71-12,618

SOLYOM, Antal Endre, 1936-

EFFECT OF METHYLTESTOSTERONE ON THE APOLIPOPROTEIN AND LIPID COMPOSITION AND ON THE APOLIPOPROTEIN METABOLISM OF CANINE SERUM LIPOPROTEINS.

The University of Oklahoma, Ph.D., 1970
Biochemistry

University Microfilms, A XEROX Company, Ann Arbor, Michigan

© 1971

ANTAL ENDRE SOLYOM

ALL RIGHTS RESERVED

Downloaded from SHAREOK. Persistent Handle URL: https://hdl.handle.net/11244/2971

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

EFFECT OF METHYLTESTOSTERONE ON THE APOLIPOPROTEIN AND LIPID COMPOSITION AND ON THE APOLIPOPROTEIN METABOLISM OF CANINE SERUM LIPOPROTEINS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

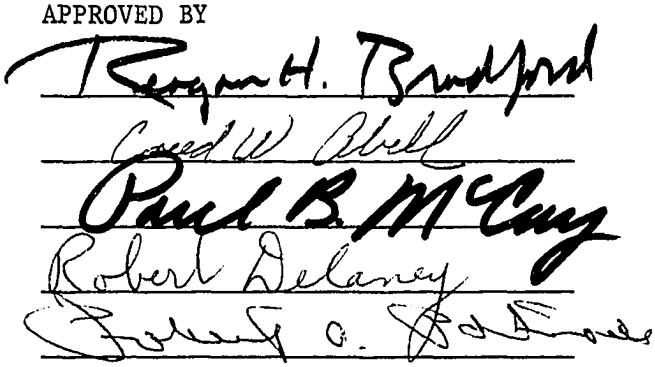
ANTAL ENDRE SOLYOM

Oklahoma City, Oklahoma

1970

EFFECT OF METHYLTESTOSTERONE ON THE APOLIPOPROTEIN AND LIPID COMPOSITION AND ON THE APOLIPOPROTEIN METABOLISM OF CANINE SERUM LIPOPROTEINS

APPROVED BY



DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

I should like to express my gratitude and appreciation to Dr. Reagan H. Bradford for his counsel and financial support (U. S. Public Health Service Grant HE-6915) during the course of this study.

I also gratefully acknowledge the support of Dr. Robert H. Furman and the Cardiovascular Section of the Oklahoma Medical Research Foundation given to me by appointment as a Cardiovascular Research Trainee (U. S. Public Health Service Grant HE-5403) and by providing the necessary research facilities and a friendly atmosphere.

I should like to thank Dr. Petar Alaupovic, and also his co-workers, especially Mr. Walter J. McConathy, for many helpful discussions.

My special thanks are due to Mrs. Jeanne Crowder and Mrs. Dell Watson, as well as to Mr. Gerald Hillerman, for valuable technical assistance and collaboration indispensible for the completion of this research. The technical help provided by several others during some phases of this research is also gratefully acknowledged.

I should also like to express my appreciation to Drs. Creed Abell, Robert Delaney, Paul McCay and Robert Patnode who served as members of the Reading Committee for this dissertation.

Finally, I am grateful to Mrs. Lynn Greene for her able assistance in the preparation and typing of this manuscript.

TABLE OF CONTENTS

		Page
LIST OF TABLES		v
LIST OF FIGURES		vii
Chapter		
I.	REVIEW OF THE LITERATURE	1
II.	INTRODUCTION	26
III.	MATERIALS AND METHODS	28
IV.	RESULTS	42
V.	DISCUSSION	105
VI.	SUMMARY	125
BIBLIOGRAPHY		129

LIST OF TABLES

Table		Page
1.	Classification and Nomenclature of Human Serum Lipoproteins	4
2.	Concentration and Composition of Lipoprotein Classes in Human Serum	5
3.	Percentage Distribution of Serum Lipids in Canine Lipoprotein Fractions	43
4.	Composition of Purified Canine Serum Lipoprotein Fractions	45
5.	Characterization of Anti-Sera Produced in Rabbits in Response to Purified Canine Serum Lipoprotein Fractions	47
6.	Distribution of Apolipoproteins in Purified Lipoprotein Fractions of Canine Serum	50
7.	Body Weight and Apolipoprotein and Lipid Concentrations in Canine Serum Lipoproteins Before and During Methyltestosterone Administration	64
8.	Effect of Methyltestosterone on Percentage Distribution of Apolipoproteins and Lipids Among Serum Lipoprotein Fractions	66 67
9.	Effect of Methyltestosterone on Composition of Purified Serum Lipoprotein Fractions	69
10.	Effect of Methyltestosterone on Amino Acid Composition of HDL Apolipoproteins	73
11.	Effect of Methyltestosterone on Concentration of Serum Protein Fractions	74
12.	Concentration of LDL and HDL Apolipoproteins and Albumin in Serum Before and During Methyltestosterone Treatment	76

LIST OF TABLES--Continued

Table		Page
13.	Rate of Incorporation of Lysine-^{14}C into LDL and HDL Apolipoproteins and Albumin Before and During Methyltestosterone Treatment	82 83
14.	Effect of Methyltestosterone on HDL:Albumin Ratio of Radioactivity Incorporated from ^{14}C-Lysine	84
15.	Effect of Methyltestosterone on Percentage Distribution of Radioactivity Recovered in Lipoprotein Fractions	85
16.	Effect of Methyltestosterone on Half Time, Turnover Rate and Total Exchangeable Pool of Apolipoprotein A in HDL (d 1.110-1.250 g/ml)	91
17.	Effect of Methyltestosterone on Half Time, Turnover Rate and Exchangeable Pools of Apolipoprotein A in Serum	96 97
18.	Effect of Methyltestosterone on Half Time, Turnover Rate and Exchangeable Pools of Albumin	103 104

LIST OF FIGURES

Figure		Page
1.	Chemical Formula of Testosterone and 17α-Methyltestosterone	18
2.	Flow Charts for Isolation of Serum Lipoproteins by Sequential Preparative Ultracentrifugation	30
3.	Quantitative Distribution of Lipids in Serum Lipoprotein Fractions	44
4.	A_1-, A_2-, A_3- and A_X-Immunoprecipitin Lines Stained with Oil Red O, Demonstrating the Presence of Lipids	48
5.	A_X-, B- and X-Immunoprecipitin Lines Stained with Oil Red O, Demonstrating the Presence of Lipids	48
6.	Immunoelectrophoretic Analysis of Purified VLDL Demonstrating A_X-, B- and X-Reactions	51
7.	Immunoelectrophoretic Analysis of Purified LDL	51
8.	Immunodiffusion Analysis of Purified LDL Preparations, Demonstrating A- and X-Reactions in Addition to the Characteristic B-Reaction	52
9.	Immunoelectrophoretic Analysis of Purified HDL Fractions Demonstrating A_1-, A_2- and A_3-Reactions	53
10.	Immunodiffusion Analysis of Purified HDL Sub-Fractions	54
11.	Immunodiffusion Analysis Demonstrating the A_X-Immunoreaction	56
12.	Immunodiffusion Analysis of Lipoprotein Fractions Separated by Gel Filtration on Sephadex G-200 Column	57

LIST OF FIGURES--Continued

Figure		Page
13.	Immunoelectrophoretic Analysis of Lipoprotein Fractions Separated by Gel Filtration on Sephadex G-200 Column Demonstrating A_X-reaction	58
14.	Immunoelectrophoretic Analysis of Lipoprotein Fractions Separated by Gel Filtration on Sephadex G-200 Column	60
15.	Analytical Polyacrylamide Electrophoresis Pattern of Lipoprotein Fractions Separated by Gel Filtration on Sephadex G-200 Column	61
16.	Effect of Methyltestosterone Treatment on Serum Lipid Levels	63
17.	Quantitative Changes in Apolipoproteins and Lipids in Serum Lipoprotein Fractions Produced by Methyltestosterone Treatment	65
18.	Immunodiffusion Analysis of the Purified Serum Lipoprotein Fraction of d < 1.110 g/ml of the Four Dogs in Which Methyltestosterone Studies were Performed	71
19.	Immunodiffusion Analysis of Purified LDL Fractions Demonstrating the Marked Diminution of the A-Precipitin Line, Relative to the B-Line, During Methyltestosterone Treatment	72
20.	Time Course and Relative Rate of ^{14}C-Lysine Incorporation into LDL and HDL Apolipoproteins and Albumin	78
21.	Time Course and Relative Rate of ^{14}C-Lysine Incorporation into LDL and HDL Apolipoproteins and Albumin	79
22.	Time Course and Relative Rate of ^{14}C-Lysine Incorporation into LDL and HDL Apolipoproteins and Albumin	80
23.	Time Course and Relative Rate of ^{14}C-Lysine Incorporation into LDL and HDL Apolipoproteins and Albumin	81
24.	Disappearance of ^{14}C-Apolipoprotein A from HDL (d 1.110-1.250 g/ml) Fraction of Serum	87

LIST OF FIGURES--Continued

Figure		Page
25.	Disappearance of ^{14}C-Apolipoprotein A from HDL (d 1.110-1.250 g/ml) Fraction of Serum	88
26.	Disappearance of ^{14}C-Apolipoprotein A from HDL (d 1.110-1.250 g/ml) Fraction of Serum	89
27.	Disappearance of ^{14}C-Apolipoprotein A from HDL (d 1.110-1.250 g/ml) Fraction of Serum	90
28.	Disappearance of ^{14}C-Apolipoprotein A from Serum	92
29.	Disappearance of ^{14}C-Apolipoprotein A from Serum	93
30.	Disappearance of ^{14}C-Apolipoprotein A from Serum	94
31.	Disappearance of ^{14}C-Apolipoprotein A from Serum	95
32.	Disappearance of ^{14}C-Albumin from Serum	99
33.	Disappearance of ^{14}C-Albumin from Serum	100
34.	Disappearance of ^{14}C-Albumin from Serum	101
35.	Disappearance of ^{14}C-Albumin from Serum	102

EFFECT OF METHYLTESTOSTERONE ON THE APOLIPOPROTEIN AND LIPID COMPOSITION AND ON THE APOLIPOPROTEIN METABOLISM OF CANINE SERUM LIPOPROTEINS

CHAPTER I

REVIEW OF THE LITERATURE

Serum Lipoproteins

Serum lipoproteins represent a heterogenous group of complex macromolecules consisting of several different lipid and protein components. Their major function apparently is to transport the water-insoluble lipids, predominantly triglycerides, although they may also have other important transport and metabolic functions.

History

By the end of the 1920's it was generally recognized that lipids were present in the euglobulin fraction of serum proteins. The first lipoprotein preparation that contained reproducible proportions of lipids and proteins was obtained from horse serum by Macheboeuf in 1929 (1). Electrophoretic separation of proteins from normal human plasma indicated that the major portions of lipids were associated with α- and β-globulin fractions (2). Fractionation of human plasma at low ionic strength in ethanol-water mixtures resulted in the precipitation of lipids into two readily separated fractions (3, 4), representing two distinct lipoprotein

classes (5).

Subjecting the serum to ultracentrifugation sedimented three protein components, i.e., albumin, globulin and X-protein (6). The X-protein complex was sensitive to changes in salt concentration and its flotation could be produced by increasing the density of the suspending medium (7). Flotation procedures were utilized for isolation and purification of this component which was characterized as β-lipoprotein. The new method of quantitative analytical ultracentrifugation for studying these low density lipoproteins (LDL) was soon developed (8), and a possible role of LDL in the pathogenesis of atherosclerotic vascular disease postulated (9). The S_f notation was then introduced for classification and characterization of serum lipoproteins according to their flotation rates in a sodium chloride solution of d 1.063 g/ml at 26° C (10, 11). Ultracentrifugation at a higher solvent density, i.e., d 1.210 g/ml permitted flotation of the high density lipoproteins (HDL). The HDL and LDL fractions isolated by ultracentrifugation corresponded with the α- and β-fractions, respectively, separated by electrophoresis (12). Immunochemical studies suggested that these two major lipoprotein classes were antigenically distinct and that these characteristics represented properties of the protein rather than the lipid moieties of the lipoproteins (13, 14).

N-terminal amino acid analyses indicated that HDL and LDL contain distinct protein moieties (15-17), inasmuch as N-terminal aspartic acid was found in HDL and glutamic acid in LDL. Additional N-terminal amino acids have also been found, predominantly in the very low density lipoproteins (VLDL, < 1.006 g/ml) and lymph chylomicrons (16-20). This observation suggested that apolipoproteins in addition to apolipoproteins

A and B, which represent the principal apolipoproteins of HDL and LDL, respectively, may be present in plasma. Investigation of this possibility led to the conclusion that a third apolipoprotein, designated apolipoprotein C, may represent a major VLDL apolipoprotein in human plasma (21, 22).

These major developments represent the framework for the present knowledge of serum lipoproteins. Much of this knowledge, particularly the more detailed structural and compositional aspects, is based on information concerning human plasma lipoproteins. This emphasis relates to the significance of lipoproteins in atherogenesis.

Classification and Nomenclature

The principal classification systems are based on hydrated densities and paper electrophoretic characteristics of lipoproteins. It has become increasingly evident during the last decade that the lipoprotein classes separated by either of these methods contain more than one protein component (Table 1). An alternative system has been proposed in which lipoproteins are classified by their apolipoprotein component into groups designated as lipoprotein A, lipoprotein B and lipoprotein C (23). However, significant new information obtained during the last two years indicates a much greater complexity in respect to the protein components than was previously recognized.

According to the density classification system, the lipoprotein spectrum consists of the five classes shown in Table 1. The concentration and composition of these lipoprotein fractions in human serum are presented in Table 2. The distribution of serum lipoproteins in males differs from that in females, inasmuch as males have higher concentrations

TABLE 1

CLASSIFICATION AND NOMENCLATURE OF HUMAN SERUM LIPOPROTEINS[a]

Hydrated Density		Flotation Range (S_f)[b]	Electrophoretic Migration on Paper	Apolipoproteins		
				N-Terminal Amino Acids		Antigenic Determinants
Density Class	Density Range (g/ml)			Major	Minor	
Chylomicrons	> 0.950	> 400	origin	Ser,Thr	Glu,Asp	C,B,A
VLDL	0.950-1.006	20-400	pre-β	Ser,Thr	Glu,Asp	C,B,A
LDL	1.006-1.063	0-20	β	Glu	Ser,Thr,Asp	B,(C),(A)
HDL	1.063-1.210	--	α	Asp	Glu,Ser,Thr	A,(B),(C)
VHDL	> 1.210	--	α	Asp	--	A

[a]Abbreviations: VLDL = very low density lipoproteins; LDL = low density lipoproteins; HDL = high density lipoproteins; VHDL = very high density lipoproteins.

[b]Negative sedimentation coefficient in Svedberg units in NaCl solution of d 1.063 g/ml at 26° C.

TABLE 2

CONCENTRATION AND COMPOSITION OF LIPOPROTEIN CLASSES IN HUMAN SERUM

Lipoprotein Class	Concentration[a] (mg/100 ml)		Composition[b] (weight percent)				
	Male	Female	Protein	Cholesterol		Phospholipid	Triglyceride
				Free	Ester		
Chylomicrons	12± 13	2± 3	2	2.1	3.9	4.3	87
VLDL	129±122	59± 63	5-12	3-5	10-13	13-20	50-60
LDL	439± 99	389± 79	22	10	36	20	12
HDL	300± 83	457±115	50	2	20	24	4
VHDL[c]	--	--	62.4	0.3	3.2	28	4.6

[a]Values obtained from ref. 120 are expressed as mean ± standard deviation.

[b]Values were obtained from ref. 46.

[c]Values were obtained from ref. 121 for the fraction of d 1.210-1.250 g/ml.

of VLDL and LDL and lower concentrations of HDL than females. Furthermore, LDL and/or VLDL levels increase with age from the third to the fifth decade, while no significant age trends are observed for HDL in either sex (24).

Structural Properties

Essentially all of the lipid can be extracted from lipoproteins with appropriate organic solvents to produce a lipid-free glycoprotein (25). This observation supports the conclusion that the major forces holding the lipid and protein components of the lipoprotein together are non-covalent. It has been suggested, however, that a small amount of fatty acid may be covalently bound to the protein (26). The lipoprotein classes are considered to be polydisperse systems which are heterogeneous in respect to particle size, hydrated density (27, 28) and protein moiety (23). Two basic types of lipoproteins have been postulated on the basis of structural features (29). One of these, the micellar type, contains less than 30% protein and includes chylomicrons, VLDL and LDL, while the other contain more than 30% protein and includes HDL and VHDL.

