972. 31, 145-160.

4. Albers, J.J., Cabana, V.G., & Hazzard, W.R. Immunoassay of human plasma apolipoprotein B. Metabolism, 1975. 24, 1339-1351.

5. Anderson, J.M., & Dietschy, J.M. Regulation of sterol synthesis in 16 tissues of rat. I. Effect of diurnal light cycling, fasting, stress, manipulation of enterohepatic circulation, and administration of chylomicrons and Triton. J. Biol. Chem., 1977. 252, 3646-3651.

6. Anderson, J.M., Turley, S.D., & Dietschy, J.M. Low and high density lipoproteins and chylomicrons as regulators of rate of cholesterol synthesis in rat liver in vivo. Proc. Natl. Acad. Sci. U.S.A., 1979. 76, 165-169.

7. Anderson, R.G.W., Goldstein, J.L., & Brown, M.S. Localization of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. Proc. Natl. Acad. Sci. U.S.A., 1976. 73, 2434-2438.

8. Anderson, R.G.W., Goldstein, J.L., & Brown, M.S. A mutation that impairs the ability of lipoprotein receptors to localise in coated pits on the cell surface of human fibroblasts. Nature, 1977. 270, 695-699.

9. Assmann, G., & Brewer, H.B. A molecular model of high density lipoproteins. Proc. Natl. Acad. Sci. U.S.A., 1974. 71, 1534-1538.

10. Assmann, G., & Brewer, H.B. Lipid-protein interactions in high density lipoproteins. Proc. Natl. Acad. Sci. U.S.A., 1974. 71, 989-993.

11. Assmann, G., Brown, B.G., & Mahley, R.W. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured swine aortic smooth muscle cells by plasma lipoproteins. Biochemistry, 1975. 14, 3996-4002.

12. Attie, A.D., Weinstein, D.B., Freeze, H.H., Pittman, R.C., & Steinberg, D. Unaltered catabolism of desialylated low-density lipoprotein in the pig and in cultured rat hepatocytes. Biochem. J., 1979. 180, 647-654.

13. auf dem Brinke, D., Hesch, R.-D., & Kohrle, J. Re-examination of the subcellular localization of thyroxine 5'-deiodination in rat liver. Biochem. J., 1979. 180, 273-279.

14. Bach, P., Hamprecht, B., & Lynen, F. Regulation of cholesterol biosynthesis in rat liver: diurnal changes of activity and influence of bile acids. Arch. Biochem. Biophys., 1969. 133, 11-21.

15. Bachorik, P.S., Kwiterovich, P.O., & Cooke, J.C. Isolation of a porcine liver plasma membrane fraction that binds low density lipoproteins. Biochemistry, 1978. 17, 5287-5299.

16. Bachorik, P.S., Livingston, J.N., Cooke, J., & Kwiterovich, P.O. The binding of low density lipoprotein by liver membrane in the pig. Biochem. Biophys. Res. Commun., 1976. 69, 927-935.

17. Baker, H.N., Delahunty, T., Gotto, A.M., & Jackson, R.L. The primary structure of high density apolipoprotein-glutamine-I. Proc. Natl. Acad. Sci. U.S.A., 1974. 71, 3631-3634.

18. Bang, I. Lipamie. Biochem. Z., 1918. 90, 383-387.

19. Bartlett, G.R. Phosphorus assay in column chromatography. J. Biol. Chem., 1959. 234, 466-468.

20. Basu, S.K., Goldstein, J.L., & Brown, M.S. Characterization of the low density lipoprotein receptor in membranes prepared from human fibroblasts. J. Biol. Chem., 1978. 253, 3852-3856.

21. Baxter, G.P., & Wallace, C.C. Changes in volume upon solution in water of the halogen salts of the alkali metals. J. Am. Chem. Soc., 1916. 38, 70-105.

22. Becker, G., & Ashwood-Smith, M.J. Endocytosis in Chinese hamster fibroblasts: inhibition by glucose. Exp. Cell Res., 1973. 82, 310-314.

23. Beg, Z.H., Allman, D.W., & Gibson, D.M. Modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity with cAMP and with protein fractions of rat liver cytosol. Biochem. Biophys. Res. Commun., 1973. 54, 1362-1369.

24. Beg, Z.H., Stonik, J.A., & Brewer, H.B., Jr. 3-Hydroxy-3-methylglutaryl coenzyme A reductase: Regulation of enzymatic activity by phosphorylation and dephosphorylation. Proc. Natl. Acad. Sci., U.S.A., 1978. 75, 3678-3682.

25. Beher, W.T., & Baker, G.D. Build up and regression of inhibitory effects of cholic acid on in vivo liver cholesterol synthesis. Proc. Soc. Exp. Biol. Med., 1959. 101, 214-217.

26. Belfrage, P., Elovson, J., & Olivecrona, T. Radioactivity in blood and liver partial glycerides, and liver phospholipids after administration to carbohydrate-fed rats of chyle containing double-labeled triglycerides. Biochim. Biophys. Acta, 1965. 106, 45-55.

27. Berg, T., Tolleshaug, H., Drevon, C.A., & Norum, K.R. Uptake and degradation of proteins in isolated rat liver cells. In H.L. Segal and D.J. Doyle (Eds.) Protein turnover and lysosome function. New York: Academic Press, 1978. pp. 417-430.

28. Bergman, E.N., Havel, R.J., Wolfe, B.M., and Bøhmer, T. Quantitative studies of the metabolism of chylomicron triglycerides and cholesterol by liver and extrahepatic tissues of sheep and dogs. J. Clin. Invest., 1971. 50, 1831-1839.

29. Berman, M., Hall, M., III, Levy, R.I., Eisenberg, S., Bilheimer, D.W., Phair, R.D., & Goebel, R.H. Metabolism of apoB and apoC lipoproteins in man: kinetic studies in normal and hyperlipoprotenemic subjects. J. Lipid Res., 1978. 19, 38-56.

30. Bernfeld, P. Interaction of plasma proteins with polyelectrolytes. Fed. Proc., 1955. 14, 182. (Abstract).

31. Berry, M.N., & Friend, D.S. High yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. J. Cell Biol., 1969. 43, 506-520.

32. Bersot, T.P., Mahley, R.W., Brown, M.S., & Goldstein, J.L. Interaction of swine lipoproteins with the low density lipoprotein receptor in human fibroblasts. J. Biol. Chem., 1976. 251, 2395-2398.

33. Bierman, E.L., & Albers, J. Regulation of low density lipoprotein receptor activity by cultured human arterial smooth muscle cells. Biochim. Biophys. Acta, 1977. 488, 152-160.

34. Bierman, E.L., Stein, O., & Stein, Y. Lipoprotein uptake and metabolism by rat aortic smooth muscle cells in tissue culture. Circ. Res., 1974. 35, 136-149.

35. Bilheimer, D.W., Stone, N.J., & Grundy, S.M. Metabolic studies in familial hypercholesterolemia. Evidence for a gene-dosage effect in vivo. J. Clin. Invest., 1979. 64, 524-533.

36. Bissell, D.M., Hammaker, L.E., & Meyer, U.A. Parenchymal cells from adult rat liver in nonproliferating monolayer culture. I. Functional studies. J. Cell Biol., 1973. 59, 722-734.

37. Blix, G., Tiselius, A., & Svensson, H. Lipids and polysaccharides in electrophoretically separated blood serum proteins. J. Biol. Chem., 1941. 137, 485-494.

38. Bloch, K. The biological synthesis of cholesterol. Science, 1965. 150, 19-28.

39. Bloch, K., & Rittenberg, D. On the utilization of acetic acid for cholesterol formation. J. Biol. Chem., 1942. 145, 625-636.

40. Blum, C.B., Levy, R.I., Eisenberg, S., Hall, M., III, Goebel, R.H., & Berman, M. High density lipoprotein metabolism in man. J. Clin. Invest., 1977. 60, 795-807.

41. Bonney, R.J. Adult liver parenchymal cells in primary culture: characteristics and cell recognition standards. In Vitro, 1974. 10, 130-142.

42. Bonney, R.J., Becker, J.E., Walker, P.R., & Potter, V.R. Primary monolayer cultures of adult rat liver parenchymal cells suitable for study of the regulation of enzyme synthesis. In Vitro, 1974. 9, 399-413.

43. Bradley, W.A., Rohde, M.F., Gotto, A.M., Jr., & Jackson, R.L. The cyanogen bromide peptides of the apoprotein of low density lipoprotein (ApoB): its molecular weight from a chemical view. Biochem. Biophys. Res. Commun., 1978. 81, 928-935.

44. Breslow, J.L., Lothrop, D.A., Clowes, A.W., & Lux, S.E. Lipoprotein regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in rat liver cell cultures. J. Biol. Chem., 1977. 252, 2726-2733.

45. Breslow, J.L., Spaulding, D.R., & Lothrop, D.A. Lipoprotein binding, degradation and regulation of sterol production in rat liver and fibroblast cell cultures. Circulation, 1975. 51 & 52, II-59. (Abstract).

46. Breslow, J.L., Spaulding, D.R., Lothrop, D.A., & Clowes, A.M. Effect of lipoprotein on 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase activity in rat liver cell culture: special suppressant effect of a lipoprotein isolated from hypercholesterolemic rat plasma. Biochem. Biophys. Res. Commun., 1975. 67, 119-125.

47. Brewer, H.B., Jr., Fairwell, T., Larue, A., Ronan, R., Houser, A., & Bronzert, T. The amino acid sequence of human ApoA-I, an apolipoprotein isolated from high density lipoproteins. Biochem. Biophys. Res. Commun., 1978. 80, 623-630.

48. Brewer, H.B., Jr., Lux, S.E., Ronan, R., & John, K.M. Amino acid sequence of human Apo-LP-gen II (ApoA-II), an apolipoprotein isolated from the high-density lipoprotein complex. Proc. Natl. Acad. Sci. U.S.A., 1972. 69, 1304-1308.

49. Brewer, H.B., Jr., Shulman, R., Herbert, P., Ronan, R., & Wehrly, K. The complete amino acid sequence of alanine apolipoprotein (apoC-III), an apolipoprotein from human plasma very low density lipoproteins. J. Biol. Chem., 1974. 249, 4975-4984.