Micellar lipoproteins are thought to consist of a hydrophobic core of triglycerides and cholesterol esters, surrounded by a hydrophilic coat of protein, phospholipid and free cholesterol. Chylomicrons, VLDL and LDL appear by electron microscopy as spherical particles with diameters of approximately 1,200 to 11,000 Å, 300 to 700 Å and 170 to 260 Å, respectively (30, 31). No specific sub-unit structure is evident. Beyond these similarities, however, there are important differences among these lipoproteins concerning the type and amount of apolipoproteins and lipids. For example, in chylomicrons and VLDL an increasing number of

protein or polypeptide components are being identified (32-34). The LDL are also heterogeneous in respect to their protein moiety (35) although proportions of apolipoproteins in LDL differ from those in chylomicrons and VLDL.

The HDL and VHDL are thought to consist of sub-units containing protein and lipid. These sub-units associate in a definite quaternary structure with a mean diameter of 74 to 98 Å, which is recognizable by the electron microscope (30). HDL is frequently sub-divided into HDL_2 (d 1.063-1.125 g/ml) and HDL_3 (d 1.125-1.210 g/ml) which are heterogeneous in respect to their protein moiety. Apolipoprotein A contains at least two non-identical polypeptides characterized by their C-terminal amino acids (R-Gln and R-Thr) in either HDL_2 or HDL_3 (36, 37). Some of the polypeptides found in HDL_2, in addition to apolipoprotein A, are possibly due to the presence of apolipoproteins B and C (38, 39). Optical measurements suggest that HDL is relatively rich in the α-helical conformation. This contrasts with native LDL, which contains a significant amount of anti-parallel chain β-structure (40-42). Measurements of circular dichroism and total amino acid content of the two major polypeptide sub-units of HDL indicate that R-Thr has a greater helical content than R-Gln (43). Since helical regions are generally on the surface of proteins rather than in the hydrophobic interior of the molecule, it is suggested that the R-Thr polypeptide might be involved to a lesser extent in lipid binding than the R-Gln polypeptide.

Despite significant progress during the last few years, present knowledge concerning the chemistry of the apolipoproteins is still insufficient for answering questions about the nature of lipid-protein inter-

actions and about those specific structural features which are necessary for lipid binding. Since the amino acid composition of the protein moieties of different lipoprotein classes does not differ significantly from that of other serum proteins, the elucidation of specific amino acid sequences of the individual peptides will probably be required to permit a better understanding of the relationship between lipoprotein structure and function.

Functions and Interrelationships

Triglycerides are a major source of fatty acids and thereby function in the physiological role of a principal energy reserve. Chylomicrons and VLDL, which are triglyceride-rich lipoproteins, appear to be primarily involved in energy metabolism. Although the role of cholesterol esters has not been defined, it is well established that free cholesterol serves as the precursor of bile acids and steroid hormones. LDL and HDL are primarily responsible for sterol transport. The main function of phospholipids is stabilization of the lipoprotein structure and of the highly apolar lipids during their transport in aqueous media. Phospholipids are concentrated at polar-apolar interfaces along with the apolipoproteins. Binding of phospholipid may be the major function of apolipoproteins which have a high affinity for phospholipid (21, 22, 44, 45).

Several lines of evidences indicate that the presence of high concentrations of triglyceride-rich VLDL affect the concentration and composition of LDL and HDL. A direct relationship has been observed between plasma levels of VLDL and the percentage content of triglyceride in HDL (46). Similarly, in vitro incubation of plasma with high levels of VLDL resulted in a significant increase in the triglyceride content

of HDL and LDL fractions (47), while a reciprocal transfer of cholesterol esters of LDL and HDL occurred. Additional evidence that triglyceride metabolism influences the concentration of lipoproteins which contain little triglyceride is provided by the observation that VLDL and LDL levels show an inverse relationship with HDL_2 (24). In vivo and in vitro studies have demonstrated that lecithin, free cholesterol and triglyceride (48, 49), but not cholesterol esters (50), undergo continual exchange among lipoprotein fractions.

The fact that lipoprotein classes share common protein or polypeptide components may represent the fundamental chemical basis for their interrelationships. Chylomicrons and VLDL probably contain the full complement of their apolipoprotein components upon release from the intestine and/or liver (51). In addition, they may pick up apolipoprotein A during circulation since both in vivo and in vitro studies show that HDL-^{131}I bind to chylomicrons (52). It is possible that the function of bound apolipoprotein A is to activate lipoprotein lipase (45, 53) and/or lecithin:cholesterol acyl transferase (LCAT) (54) and thereby play a role in the metabolism of these lipoproteins. After in vivo heparin administration, chylomicrons and VLDL are rapidly degraded into smaller lipoprotein products which have ultracentrifugal flotation properties similar to LDL and HDL (55). While the chemical properties of the LDL products are similar to those of normally occurring LDL, the HDL products contain an appreciable amount of phospholipid and less of other lipid components which normally occur in HDL (56). The lipid composition of the HDL product may be normalized by a sequence in which cholesterol ester is formed by an LCAT-catalyzed reaction between lecithin and free cholesterol. The cho-

lesterol ester formed in HDL from lecithin and free cholesterol, which was transferred from chylomicrons or VLDL to HDL during lipolysis, is subsequently transferred back into the VLDL. The possibility that this LCAT-catalyzed reaction involving HDL is a significant pathway for cholesterol ester formation is supported by the fact that LCAT inhibitors markedly reduce this transfer in vitro, and that newly synthesized cholesterol ester appears most rapidly in HDL (57, 58). A precursor-product relationship between the VLDL and LDL is suggested by tracer studies in vivo using protein-labeled lipoproteins (52, 59), and by investigations evaluating changes in lipoprotein distributions during metabolic (60) and pharmacologic (61, 62) alterations in which substantial shifts in plasma lipoprotein distributions were characterized by marked decreases in VLDL and increases in LDL and HDL levels.

The function of apolipoprotein B appears to be its essential role in the formation and secretion of chylomicrons and VLDL by the intestine and liver. Evidence favoring this conclusion is provided by studies of abetalipoproteinemia, a human genetic disorder (63), as well as by response to agents such as puromycin (64) or orotic acid (65) which inhibit synthesis of this protein.

Serum lipoproteins may play an important role in maintaining red blood cell membranes and probably other tissue membranes in a functional state. It has long been known that the free cholesterol of plasma is in rapid equilibrium with the free cholesterol of the erythrocytes (48, 66, 67). In addition, it has been reported that phospholipid exchange also occurs between erythrocytes and lipoproteins (68, 69). Lecithin and sphingomyelin in HDL and LDL and lysolecithin in VHDL are involved in

this process. It is possible that new lecithin molecules for the red cells are derived through an exchange of red cell lecithin for lysolecithin from serum (70). The red cell abnormalities in a number of diseases (abetalipoproteinemia, α-lipoprotein deficiency and LCAT deficiency) most likely reflect an imbalance in the serum lipoprotein system. Furthermore, patients with abetalipoproteinemia frequently show neurological defects associated with partial demyelination, and in some cases of α-lipoprotein deficiency peripheral neuropathies have been observed. These observations may reflect the dependence of tissue membranes on plasma lipoproteins. This proposed interrelationship is consistent with the fact that different cellular and subcellular membranes have been found to exchange cholesterol with serum lipoproteins (71).

The transport of carotenoids by serum lipoproteins (72-74) represents another important biological function (75).

Synthesis and Catabolism of Apolipoproteins

Many of the details of protein synthesis, as well as pathways for synthesis and breakdown of different lipid components, are now well-known. However, little information is available to provide a reasonably clear concept of the formation and degradation of lipoproteins as a macromolecular entity. The limited knowledge in this field may be due to several possible reasons, including the incomplete characterization of the chemistry and number of apolipoprotein polypeptides and the large number and poorly defined interrelations and interconversions of lipoprotein components. The possible models suggested for lipoprotein biosynthesis (76) are built around the concept that the protein moiety plays a cardinal role in this process. This thesis is consistent with structural consid-

erations discussed above, as well as the observation that the interaction of highly specific protein with reconstituted lipid-bilayer membranes produces profound alterations in the electrical characteristics of the lipid-bilayer (77). The observation that individual lipoprotein components have distinct turnover rates (78) favors the conclusion that serum lipoprotein metabolism resembles that of cellular membranes (79). Furthermore, the turnover of triglyceride, cholesterol ester and other components of individual lipoprotein fractions depends on the lipoprotein class in which the component is transported (58). Additional studies will be required to determine whether this reflects the influence of apolipoprotein or particle size of individual lipoproteins.

It has been generally accepted that all plasma proteins except gamma-globulins are synthesized in the liver (80). The intestine may serve as an extra-hepatic site for apolipoprotein synthesis, however, insofar as chylomicron formation is considered. Results from in vivo studies in dogs fed labeled amino acids have suggested an intestinal origin for at least a part of the chylomicron protein (81). ^{14}C-leucine was incorporated into chylomicrons by rat intestinal mucosa (82, 83). Furthermore, little radioactivity was incorporated into VLDL in hepatectomized dogs and none was observed following evisceration (84). These studies also indicate that in the synthesis of lipoprotein classes other than chylomicrons the intestine does not play any significant role.

Experiments with rat liver slices and with perfused livers demonstrated the net synthesis of LDL apolipoprotein, which was shown by a quantitative precipitin method to be identical with that circulating in the blood (85), and of HDL apolipoprotein, which was similar by peptide

finger printing and labeling technique to that found in plasma HDL (86).

Amino acid incorporation studies of the synthesis of LDL and HDL apolipoproteins, in various laboratory animals and experimental conditions (87-89), have repeatedly demonstrated that LDL apolipoprotein has a higher specific radioactivity, and presumably a greater turnover rate, than HDL apolipoprotein. In studies with perfused rat livers in which mevalonic acid served as precursor (50), LDL cholesterol esters had a more rapid turnover than those in HDL. Studies in rabbits of the disappearance of LDL and HDL containing ^{14}C-labeled protein indicated no significant metabolic interconversion between them (87). Similarly, studies in man with ^{131}I-labeled lipoproteins have demonstrated that LDL apolipoprotein had a shorter half time than that of HDL, and that labeled LDL peptides were not found subsequently in HDL (90).

It has been shown that the intracellular site of apolipoprotein synthesis is in the microsomal fraction (91) and that isolated rat liver ribosomes, in an essentially lipid-free system, but not mitochondria or kidney microsomes can synthesize the LDL and HDL apolipoproteins (92). The labeled peptides can be removed from the ribosomes and combined with lipid to form lipoprotein. The tentative conclusion from these experiments is that the presence of all lipid classes in lipoproteins is not required for the synthesis of the protein moiety, although their role in the release of polypeptides from the ribosomes is not clear. Similarly, information is lacking concerning the attachment of the small amount of covalently-bound carbohydrate found in apolipoproteins and the role it may play in the lipid binding, i.e., in the overall configuration of the apolipoprotein and the assembly of the lipoprotein, or possibly in the

transport from the cell (93) in which the apolipoprotein is formed. The presence of a small amount of covalently-bound fatty acids in lipoproteins (26) adds another facet to the problem.

Electron microscopic studies in perfused rat liver showed that soon after raising the free fatty acid concentration in the perfusate, strongly osmophilic bodies of 300-800 Å, probably representing VLDL, appeared in the smooth endoplasmic reticulum and Golgi apparatus and later in the space of Disse (94, 95). These observations suggested that the Golgi apparatus participated in the formation and secretion of VLDL. Recently, in similar studies the Golgi apparatus has been isolated and the presence of all apolipoprotein components of circulating VLDL has been demonstrated by chemical analyses (51).

Although several factors are known to influence the synthesis of apolipoproteins, the regulation of serum lipoprotein synthesis is far from being understood. Most of the agents, or conditions, known to produce fatty liver interfere with apolipoprotein synthesis. Ethionine (96) or puromycin (97) administration decreased the ability of liver slices to incorporate leucine into LDL and HDL. Carbon tetrachloride poisoning resulted in a marked decrease of leucine (98) or lysine (89) incorporation into lipoproteins. In perfused livers from orotic acid-fed rats, LDL production was inhibited without altering the release of HDL, albumin and other proteins (99). Actinomycin treatment caused rapid depression of VLDL and LDL levels in the rat while HDL and other serum proteins were less affected. The incorporation of lysine into LDL was decreased most, although a reduced incorporation into HDL and albumin was also observed (100). On the other hand, triglyceride feeding (101) or an increased

free fatty acid influx into the perfused liver (102) resulted in a greater production of triglyceride-rich VLDL with an increased incorporation of amino acids into VLDL but not HDL apolipoproteins. Puromycin markedly depressed the production of VLDL by livers perfused with fatty acid-rich medium by inhibiting protein, but not triglyceride, synthesis (94).

These studies indicate that the liver is the major source of all apolipoproteins and that their unimpaired synthesis is essential for normal lipid transport and metabolism. On the other hand, the mechanisms and sites of apolipoprotein degradation are entirely unknown. Although lipoproteins may be completely assembled as a result of de novo synthesis in the liver prior to leaving the cell, the different turnover rates of their components clearly indicate that they are not degraded as a whole. The fact that apolipoproteins have longer half times than the lipid components except cholesterol may be related to the exchanges of lipids between circulating lipoproteins and cell membranes, and might also accomodate yet unproven concepts of recycling apolipoproteins (53, 103).

Species Differences

The transport functions of plasma lipoproteins in other mammalian species are presumed to be similar to those in man. However, it has long been recognized that the distribution among the lipoprotein spectrum was different in most animals when compared with man (12, 104, 105). The pig and monkey were found to have a lipoprotein distribution pattern similar to that of man, while a dissimilar distribution was observed in the rat, rabbit, chicken, dog, cat, guinea pig and hamster. The guinea pig has almost no HDL, while other animals differ from humans mainly in that the HDL fraction contains a significant proportion of their lipid. More recently,

the bison (106), cow (107), marine mammals (108) and some migratory birds (74) were also found to differ from the pattern in human subjects. Furthermore, some of these studies indicated that species differences also exist in respect to the lipid composition of the lipoprotein classes. Paper electrophoretic (109) and zonal ultracentrifugal (110) studies of lipoprotein distributions of several animal species confirm the previous findings.

Remarkable similarities were found in the immunological characteristics of LDL from human and animal sera. LDL from chicken serum are the only LDL in the many species examined which have failed to show any cross-reaction with human LDL (111-113). Comparable information is not available, however, regarding the immunological characteristics of HDL in different animal species. In general, few reports have appeared concerning the distribution and chemical characteristics of specific apolipoproteins. Detailed analyses of lipoproteins and apolipoproteins have been reported for the pig (114-116) and the rat (51, 117-119). On the basis of these studies, it appears that an apolipoprotein pattern which is similar in complexity to that found in human lipoproteins can be expected in other animal species. Beyond these superficial similarities in chemistry and structure, however, specific characteristics in components and/or composition of apolipoproteins should be found to explain the species differences in lipoprotein distribution and composition, as well as in some aspects of lipid transport and metabolism (74).

Methyltestosterone

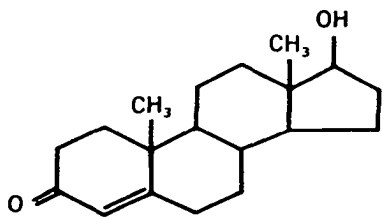
Soon after the chemical structure of the male sex hormone, testosterone, was elucidated and the chemical synthesis achieved in 1935,

a number of analogs and derivatives were prepared. Methyltestosterone (17α-methyl-17β-hydroxyandrosta-4-en-3-one, Figure 1) is unique among 17α-substituted alkyl derivatives in retaining androgenic potency when given orally.

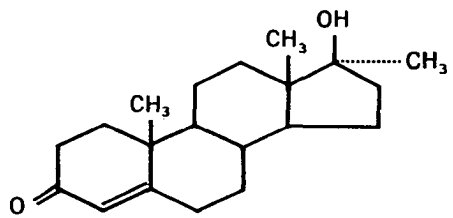
Although testosterone is effective when given parenterally, it is practically inactive by oral administration (122, 123). This is due to the rapid inactivation of testosterone in the liver (124). Earlier investigations have shown that 17-ketosteroid excretion is markedly increased by testosterone, but is either unaltered or lowered by methyltestosterone (125-127). This observation was supported by in vitro experiments with liver showing that no 17-ketosteroid was formed from methyltestosterone (128), and by the finding that 17α-alkylation lowers the ease with which 17β-hydroxyl groups may be oxidized (127, 129). Thus, substitution of an alkyl group in the 17α-position prevents the oxidation of the 17β-hydroxy group of testosterone (by the 17β-hydroxysteroid dehydrogenase), thereby impeding and directing to other pathways the metabolism of methyltestosterone. Methyltestosterone was introduced into clinical medicine in 1939 (123) and has become the prototype of orally active androgens and synthetic anabolic steroids.