50. Brown, M.S., Brunschede, G.Y., & Goldstein, J.L. Inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase *in vitro*. J. Biol. Chem., 1975. 250, 2502-2509.

51. Brown, M.S., Dana, S.E., & Goldstein, J.L. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts by lipoproteins. Proc. Natl. Acad. Sci. U.S.A., 1973. 70, 2162-2166.

52. Brown, M.S., & Goldstein, J.L. Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. Proc. Natl. Acad. Sci. U.S.A., 1974. 71, 788-792.

53. Brown, M.S., & Goldstein, J.L. Regulation of the activity of the low density lipoprotein receptor in human fibroblasts. Cell, 1975. 6, 307-316.

54. Brown, M.S., & Goldstein, J.L. Receptor-mediated control of cholesterol metabolism. Study of human mutants has disclosed how cells regulate a substance that is both vital and lethal. Science, 1976. 191, 150-154.

55. Brown, M.S., & Goldstein, J.L. Receptor-mediated endocytosis: Insights from the lipoprotein receptor system. Proc. Natl. Adac. Sci. U.S.A., 1979. 76, 3330-3337.

56. Brown, M.S., Goldstein, J.L., & Dietschy, J.M. Active and inactive forms of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver of the rat. Comparison with the rate of cholesterol synthesis in different physiological states. J. Biol. Chem., 1979. 254, 5144-5149.

57. Brown, W.V., & Baginsky, M.L. Inhibition of lipoprotein lipase by an apolipoprotein of human very low density lipoprotein. Biochem. Biophys. Res. Commun., 1972. 46, 375-381.

58. Bruno, G.A., & Christian, J.E. Correction for quenching associated with liquid scintillation counting. Anal. Chem., 1961. 33, 650-651.

59. Bucher, N.L.R., McGarrahan, K., Gould, E., & Loud, A.V. Cholesterol biosnythesis in preparations of liver from normal, fasting, x-irradiated, cholesterol-fed, Triton, or Δ^4-cholesten-3-one-treated rats. J. Biol. Chem., 1959. 234, 262-269.

60. Burstein, M., & Samaille, J. Clarification du sérum lipémique par l'heparine in vitro. C.R. Acad. Sci., 1955. 241, 664-665.

61. Burstein, M., & Scholnick, H.R. Lipoprotein-polyanion-metal interactions. Advan. Lipid Res., 1973. 11, 67-108.

62. Calvert, G.D., Scott, P.J., & Sharpe, D.N. The plasma and tissue turnover and distribution of two radio-iodine-labelled pig plasma low density lipoproteins. Atherosclerosis, 1975. 22, 601-628.

63. Capuzzi, D.M., Rostron, P.L., & Langer, T. Catabolism of ^{125}I-labeled plasma lipoproteins by isolated hepatocytes. Fed. Proc., 1975. 34, 500. (Abstract).

64. Capuzzi, D.M., Sparks, C.E., & DeHoff, J.L. Effect of residual enzymes on degradation of radioiodinated VLDL by collagenase-dispersed hepatocytes. Biochem. Biophys. Res. Commun., 1979. 90, 587-595.

65. Carew, T.E., Hayes, S.B., Kochinsky, T., & Steinberg, D. A mechanism by which high density lipoproteins may slow the atherogenic process. Lancet, 1976. 1, 1315-1318.

66. Carrella, M., & Cooper, A.D. High affinity binding of chylomicron remnants to liver liver plasma membranes. Proc. Natl. Acad. Sci. U.S.A., 1979. 76, 338-342.

67. Castelli, W.P., Doyle, J.T., Gordon, T., Hames, C.G., Hjortland, M., Hulley, S.B., Kagan, A., & Zukel, W.J. HDL cholesterol and other lipids in coronary heart disease. Circulation, 1977. 55, 767-772.

68. Chen, C.H., & Aladjem, F. Subunit structure of the apoprotein of human serum low density lipoproteins. Biochem. Biophys. Res. Commun., 1974. 60, 549-554.

69. Chen, C.H., & Aladjem, F. Further studies on the subunit structure of human serum low density lipoproteins. Biochem. Med., 1978. 19, 178-187.

70. Chick, H. The apparent formation of euglobulin from pseudo-globulin and a suggestion as to the relationship between these two proteins in serum. Biochem. J., 1914. 8, 404-420.

71. Chopra, I.J. A study of extrathyroidal conversion of thyroxine (T_4) to 3,3',5-triiodothyronine (T_3) in vitro. Endocrinology, 1977. 101, 453-463.

72. Chow, J.C., Higgins, M.J.P., & Rudney, H. The inhibitory effect of ATP on HMG CoA reductase. Biochem. Biophys. Res. Commun., 1975. 63, 1077-1084.

73. Cohn, Z.A., & Ehrenreich, B.A. The uptake, storage and intracellular hydrolysis of carbohydrates by macrophages. J. Exp. Med., 1969. 129, 201-225.

74. Cooper, A.D. The metabolism of chylomicron remnants in the isolated perfused rat liver. Biochim. Biophys. Acta, 1977. 488, 464-474.

75. Cooper, A.D., & Yu, P.Y.S. Rates of removal and degradation of chylomicron remnants by isolated perfused rat liver. J. Lipid Res., 1978. 19, 635-643.

76. Curry, M.D., McConathy, W.J., Alaupovic, P., Ledford, J.H., & Popovic, M. Determination of human apolipoprotein E by electroimmunoassay. Biochim. Biophys. Acta, 1976. 439, 413-425.

77. Dangerfield, W.G., & Smith, E.B. An investigation of serum lipids and lipoproteins by paper electrophoresis. J. Clin. Pathol., 1955. 8, 132-139.

78. Davis, R.A., Engelhorn, S.C., Pangburn, S.H., Weinstein, D.B., & Steinberg, D. Very low density lipoprotein synthesis and secretion by cultured rat hepatocytes. J. Biol. Chem., 1979. 254, 2010-2016.

79. Day, C.E., & Levy, R.S. Molecular structure of serum lipoproteins. J. Theor. Biol., 1969. 23, 387-399.

80. Dean, R.T. Lysosomes and membrane recycling. A hypothesis. Biochem. J., 1977. 168, 603-605.

81. Deckelbaum, R.J., Shipley, G.G., & Small, D.M. Structure and interactions of lipids in human plasma low density lipoproteins. J. Biol. Chem., 1977. 252, 744-754.

82. deLalla, O.F., & Gofman, J.W. Ultracentrifugal analysis of serum lipoproteins. Meth. Biochem. Anal., 1954. 1, 459-478.

83. Dietschy, J.M., & Siperstein, M.D. Effect of cholesterol feeding and fasting on sterol synthesis in seventeen tissues of the rat. J. Lipid Res., 1967. 8, 97-102.

84. Dietschy, J.M., & Wilson, J.D. Cholesterol synthesis in the squirrel monkey: relative rates of synthesis in various tissues and mechanisms of control. J. Clin. Invest., 1968. 47, 166-174.

85. Dietschy, J.M., & Wilson, J.D. Regulation of cholesterol metabolism. N. Engl. J. Med., 1970. 82, 1128-1138.

86. Dittmer, D.S. (Ed.) Blood and other body fluids. Washington, D.C.: Federation of American Societies for Experimental Biology, 1961. (page 5)

87. Drevon, C.A., Berg, T., & Norum, K.R. Uptake and degradation of cholesterol ester-labelled rat plasma lipoproteins in purified rat hepatocytes and nonparenchymal cells. Biochim. Biophys. Acta, 1977. 487, 122-136.

88. Dugan, R.E., & Porter, J.W. Hormonal regulation of cholesterol synthesis. In G. Litwach (Ed.) Biochemical actions of hormones, vol. IV. New York, NY: Academic Press, 1977. pp. 197-247.

89. Dugan, R.E., Slakey, L.L., Briedis, A.V., & Porter, J.W. Factors affecting the diurnal variation in the level of β-hydroxy-β-methylglutaryl coenzyme A reductase and cholesterol-synthesizing activity in rat liver. Arch. Biochem. Biophys., 1972. 152, 21-27.

90. Dujovne, C.A., Shoeman, D., & Bianchine, J. Experimental bases for the different hepatotoxicity of erythromycin preparations in man. J. Lab. Clin. Med., 1972. 79, 832-844.

91. Duncan, R., & Pratten, M.K. Membrane economics in endocytic systems. J. Theor. Biol., 1977. 66, 727-735.

92. Economou, S.G., Tews, B.J., Taylor, C.B., & Cox, G.E. Studies of lipid metabolism in dogs with altered biliary physiology. Surg. Forum, 1957. 8, 218-221.

93. Edelstein, C., Lim, C.T., & Scanu, A.M. The serum high density lipoproteins of Macacus rhesus. II. Isolation, purification, and characterization of their two major polypeptides. J. Biol. Chem., 1973. 248, 7653-7660.

94. Edwards, P.A. Effect of plasma lipoproteins and lecithin-cholesterol dispersions on the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase of isolated rat hepatocytes. Biochim. Biophys. Acta, 1975. 409, 39-50.

95. Edwards, P.A. The influence of catecholamines and cyclic AMP on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and lipid biosynthesis in isolated rat hepatocytes. Arch. Biochem. Biophys., 1975. 170, 188-203.

96. Edwards, P.A., Fogelman, A.M., & Popják, G. A direct relationship between the amount of sterol lost from rat hepatocytes and the increase in activity of HMG-CoA reductase. Biochem. Biophys. Res. Commun., 1976. 68, 64-69.

97. Edwards, P.A., & Gould, R.G. Turnover rate of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase as determined by use of cycloheximide. J. Biol. Chem., 1972. 247, 1520-1524.

98. Edwards, P.A., Lemongello, D., & Fogelman, A.M. The effect of glucagon, norepinephrine, and dibutyryl cyclic AMP on cholesterol efflux and on the activity of 3-hydroxy-3-methylglutaryl CoA reductase in rat hepatocytes. J. Lipid Res., 1979. 20, 2-7.

99. Edwards, P.A., Popják, G., Fogelman, A.M., & Edmond, J. Control of 3-hydroxy-3-methylglutaryl coenzyme A reductase by endogenously synthesized sterols in vitro and in vivo. J. Biol. Chem., 1977. 252, 1057-1063.

100. Eisenberg, S. Metabolism of very low density lipoproteins. In H. Greten (Ed.) Lipoprotein metabolism. Berlin: Springer-Verlag, 1976. pp. 32-43.

101. Eisenberg, S., Bilheimer, D.W., & Levy, R.I. The metabolism of very low density lipoproteins. II. Studies on the transfer of apolipoproteins between plasma lipoproteins. Biochim. Biophys. Acta, 1972. 280, 94-104.

102. Eisenberg, S., Bilheimer, D.W., Levy, R.I., & Lindgren, F.T. On the conversion of human plasma very low density lipoprotein to low density lipoprotein. Biochim. Biophys. Acta, 1973. 326, 361-377.

103. Eisenberg, S., & Levy, R.I. Lipoprotein metabolism. Advan. Lipid Res., 1975. 13, 1-89.

104. Eisenberg, S., & Rachmilewitz, D. Metabolism of rat plasma very low density lipoprotein. I. Fate in incubation of the whole lipoprotein. Biochim. Biophys. Acta, 1973. 326, 378-390.

105. Eisenberg, S., & Rachmilewitz, D. Metabolism of rat plasma very low density lipoprotein. II. Fate in circulation of apoprotein subunits. Biochim. Biophys. Acta, 1973. 326, 391-405.

106. Eisenberg, S., & Rachmilewitz, D. Interaction of rat plasma very low density lipoprotein with lipoprotein lipase-rich (postheparin) plasma. J. Lipid Res., 1975. 16, 341-351.

107. Eisenberg, S., Windmueller, H.G., & Levy, R.I. Metabolic fate of rat and human apolipoproteins in the rat. J. Lipid Res., 1973. 14, 446-458.

108. Fairbanks, G., Steck, T.L., & Wallach, D.H.F. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry, 1971. 10, 2606-2617.

109. Felker, T.E., Fainaru, M., Hamilton, R.L., & Havel, R.J. Secretion of the arginine-rich and A-I apolipoproteins by isolated perfused rat liver. J. Lipid Res., 1977. 18, 465-473.

110. Felts, J.M., Itakura, H., & Crane, R.T. The mechanism of assimilation of constituents of chylomicrons, very low density lipoproteins and remnants. Biochem. Biophys. Res. Commun., 1975. 66, 1467-1475.

111. Fidge, N.H., & Foxman, C.J. In vivo transformation of rat plasma very low density lipoprotein into higher density lipoproteins. Austral. J. Exp. Biol. Med. Sci., 1971. 49, 581-593.

112. Fidge, N.H., & Poulis, P. Studies on the radioiodination of very low density lipoprotein obtained from different mammalian species. Clin. Chim. Acta, 1974. 52, 15-26.

113. Fidge, N.H., & Poulis, P. Metabolic heterogeneity in the formation of low density lipoprotein from very low density lipoprotein in the rat: evidence for the independent production of a low density lipoprotein subfraction. J. Lipid Res., 1978. 19, 342-349.

114. Fielding, C.J., Shore, V.G., & Fielding, P.E. A protein cofactor of lecithin:cholesterol acyltransferase. Biochem. Biophys. Res. Commun., 1972. 46, 1493-1498.

115. Filipovic, I., Schwarzmann, G., Mraz, W., Wiegandt, H., & Buddecke, E. Sialic-acid content of low-density lipoproteins controls their binding and uptake by cultured cells. Eur. J. Biochem., 1979. 93, 51-55.

116. Finer, E.G., Henry, R., Leslie, R.B., & Robertson, R.N. NMR studies of pig low- and high-density serum lipoproteins. Biochim. Biophys. Acta, 1975. 380, 320-337.

117. Fisher, W.R., Hammond, M.G., Mengel, M.C., & Warmke, G.L. A genetic determinant of the phenotypic variance of the molecular weight of low density lipoprotein. Proc. Natl. Acad. Sci. U.S.A., 1975. 72, 2347-2351.

118. Fisher, W.R., Hammond, M.G., & Warmke, G.L. Measurements of the molecular weight variability of plasma low density lipoproteins among normals and subjects with hyper-β-lipoproteinemia. Demonstration of macromolecular heterogeneity. Biochemistry, 1972. 11, 519-525.

119. Fless, G.M., & Scanu, A.M. Physicochemical characterization of rhesus low density lipoproteins. Biochemistry, 1975. 14, 1783-1790.

120. Florén, C.-H., & Nilsson, Å. Binding, interiorization and degradation of cholesteryl ester-labelled chylomicron-remnant particles by rat hepatocyte monolayers. Biochem. J., 1977. 168, 483-494.

121. Florén, C.-H., & Nilsson, Å. Degradation of chylomicron remnant cholesteryl ester by rat hepatocyte monolayers. Inhibition by chloroquine and colchicine. Biochem. Biophys. Res. Commun., 1977. 74, 520-528.

122. Florén, C.-H., & Nilsson, Å. Uptake and degradation of iodine-labelled chylomicron remnant particles by monolayers of rat hepatocytes. Biochem. J., 1978. 174, 827-838.

123. Florén, C.-H., & Nilsson, Å. Some properties of the chylomicron remnant uptake by rat hepatocyte monolayers. Scand. J. Gastroenterol., 1979. 14, 281-287.

124. Folch, J., Lees, M., & Sloane Stanley, G.H. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 1957. 226, 497-509.

125. Forte, T., & Nichols, A.V. Application of electron microscopy to the study of plasma lipoprotein structure. Advan. Lipid Res., 1972. 10, 1-41.

126. Fredrickson, D.S., Levy, R.I., & Lees, R.S. Fat transport in lipoproteins--an integrated approach to mechanisms and disorders. N. Engl. J. Med., 1967. 276, 148-156.

127. Fredrickson, D.S., Loud, A.V., & Hinkleman, B.T. The effect of ligation of the common bile duct on cholesterol synthesis in the rat. J. Exp. Med., 1954. 99, 43-53.

128. Furman, R.H., Sanbar, S.S., Alaupovic, P., Bradford, R.H., & Howard, R.P. Studies on the metabolism of radioiodinated human serum alpha lipoprotein in normal and hyperlipidemic subjects. J. Lab. Clin. Med., 1964. 63, 193-204.

129. Gaertner, U. Die Regulation der 3-Hydroxy-3-Methylglutaryl-Coenzym-A-Reduktase von Hepatozyten in primärer Zellkultur durch Low Density Lipoprotein. Ztsch. Gastroenterol., 1978. 16, 251-256.

130. Ganesan, D., & Bass, H.B. Isolation of C-I and C-II activated lipoprotein lipases and protamine insensitive triglyceride lipase by heparin sepharose affinity chomatography. FEBS Lett., 1975. 53, 1-4.

131. Ganesan, D., & Bradford, R.H. Isolation of apolipoprotein-free lipoprotein lipase from human post-heparin plasma. Biochem. Biophys. Res. Commun., 1971. 43, 544-549.

132. Gitlin, D., Cornwell, D.G., Nakasato, D., Oncley, J.L., Hughes, W.L., Jr., & Janeway, C.A. Studies on the metabolism of plasma proteins in the nephrotic syndrome. II. The lipoproteins. J. Clin. Invest., 1958. 37, 172-184.

133. Gjone, E. Familial lecithin:cholesterol acyltransferase deficiency--a clinical survey. Scand. J. Clin. Lab. Invest., 1974. 33, Suppl. 137, 73-82.

134. Glangeaud, M.C., Eisenberg, S., & Olivecrona, T. Very low density lipoprotein: dissociation of apolipoprotein C during lipoprotein lipase induced lipolysis. Biochim. Biophys. Acta, 1977. 486, 23-35.

135. Glomset, J., Janssen, E., Kennedy, R., & Dobbins, J. Role of plasma lecithin:cholesterol acyltransferase in the metabolism of high density lipoproteins. J. Lipid Res., 1966. 7, 638-648.

136. Gofman, J.W., Glazier, F., Tamplin, A., Strisower, B., & deLalla, O. Lipoproteins, coronary heart disease, and atherosclerosis. Physiol. Rev., 1954. 34, 589-607.

137. Gofman, J.W., Lindgren, F.T., & Elliot, H. Ultracentrifugal studies of lipoproteins of human serum. J. Biol. Chem., 1949. 179, 973-979.

138. Goldstein, J.L., Anderson, R.G.W., & Brown, M.S. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature, 1979. 279, 679-685.

139. Goldstein, J.L., Basu, S.K., Brunschede, G.Y., & Brown, M.S. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. Cell, 1976. 7, 85-95.

140. Goldstein, J.L., & Brown, M.S. Familial hypercholesterolemia: identification of a defect in the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity associated with overproduction of cholesterol. Proc. Natl. Acad. Sci. U.S.A., 1973. 70, 2804-2808.

141. Goldstein, J.L., & Brown, M.S. Binding and degradation of low density lipoproteins by cultured human fibroblasts. J. Biol. Chem., 1974. 249, 5153-5162.

142. Goldstein, J.L., & Brown, M.S. The LDL pathway in human fibroblasts: a receptor mediated mechanism for the regulation of cholesterol metabolism. Curr. Topics Cell. Reg., 1976. 11, 147-181.

143. Goldstein, J.L., & Brown, M.S. The low-density lipoprotein pathway and its relation to atherosclerosis. Annu. Rev. Biochem., 1977. 46, 897-930.

144. Gordon, T.M., Castelli, M., Hjortland, M., Kannel, W.B., & Dawber, T. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am. J. Med., 1977. 62, 707-714.

145. Gotto, A.M., Jr., Levy, R.I., John, K., & Fredrickson, D.S. On the protein defect in abetalipoproteinemia. N. Engl. J. Med., 1971. 284, 813-818.