Biological Effects of Androgens

Androgens promote general body growth (hence their designation as anabolic steroids) and also produce masculinizing effects. Anabolism means a preponderance of protein synthesis, a constructive metabolism which is recognized by a positive nitrogen balance. The androgenic effect differs from the anabolic effect primarily in its location. Androgenicity refers to an anabolic effect on the sex organs and on cell differentia-



TESTOSTERONE



METHYLTESTOSTERONE

Fig. 1 - Chemical formula of testosterone and 17α-methyltestosterone.

tion, while in the common usage of the term an anabolic effect signifies the extragenital stimulation of protein synthesis.

Testosterone is one of the most potent anabolic steroids known. Studies on the incorporation of amino acids into tissue protein indicated that testosterone does not slow down protein breakdown but rather stimulates protein synthesis (130-132). Similarly, investigation of the dynamics of protein metabolism in man showed that anabolic steroids did not inhibit protein degradation, but stimulated protein synthesis exclusively (133). At the molecular level two fundamental activities have been demonstrated as influenced by anabolic steroids, i.e., an increase of the ribonucleic acid (RNA) content of cells and an increase in the activities of amino acid activating enzymes (134-136). Testosterone is bound to a protein component of the chromatin at the site of active RNA synthesis within the nuclei of accessory sex tissues (137). This occurs after conversion of testosterone by nuclear 5α-reductase into the more potent androgen, dihydrotestosterone, which appears to be the active form of testosterone in these tissues (138). However, in the same study dihydrotestosterone formation in non-target tissues, e.g. liver and muscle, was negligible. Polar metabolites other than dihydrotestosterone were formed when testosterone was incubated with liver nuclei or liver cytoplasm. These results indicate that the metabolic actions of androgens are mediated possibly through different metabolites in different tissues. This is in agreement with the fact that chemical modifications of testosterone resulted in significant dissociations of the anabolic and androgenic effects.

Investigations into the effect of androgens on the concentrations

of total protein and protein fractions in serum have yielded inconclusive results. The influence of anabolic steroids on serum protein fractions has not been evaluated adequately in healthy subjects and laboratory animals. No characteristic change in serum total protein concentration has been observed during testosterone treatment of patients with a variety of diseases (139). Administration of either natural androgens or synthetic anabolic steroids, however, produced an increase in the α_2-globulin fraction (140, 141). Methyltestosterone administration to human subjects decreased the thyroxine-binding capacity of the thyroxine-binding globulin, suggesting that the concentration of this protein in serum was lower (142). Norethandrolone produced a similar response in thryoxine-binding globulin, but increased the thyroxine-binding capacity and the concentration of the thyroxine-binding pre-albumin fraction (143).

Effects on Lipid Metabolism

Sex differences in the development of atherosclerosis as well as the resistance of male castrates to coronary heart disease have long been recognized. Although sex differences exist in respect to serum lipid and lipoprotein concentrations, and administration of gonadal hormones characteristically alters the lipid levels and lipoprotein patterns in the serum (144), it is not at all clear what is the significance for atherogenesis of these effects of gonadal hormones (145). In general, the administration of androgens to subjects with normal serum lipid levels diminishes the concentration of HDL and to a lesser extent increases the concentration of LDL, which are changes opposite to those elicited by estrogens (146-148). Although serum cholesterol levels may not change significantly in healthy subjects, in lipemic patients the hypocholes-

terolemic effect of methyltestosterone and other androgens has been well documented (149-152). In the dog, testosterone or methyltestosterone administration lowers the concentration of both HDL and LDL (153) and reduces serum cholesterol, phospholipid and triglyceride levels (153-155).

These data clearly indicate that methyltestosterone and testosterone exert a similar effect on serum lipoproteins. The mechanism whereby these effects are produced has not been determined. The observation that methyltestosterone decreased the incorporation of acetate into serum cholesterol (156) may provide an explanation for the changes in cholesterol metabolism, but this explanation is inadequate to account for the marked decrease which was demonstrated in all three major lipid classes. A major fecal metabolite of methyltestosterone, i.e., 17α-methyl-5α-androstane-3β,17β-diol, has been found recently to reproduce completely the hypocholesterolemic effect of methyltestosterone (157) in dogs. These results suggest that there is no direct relationship between the androgenicity and the hypocholesterolemic activity, inasmuch as the metabolite appeared to be a much weaker androgen than the parent compound (158). The possibility that the metabolite isolated from the feces of methyltestosterone-treated dogs (159) may be the active compound still leaves unanswered the question concerning the mechanism of action of methyltestosterone.

Methyltestosterone has also been found to lower serum cholesterol concentrations in rabbits (160), although this agent had no effect in preventing hypercholesterolemia, tissue lipid deposits or aortic and coronary atherosclerosis in cholesterol-fed rabbits, nor did it modify previously developed lesions (161). Reports describing a hypocholesterolemic effect

in rats (162) were not confirmed by others (163). In the latter study, methyltestosterone produced a significant decrease in hepatic cholesterol synthesis, but this response was compensated by a significant increase in intestinal cholesterol synthesis and total cholesterol biosynthesis in these tissues remained unaltered. The oxidation of cholesterol to biliary and fecal bile acids was not increased significantly in rats treated with methyltestosterone (164).

Prolonged administration of high doses of testosterone or 17α-methylandrost-5-ene-3β,17β-diol has been reported to decrease the total fat content of the body (165-167). On the other hand, testosterone treatment of starved female rats caused a marked increase in plasma free fatty acid concentrations, suggesting the possibility of a direct fat-mobilizing activity of the androgens (168). These observations and others, i.e., that during puberty the subcutaneous fat decreases in males but not in females and that eunuchs have a tendency to obesity, seem to warrant investigations on the role androgens may play in adipose tissue metabolism. At the present, however, such information is not available.

Side-effects and Metabolism of Methyltestosterone

Although the biological effects of methyltestosterone are generally equivalent to those of testosterone, even insofar as producing an apparent feed-back regulation of gonadotropin secretion and testosterone production (169), the effects of methyltestosterone on creatine metabolism and on the conjugative or excretory functions of the liver differ from those of the natural hormone. Testosterone lowers the hypercreatinuria caused by castration (170), or by thyroxine administration (171), and reduces the excretion of creatine and creatinine in normal persons as well

(172). On the other hand, methyltestosterone produces a pronounced increase in urinary excretion and serum levels of creatine (171, 173) and guanidinoacetate (173, 174), its physiologic precursor. In the absence of any change in kidney threshold, it is possible that the hypercreatinuria is due to increased creatine synthesis. Arginine and methionine are required in the formation of guanidinoacetate and its subsequent methylation to form creatine. The possibility therefore exists that methyltestosterone may increase the utilization of these two amino acids for creatine synthesis. For this reason, it appears unwise to consider the use of either of these two essential amino acids or glycine as precursors in studying the effect of methyltestosterone on protein synthesis.

The original observation (175) suggesting that methyltestosterone might cause obstructive jaundice led to the recognition that prolonged administration of 17α-alkyl substituted steroids can cause hepatic dysfunction due to intrahepatic cholestasis (176). In addition to development of icterus, these agents may produce acholic feces, an increase in serum bilirubin concentration, impaired removal of bromsulphalein and an increase in the serum activities of alkaline phosphatase and glutamic-oxalacetic transaminase. No direct relationship has been found between the duration or dosage of methyltestosterone treatment and the appearance of these side-effects. On the other hand, the prognosis is generally good, i.e., complete recovery usually occurs after methyltestosterone administration is suspended. The hepatocytes exhibit only minor histological changes and remain viable (177, 178). Electron microscopic observations led to the conclusion that the primary damage occurs in the canalicular membranes of liver cells (179). Alternatively, continuous administration

of methyltestosterone for years may lead to biliary cirrhosis (180).

It is likely that these special side-effects of methyltestosterone are due to its being metabolized in a different way than testosterone. However, while the intermediary metabolism of natural androgens has been elucidated in practically all details (181), information regarding the metabolism of methyltestosterone is incomplete. The intermediary metabolism of testosterone proceeds essentially via three main reactions:

1. 17β-hydroxysteroid dehydrogenase: oxidation of the 17β-hydroxy group resulting in 17-ketosteroid formation (androstenedione),

2. Δ^4-5α- or Δ^4-5β-reductase: reduction of the 4,5-double bond in ring A, and

3. 3α- and 3β-hydroxysteroid dehydrogenase: formation of the 3-hydroxy-5-androstan-17-one metabolites (4 isomers).

It has long been known, as mentioned above, that methyltestosterone does not undergo the first reaction. However, from the available information on some of the metabolites of methyltestosterone it would appear that its metabolism by the other two reactions is not impaired. After the administration of labeled methyltestosterone to human subjects, 17α-methyl-5α-androstane-3α,17β-diol and its 5β:3α isomer were isolated in the feces and urine, in addition to unchanged methyltestosterone and unidentified polar compounds (182, 183). Methyltestosterone disappeared from the plasma with a half time of about 2.5 hr, while plasma conjugated steroids containing radioactivity administered as methyltestosterone began to increase soon after the injection and reached a plateau within 2 to 6 hr. Furthermore, about three-quarters of the urine radioactivity was found in the conjugated steroid fraction, in which the por-

tion esterified with glucuronic acid exceeded that with sulphuric acid. The excretion of radioactivity in urine and feces also appeared to be slow, inasmuch as only 50% of the injected dose had been excreted after 4 days (183). These results indicate that methyltestosterone is metabolized significantly more slowly than testosterone, which disappears from plasma with a half time of about 30 min (184). In addition, methyltestosterone and its metabolites are conjugated to a lesser extent, although qualitatively these metabolites resemble those of testosterone (185). Renal clearance of methyltestosterone and its metabolites may also be lower. The fact that clearly the greater portion of the metabolites were hydroxylated and reduced in ring A differentiates the metablism of methyltestosterone from that of most other steroid hormones.

In the dog, the metabolic fate of methyltestosterone appears to be similar in many respects to that in man (159). The same metabolites could be identified in the feces and urine in both species. However, in the dog more than 95% of the dose of radioactivity administered daily was recovered in urine and feces. No appreciable amount of labeled material could be detected in the tissues as early as 24 hr after the last injection. This indicates that methyltestosterone is metabolized and excreted at a more rapid rate in the dog than in man. The metabolic clearance rate of testosterone was also found to be higher in dogs than in man (186).

CHAPTER II

INTRODUCTION

The present study was undertaken to evaluate the mechanism whereby androgens lower the concentrations of serum lipids and lipoproteins. Interest in this research problem was prompted in part by the significance of gonadal hormones in relation to atherosclerosis and heart disease. Furthermore, results obtained in this study should provide additional knowledge concerning the role of gonadal hormones in the regulation of lipid transport and metabolism.

The dog was selected as the laboratory animal for these studies in order that each animal might serve as his own control and studies could be performed in which a series of blood samples are obtained from each animal. While many studies of lipid transport and metabolism in which a relatively large laboratory animal is desirable have been performed in the dog, limited information is available concerning the characteristics of canine serum lipoproteins, particularly the distribution of apolipoproteins among the lipoproteins. In the present study, a series of canine serum lipoprotein fractions was isolated by sequential preparative ultracentrifugation. The apolipoprotein and lipid composition of the purified lipoprotein fractions was then characterized by immunochemical and quantitative chemical analyses.

Previous studies have shown that the administration of methyl-

testosterone significantly lowers the concentration of cholesterol, phospholipid and triglyceride in serum HDL and LDL. All serum lipids are decreased to approximately the same degree. In view of the major role of apolipoproteins in the formation and structure of lipoproteins, the possibility exists that methyltestosterone exerts its effect on lipoproteins through a primary effect on apolipoprotein metabolism.

Studies were undertaken to characterize the changes elicited by methyltestosterone in apolipoproteins of serum lipoproteins. The apolipotein and lipid distribution in serum lipoproteins, as well as the composition of purified lipoprotein fractions were determined in serum samples obtained before and during methyltestosterone administration. Quantitative data were also obtained for serum albumin and conventional globulin fractions. Metabolic studies were performed in which the effect of methyltestosterone on the incorporation of ^{14}C-lysine into the apolipoproteins of serum HDL and LDL and into serum albumin was evaluated. In addition, measurements were obtained of the turnover of HDL apolipoprotein following the administration of purified HDL containing ^{14}C-labeled apolipoprotein and of albumin turnover following ^{14}C-albumin administration, in studies performed before and during methyltestosterone treatment. Turnover rates, pool sizes and related metabolic values for these two proteins were calculated.

CHAPTER III

MATERIALS AND METHODS

Animals

Mature mongrel dogs were maintained on a commercial dog food with an approximate caloric composition of 28% protein, 18% fat and 54% carbohydrate (Superior Tast-Tee Chunks Dog Food, Superior Feeds, Oklahoma City, Okla.). Body weight was monitored weekly and diet ration was adjusted to maintain body weight constant.

A group of ten dogs (9 males, 1 female) was used in studies of the apolipoprotein and lipid distribution in serum lipoproteins and the composition of purified lipoprotein fractions. After a 20-hr fast, the dogs were anesthetized with sodium pentobarbital (30 mg per kg, i.v.) and 150 to 200 ml of blood was obtained through a femoral vessel.

Another group of four dogs (3 males, 1 female) was used in the methyltestosterone studies, in which apolipoprotein and lipid distribution in serum lipoproteins and the composition of purified lipoprotein fractions were analyzed and incorporation and turnover studies were performed before and during methyltestosterone administration. Blood samples were obtained at weekly intervals from each dog for analyses of serum cholesterol, phospholipid, triglyceride, total protein and individual protein fractions. An additional 200 ml of blood for lipoprotein analysis was

obtained from each dog after a 20-hr fast. Liver function was evaluated by measurements of serum bilirubin concentration and bromsulphalein retention. Methyltestosterone, 200 mg per day 5 days per week, was then administered to each dog and studies similar to those during the control period were performed after serum lipid levels had stabilized at a new low level characteristically produced by methyltestosterone. Incorporation and turnover studies were performed no earlier than 7 weeks after initiation of methyltestosterone administration, to insure that a metabolic steady state had been attained. Immediately before and during these metabolic studies, 200 mg of methyltestosterone was administered daily.