146. Gould, R.G. Lipid metabolism and atherosclerosis. Am. J. Med., 1951. 11, 209-227.

147. Granda, J.L., & Scanu, A. Solubilization and properties of the apoproteins of the very low- and low-density lipoproteins of human serum. Biochemistry, 1966. 5, 3301-3308.

148. Gross, W. Experimentlle Untersuchungen über den Zusammenhang zwischen histologischen Veränderungen und Funktionsstörungen der Nieren. Beitr. path. Anat. u. allg. Path., 1911. 51, 528-535.

149. Guo, L.S.S., Meng, M., Hamilton, R.L., & Ostwald, R. Changes in the plasma lipoprotein-apoproteins of guinea pigs in response to dietary cholesterol. Biochemistry, 1977. 16, 5807-5812.

150. Gurd, F.R.N., Oncley, J.L., Edsall, J.T., & Cohn, E.J. A review of the concept of the α- and β-lipoproteins of human plasma. Discuss. Faraday Soc., 1949. 6, 70-74.

151. Hamilton, R.L., Williams, M.C., Fielding, C.J., & Havel, R.J. Discordal bilayer structure of nascent high density lipoproteins from perfused rat liver. J. Clin. Invest., 1976. 58, 667-680.

152. Hardy, W.B. Colloidal solutions. The globulins. J. Physiol. (Lond.), 1905. 33, 251-337.

153. Haslam, H.C. Separation of proteins. III. Globulins. Biochem. J., 1913. 7, 492-516.

154. Hatch, F.T., & Lees, R.S. Practical methods for plasma lipoprotein analysis. Advan. Lipid Res., 1968. 6, 1-67.

155. Hatch, F.T., Lindgren, F.T., Adamson, G.L., Jensen, L.C., Wong, W.W., & Levy, R.I. Quantitative agarose gel electrophoresis of plasma lipoproteins: a simple technique and two methods for standardization. J. Lab. Clin. Med., 1973. 81, 946-960.

156. Havel, R.S., Eder, H.A., & Bragdon, J.P. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest., 1955. 34, 1345-1353.

157. Havel, R.J., Fielding, C.J., Olivecrona, T., Shore, V.G., Fielding, P.E., & Egelrud, T. Co-factor activity of protein components of human very low density lipoproteins in the hydrolysis of triglycerides by lipoprotein lipase from different sources. Biochemistry, 1973. 12, 1828-1833.

158. Havel, R.J., Shore, V.G., Shore, B., & Bion, D.M. The role of glycopeptides of human serum lipoproteins in the activation of lipoprotein lipase. Circulation Res., 1970. 27, 595-600.

159. Hay, R.V., Pottenger, L.A., Reingold, A.L., Getz, G.S., & Wissler, R.W. Degradation of ^{125}I-labelled serum low density lipoprotein in normal and estrogen-treated male rats. Biochem. Biophys. Res. Commun., 1971. 44, 1471-1477.

160. Herbert, P.N., Windmueller, H.B., Bersot, T.P., & Shulman, R.S. Characterization of the rat apolipoproteins. I. The low molecular weight proteins of rat plasma high density lipoproteins. J. Biol. Chem., 1974. 249, 5718-5724.

161. Higgins, M., & Rudney, H. Regulation of rat liver β-hydroxy-β-methylglutaryl-CoA reductase activity by cholesterol. Nature New Biol., 1973. 246, 60-61.

162. Ho, H.K., Brown, M.S., Bilheimer, D.W., & Goldstein, J.L. Regulation of low density lipoprotein receptor activity in freshly isolated human lymphocytes. J. Clin. Invest., 1976. 58, 1465-1474.

163. Illingworth, D.R. Metabolism of lipoproteins in nonhuman primates. Studies on the origin of low density lipoprotein apoprotein in the plasma of the squirrel monkey. Biochim. Biophys. Acta, 1975. 388, 38-51.

164. Imaizumi, K., Havel, R.J., Fainaru, M., & Vigne, J.-L. Origin and transport of the A-1 and arginine-rich apolipoproteins in mesenteric lymph of rats. J. Lipid Res., 1978. 19, 1038-1046.

165. Ingebritsen, T.S., Lee, H., Parker, R.A., & Gibson, D.M. Reversible modulation of the activities of both liver microsomal hydroxymethylglutaryl coenzyme A reductase and its inactivating enzyme. Evidence for regulation by phosphorylation-dephosphorylation. Biochem. Biophys. Res. Commun., 1978. 81, 1268-1277.

166. Innerarity, T.L., & Mahley, R.W. Enhanced binding by cultured human fibroblasts of apo-E containing lipoproteins as compared with low density lipoproteins. Biochemistry, 1978. 17, 1440-1447.

167. Innerarity, T.L., Pitas, R.E., & Mahley, R.W. Binding of the arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. J. Biol. Chem., 1979. 254, 4186-4190.

168. Jackson, R.L., Baker, H.N., Gilliam, F.B., & Gotto, A.M., Jr. Primary structure of very low density apolipoprotein C-II of human plasma. Proc. Natl. Acad. Sci. U.S.A., 1977. 74, 1942-1945.

169. Jackson, R.L., Morrisett, J.D., & Gotto, A.M. The mechanism of lipid-binding by plasma lipoproteins. Mol. Cell. Biochem., 1975. 6, 43-50.

170. Jackson, R.L., Morrisett, J.D., & Gotto, A.M., Jr. Lipoprotein structure and metabolism. Physiol. Rev., 1976. 56, 259-316.

171. Jackson, R.L., Sparrow, J.T., Baker, H.N., Morrisett, J.D., Taunton, O.D., & Gotto, A.M., Jr. The primary structure of apolipoprotein-serine. J. Biol. Chem., 1974. 249, 5308-5313.

172. Jacques, P.J. Endocytosis. In J.T. Dingle & H.B. Fell (Eds.) Lysosomes in biology and pathology. Vol. 2. Amsterdam: North Holland Publishing Co., 1969. pp. 362-420.

173. Jeejeebhoy, K.N., Ho, J., Breckenridge, C., Bruce-Robertson, A., Steiner, G., & Jeejeebhoy, J. Synthesis of VLDL by isolated rat hepatocytes in suspension. Biochem. Biophys. Res. Commun., 1975. 66, 1147-1153.

174. Kahn, C.R. Membrane receptors for polypeptide hormones. In E.D. Korn (Ed.) Methods in membrane biology. Vol. 3. New York: Plenum Press, 1975. pp. 81-146.

175. Kandutsch, A.A., & Saucier, S.E. Prevention of cyclic and Triton-induced increases in hydroxymethylglutaryl coenzyme A reductase and sterol synthesis by puromycin. J. Biol. Chem., 1969. 244, 2299-2305.

176. Kane, J.P., Richards, E.G., & Havel, R.J. Subunit heterogeneity in human serum beta lipoprotein. Proc. Natl. Acad. Sci. U.S.A., 1970. 66, 1075-1082.

177. Kane, J.P., Sata, T., Hamilton, R.L., & Havel, R.J. Apoprotein composition of very low density lipoproteins of human serum. J. Clin. Invest., 1975. 56, 1622-1634.

178. Kaplan, M.M., & Utiger, R.D. Iodothyronine metabolism in rat liver homogenates. J. Clin. Invest., 1978. 61, 459-471.

179. Katz, N.R., Nuack, M.A., & Wilson, P.T. Induction of glucokinase by insulin under the permissive action of dexamethasone in primary rat hepatocyte cultures. Biochem. Biophys. Res. Commun., 1979. 88, 23-29.

180. Keys, A., Michelson, O., Miller, E.V.O., Hayes, E.R., & Todd, R.L. Concentration of cholesterol in blood serum of normal man and its relation to age. J. Clin. Invest., 1950. 29, 1347-1353.

181. Kinsell, L.W., Partridge, J., Boling, L., Margen, S., & Michaels, G. Dietary modification of serum cholesterol and phospholipid levels. J. Clin. Endocrinol. Metab., 1952. 12, 909-913.

182. Kobayashi, Y., & Maudsley, D.V. Biological applications of liquid scintillation counting. New York: Academic Press, 1974. (pages 143-144)

183. Kostner, G. Studies on the cofactor requirement for lecithin:cholesterol acyltransferase. Scand. J. Clin. Lab. Invest., 1974. 33, Suppl. 137, 19-21.

184. Kostner, G.M. Studies on the composition and structure of human serum lipoproteins. Isolation and partial characterization of apolipoprotein A-III. Biochim. Biophys. Acta, 1974. 336, 383-395.

185. Kostner, G., & Holasek, A. Investigation and quantitation of the apolipoprotein from human chyle chylomicrons. Biochemistry, 1972. 11, 1217-1223.

186. Kovanen, P.T., Faust, J.R., Brown, M.S., & Goldstein, J.L. Low density lipoprotein receptors in bovine adrenal cortex. I. Receptor-mediated uptake of low density lipoprotein and utilization of its cholesterol for steroid synthesis in cultured adrenocortical cells. Endocrinology, 1979. 104, 599-609.

187. Krebs, H.A., Cornell, N.W., Lund, P., & Hems, R. Isolated liver cells as experimental material. In F. Lundquist & N. Tygstrup (Eds.) Regulation of hepatic metabolism. New York: Academic Press, 1974. pp. 726-759.

188. Krishnaiah, K.V., & Wiegandt, H. Demonstration of protease-like activity in human serum low density lipoprotein. FEBS Lett., 1974. 40, 265-268.

189. Kushwaha, R.S., & Hazzard, W.R. Catabolism of very low density lipoproteins in the rabbit: effect of changing composition and pool size. Biochim. Biophys. Acta, 1978. 528, 176-189.

190. Laggner, P. Physicochemical characterization of low-density lipoproteins. In C.E. Day & R.S. Levy (Eds.) Low density lipoproteins. New York: Plenum Press, 1976. pp. 49-69.

191. Laggner, P., Degovics, G., Müller, K.W., Glatter, O., Kratky, O., Kostner, G., & Holasek, A. Molecular packing and fluidity of lipids in human serum low density lipoproteins. Hoppe Seylers Z. Physiol. Chem., 1977. 358, 771-778.

192. Laggner, P., Müller, K., Kratky, O., Kostner, G., & Holasek, A. X-ray small angle scattering on human plasma lipoproteins/low density lipoprotein, high density lipoprotein. J. Colloid Interface Sci., 1976. 55, 102-108.

193. Lakshmanan, M.R., Meusing, R.A., Cook, G.A., & Veech, R.L. Regulation of lipogenesis in isolated hepatocytes by triglyceride-rich lipoproteins. J. Biol. Chem., 1977. 252, 6581-6584.

194. Langer, T., Strober, W., & Levy, R.I. The metabolism of low density lipoprotein in familial type II hyperlipoproteinemia. J. Clin. Invest., 1972. 51, 1528-1536.

195. LaRosa, J.C., Levy, R.I., Herbert, P.N., Lux, S.E., & Fredrickson, D.S. A specific apoprotein activator for lipoprotein lipase. Biochem. Biophys. Res. Commun., 1970. 41, 57-62.

196. Lee, D.M., & Alaupovic, P. Physicochemical properties of low-density lipoproteins of normal human plasma. Biochem. J., 1974. 137, 155-167.

197. Lees, R.S., & Hatch, R.T. Sharper separation of lipoprotein species by paper electrophoresis in albumin-containing buffer. J. Lab. Clin. Med., 1963. 61, 518-528.

198. Levy, R.S., Lynch, A.C., McGee, E.D., & Mehl, J.W. Amino acid composition of the proteins from chylomicrons and human serum lipoproteins. J. Lipid Res., 1967. 8, 463-472.

199. Lin, M.C., Nicosia, S., & Rodbell, M. Effects of iodination of tyrosyl residues on the binding and action of glucagon at its receptor. Biochemistry, 1976. 15, 4537-4548.

200. Lin, R.C., & Snodgrass, P.J. A steady state of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured rat liver cells and the increase of its activity by dexamethasone. FEBS Lett., 1977. 83, 89-92.

201. Lineweaver, H., & Burk, D. The determination of enzyme dissociation constants. J. Am. Chem. Soc., 1934. 56, 658-666.

202. Linn, T.C. The effect of cholesterol feeding and fasting upon β-hydroxy-β-methylglutaryl coenzyme A reductase. J. Biol. Chem., 1967. 242, 990-993.

203. Lipp, K., & Wiegandt, H. Studies on the B-protein of human serum β-lipoprotein using SDS-polyacrylamide gel electrophoresis. Hoppe Seylers Z. Physiol. Chem., 1973. 354, 262-266.

204. Lowry, D.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. Protein measurement with Folin phenol reagent. J. Biol. Chem., 1951. 193, 265-275.

205. Lübke, K., Schillinger, E., & Töpert, M. Hormone receptors. Angew. Chem. Int. Ed. Engl., 1976. 15, 741-748.

206. Lux, S.E., Hirz, R.V., Shrager, R.I., & Gotto, A.M. The influence of lipid on the conformation of human plasma high density apolipoproteins. J. Biol. Chem., 1972. 247, 2598-2606.

207. Macheboeuf, M.A. Recherches sur les phosphoaminolipides et les stérides du sérum et du plasma sanguins. I. Entraînement des phospholipides, des stérols et des stérides par les diverses fractions au cours du fractionnement des protéide du serum. Bull. Soc. Chim. Biol., 1929. 11, 268-293.

208. Macheboeuf, M., & Rebeyrotte, P. Studies on lipoprotein cenapses of horse serum. Discuss. Faraday Soc., 1949. 6, 62-68.

209. Maciel, R.M.B., Ozawa, Y., & Chopra, I.J. Subcellular localization of thyroxine and reverse triiodothyronine outer ring monodeiodinating activities. Endocrinology, 1979. 104, 365-371.

210. Mahley, R.W. Alterations in plasma lipoproteins induced by cholesterol feeding in animals including man. In J.M. Dietschy, A.M. Gotto, Jr., & J.A. Ontka (Eds.) Disturbances in lipid and lipoprotein metabolism. Bethesda, Md.: American Physiological Society, 1978. pp. 181-197.

211. Mahley, R.W., Bersot, T.P., & Leguire, V.S. Identity of very low density lipoprotein apoproteins of plasma and liver Golgi apparatus. Science, 1970. 168, 380-382.

212. Mahley, R.W., & Holcombe, K.S. Alterations of the plasma lipoproteins and apoproteins following cholesterol feeding in the rat. J. Lipid Res., 1977. 18, 314-323.

213. Mahley, R.W., & Innerarity, T.L. Interaction of canine and swine lipoproteins with the low density lipoprotein receptor of fibroblasts as correlated with heparin/manganese precipitability. J. Biol. Chem., 1977. 252, 3980-3986.

214. Mahley, R.W., Innerarity, T.L., Pitas, R.E., Weisgraber, K.H., Brown, J.H., & Gross, E. Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B-apoproteins. J. Biol. Chem., 1977. 252, 7229-7287.

215. Mahley, R.W., Innerarity, T.L., Weisgraber, K.H., & Oh, S.Y. Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective chemical modification of lysine residues of apoproteins. J. Clin. Invest., 1979. 64, 743-750.

216. Mahley, R.W., Weisgraber, K.H., & Innerarity, T. Canine lipoproteins and atherosclerosis. II. Characterization of the plasma lipoproteins associated with atherogenic and nonatherogenic hyperlipidemia. Circulation Res., 1974. 35, 722-733.

217. Mahley, R.W., Weisgraber, K.H., Innerarity, T., & Brewer, H.B. Characterization of the plasma lipoproteins and apoproteins of the Erythrocebus patas monkey. Biochemistry, 1976. 15, 1928-1933.

218. Mahley, R.W., Weisgraber, K.H., Innerarity, T., Brewer, H.B., & Assmann, G. Swine lipoproteins and atherosclerosis. Changes in the plasma lipoproteins and apoproteins induced by cholesterol feeding. Biochemistry, 1975. 14, 2817-2823.

219. Margolis, S., & Langdon, R.G. Studies on human serum β_1-lipoprotein. J. Biol. Chem., 1966. 241, 469-476.

220. Marsh, J.B. Lipoproteins in a nonrecirculating perfusate of rat liver. J. Lipid Res., 1974. 15, 544-550.

221. Marsh, J.B. Apoproteins of the lipoproteins in a nonrecirculating perfusate of rat liver. J. Lipid Res., 1976. 17, 85-90.

222. Mateu, L., Tardieu, A., Aggerbech, L., & Scanu, A.M. Small angle X-ray scattering of human serum low density lipoprotein. In R.M.S. Smellie (Ed.) Plasma lipoproteins. London: Academic Press, 1971. pp. 87-88.

223. Mateu, L., Tardieu, A., Luzzati, V., Aggerbech, L., & Scanu, A.M. On the structure of human serum low density lipoprotein. J. Mol. Biol., 1972. 70, 105-116.

224. McConathy, W.J., & Alaupovic, P. Isolation and partial characterization of apolipoprotein D: a new protein moiety of the human plasma lipoprotein system. FEBS Lett., 1973. 37, 178-182.

225. McConathy, W.J., & Alaupovic, P. Studies on the isolation and partial characterization of apolipoprotein D and lipoprotein D in human plasma. Biochemistry, 1976. 15, 515-520.

226. McFarlane, A.S. Efficient trace-labelling of proteins with iodine. Nature, 1958. 182, 53.

227. McFarlane, A.S. In vivo behavior of I^{131}-fibrinogen. J. Clin. Invest., 1963. 42, 346-361.

228. Miller, L.L., Bly, C.G., Watson, M.L., & Bale, W.F. The dominant role of the liver in plasma protein synthesis. A direct study of the isolated perfused rat liver with the aid of lysine-ϵ-^{14}C. J. Exp. Med., 1951. 94, 431-453.

229. Miller, N.E. Induction of low density lipoprotein receptor synthesis by high density lipoprotein in cultures of human skin fibroblasts. Biochim. Biophys. Acta, 1978. 529, 131-137.

230. Miller, N.E., Thelle, D.S., Førde, O.H., & Mjøs, O.D. The Tromsø Heart Study. High density lipoprotein and coronary heart disease: a prospective case-control study. Lancet, 1977. 1, 965-967.

231. Miller, N.E., Weinstein, D.B., & Steinberg, D. Binding, internalization, and degradation of high density lipoproteins by cultured normal human fibroblasts. J. Lipid Res., 1977. 18, 438-450.

232. Morrisett, J.D., Jackson, R.L., & Gotto, A.M., Jr. Lipoproteins: structure and function. Annu. Rev. Biochem., 1975. 44, 183-207.

233. Morrisett, J.D., Jackson, R.L., & Gotto, A.M., Jr. Lipid-protein interactions in the plasma lipoproteins. Biochim. Biophys. Acta, 1977. 472, 93-133.

234. Motulsky, A.G. The genetic hyperlipidemias. N. Engl. J. Med., 1976. 294, 823-827.

235. Müller, K., Laggner, P., Glatter, O., & Kostner, G. The structure of human-plasma low-density lipoprotein B. An x-ray small-angle scattering study. Eur. J. Biochem., 1978. 82, 73-90.

236. Müller, P., Singh, A., Orci, L., & Jeanrenaud, B. Secretory processes, carbohydrate and lipid metabolism in isolated mouse hepatocytes. Aspects of regulation by glucagon and insulin. Biochim. Biophys. Acta, 1976. 428, 480-494.

237. Murphy, J.R. Erythrocyte metabolism. III. Relationship of energy metabolism and serum factors to the osmotic fragility following incubation. J. Lab. Clin. Med., 1962. 60, 86-109.

238. Nakai, T., Otto, P.S., Kennedy, D.L., & Whayne, T.F., Jr. Rat high density lipoprotein subfraction (HDL_3) uptake and catabolism by isolated rat liver parenchymal cells. J. Biol. Chem., 1976. 251, 4914-4921.

239. Nakai, T., & Whayne, T.F., Jr. Catabolism of canine apolipoprotein A-I, purification, catabolic rate, organs of catabolism and liver subcellular catabolic site. J. Lab. Clin. Med., 1976. 88, 63-80.