Preparative and Analytical Procedures

Isolation and Purification of Lipoprotein Fractions

Sequential preparative ultracentrifugation was used to isolate lipoprotein fractions in the following density ranges: d < 1.006 g/ml, very low density (VLDL); d 1.006-1.019 g/ml and 1.019-1.063 g/ml, low density (LDL); d 1.063-1.110, 1.110-1.160, 1.110-1.210 and 1.160-1.210 g/ml, high density (HDL); and d 1.210-1.250 g/ml, very high density (VHDL) (Figure 2, scheme A). Seven ml of serum in a polyallomer tube was overlayered with 4 ml of sodium bromide solution, d 1.006 g/ml, and centrifuged at 4° C in a type 50 rotor for 22 hr at 105,000 x g in a Spinco Model L or L-2 preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). After removing the top 2 ml from the tube with the aid of a tube slicer, the infranatant portion was adjusted to a solution density corresponding to a solvent density of 1.019 g/ml by adding solid sodium bromide. A similar sequence of centrifugation followed by removal

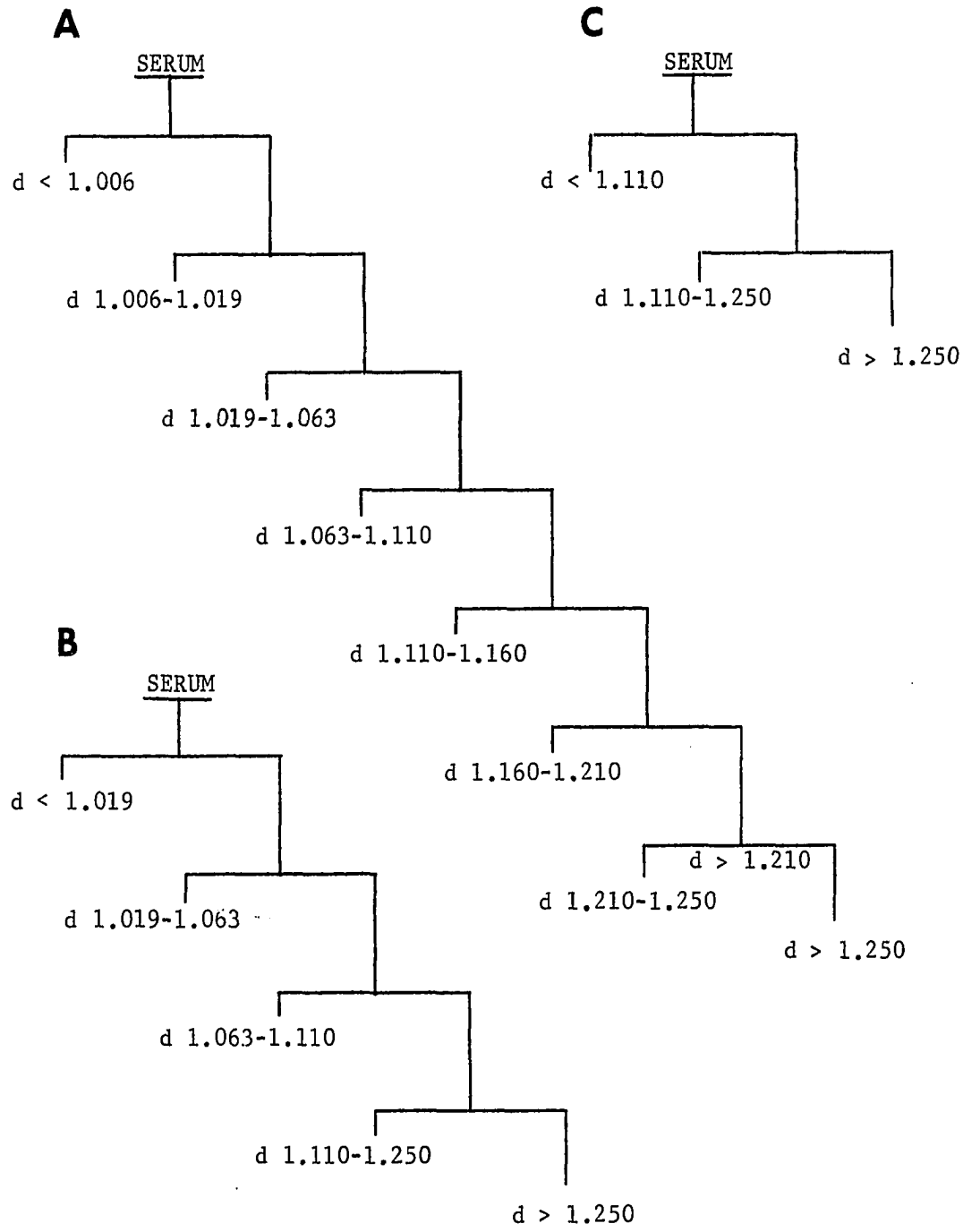


Fig. 2 - Flow charts for isolation of serum lipoproteins by sequential preparative ultracentrifugation.

of the supernatant fraction and adjustment of the solvent density of the infranatant portion to the next higher density was utilized to obtain the series of lipoprotein fractions, except that the fraction of d 1.210-1.250 g/ml was isolated by ultracentrifugation for 44 hr at 105,000 x g.

Isolation of lipoprotein fractions of broader density ranges (Figure 2, schemes B and C) followed the procedure outlined above, except that 11 ml of serum per tube was adjusted to the appropriate solution density by adding solid sodium bromide. All lipoprotein fractions of d < 1.110 g/ml (Figure 2, scheme B) were isolated by ultracentrifugation for 22 hr, with the exception that in instances in which this segment of the lipoprotein spectrum was obtained in one step ultracentrifugation was repeated at the same density for an additional 22 hr (Figure 2, scheme C). The fraction of d 1.110-1.250 g/ml was isolated in either instance by centrifugation for 44 hr.

Samples of each lipoprotein fraction, as well as of the infranatant portion obtained at d 1.210 or 1.250 g/ml, were removed for lipid and immunochemical analyses. Lipid recoveries in the lipoprotein fractions exceeded 80% of serum values. The remainder of each lipoprotein fraction was purified by resuspending the fraction in sodium bromide solution of appropriate density and centrifuging for 44 hr at 105,000 x g. After two washings, the lipoprotein fractions of d < 1.110 g/ml were found to be albumin-free by immunochemical analysis. One additional washing was necessary to provide an albumin-free preparation of fractions of d > 1.110 g/ml. Samples of the purified lipoprotein fractions were obtained for lipid, protein and immunochemical analyses.

Lipid Analysis

Lipoprotein fractions were extracted with a mixture of chloroform and methanol (2:1, v/v) according to the method of Folch and co-workers (187). The extract was evaporated in vacuo at room temperature, the lipid residue dissolved in chloroform and samples taken for lipid analyses. Cholesterol was determined by the Sperry and Webb modification (188) of the Schoenheimer-Sperry method. Lipid phosphorus was analyzed by the method of Fiske and Subbarow (189), after wet digestion by the procedure of Youngburg and Youngburg (190). The factor 25 was used to convert lipid phosphorus to "phospholipid". Triglyceride was determined by the method of Van Handel and Zilversmit (191), using tripalmitin as standard, or by the fluorometric method of Kessler and Lederer as applied to the Auto-analyzer (192).

Protein Analysis

Total protein in serum was determined by the biuret reaction (193). Serum protein fractions were separated by electrophoresis in a Beckman Microzone cell using cellulose acetate, stained with Ponceau S dye and quantitated by densitometric analysis in the Analytrol (Beckman Instruments, Inc.).

Protein in purified lipoprotein fractions was determined by the Lowry method (194), using canine serum albumin (Fraction V, Mann Research Laboratories, Inc., New York, N. Y.) as standard.

Delipidization Procedures

Samples of the purified lipoprotein fractions were dialyzed against changes of 0.15 M sodium chloride for a minimum of 44 hr at 4° C.

The dialyzed samples of HDL were then extracted with a mixture of ethanol and diethyl ether (3:1, v/v), followed by diethyl ether, according to the method of Scanu and co-workers (25) for total delipidization of lipoproteins. Partially delipidized lipoproteins were obtained using the method of Gustafson (195), in which lyophilized lipoprotein fractions are extracted repeatedly with n-heptane. After delipidization, the protein residue was dissolved in a buffer containing 0.1 M Tris-hydrochloride, pH 8, or borate, pH 9. Most of the protein present in HDL, but little of that in LDL, was recovered following total delipidization.

Preparation of Anti-Sera

Rabbits were injected intraperitoneally with purified lipoprotein fractions, i.e., VLDL (1-5 mg of protein), LDL of d 1.019-1.063 g/ml (8-24 mg of protein) and HDL of d 1.110-1.210 or 1.160-1.210 g/ml (10-60 mg of protein), as well as totally delipidized HDL of d 1.110-1.210 g/ml (10 mg of protein), mixed with complete Freund adjuvant (1:1, v/v). The rabbits were subsequently bled by cardiac puncture, usually at weekly intervals, and received no additional antigen unless the first series of injections failed to induce antibody production within 6 weeks. After the addition of 0.01% merthiolate, serum samples containing antibodies were divided into small portions and stored frozen at -10° C. The following commercial anti-sera were also utilized: rabbit anti-canine serum and anti-canine γ-globulin (Mann Research Laboratories, Inc.), and rabbit anti-human serum, anti-human α_1-lipoprotein and anti-human β-lipoprotein (Hoechst Pharmaceutical Co., Kansas City, Mo.).

Immunochemical Analyses

Double diffusion in gel, according to the Ouchterlony method (196), was performed at room temperature in high humidity chambers, using plates prepared with 1% Difco special noble agar in Veronal buffer, pH 8.6, containing 0.01% merthiolate. Development of precipitin lines was observed for a period of 4 days, although the process was usually completed within 24 hr.

Immunoelectrophoresis on agar plates was performed in Veronal buffer, pH 8.6, at room temperature, using 190 V and 25 mA for 60 min, according to the technique of Grabar and Williams as modified for microscopic slides by Scheidegger (197). Precipitin bands were recorded by direct photography of the plates and/or by staining of the immunoprecipitin lines for protein or lipid. Prior to staining, the plates were washed for 48 to 72 hr with several changes of 0.15 M sodium chloride and then distilled water. Lipids were stained with Oil Red O and protein with Amido Black B.

Gel Filtration Procedure

Samples of Sephadex G-200 were equilibrated in a buffer solution containing 0.1 M Tris-hydrochloride and 0.15 M sodium chloride, pH 8.0. This suspension was added to a 6-cm diameter column until a gel height of approximately 140 cm was attained. Lipoprotein fractions which had been dialyzed against the Tris-hydrochloride-sodium chloride buffer or serum samples were applied to the top of the gel and elution carried out at 4° C using the buffer solution. A flow rate of approximately 50 ml per hr was maintained and the eluate collected in 10.5-ml fractions. Each fraction was analyzed for optical density at 280 mμ using a Beckman Model DU spec-

trophotometer. The fractions common to a segment of the protein elution pattern were combined and subjected to ultrafiltration on Millipore XM-50 membranes (Millipore Filter Corp., Bedford, Mass.) as necessary to obtain uniform protein concentrations. Immunochemical analyses of each of the combined fractions were then performed.

Disc Electrophoresis

Samples containing 0.1 to 0.4 mg of protein were applied to a standard gel, prepared from premixed solutions, containing 7% polyacrylamide, pH 8.9, in 5 x 76 mm tubes. Electrophoresis in 8 M urea was performed using the Canalco Model 6 apparatus (Canal Industrial Corp., Rockville, Md.), in which 5 mA per tube was applied for 30 min. Samples were stained either for lipid with Sudan Black B applied prior to electrophoresis or for protein with Amido Black B applied after electrophoresis. In several studies, reagents of the QDL-Kit (Canal Industrial Corp.) were used to permit comparison of results obtained with those from conventional lipoprotein analysis.

Amino Acid Analysis

Lipoprotein samples containing approximately 2 mg of protein were hydrolyzed with 5.7 N HCl in evacuated tubes at 110° C for 24 hr or 72 hr. To each sample, 0.125 μmoles of norleucine was added as internal standard. The hydrolysates were extracted with chloroform three times to remove lipids. Amino acids in the chloroform extracts were recovered by three washings with distilled water. These washings were combined with the lipid-free hydrolysate and evaporated to dryness <u>in vacuo</u>. The residue was redissolved in 2 ml of 0.2 N citrate buffer, pH 2.2 and filtered through a

sintered glass filter. Samples of the filtrate were obtained for amino acid analysis.

Chromatography of neutral and acidic amino acids was performed on a 52-cm column of Beckman ion exchange resin PA-28. Elution with Aminex citrate buffers (Bio-Rad Laboratories, Richmond, Calif.) was completed within 65 min (pH 3.25) or 105 min (pH 4.25). Basic amino acids were separated on a 6-cm column of Beckman ion exchange resin PA-35. Elution with Aminex citrate buffer was completed in 60 min (pH 5.28). The Beckman Model 120C amino acid analyzer was calibrated using an amino acid reference mixture. Values obtained in the 24-hr and 72-hr hydrolysates were extrapolated to zero time.

Metabolic Studies

Incorporation Studies

Each of the four dogs, after a 20-hr fast, was given intravenously 175 μCi of L-lysine-U.L.-^{14}C (New England Nuclear Corp., Boston, Mass.). The ^{14}C-lysine used in all incorporation studies was obtained from a single lot. Blood samples, each of approximately 40 ml, were collected before and at intervals of 0.5, 1, 2, 4, 8 and 12 hr after isotope injection. A small portion of the samples obtained at 0.5 and 12 hr was used for hematocrit determination. Lipoproteins and albumin were isolated from the remainder of these and all other samples. The dogs remained fasting throughout the study.

Lipoprotein fractions were isolated from the serum according to scheme B in Figure 2. Purified fractions of d 1.019-1.063 g/ml (LDL) and d 1.110-1.250 g/ml were subjected to total delipidization. The lipid-free

protein residue was dissolved in 2 ml of 0.1 N NaOH and samples obtained for analysis of protein and radioactivity. Specific radioactivities were calculated as DPM per mg of protein.

Albumin was isolated from the infranatant fraction of d > 1.250 g/ml, using the trichloroacetic acid-ethanol extraction procedure described by Schwert (198). Proteins were precipitated with 10% trichloroacetic acid, washed twice with 5% trichloroacetic acid and extracted with 95% ethanol. The ethanol extract was dialyzed for 48 hr against several changes of 0.15 M sodium chloride and then distilled water. After removing a small amount of denatured protein by centrifugation, the albumin fraction was lyophilized, re-dissolved in 2 ml of 0.15 M sodium chloride and analyzed immunochemically. Samples were then obtained for determinations of protein and radioactivity and specific radioactivity values calculated, as described above.

Turnover Studies

One of the male dogs used in earlier studies served as donor of biologically labeled proteins. While fasting, this dog was injected with 5 mCi of L-lysine-U.L.-^{14}C and, after 6 hr, approximately 600 ml of blood was obtained for isolation of HDL and albumin.

The HDL fraction of d 1.110-1.250 g/ml was isolated according to scheme C in Figure 2 and purified. After dialysis for 48 hr against several changes of 0.15 M sodium chloride, immunochemical analysis demonstrated only HDL apolipoproteins, i.e., those producing A_1-, A_2- and A_3-immunoreactions. Samples of the purified HDL fraction were obtained for determination of protein and radioactivity, as well as for delipidization with chloroform and methanol (2:1, v/v). Radioactivity in the lipid ex-

tract represented less than 2% of the total lipoprotein radioactivity. The remainder of the purified HDL fraction was administered to dogs in which turnover measurements were performed. Although it appears that HDL are relatively stable lipoproteins, this fraction was maintained at 4° C throughout the preparative procedures and re-injection was accomplished within 48 hr after completion of dialysis.

Albumin was isolated from the infranatant fraction of d > 1.250 g/ml by the trichloroacetic acid-ethanol extraction procedure described above. After dialysis against 0.15 M sodium chloride, the albumin solution was found consistently to contain traces of apolipoprotein A. This contaminant was removed by gel filtration on a Sephadex G-200 column equilibrated with a buffer solution containing 0.1 M Tris-hydrochloride and 0.15 M sodium chloride, pH 8.0. The eluate containing albumin was concentrated by ultrafiltration on Millipore XM-50 membranes and then dialyzed against 0.15 M sodium chloride and distilled water. Immunochemical analysis of the purified albumin fraction demonstrated no evidence of apolipoproteins or other globulins. Samples of the purified albumin fraction were obtained for protein and radioactivity analyses and for chloroform-methanol extraction. Less than 2% of the albumin fraction radioactivity was removed in the chloroform-methanol extract. The remainder of the purified albumin fraction was lyophilized and stored in a desiccator until re-injected.

A sample of the purified HDL fraction containing ^{14}C-labeled apolipoprotein (45 X 10^3 DPM per kg; 20 X 10^3 DPM per mg of protein) or purified ^{14}C-labeled albumin (50 X 10^3 DPM per kg; 6.9 X 10^3 DPM per mg of protein) was injected intravenously over a 2-min interval into each of

the four dogs following a 20-hr fast. Blood samples were collected before (45 ml) and at intervals of 10 and 20 min (5 ml each) and 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 7 days (40 ml each) after injection of the labeled protein. Lipoprotein fractions were isolated according to scheme C in Figure 2. In studies in which ^{14}C-labeled HDL were injected, the fractions of d < 1.110 g/ml and d 1.110-1.250 g/ml were purified until albumin-free and then delipidized, analyzed for protein and radioactivity and specific radioactivity calculated. The infranatant fraction of d > 1.250 g/ml was dialyzed against 0.15 M sodium chloride and analyzed for radioactivity. In studies in which ^{14}C-labeled albumin was administered, the infranatant fraction of d > 1.250 g/ml was used for isolation of albumin following procedures described above.

Radioactivity Analyses

Measurements were performed using the Packard Tri-Carb liquid scintillation spectrometer equipped with external standardization (Packard Instrument Co., Downers Grove, Ill.). In the incorporation studies, 1 ml of NCS (Nuclear Chicago Corp., Des Plaines, Ill.) and 15 ml of diluted Permafluor (Packard Instrument Co.) liquid scintillation solution were added to vials containing the sample in 0.2 ml of 0.1 N NaOH. In the turnover studies, a maximum of 1.0 ml of sample was added to vials containing 20 ml of Gordon's scintillation mixture (199). Radioactivity values are presented as disintegrations per min (DPM).

Calculations

Radioactivity values were adjusted, where applicable, for the radioactivity removed in preceding blood samples during the course of a

study. Data obtained in the turnover studies were not corrected, however, for re-incorporation of ^{14}C-lysine, since this must be negligible due to the relatively long turnover times of the proteins studied and to the observation that less than 2% of amino acids injected intravenously are recovered in plasma proteins (200).

Plasma volume (intravascular space) was calculated by the isotopic dilution technique using plasma radioactivity values at 10 min after injection of the labeled protein. Values were obtained by the equation: plasma volume (ml) = total radioactivity injected (DPM)/plasma radioactivity (DPM) per ml. Half time (T/2) was calculated from the exponential slope representing disappearance of protein radioactivity from plasma. Radioactivity values obtained between the third and seventh day were used in calculating this slope using a linear regression equation (201). The total volume of distribution (total exchangeable space) was estimated by extrapolating the disappearance curve of protein radioactivity in plasma to zero time, according to the method of Sterling (202). Turnover time (T_t) was calculated by the formula 1.44 x T/2. The total exchangeable pool (i.e., "body pool", expressed as mg per kg body weight) of the protein was calculated using the total exchangeable space (ml), the serum concentration of the protein (mg per ml) and body weight (kg). The replacement rate (i.e., absolute turnover rate, expressed as mg per kg per day) was calculated by multiplying the total exchangeable pool by the fractional turnover time ($1/T_t$, day^{-1}).

Liver Function Tests

Bromsulphalein (Hynson, Westcott and Dunning, Inc., Baltimore, Md.) was injected intravenously at a dose of 5 mg per kg. Serum samples

obtained after 15, 30 and 45 min were analyzed using the method of Rosenthal and White (203).

Bilirubin in serum was determined according to the method of Malloy and Evelyn, as modified by Kingsley and co-workers (204).

CHAPTER IV

RESULTS

Apolipoprotein and Lipid Composition of Serum Lipoprotein Fractions

Distribution of Serum Lipids in Lipoprotein Fractions

The percentage distribution of serum cholesterol, phospholipid and triglyceride among the lipoprotein fractions was obtained using results of lipid analyses of the fractions prior to purification and the results were corrected for the actual recovery (Table 3). Lipoproteins of $d > 1.063$ g/ml contained approximately 85% of the cholesterol and 90% of the phospholipid present in serum. Lipoproteins of $d < 1.063$ g/ml contained only 15% of the serum total cholesterol and 10% of the serum phospholipid, but more than 60% of the serum triglyceride. The corresponding quantitative data are shown in Figure 3.

The lipid and protein composition of purified serum lipoprotein fractions is presented in Table 4. The content of protein increased and the ratio of phospholipid to protein decreased with increasing hydrated density of the lipoproteins.

Immunochemical Characterization of Purified Serum Lipoprotein Fractions

The apolipoproteins in purified lipoprotein fractions were investigated using anti-serum produced against purified canine serum lipo-

TABLE 3

PERCENTAGE DISTRIBUTION OF SERUM LIPIDS IN CANINE LIPOPROTEIN FRACTIONS[a]

Serum Lipid	Lipoprotein Fraction (g/ml)						
	< 1.006	1.006–1.019	1.019–1.063	1.063–1.110	1.110–1.160	1.160–1.210	> 1.210
	VLDL	← LDL →		← HDL →			VHDL
Cholesterol	1.7±0.9 (3)[b]	0.7±0.5 (6)	12.7±2.7 (6)	22.9±5.8 (8)	37.0±9.8 (7)	19.3±3.8 (6)	5.8±1.6 (7)
Phospholipid	1.1±0.5 (4)	0.4±0.1 (6)	6.2±3.3 (6)	22.4±5.7 (8)	33.8±7.1 (5)	22.0±6.0 (8)	13.8±3.0 (8)
Triglyceride	20.5±7.2 (5)	6.8±6.4 (6)	35.6±6.0 (6)	6.1±4.3 (8)	17.4±8.8 (6)	9.3±2.8 (8)	4.0±2.4 (8)

[a]Values are presented as the mean ± standard error. The lipoprotein fractions were isolated according to scheme A in Figure 2.

[b]Number of samples analyzed.

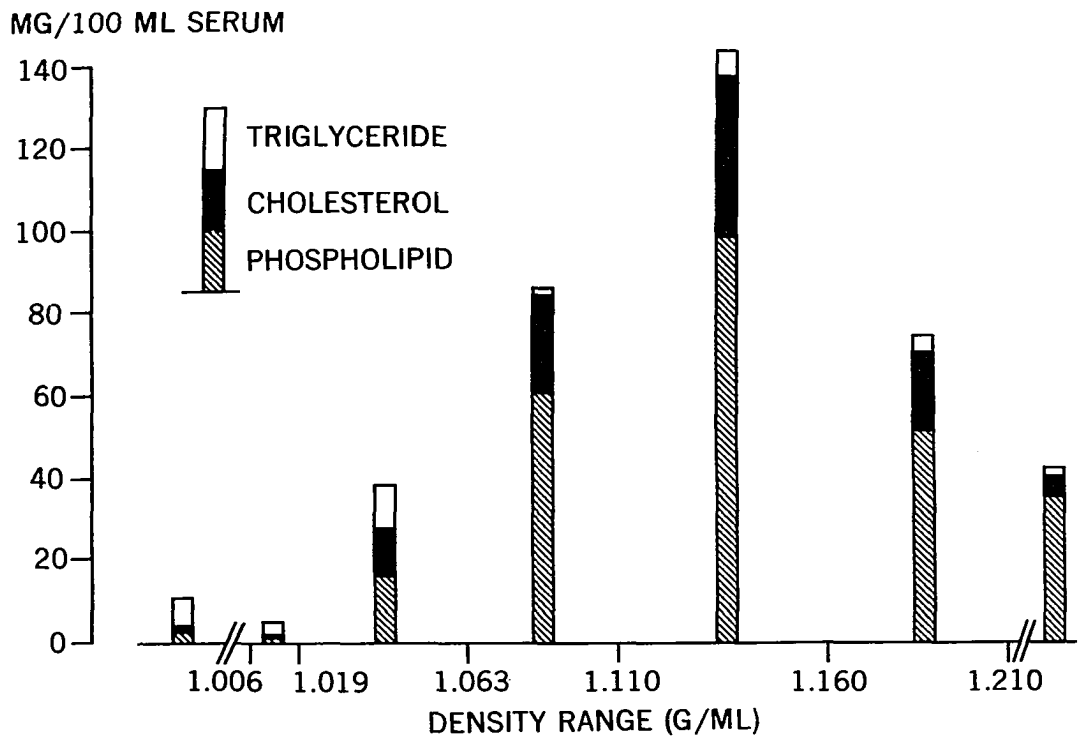


Fig. 3 - Quantitative distribution of lipids in serum lipoprotein fractions.

TABLE 4

COMPOSITION OF PURIFIED CANINE SERUM LIPOPROTEIN FRACTIONS[a]

Lipoprotein Fraction	No. of Samples	Percent of Protein and Lipids				Phospholipid / Protein
		Protein	Cholesterol	Phospholipid	Triglyceride	
VLDL						
d < 1.006	3	12.3±4.4	9.0±0.8	18.3±5.9	60.2±10.7	1.50±0.08
LDL						
d 1.019-1.063	7	30.9±2.8	22.1±5.8	31.0±6.1	16.2± 5.8	1.02±0.21
HDL						
d 1.063-1.110	10	34.8±3.4	19.6±3.5	37.7±3.5	7.9± 3.6	1.10±0.18
d 1.110-1.160	8	46.7±3.4	17.2±1.8	33.4±1.7	2.8± 0.3	0.72±0.08
d 1.160-1.210	8	50.2±1.4	12.8±1.1	34.3±2.1	2.7± 0.7	0.69±0.05
VHDL						
d 1.210-1.250	7[b]	--	--	--	--	0.47±0.09

[a]Values, presented as the mean ± standard error, are expressed as percent of the sum of the weights of protein and lipids in each sample.

[b]Analyses of protein and lipid phosphorus only are available, due to the very limited amounts of these samples.

protein fractions. Each anti-serum preparation was characterized by the immunoprecipitin lines observed when reacted with canine serum, plasma or purified serum lipoprotein fractions (Table 5). Anti-serum obtained from rabbits given purified HDL fractions of d 1.110-1.210 g/ml or 1.160-1.210 g/ml, when reacted with serum gave three immunoprecipitin lines, designated A_1, A_2 and A_3 (Figure 4). In some cases a fourth immunoprecipitin line, designated A_X, was also obtained. The A_1- and A_2-precipitin lines had α_1-mobility on immunoelectrophoresis, while the A_3-line had α_2-mobility. The A_X-precipitin reaction was observed as an arc which migrated toward the cathode.

Anti-serum obtained following the administration of purified LDL (d 1.019-1.063 g/ml) gave a single immunoprecipitin line, designated B, when reacted with serum. This reaction was characterized by immunodiffusion analyses as a line which, after 48 hr of development, appeared to have forked ends. During immunoelectrophoresis, the B-reaction migrated as a diffuse immunoprecipitin arc to the α_2- or β-region (the B-reaction resembled that obtained with anti-serum to purified VLDL shown in Figure 5).

Anti-serum to purified VLDL contained antibodies which reacted with serum to give the B- and A_X-reactions and also a third immunoprecipitin line, the X-reaction (Figure 5). The X-precipitin line had an α_2-migration on immunoelectrophoresis and in rare instances appeared as a double line. The X-precipitin reaction was non-identical to the A_3-reaction.

Rabbit anti-canine serum gave a B-reaction and, in some preparations, an A_1-reaction when reacted with purified LDL or HDL. Rabbit

TABLE 5

CHARACTERIZATION OF ANTI-SERA PRODUCED IN RABBITS IN RESPONSE TO PURIFIED CANINE SERUM LIPOPROTEIN FRACTIONS

Anti-Serum	Antigenic Components					
	A_1	A_2	A_3	A_x	B	X
anti-HDL[a]	+	+	+	+	-	-
anti-LDL	-	-	-	-	+	-
anti-VLDL	-	-	-	+	+	+

[a]Produced by injecting purified HDL fractions of d 1.110-1.210 or 1.160-1.210 g/ml.

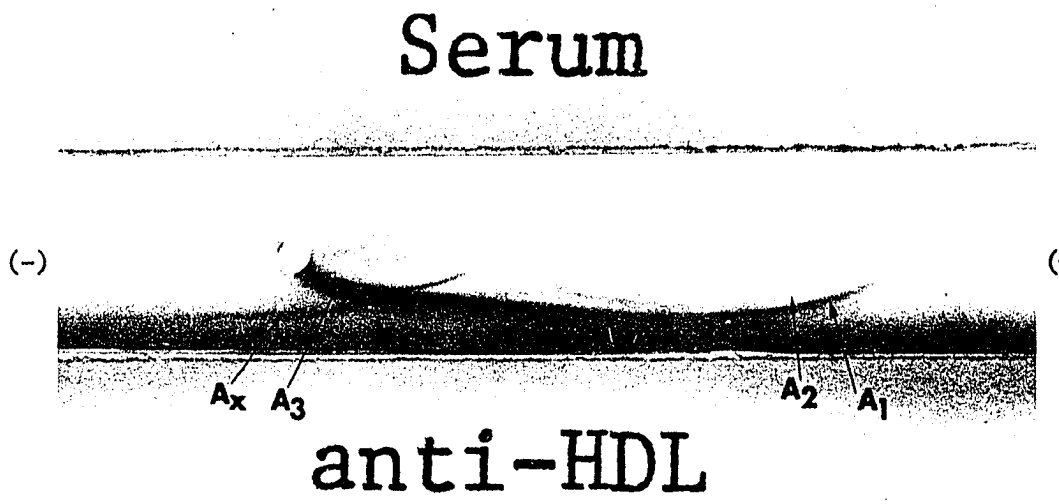


Fig. 4 - A_1-, A_2-, A_3- and A_x-immunoprecipitin lines stained with Oil Red O, demonstrating the presence of lipids.

Anti-HDL serum containing these antibodies was produced in rabbits following the administration of purified dog HDL.

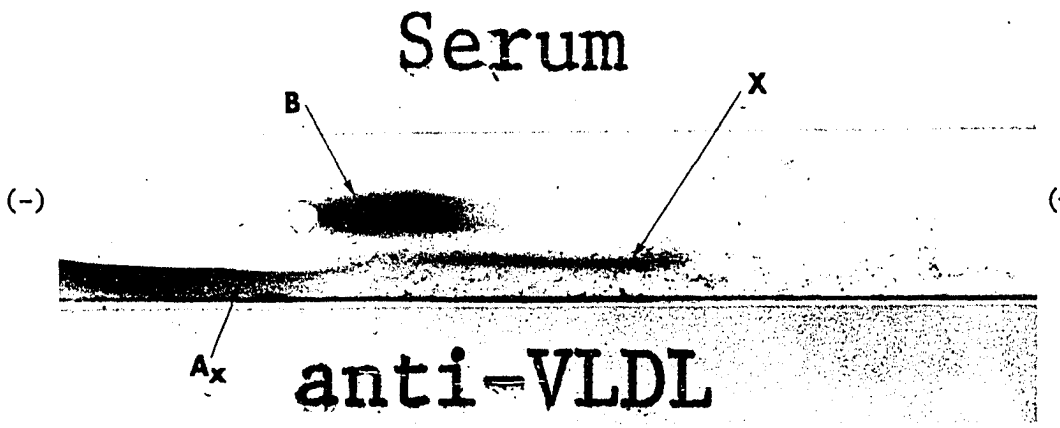


Fig. 5 - A_x-, B- and X-immunoprecipitin lines stained with Oil Red O, demonstrating the presence of lipids.

Anti-VLDL serum containing these antibodies was produced in rabbits following the administration of purified dog VLDL.

anti-canine γ-globulin serum reacted with canine serum to produce at least two precipitin lines, one of which was the A_X-reaction.

The distribution of apolipoproteins in purified lipoprotein fractions, as demonstrated by immunochemical analysis, is presented in Table 6. Precipitin lines characteristic of the A_X-, B- and X-immunoreactions were observed when purified VLDL was reacted with anti-VLDL (Figure 6). Although several purified LDL preparations gave only the B-reaction (Figure 7), evidence of apolipoprotein heterogeneity in the fractions isolated in the LDL density range was observed in analyses of other purified LDL preparations in which A- and X-reactions were also demonstrated (Figure 8).

Precipitin lines indicative of A_1-, A_2- and A_3-reactions were observed in purified HDL (Figure 9). The A_1-precipitin line, which was observed as the dominant reaction in this fraction, and the A_2-reaction were demonstrated in all HDL preparations. The A_3-reaction could be demonstrated only in instances in which the concentration of HDL in the preparation employed as antigen was similar to that in serum. Only the A_1-precipitin line was detectable after dilution of HDL. Some purified HDL fractions of d 1.063-1.110 g/ml gave B- and X-reactions. In very rare instances, the X-precipitin line was also found in purified HDL of d 1.110-1.160 g/ml. The proportions of B- and X-reacting components decreased with increasing density, whereas the proportion of those characterized by A_1, A_2 and A_3 increased (Figure 10).

A_3- and A_X-immunoreactions were demonstrated consistently in the infranatant portion obtained following ultracentrifugation of serum at d 1.250 g/ml. The A_X-reaction was also found in unpurified, but not

TABLE 6

DISTRIBUTION OF APOLIPOPROTEINS IN PURIFIED LIPOPROTEIN FRACTIONS OF CANINE SERUM

Lipoprotein Fraction	Immunoprecipitin Reactions[a]					
	A_1	A_2	A_3	A_x	B	X
VLDL						
d < 1.006	-	-	-	+	+	+
LDL[b]						
d 1.019-1.063	±	±	-	-	+	±
HDL						
d 1.063-1.110	+	+	+	-	±	±
d 1.110-1.160	+	+	+	-	-	-
d 1.160-1.210	+	+	+	-	-	-
VHDL						
d 1.210-1.250	+	+	+	-	-	-
d > 1.250	-	-	+	+	-	-
Fresh serum or plasma	+	+	+	+	+	+

[a]The following symbols are used to indicate the immunoprecipitin reactions observed: + = reaction demonstrated consistently; ± = reaction demonstrated but not consistently; - = no reaction detected.