240. Nakaya, N., Chung, B.H., Patsch, J.R., & Taunton, O.D. Synthesis and release of low density lipoproteins by the isolated perfused pig liver. J. Biol. Chem., 1977. 252, 7530-7533.

241. Nerking, J. Über Fetlerweissverbindungen. Pflugers Arch. ges. Physiol., 1901. 85, 330-344.

242. Nervi, F.O., & Dietschy, J.M. The mechanisms of and the interrelationships between bile acid and chylomicron-mediated regulation of hepatic cholesterol synthesis in the liver of the rat. J. Clin. Invest., 1978. 61, 895-909.

243. Nichols, A.V., & Smith, L. Effect of very low-density lipoproteins on lipid transfer in incubated serum. J. Lipid Res., 1965. 6, 206-210.

244. Nilsson, Å. Effects of anti-microtubular agents and cycloheximide on the metabolism of chylomicron cholesteryl esters by hepatocyte suspensions. Biochem. J., 1977. 162, 367-377.

245. Noble, R.P. Electrophoretic separation of plasma lipoproteins in agarose gel. J. Lipid Res., 1968. 9, 693-700.

246. Noel, S.-P., D.J., & Rubinstein, D. An in vitro model for the catabolism of rat chylomicrons. Biochem. Biophys. Res. Commun., 1975. 63, 764-772.

247. Noel, S.-P., & Rubinstein, D. Secretion of apolipoproteins in very low density and high density lipoproteins by perfused rat liver. J. Lipid Res., 1974. 15, 301-308.

248. Nordby, G., Berg, T., Nilsson, M., & Norum, K.R. Secretion of lecithin:cholesterol acyltransferase from isolated rat hepatocytes. Biochim. Biophys. Acta, 1976. 450, 69-77.

249. Nordstrom, J.L., Rodwell, V.W., & Mitschelen, J.J. Interconversion of active and inactive forms of rat liver hydroxymethylglutaryl-CoA reductase. J. Biol. Chem., 1977. 252, 8924-8934.

250. Olofsson, S.-O., McConathy, W.J., & Alaupovic, P. Isolation and partial characterization of a new acidic apolipoprotein (apolipoprotein F) from high density lipoproteins of human plasma. Biochemistry, 1978. 17, 1032-1036.

251. Oncley, J.L. In J. Folch Pi & H. Bauer (Eds.) Brain lipids and lipoproteins and leukodystrophies. Amsterdam: Elsevier, 1963. p. 5.

252. Oncley, J.L., & Harvie, R.N. Lipoproteins--a current perspective of methods and concepts. Proc. Natl. Acad. Sci. U.S.A., 1969. 64, 1107-1118.

253. Oncley, J.L., Walton, K.W., & Cornwell, D.G. A rapid method for the bulk isolation of β lipoproteins from human plasma. J. Am. Chem. Soc., 1957. 79, 4666-4671.

254. Osborne, J.C., Jr., & Brewer, H.B., Jr. The plasma lipoproteins. Advan. Protein Chem., 1977. 31, 253-337.

255. Ostlund, R.E., Jr., Pfleger, B., & Schonfeld, G. Role of microtubules in low density lipoprotein processing by cultured cells. J. Clin. Invest., 1979. 63, 75-84.

256. Pangburn, S.H., & Weinstein, D.B. Degradation of human low density (LDL) and high density (HDL) lipoproteins by cultured hepatocytes. Fed. Proc., 1978. 37, 1482. (Abstract).

257. Pappenheimer, A.M. Experimental studies upon lymphocytes. J. Exp. Med., 1917. 25, 633-650.

258. Phair, R.D., Hall, M., III, Bilheimer, D.W., Levy, R.I., Goebel, R., & Berman, M. Modeling lipoprotein metabolism in man. In Proceedings of the 1976 Summer Computer Simulation Conference. LaJolla, Calif.: Simulations Councils, Inc., 1976. Quoted by Berman et al. (29).

259. Pitas, R.E., Innerarity, T.L., Arnold, K.S., & Mahley, R.W. Rate and equilibrium constants for binding of apo-E HDL_c (a cholesterol-induced lipoprotein) and low density lipoproteins to human fibroblasts: Evidence for multiple receptor binding of apo-E HDL_c. Proc. Natl. Acad. Sci. U.S.A., 1979. 76, 2311-2315.

260. Pittman, R.C., Attie, A.D., Carew, T.E., & Steinberg, D. Tissue sites of degradation of low density lipoprotein: Application of a method for determining the fate of plasma proteins. Proc. Natl. Acad. Sci. U.S.A., 1979. 76, 5345-5349.

261. Pittman, R.C., Green, S.R., Attie, A.D., & Steinberg, D. Radiolabeled sucrose covalently linked to protein. A device for quantifying degradation of plasma proteins catabolized by lysosomal mechanisms. J. Biol. Chem., 1979. 254, 6876-6879.

262. Pittman, R.C., & Steinberg, D. A new approach for assessing cumulative lysosomal degradation of proteins or other macromolecules. Biochem. Biophys. Res. Commun., 1978. 81, 1254-1259.

263. Pliam, N.B., & Goldfine, I.D. High affinity thyroid hormone binding sites on purified rat liver plasma membranes. Biochem. Biophys. Res. Commun., 1977. 79, 166-172.

264. Pollard, H.B., & Devi, S.K. Construction of a three-dimensional iso-density map of the low-density lipoprotein particle from human serum. Biochem. Biophys. Res. Commun., 1971. 44, 593-599.

265. Pollard, H., Scanu, A.M., & Taylor, E.W. On the geometrical arrangement of the protein subunits of human serum low-density lipoprotein: evidence for a dodecahedral model. Proc. Natl. Acad. Sci. U.S.A., 1969. 64, 304-310.

266. Portman, O.W., & Alexander, M. Changes in arterial subfractions with aging and atherosclerosis. Biochim. Biophys. Acta, 1972. 260, 460-474.

267. Portman, O.W., Alexander, M., & Kannan, R. The transport of control and hyperlipidemic low density lipoproteins from plasma to liver of rabbits. Atherosclerosis, 1979. 32, 333-345.

268. Portman, O.W., Alexander, M., Soltys, P., Illingworth, D.R., & Tanaka, N. Species and dietary effects on lipoprotein apoprotein metabolism in vivo. In H. Peeters (Ed.) Protides of the biological fluids. New York: Pergamon Press, 1978. pp. 143-150.

269. Portman, O.W., Alexander, M., Tanaka, N., & Soltys, P. The effects of dietary fat and cholesterol on the metabolism of plasma LDL apoproteins in squirrel monkeys. Biochim. Biophys. Acta, 1976. 450, 185-196.

270. Portman, O.W., Illingworth, D.R., & Alexander, M. The effects of hyperlipidemia on lipoprotein metabolism in squirrel monkeys and rabbits. Biochim. Biophys. Acta, 1975. 398, 55-71.

271. Rachmilewitz, P., Stein, O., Roheim, P.S., & Stein, Y. Metabolism of iodinated high density lipoproteins in the rat. II. Autoradiographic localization in the liver. Biochim. Biophys. Acta, 1972. 270, 414-425.

272. Radding, C.M., & Steinberg, D. Studies on the synthesis and secretion of serum lipoproteins by rat liver slices. J. Clin. Invest., 1960. 39, 1560-1569.

273. Ragland, J.B., Bertram, P.D., & Setesin, S.M. Identification of nascent high density lipoproteins containing arginine-rich protein in human plasma. Biochem. Biophys. Res. Commun., 1978. 80, 81-88.

274. Ragland, J.B., Hawkins, H.L., & Setesin, S.M. Arginine rich protein (ARP) in nascent high density lipoprotein of alcoholic hepatitis (AH): A model to study lipoprotein metabolism. Circulation, 1976. 54, Suppl. II, 0100. (Abstract).

275. Redgrave, T.G. Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. J. Clin. Invest., 1970. 49, 465-471.

276. Rehnborg, C.S., & Nichols, A.V. The fate of cholesterol esters in human serum incubated in vitro at 38 degrees. Biochim. Biophys. Acta, 1964. 84, 596-603.

277. Reichl, D., Myant, N.B., & Pflug, J.J. Concentration of lipoproteins containing apolipoprotein B in human peripheral lymph. Biochim. Biophys. Acta, 1977. 489, 98-105.

278. Reichl, D., Postiglione, A., Myant, N.B., Pflug, J.J., & Press, M. Observations on the passage of apoproteins from plasma lipoproteins into peripheral lymph in two men. Clin. Sci. Mol. Med., 1975. 49, 419-426.

279. Rhoads, G., Gulbrandsen, C.L., & Kagan, A. Serum lipoproteins and coronary heart disease in a population study of Hawaii Japanese men. N. Engl. J. Med., 1976. 294, 293-295.

280. Rodbard, D. Mathematics of hormone-receptor interaction. I. Basic principles. In B.W. O'Malley & R.A. Means (Eds.) Receptors for reproductive hormones. New York: Plenum Publishing Corp., 1973. pp. 289-326.

281. Rodbell, M. N-Terminal amino acid and lipid composition of lipoproteins from chyle and plasma. Science, 1958. 127, 701-702.

282. Roheim, P.S., Rachmilewitz, D., Stein, O., & Stein, Y. Metabolism of iodinated high density lipoproteins in the rat. I. Half-life in the circulation and uptake by organs. Biochim. Biophys. Acta, 1971. 248, 315-329.

283. Rudel, L.L., Lee, J.A., Morris, M.D., & Felts, J.M. Characterization of plasma lipoproteins separated and purified by agarose-column chromatography. Biochem. J., 1974. 139, 89-95.

284. Savage, C.R., Jr., & Bonney, R.J. Extended expression of differentiated function in primary cultures of adult liver parenchymal cells maintained on nitrocellulose filters. Exp. Cell Res., 1978. 114, 307-315.

285. Scanu, A.M. Structural studies on serum lipoproteins. Biochim. Biophys. Acta, 1972. 265, 471-508.

286. Scanu, A., & Hughes, W.L. Further characterization of the human serum D 1.063-1.21, α_1 lipoprotein. J. Clin. Invest., 1962. 41, 1681-1689.