[b]The LDL fraction of d 1.006-1.019 g/ml contained very small amounts of lipoproteins which were insufficient for reliable analysis of the purified fraction.

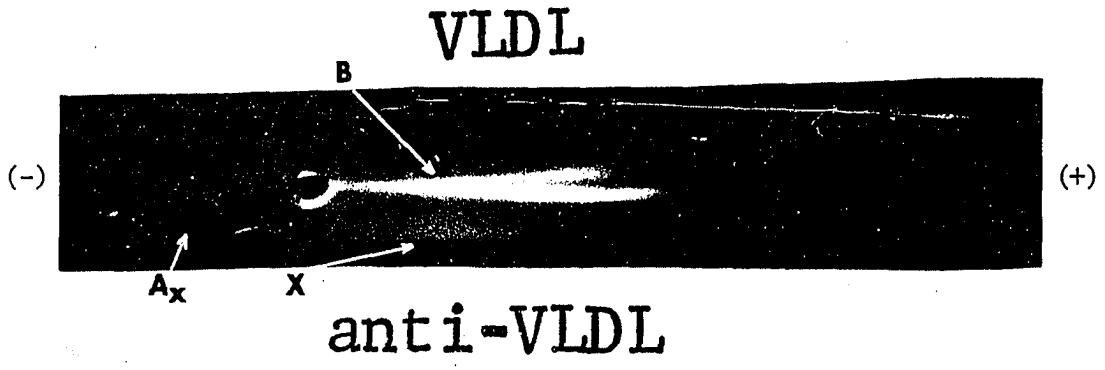


Fig. 6 - Immunoelectrophoretic analysis of purified VLDL demonstrating A_X-, B- and X-reactions.

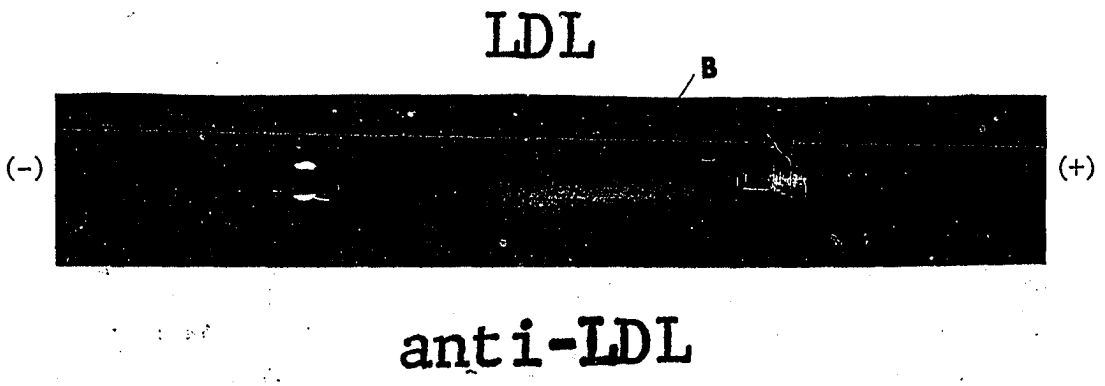
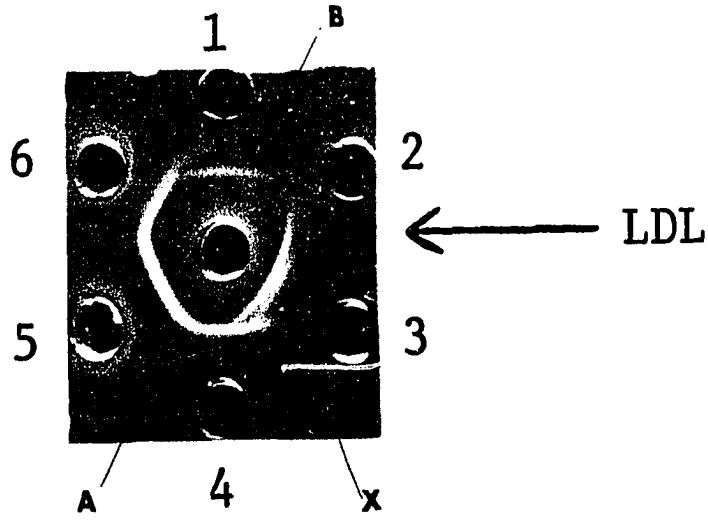


Fig. 7 - Immunoelectrophoretic analysis of purified LDL.

Antibodies producing the B-immunoprecipitin reaction when reacted with LDL were found in anti-LDL.



1. anti-CANINE SERUM
2. anti-GAMMA-GLOBULIN
3. anti-VLDL
4. anti-HDL (3)
5. anti-HDL (10)
6. anti-HDL (9)

Fig. 8 - Immunodiffusion analysis of purified LDL preparations, demonstrating A- and X-reactions in addition to the characteristic B-reaction.

In parenthesis are the identification code numbers of rabbits from which the anti-sera were obtained.

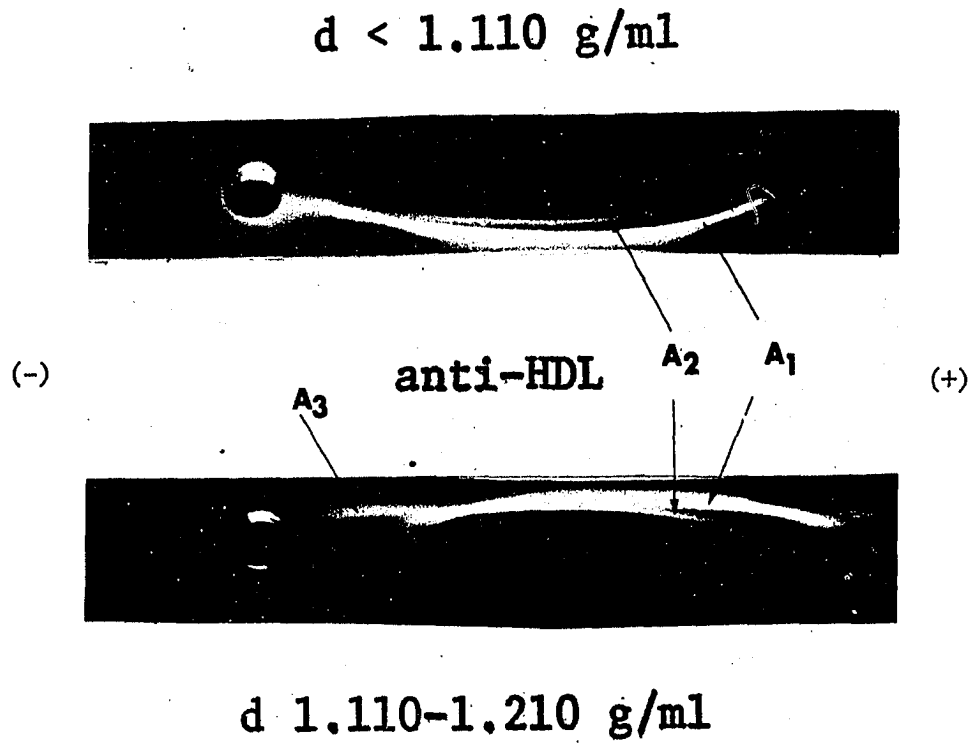


Fig. 9 - Immunoelectrophoretic analysis of purified HDL fractions demonstrating A_1-, A_2- and A_3-reactions.

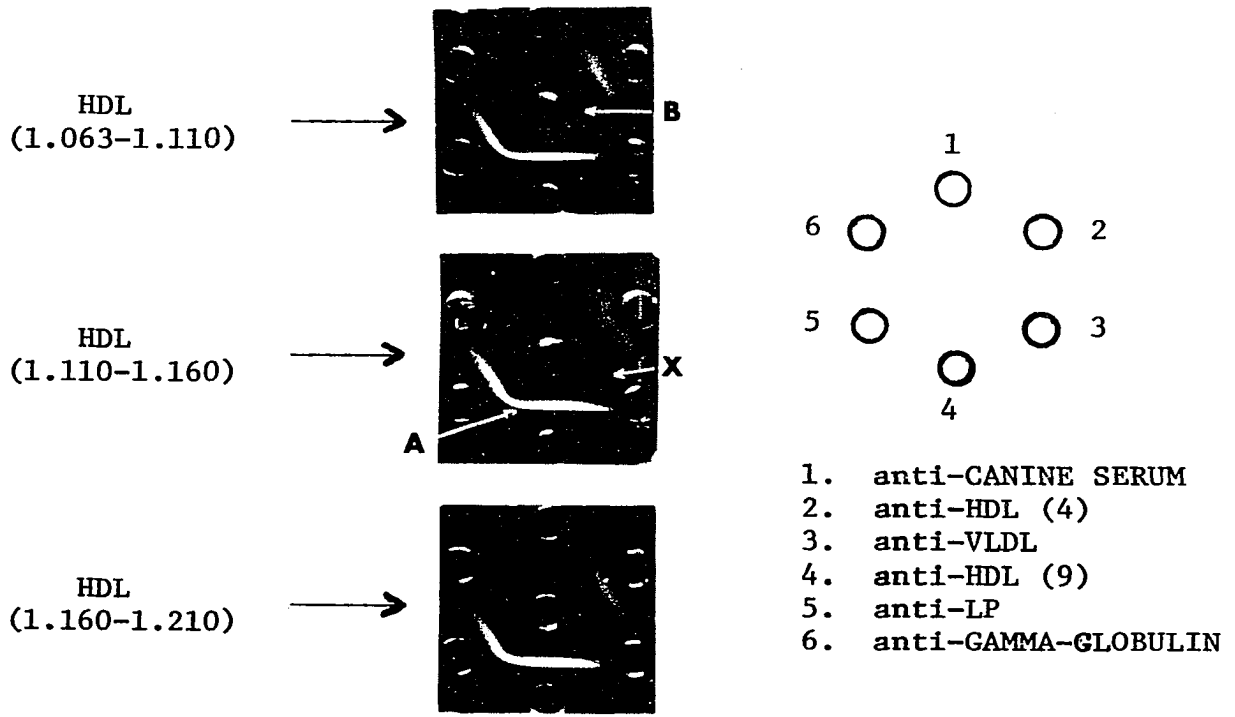


Fig. 10 - Immunodiffusion analysis of purified HDL subfractions.

B- and X-reactions were demonstrated in the subfraction of d 1.063-1.110 g/ml, but only a faint X-immunoprecipitin line in the subfraction of d 1.110-1.160 g/ml, in addition to the characteristic A -reaction. The HDL subfraction of d 1.160-1.210 g/ml gave only the A-reaction. In parenthesis are the identification code numbers of rabbits from which the anti-sera were obtained.

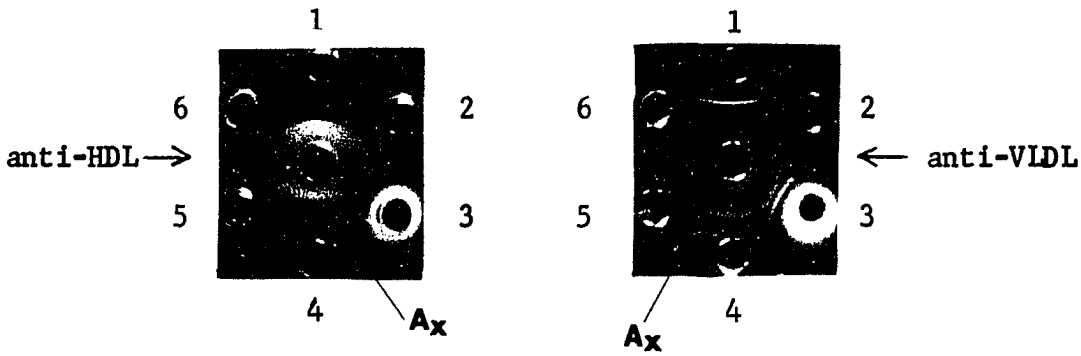
purified HDL preparations, as well as in the washings obtained during ultracentrifugal purification of HDL (Figure 11).

Each precipitin line was obtained with fresh serum or plasma. These precipitin lines generally reacted with Oil Red O, except that in several instances the A_X-line either failed to react or the positive reaction was limited to its anodal end. Although delipidization of the lipoprotein fractions resulted in loss of reactivity with Oil Red O, the immunoprecipitin reactions were clearly evident in all instances except for the B-reaction, which was not observed after total delipidization due to apparent very limited water solubility of apolipoprotein B.

Immunochemical Characterization of Lipoproteins Isolated by Gel Filtration

Two major peaks and one minor peak were observed when purified lipoproteins of d < 1.250 g/ml were separated by gel filtration on Sephadex G-200 (Figures 12 and 13). Immunochemical analyses of the lipoproteins in Fractions 1 and 2 eluted from the column demonstrated primarily a B-reaction, although A_1-, A_2-, A_3-, A_X- and X-reactions were also observed. Lipoproteins which exhibited A_1-, A_2- and A_3-immunoreactions, but failed to give A_X-, B- and X-reactions, were eluted in Fractions 4 to 6. The immunologic characteristics of the lipoproteins eluted in the first peak are similar to lipoproteins of d < 1.110 g/ml, while the lipoproteins eluted subsequently resemble HDL of d > 1.110 g/ml.

When whole serum or lipoprotein-free serum, i.e., serum from which lipoproteins of d < 1.250 g/ml had been removed, was subjected to gel filtration, the first two of the three major peaks observed coincided in their elution pattern to the two major peaks isolated from the purified



1. SERUM
2. SERUM (1:100)
3. VLDL (PURIFIED)
4. HDL (UNPURIFIED)
5. d > 1.250 (1:100)
6. INFRANATANT FROM HDL PURIFICATION

Fig. 11 - Immunodiffusion analysis demonstrating the A_X-immunoreaction.

An identical A_X-precipitin line was observed in fresh serum, purified VLDL, unpurified HDL, the washings obtained during purification of HDL and the serum sample from which lipoproteins of d < 1.250 g/ml had been removed. Antibodies producing the A_X-reaction were present in anti-VLDL and anti-HDL serum.

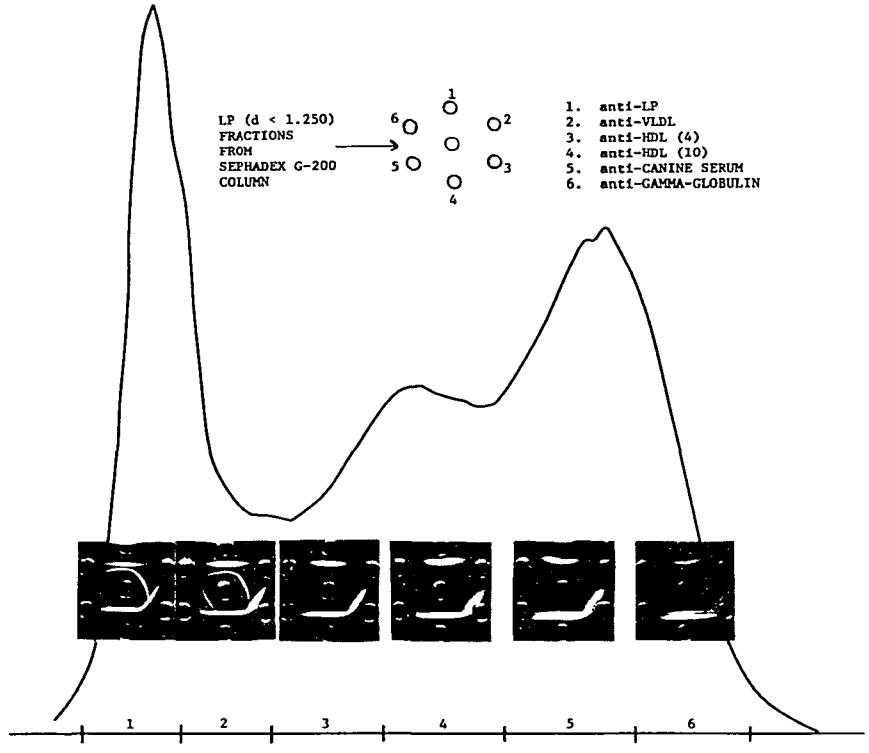


Fig. 12 - Immunodiffusion analysis of lipoprotein fractions separated by gel filtration on Sephadex G-200 column.

The albumin-free preparation of lipoproteins (d < 1.250 g/ml) applied to this column contained 131 mg of protein. Elution volumes: prior to Fraction 1, 868 ml; Fractions 1-6 were 116, 95, 116, 126, 137 and 116 ml, respectively (void volume, 952 ml).

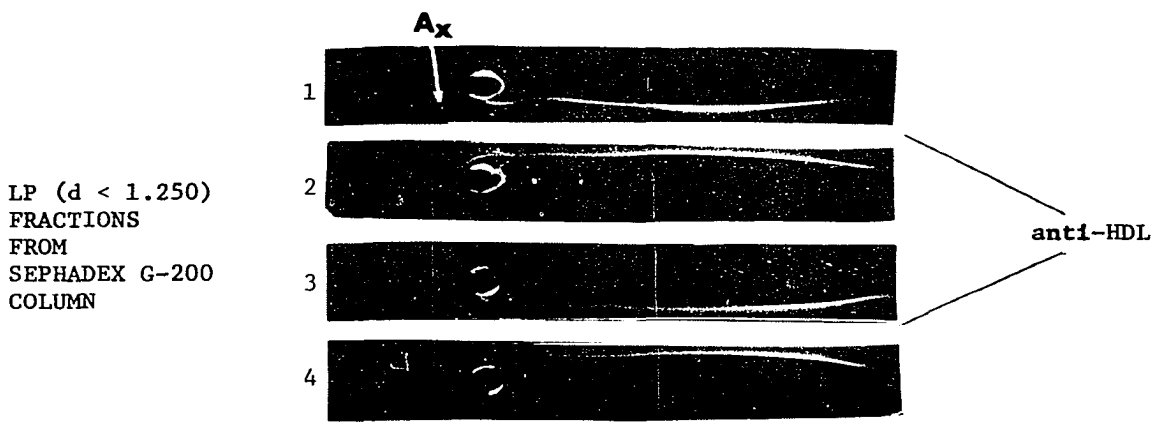


Fig. 13 - Immunoelectrophoretic analysis of lipoprotein fractions separated by gel filtration on Sephadex G-200 column demonstrating A_X-reaction.

Identification of fractions can be seen on Fig. 12.

lipoprotein fraction of d < 1.250 g/ml. Immunoreactions characteristic of γ-globulins were observed with the first and second major peaks, while albumin was found in the third major peak. The immunoreactions of lipoproteins in the first two peaks obtained from whole serum were similar to those demonstrated in the corresponding peaks described in the preceding paragraph, with the exception that the A_X-reaction was also demonstrated in the second peak isolated from whole serum. Lipoprotein immunoreactions in the eluate obtained from lipoprotein-free serum were limited to an A_X-reaction in the first two peaks and almost negligible A_1-, A_2- and A_3-reactions in the second peak.

Gel filtration of partially delipidized preparations of purified lipoprotein fractions indicated similar order of elution of apolipoprotein components as described above for native lipoproteins. However, after partial delipidization it was more apparent that the proportion of the A_3-reacting component, relative to the A_1- and A_2-components, increased in the fractions eluted later (Figure 14). The disc electrophoretic pattern of these fractions in 7% polyacrylamide gel revealed several protein bands to which immunochemical characteristics of the fractions could be related only to a limited extent (Figure 15). Although differences among fractions in the proportions of the bands related to the A-antigenic components could be observed, the bands did not appear to represent separated apolipoproteins of A_1-, A_2- or A_3-antigenic components. Similar conclusions were reached when intact or partially delipidized lipoprotein fractions were subjected to disc electrophoresis using the larger pore-size gel designed for routine lipoprotein analysis (QDL-Kit).

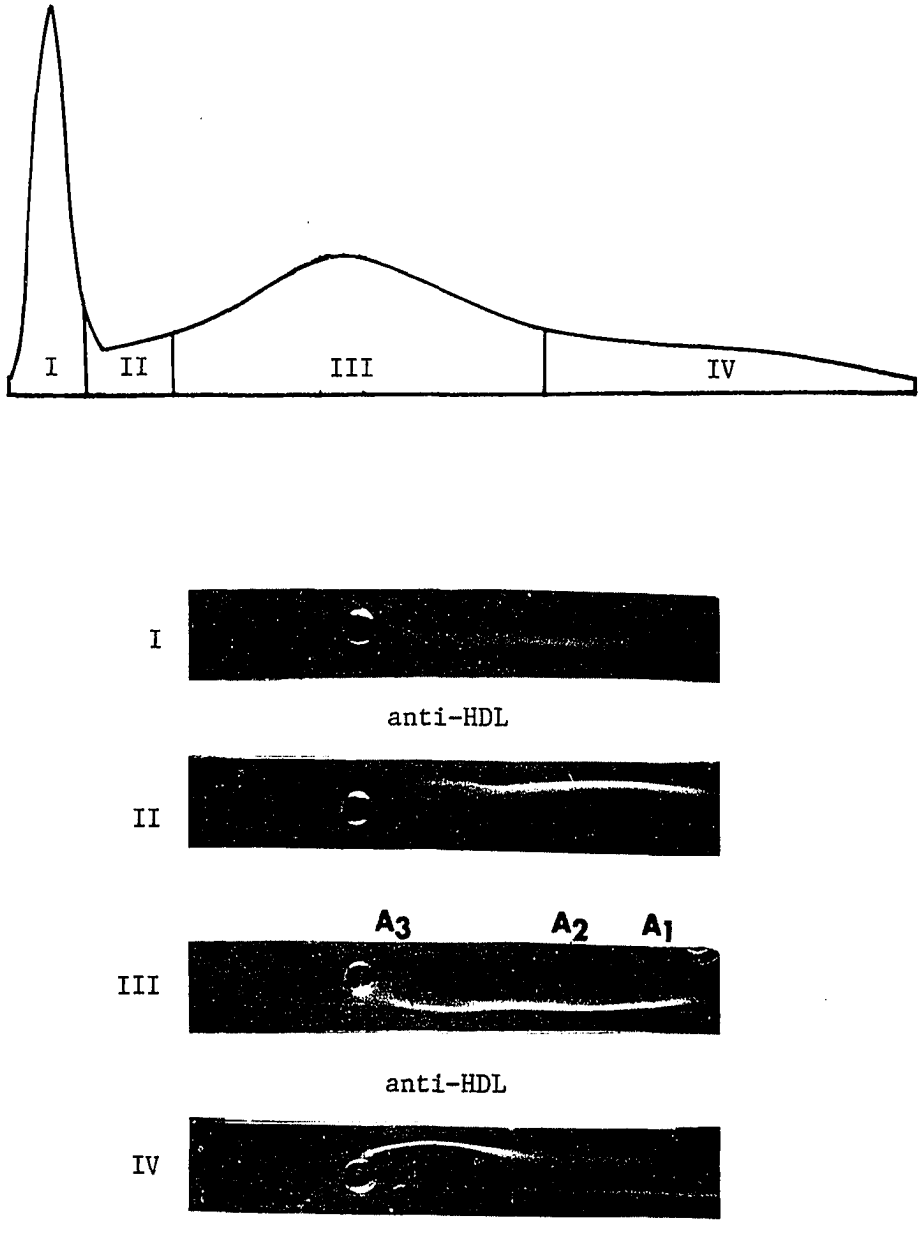


Fig. 14 - Immunoelectrophoretic analysis of lipoprotein fractions separated by gel filtration on Sephadex G-200 column.

A partially delipidized preparation of purified lipoproteins ($d < 1.110$ g/ml) was applied to the column. The first peak (I) eluted with the void volume. The fractions were subjected to immunoelectrophoresis in uniform protein concentration (1 mg/ml). Note the apparent differences between the proportions of apolipoprotein components characterized by the A_1-, A_2- and A_3-immunoprecipitin lines.

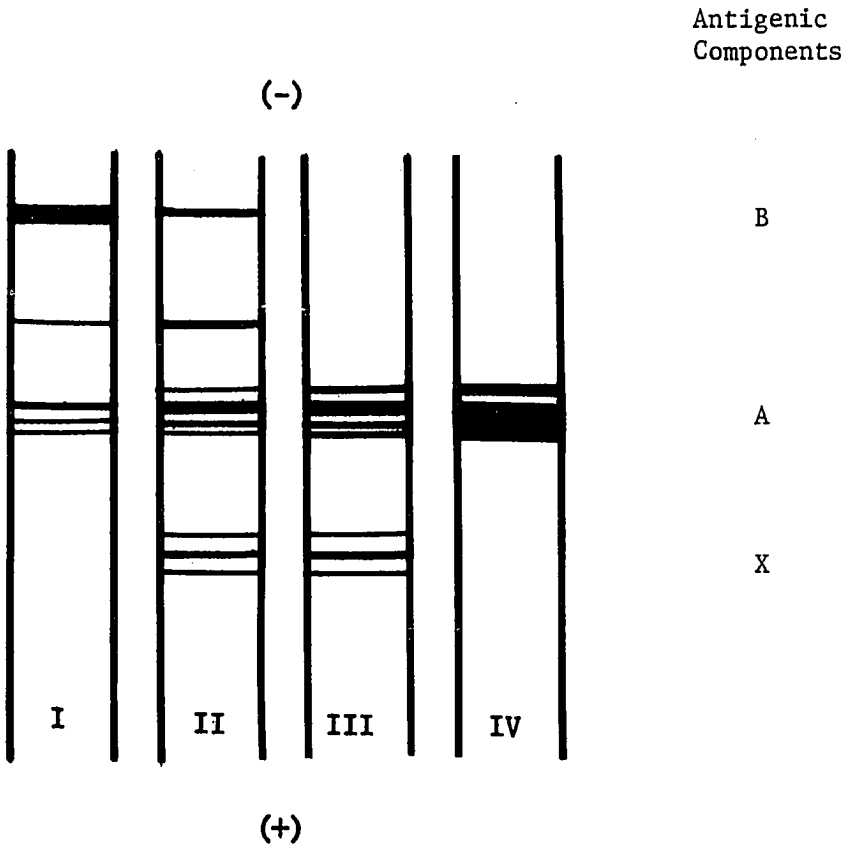


Fig. 15 - Analytical polyacrylamide electrophoresis pattern of lipoprotein fractions separated by gel filtration on Sephadex G-200 column.

Disc electrophoresis in 7% polyacrylamide gel, pH 8.9, containing 8 M urea; gels were stained with Amido Black 10B. After electrophoretic separation disc segments containing unstained gels were embedded in agar and subjected to immunochemical analyses.

Fractions I through IV are identified in Fig. 14. These fractions gave the following immunoreactions:

	A_1	A_2	A_3	A_x	B	X
Fraction I	+	+	+	+	+	-
Fraction II	+	+	+	+	+	+
Fraction III	+	+	+	-	-	+
Fraction IV	+	+	+	-	-	-

Effect of Methyltestosterone on the Apolipoprotein and Lipid Composition of Serum Lipoprotein Fractions

No change in body weight was observed during methyltestosterone administration and the dogs remained in good health throughout the study. A relatively constant new low level of serum lipids was attained after 4 weeks of methyltestosterone administration, although a downward trend was evident within 1 or 2 weeks. Changes in the opposite direction occurred at the same rate after the termination of treatment (Figure 16). Methyltestosterone produced a statistically significant reduction ($p < 0.001$) in serum total cholesterol, phospholipid and triglyceride concentrations in each dog (Table 7). The cholesterol to phospholipid ratio was decreased by methyltestosterone to an average value of 86% of control. Methyltestosterone also lowered the apolipoprotein concentration to a mean value of 40% of control.

The concentrations of apolipoproteins and lipids were lower in all lipoprotein fractions during methyltestosterone administration (Figure 17). This reduction was quantitatively greatest in the HDL fraction of d 1.110-1.250 g/ml, which comprises the major lipoprotein fraction in dog plasma.

The percentage distribution of apolipoproteins and lipids among the lipoprotein fractions is presented in Table 8. A greater proportion of each component, particularly cholesterol and triglyceride, was found in the fraction of $d < 1.019$ g/ml during methyltestosterone administration. Methyltestosterone also increased the proportion of total serum triglyceride found in the LDL fraction, but decreased the proportions of total apolipoprotein, cholesterol and phospholipid. The HDL fraction of d 1.063-1.110 g/ml contained a slightly greater portion of the total apo-

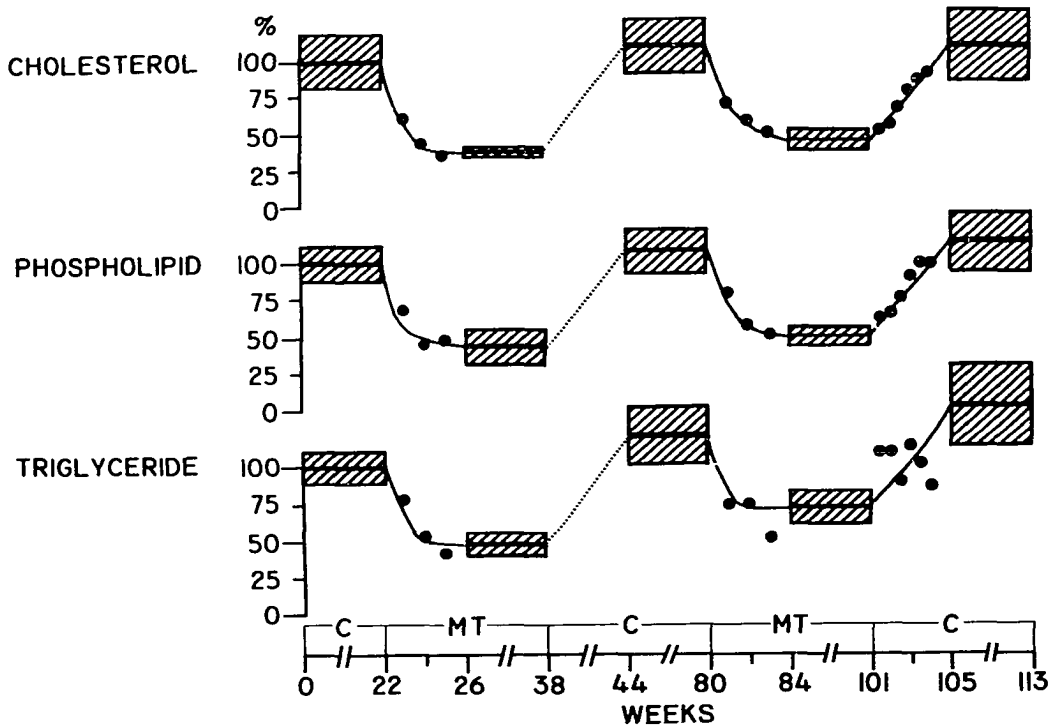


Fig. 16 - Effect of methyltestosterone treatment on serum lipid levels.

C = control period, MT = methyltestosterone treatment. 100% = serum lipid levels of each dog during the first control period. Shaded area = mean ± standard error of four dogs in steady state condition. Solid and dotted lines = transitory periods between two steady states with or without lipid determinations, respectively.

TABLE 7

BODY WEIGHT AND APOLIPOPROTEIN AND LIPID CONCENTRATIONS IN CANINE SERUM LIPOPROTEINS BEFORE AND DURING METHYLTESTOSTERONE ADMINISTRATION[a]

Dog	Body Weight (kg)	Lipoprotein Components (mg/100 ml)			
		Cholesterol	Phospholipid	Triglyceride	Protein[b]
104 (Male)					
Control	23.3±0.5 (49)[c]	249.5±2.8 (44)	442.5± 7.5 (40)	49.1±2.1 (42)	494
Methyltestosterone	23.4±0.1 (21)	78.0±3.7 (19)	182.5±10.0 (19)	25.4±2.1 (19)	223
Difference[d]	n.s.	$p < 0.001$	$p < 0.001$	$p < 0.001$	
181 (Female)					
Control	22.4±0.2 (47)	177.0±4.6 (41)	335.0± 7.5 (40)	32.9±1.9 (42)	416
Methyltestosterone	22.5±0.1 (21)	66.7±2.2 (19)	155.0± 7.5 (18)	16.4±1.3 (19)	175
Difference	n.s.	$p < 0.001$	$p < 0.001$	$p < 0.001$	
233 (Male)					
Control	24.9±0.1 (48)	114.8±2.0 (44)	255.0± 5.0 (44)	28.5±1.3 (41)	432
Methyltestosterone	25.3±0.1 (13)	48.8±1.9 (12)	122.5± 5.0 (12)	12.2±1.0 (12)	167
Difference	n.s.	$p < 0.001$	$p < 0.001$	$p < 0.001$	
236 (Male)					
Control	18.8±0.1 (37)	124.3±3.9 (36)	272.5± 7.5 (34)	35.9±1.6 (34)	524
Methyltestosterone	18.3±0.1 (12)	55.1±3.0 (12)	122.5± 5.0 (12)	16.2±0.9 (12)	204
Difference	n.s.	$p < 0.001$	$p < 0.001$	$p < 0.001$	

[a]Values presented are the mean ± standard error.