287. Scanu, A., Pollard, H., & Reader, W. Properties of human serum low density lipoproteins after modification by succinic anhydride. J. Lipid Res., 1968. 9, 342-348.

288. Scanu, A.M., & Wisdom, C. Serum lipoproteins: structure and function. Annu. Rev. Biochem., 1972. 41, 703-730.

289. Scatchard, G. The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci., 1949. 51, 660-672.

290. Schaefer, E.J., Eisenberg, S., & Levy, R.I. Lipoprotein apoprotein metabolism. J. Lipid Res., 1978. 19, 667-687.

291. Schaefer, E.J., Jenkins, L.L., & Brewer, H.B., Jr. Human chylomicron apolipoprotein metabolism. Biochem. Biophys. Res. Commun., 1978. 80, 405-412.

292. Schonfeld, G., Bell, E., & Alpers, D.H. Intestinal apoproteins during fat absorption. J. Clin. Invest., 1978. 61, 1539-1550.

293. Schulz, F.N. Über den Fettgehalt des Blutes beim Hunger. Pflügers Arch. ges. Physiol., 1897. 65, 299-307.

294. Scott, P.J., & Hurley, P.J. The distribution of radioiodinated serum albumin and low-density lipoprotein in tissues and the arterial wall. Atherosclerosis, 1970. 11, 77-103.

295. Segrest, J.P., Jackson, R.L., Morrisett, J.D., & Gotto, A.M. A molecular theory of lipid-protein interactions in the plasma lipoproteins. FEBS Lett., 1974. 38, 247-253.

296. Shapiro, D.J., & Rodwell, V.W. Fine structure of the cyclic rhythm of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Differential effects of cholesterol feeding and fasting. Biochemistry, 1972. 11, 1042-1045.

297. Shelburne, F.A., & Quarfordt, S.H. A new apoprotein of human plasma very low density lipoproteins. J. Biol. Chem., 1974. 249, 1428-1433.

298. Shepherd, J., Bicker, S., Lorimer, A.R., & Packard, C.J. Receptor-mediated low density lipoprotein catabolism in man. J. Lipid Res., 1979. 20, 999-1006.

299. Sherrill, B.C., & Dietschy, J.M. Characterization of the sinusoidal transport process responsible for uptake of chylomicrons by the liver. J. Biol. Chem., 1978. 253, 1859-1867.

300. Shimidzu, Y. Ein Beitrag zur Kumagawa-Sutoschen Fettbestimmungsmethode. Biochem. Z., 1910. 28, 237-273.

301. Shireman, R.B., & Fisher, W.R. The absence of a role for the carbohydrate moiety in the binding of apolipoprotein B to the low density lipoprotein receptor. Biochim. Biophys. Acta, 1979. 572, 537-540.

302. Shireman, R., Kilgore, L.L., & Fisher, W.R. Solubilization of apolipoprotein B and its specific binding by the cellular receptor for low density lipoprotein. Proc. Natl. Acad. Sci. U.S.A., 1977. 74, 5150-5154.

303. Shore, B. C- and N-terminal amino acids of human serum lipoproteins. Arch. Biochem. Biophys., 1957. 71, 1-10.

304. Shore, B., & Shore, V. Isolation and characterization of polypeptides of human serum lipoproteins. Biochemistry, 1969. 8, 4510-4516.

305. Shore, B., & Shore, V. An apolipoprotein preferentially enriched in cholesterol ester-rich very low density lipoproteins. Biochem. Biophys. Res. Commun., 1974. 58, 1-7.

306. Shore, V.G., Shore, B., & Hart, R.G. Changes in apolipoproteins and properties of rabbit very low density lipoprotein on induction of cholesterolemia. Biochemistry, 1974. 13, 1579-1585.

307. Shulman, R.S., Herbert, P.N., Wehrly, K., & Fredrickson, D.S. The complete amino acid sequence of C-1 (apoLp Ser) an apolipoprotein from human very low density lipoproteins. J. Biol. Chem., 1975. 250, 182-190.

308. Sigurdsson, G., Nicoll, A., & Lewis, B. Conversion of very low density lipoprotein to low density lipoprotein. A metabolic study of apolipoprotein B kinetics in human subjects. J. Clin. Invest., 1975. 56, 1481-1490.

309. Sigurdsson, G., Noel, S.-P., & Havel, R.J. Catabolism of the apoprotein of low density lipoproteins by the isolated perfused rat liver. J. Lipid Res., 1978. 19, 628-634.

310. Sigurdsson, G., Noel, S.-P., & Havel, R.J. Quantification of the hepatic contribution to the catabolism of high density lipoproteins in rats. J. Lipid Res., 1979. 20, 316-324.

311. Simons, K., & Helenius, A. Heterogeneity of maleylated and partially delipidated low density lipoproteins. Ann. Med. exp. Fenn., 1969. 47, 48-51.

312. Simons, K., & Helenius, A. Effect of sodium dodecyl sulfate on human plasma low density lipoproteins. FEBS Lett., 1970. 7, 59-62.

313. Siperstein, M.D. The homeostatic control of cholesterol synthesis in liver. Am. J. Clin. Nutr., 1960. 8, 645-650.

314. Siperstein, M.D., & Fagan, V.M. Feedback control of mevalonate synthesis by dietary cholesterol. J. Biol. Chem., 1966. 241, 602-609.

315. Smith, E.B., & Slater, R.S. The chemical and immunological assay of low density lipoproteins extracted from human aortic intima. Atherosclerosis, 1970. 11, 417-438.

316. Smith, E.B., & Slater, R.S. Relationship between low-density lipoprotein in aortic intima and serum-lipid levels. Lancet, 1972. 1, 463-469.

317. Smith, R., Dawson, J.R., & Tanford, C. The size and number of polypeptide chains in human serum low density lipoprotein. J. Biol. Chem., 1972. 247, 3376-3381.

318. Sniderman, A.D., Carew, T.E., Chandler, J.G., & Steinberg, D. Paradoxical increase in rate of catabolism of low density lipoproteins after hepatectomy. Science, 1974. 183, 526-528.

319. Soltys, P.A., & Portman, O.W. Low density lipoprotein receptors and catabolism in primary cultures of rabbit hepatocytes. Biochim. Biophys. Acta, 1979. 574, 505-520.

320. Soutar, A.C., Garner, C., Baker, H.N., Sparrow, J.T., Jackson, R.L., Gotto, A.M., & Smith, L.C. The effects of apolipoproteins on lecithin:cholesterol acyltransferase. Biochemistry, 1975. 14, 3057-3064.

321. Stanbury, J.B. Deiodination of iodinated amino acids. Ann. N.Y. Acad. Sci., 1960. 86, 417-439.

322. Stange,E., Agostini, B., & Papenberg, J. Changes in rabbit lipoprotein properties by dietary cholesterol, and saturated and polyunsaturated fats. Atherosclerosis, 1975. 22, 125-148.

323. Stein, O., Rachmilewitz, D., Sanger, L., Eisenberg, S., & Stein, Y. Metabolism of iodinated very low density lipoprotein in the rat. Autoradiographic localization in the liver. Biochim. Biophys. Acta, 1974. 360, 205-216.

324. Stein, O., Weinstein, D.B., Stein, Y., & Steinberg, D. Binding, internalization, and degradation of low density lipoprotein by normal human fibroblasts and by fibroblasts from a case of homozygous familial hypercholesterolemia. Proc. Nat. Acad. Sci. U.S.A., 1976. 73, 14-18.

325. Steinberg, D.E., Carew, T.E., Chandler, J.G., & Sniderman, A.D. The role of the liver in metabolism of plasma lipoproteins. In F. Lundquist & N. Tygstrup (Eds.) Proceedings of the Alfred Benzon Symposium VI. New York: Academic Press, 1974. pp. 144-156.

326. Steinberg, D., Nestel, P.J., Weinstein, D.B., Remaut-Desmeth, M., & Chang, C.M. Interactions of native and modified human low density lipoproteins with human skin fibroblasts. Biochim. Biophys. Acta, 1978. 528, 199-212.

327. Steinman, R.M., Brodie, S.E., & Cohn, Z.A. Membrane flow during pinocytosis. A stereologic analysis. J. Cell Biol., 1976. 68, 665-687.

328. Stuhrmann, H.B., Tardieu, A., Mateu, L., Sardet, C., Luzzati, V., Aggerback, L., & Scanu, A.M. Neutron scattering study of human low density lipoprotein. Proc. Natl. Acad. Sci. U.S.A., 1975. 72, 2270-2273.

329. Sugano, M., & Portman, O.W. Fatty acid specificities and rates of cholesterol esterification in vivo and in vitro. Arch. Biochem. Biophys., 1964. 107, 341-351.

330. Suri, B.S., Targ, M.E., & Robinson, D.S. The metabolic conversion of very-low-density lipoprotein into low density lipoprotein by the extrahepatic tissues of the rat. Biochem. J., 1979. 178, 455-466.

331. Swaney, J.B., Reese, H., & Eder, H.A. Polypeptide composition of rat high density lipoprotein: characterization by SDS-gel electrophoresis. Biochem. Biophys. Res. Commun., 1974. 59, 513-519.

332. Takeuchi, N., Katayama, Y., Koga, M., Yamamura, Y., Yamaguchi, Y., & Uchida, K. Cholesterol uptake from serum and cholesterol-phospholipid dispersions by isolated rat hepatocytes in vitro. J. Clin. Lab. Med., 1978. 92, 228-238.

333. Tall, A.R., Small, D.M., Atkinson, D., & Rudel, L.L. Studies on the structure of low density lipoproteins isolated from Macaca fascicularis fed an atherogenic diet. J. Clin. Invest., 1978. 62, 1354-1363.

334. Tardieu, A., Mateu, L., Sardet, C., Weiss, B., Luzzati, V., Aggerback, L., & Scanu, A.M. Structure of human serum lipoproteins in solution. II. Small-angle x-ray scattering study of HDL and LDL. J. Mol. Biol., 1976. 101, 129-153.

335. Taylor, C.B., & Gould, R.G. Effect of dietary cholesterol on rate of cholesterol synthesis in the intact animal measured by means of radioactive carbon. Circulation, 1950. 2, 467-468. (Abstract).

336. Tolleshaug, H., Berg, T., Nilsson, M., & Norum, K.R. Uptake and degradation of ^{125}I-labelled asialo-fetuin by isolated rat hepatocytes. Biochim. Biophys. Acta, 1977. 499, 73-84.

337. Tolman, K.G., Peterson, P., Gray, P., & Hammar, S.P. Hepatotoxicity of salicylates in monolayer cell cultures. Gastroenterology, 1978. 74, 205-208.

338. Tompkins, G.M., & Chaikoff, I.L. Cholesterol synthesis by liver. I. Influence of fasting and of diet. J. Biol. Chem., 1952. 196, 569-573.

339. Utermann, G., Hees, M., & Steinmetz, A. Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinaemia in man. Nature, 1977. 269, 604-607.

340. Van Berkel, Th.J.C., Koster, J.F., & Hülsmann, W.C. High density lipoprotein and low density lipoprotein catabolism by human liver and parenchymal and non-parenchymal cells from rat liver. Biochim. Biophys. Acta, 1977. 486, 586-589.

341. Van Berkel, Th.J.C., & van Tol, A. In vivo uptake of human and rat low density and high density lipoprotein by parenchymal and nonparenchymal cells from rat liver. Biochim. Biophys. Acta, 1978. 530, 299-304.

342. Van Berkel, Th.J.C., & van Tol, A. Role of parenchymal and non-parenchymal rat liver cells in the uptake of cholesterol-ester-labeled serum lipoproteins. Biochem. Biophys. Res. Commun., 1979. 89, 1097-1101.

343. Van Berkel, Th.J.C., van Tol, A., & Koster, J.F. Iodine labeled human and rat low-density and high-density lipoprotein degradation by human liver and parenchymal and non-parenchymal cells from rat liver. Biochim. Biophys. Acta, 1978. 529, 138-146.

344. Van Bruggen, J.J., Hutchens, T.T., Claycomb, C.K., Cathey, W.J., & West, E.S. The effect of fasting upon lipogenesis in the intact rat. J. Biol. Chem., 1952. 196, 389-394.

345. Van Tol, A., van Gent, T., & van 't Hooft, F.M. Low density lipoprotein catabolism in the liver: decrease of fractional catabolic rate after partial hepatectomy. In H. Peeters (Ed.) Protides of the biological fluids. New York, Pergamon Press, 1978. pp. 197-200.

346. Van Tol, A., van Gent, T., van 't Hooft, F.M., & Vlaspolder, F. High density lipoprotein catabolism before and after partial hepatectomy. Atherosclerosis, 1978. 29, 439-448.

347. Van Tol, A., van 't Hooft, F.M., and van Gent, T. Discrepancies in the catabolic pathways of rat and human low density lipoproteins as revealed by partial hepatectomy in the rat. Atherosclerosis, 1978. 29, 449-457.

348. Vermeer, R.J., Koster, J.F., Emeis, J.J., Reman, F.C., & De Bruijn, W.C. Binding of unmodified low-density lipoproteins to human fibroblasts. An investigation by immunoelectron microscopy. Biochim. Biophys. Acta, 1979. 553, 169-174.

349. Vickrey, H.M., Joseph, R.R., & McCann, D.S. Continuous culture of normal, adult mammalian hepatocytes exhibiting parenchymal functions. In Vitro, 1979. 15, 120-127.

350. Vlodavsky, I., Fielding, P.E., Fielding, C.J., & Gospodarowicz, D. Role of contact inhibition in the regulation of receptor-mediated uptake of low density lipoprotein in cultured vascular endothelial cells. Proc. Natl. Acad. Sci. U.S.A., 1978. 75, 356-360.

351. Wagner, R., Rosenberg, M., & Estensen, R. Endocytosis in Chang liver cells. J. Cell Biol., 1971. 50, 804-817.

352. Walton, K.W. Pathogenic mechanisms in atherosclerosis. Am. J. Cardiol., 1975. 35, 542-558.

353. Weibel, E.R., Staubli, W., Gnagi, H.R., & Hess. F.A. Correlated morphometric and biochemical studies on the liver cell. I. Morphometric model, stereologic methods, and normal morphometric data for rat liver. J. Cell Biol., 1969. 42, 68-91.

354. Weinhouse, S. Regulation of glucokinase in liver. Curr. Top. Cell. Regul., 1976. 11, 1-50.

355. Weis, H.J., & Dietschy, J.M. Failure of bile acids to control hepatic cholesterogenesis: evidence for endogenous cholesterol feedback. J. Clin. Invest., 1969. 48, 2398-2408.

356. Weisbraber, K.H., Innerarity, T.L., & Mahley, R.W. Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. J. Biol. Chem., 1978. 253, 9053-9062.

357. Weisgraber, K.H., Mahley, R.W., & Assmann, G. The rat arginine-rich apolipoprotein and its redistribution following injection of iodinated lipoproteins into normal and hypercholesterolemic rats. Atherosclerosis, 1977. 28, 121-140.

358. White, A., Handler, P., Smith, E.L., Hill, R.L., & Lehman, I.R. Principles of biochemistry. (6th Ed.) New York: McGraw Hill, 1978. (pages 738-739)

359. Windmueller, H.G., Herbert, P.N., & Levy, R.I. Biosynthesis of lymph and plasma lipoprotein apoprotein by isolated perfused rat liver and intestine. J. Lipid Res., 1973. 14, 215-223.

360. Wroblewski, F., & LaDue, J.S. Lactic dehydrogenase activity in blood. Proc. Soc. Exp. Biol. Med., 1955. 90, 210-213.

361. Wu, A.L., & Windmueller, H.G. Identification of circulating apolipoprotein synthesized by rat small intestine in vivo. J. Biol. Chem., 1978. 253, 2525-2528.

362. Yeagle, P.L., Langdon, R.G., & Martin, R.B. Phospholipid-protein interactions in human low density lipoprotein detected by ^{31}P nuclear magnetic resonance. Biochemistry, 1977. 16, 3487-3491.

363. Yeagle, P.L., Martin, R.B., Pottenger, L., & Langdon, R.G. Location and interactions of phospholipid and cholesterol in human low density lipoprotein from ^{31}P nuclear magnetic resonance. Biochemistry, 1978. 17, 2707-2710.

364. Yip, D.K., & Auersperg, N. The dye-exclusion test for cell viability: persistence of differential staining following fixation. In Vitro, 1972. 7, 323-329.

365. Yoffey, J.M., & Courtice, F.C. Lymphatics, lymph and the lymphomyeloid complex. New York: Academic Press, 1970. (pages 105-126)

366. Zaleski, J., & Bryła, J. Effects of oleate, palmitate, and octanoate on gluconeogenesis in isolated rabbit liver cells. Arch. Biochem. Biophys., 1977. 183, 553-562.

367. Zaleski, J., & Bryła, J. Effect of alloxan-diabetes on gluconeogenesis and ureogenesis in isolated rabbit liver cells. Biochem. J., 1978. 176, 563-568.

368. Zaleski, J., & Bryla, J. Rate-limiting factors in the utilization of pyruvate for gluconeogenesis in rabbit hepatocytes. Int. J. Biochem., 1979. 10, 241-246.

369. Zilversmit, D.B. Formation and transport of chylomicrons. Fed. Proc., 1967. 26, 1599-1605.

370. Zilversmit, D.B. Mechanisms of cholesterol accumulation in the arterial wall. Am. J. Cardiol., 1975. 35, 559-566.

LIST OF COMMERCIAL SUPPLIERS

Short Name Used in Text	Full Name of Company
AMR	Advanced Metals Research Corp. Bedford, Massachusetts
Amersham/Searle	Amersham/Searle Arlington Heights, Illinois
Beckman	Beckman Instruments Palo Alto, California
Behringwerke	Behringwerke Marburg, Germany
Biorad	Biorad Laboratories Richmond, California
Calbiochem	Calbiochemicals San Diego, California
Dr. G. Grübler & Co.	Dr. G. Grübler & Co. Leipzig, Germany
E M Laboratories	E M Laboratories Elmsford, New York
Falcon	Falcon, Div. Becton, Dickinson and Co. Oxnard, California
Farrand	Farrand Optical Co. Bronx, New York
Gelman	Gelman Instrument Co. Ann Arbor, Michigan
Gibco	Grand Island Biological Co. Grand Island, New York
Gilford	Gilford Instrument Laboratories, Inc. Oberlin, Ohio
Grand Ronde Rabbitry	Grand Ronde Rabbitry Grand Ronde, Oregon
Holly Hill Laboratories	Holly Hill Laboratory Hillsboro, Oregon
LKB	LKB Instruments, Inc. Bromma, Sweden

Microbiological Associates	Microbiological Associates Walkerville, Maryland
New England Nuclear	New England Nuclear Boston, Massachusetts
Packard	Packard Instruments Downers Grove, Illinois
Pfizer	Pfizer, Inc. New York, New York
Pharmacia	Pharmacia Piscataway, New Jersey
Purina®	Ralston Purina Company St. Louis, Missouri
Schwarz Mann	Schwarz Mann Orangeburg, New York
Sigma	Sigma Chemical Company St. Louis, Missouri
Tetko	Tetko Industries Elmsford, New York

ABBREVIATIONS

apo-A	apolipoprotein-A
apo-B	apolipoprotein-B
apo-C	apolipoprotein-C
apo-D	apolipoprotein-D
apo-E	apolipoprotein-E
apo-F	apolipoprotein-F
apo-LDL	the apoprotein of low density lipoprotein
EDTA	ethylenediaminediaminetetraacetic acid
FCR	fractional catabolic rate
FH	familial hypercholesterolemia
HDL	high density lipoprotein
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDH	lactic dehydrogenase
LDL	low density lipoprotein
leu	leucine
Lp	lipoprotein family
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TMU	1,1,3,3-tetramethylurea
Tris	Tris(hydroxymethyl)amino methane
VLDL	very low density lipoprotein