[b]Apolipoprotein values were calculated using the phospholipid:protein ratio for the purified lipoprotein fractions (see Table 9).

[c]Number of observations or analyses.

[d]Statistical significance of difference between values obtained during control and methyltestosterone treatment periods is indicated (n.s. = not significant, i.e., $p \geq 0.05$).

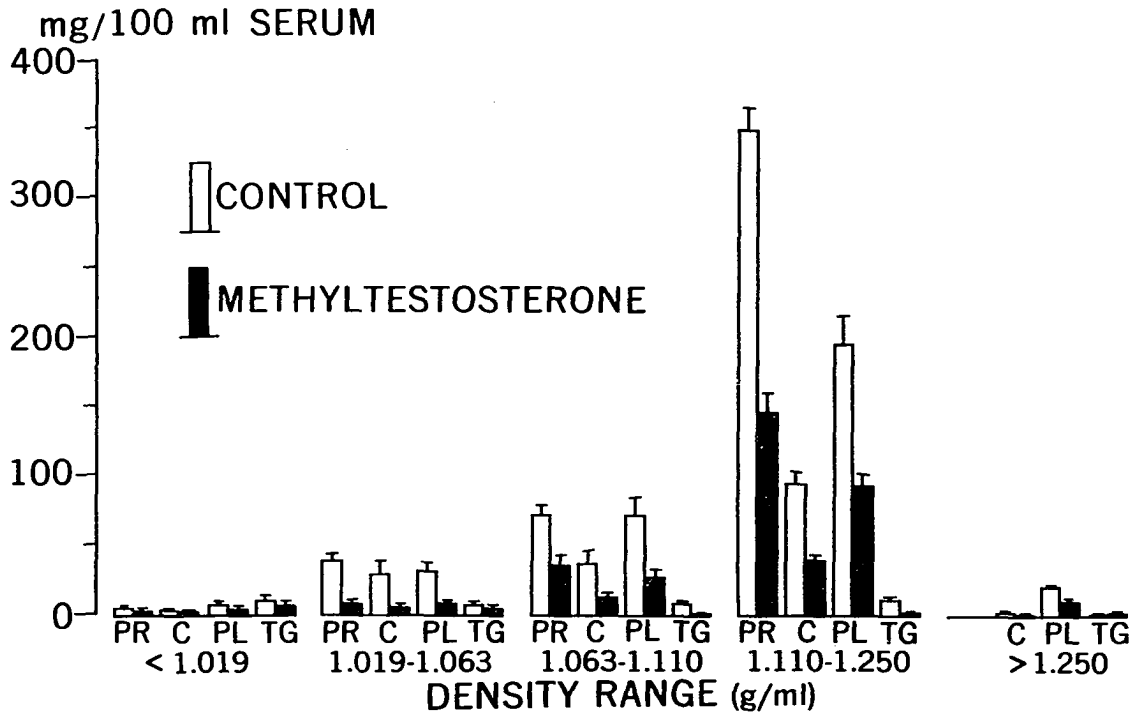


Fig. 17 - Quantitative changes in apolipoproteins and lipids in serum lipoprotein fractions produced by methyltestosterone treatment.

PR = protein, C = cholesterol, PL = phospholipid, TG = triglyceride.

TABLE 8

EFFECT OF METHYLTESTOSTERONE ON PERCENTAGE DISTRIBUTION OF APOLIPOPROTEINS AND LIPIDS AMONG SERUM LIPOPROTEIN FRACTIONS[a]

Lipoprotein Fraction	Lipoprotein Component	
	Protein[b]	Cholesterol[c]
d < 1.019		
Control	1.1±0.2	2.5±0.8
Methyltestosterone	1.8±0.8	5.0±0.6
Difference[d]	n.s.	p < 0.05
d 1.019-1.063		
Control	8.7±0.7	16.5±2.4
Methyltestosterone	4.4±1.4	8.5±1.3
Difference	p < 0.05	p < 0.05
d 1.063-1.110		
Control	15.4±0.5	21.8±1.4
Methyltestosterone	18.3±4.2	19.9±1.8
Difference	n.s.	n.s.
d 1.110-1.250		
Control	74.9±0.8	58.5±3.4
Methyltestosterone	75.6±2.8	65.7±3.1
Difference	n.s.	n.s.
d > 1.250		
Control	--	0.9±0.1
Methyltestosterone	--	1.0±0.1
Difference	--	n.s.

[a]Values from the four dogs are presented as the mean ± standard error.

[b]Apolipoprotein values were calculated using the phospholipid concentration in the unwashed lipoprotein fraction and the phospholipid to protein ratio of the purified lipoprotein fraction. The protein content in the lipoprotein fraction is presented as a percentage of the total amount recovered in the fractions of d < 1.250 g/ml.

[c]The content of each lipid in the unwashed lipoprotein fraction is presented as a percentage of the total amount of that lipid recovered in all fractions.

[d]Statistical significance of difference between values obtained during control and methyltestosterone treatment periods is indicated (n.s. = not significant, i.e., $p \geq 0.05$).

TABLE 8--Continued

Phospholipid[c]	Triglyceride[c]
1.9±0.4	26.5±4.8
2.8±0.7	36.7±5.2
n.s.	n.s.
9.5±1.3	21.8±4.3
6.1±1.0	32.4±6.6
n.s.	n.s.
21.9±2.3	21.6±2.2
19.3±2.5	7.9±0.8
n.s.	$p < 0.01$
60.3±2.0	28.4±2.7
65.0±2.6	12.9±1.1
n.s.	$p < 0.01$
6.5±0.6	1.8±0.1
6.9±0.7	8.2±2.0
n.s.	$p < 0.02$

lipoprotein, cholesterol and phospholipid. The HDL fraction of d 1.063-1.110 g/ml contained a slightly greater portion of the total apolipoproteins in serum lipoproteins during methyltestosterone administration, but less of the total serum triglyceride was found in this fraction. Methyltestosterone increased slightly the proportions of cholesterol and phospholipid contained in the principal plasma lipoprotein fraction, i.e., the HDL fraction of d 1.110-1.250 g/ml, and decreased the proportion of total serum triglyceride. The proportions of total serum cholesterol and phospholipid remaining in the d > 1.250 g/ml fraction was essentially unchanged by methyltestosterone. Although a statistically significant increase was found in the proportion of total serum triglyceride contained in the d > 1.250 g/ml fraction, this observation should be interpreted with caution inasmuch as the amounts of triglyceride available in this fraction for analysis were very small and hence the accuracy of these determinations may be questioned.

Results presented in Table 9 demonstrate that the apolipoprotein represented relatively less of each lipoprotein fraction during methyltestosterone administration. The phospholipid to protein ratio increased in all but the d < 1.019 g/ml fraction. Triglyceride constituted a greater proportion of those lipoproteins of d < 1.110 g/ml during methyltestosterone administration.

The purified lipoprotein fractions obtained from each dog during the control period gave similar immunoprecipitin reactions to those described in the preceding section of this Chapter. The purified HDL fraction of d 1.110-1.250 g/ml gave A_1-, A_2- and A_3-reactions. A_1- and A_2-reactions were the major precipitin lines found in the purified HDL frac-

TABLE 9

EFFECT OF METHYLTESTOSTERONE ON COMPOSITION OF PURIFIED SERUM LIPOPROTEIN FRACTIONS[a]

Lipoprotein Fraction	Percent of Lipoprotein Components				Phospholipid / Protein
	Protein	Cholesterol	Phospholipid	Triglyceride	
d < 1.019					
Control	15.2±1.8	7.2±1.2	22.4±4.7	55.3±6.2	1.50±0.36
Methyltestosterone	10.9±2.7	9.5±1.2	14.0±2.1	65.7±5.1	1.45±0.25
d 1.019-1.063					
Control	31.5±1.7	20.3±4.2	27.7±3.0	20.6±6.8	0.88±0.09
Methyltestosterone	25.3±6.2	15.4±2.8	26.6±2.8	32.7±5.6	1.28±0.35
d 1.063-1.110					
Control	38.3±4.0	18.3±1.8	36.6±3.5	6.8±1.5	1.02±0.19
Methyltestosterone	34.9±7.6	17.1±5.4	32.7±8.3	10.6±3.4	1.12±0.37
d 1.110-1.250					
Control	55.1±2.7	12.5±1.0	30.3±2.1	2.2±0.3	0.56±0.06
Methyltestosterone	52.2±0.4	12.2±0.5	33.8±0.8	1.8±0.2	0.65±0.02

[a]Values from the four dogs are presented as the mean ± standard error. In each instance the difference between values obtained during control and methyltestosterone periods is not statistically significant, i.e., $p \geq 0.05$.

tion of 1.063-1.110 g/ml. In addition, a minor immunoprecipitin line characteristic of the B-reaction was observed in the HDL fraction of d 1.063-1.110 g/ml in each dog. The X-reaction was also demonstrated in this fraction from Dog 104. The purified LDL fraction gave a B-reaction, as well as A- and X-reactions representing minor immunoprecipitin lines. The purified fraction of d < 1.019 g/ml gave a B-reaction and a less intense X-reaction. Individual variations in the proportions of the apolipoproteins present in the purified lipoprotein fraction of d < 1.110 g/ml are shown in Figure 18. Methyltestosterone reduced the intensity of the A-reactions. Although apparent in the immunoprecipitin reaction of the HDL fraction, this response to methyltestosterone was most obvious in the marked diminution of the A-reaction in the purified LDL fraction (Figure 19). Proportionate changes in the B- and X-reactions were not observed.

Analysis of the amino acid composition of apolipoproteins in the purified HDL fraction of d 1.110-1.250 g/ml suggested no significant qualitative change in the protein in response to methyltestosterone administration (Table 10). Although some values for a few amino acids were different before and during methyltestosterone treatment, this observation should be interpreted with caution inasmuch as the values represented single determinations. Similar analyses were not attempted on other lipoprotein fractions due to the apolipoprotein heterogeneity of these fractions.

Methyltestosterone had no effect on total serum protein concentration (Table 11). Nevertheless, methyltestosterone produced a significant increase in albumin and a decrease in the α_1- and β-globulin fractions. Neither bromsulphalein retention nor serum bilirubin concentration was altered by methyltestosterone administration.

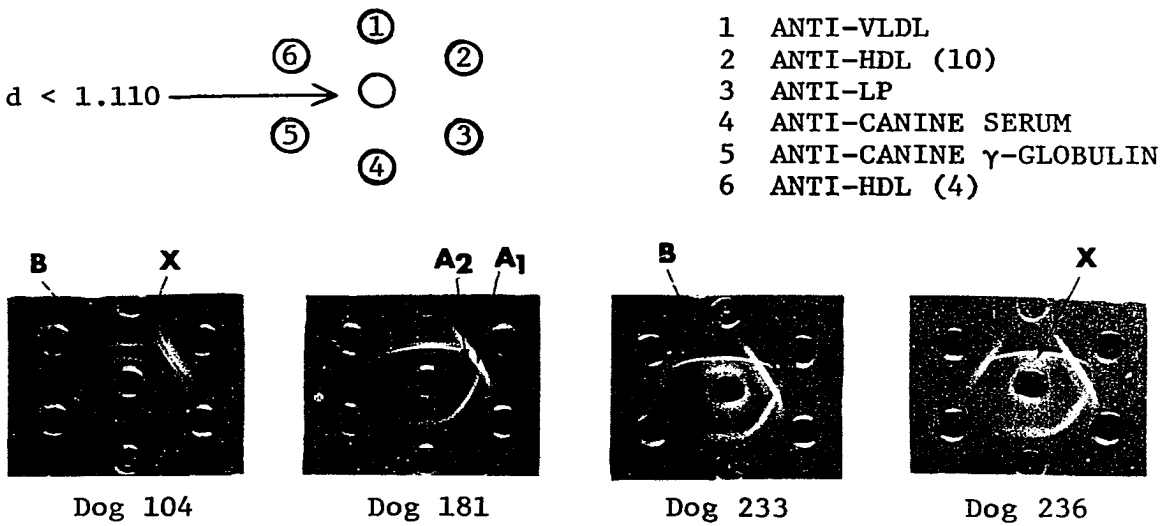


Fig. 18 - Immunodiffusion analysis of the purified serum lipoprotein fraction of d < 1.110 g/ml of the four dogs in which methyltestosterone studies were performed.

The fractions were isolated according to scheme C in Fig. 2. The concentration of the purified fractions was 4 mg/ml. Note the individual variations in apolipoprotein components by comparing the relative positions and intensities of the A-, B- and X-immunoprecipitin lines. In the analyses presented, the failure to demonstrate an A-precipitin line in reactions with well number 6 for Dog 104 and with wells number 3 and 4 in each dog is due to excessive antigen in relation to antibody concentration.

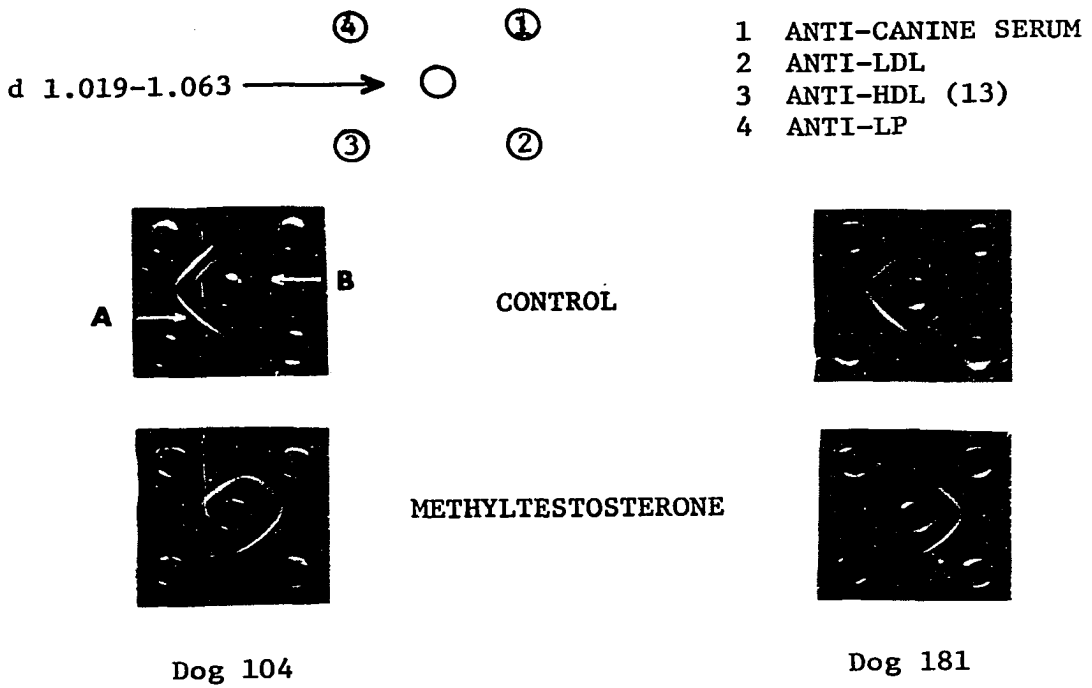


Fig. 19 - Immunodiffusion analysis of purified LDL fractions demonstrating the marked diminution of the A-precipitin line, relative to the B-line, during methyltestosterone treatment.

TABLE 10

EFFECT OF METHYLTESTOSTERONE ON AMINO ACID COMPOSITION OF HDL APOLIPOPROTEINS[a]

Amino Acid	Control		Methyltestosterone	
	(104 & 181)[b]	(233 & 236)	(104 & 181)	(233 & 236)
Alanine	78.8	89.0	83.7	88.1
Arginine	46.7	40.3	50.5	40.8
Aspartic acid	71.1	74.6	70.2	74.2
Cystine/2	0.0	0.0	0.0	tr.
Glutamic acid	164.4	171.2	167.5	173.7
Glycine	41.9	41.6	37.0	36.1
Histidine	8.3	0.0	7.2	4.4
Isoleucine	6.6	6.9	7.2	8.5
Leucine	112.3	117.3	112.8	126.3
Lysine	45.3	48.4	47.9	37.6
Methionine	3.5	2.3	3.0	3.3
Phenylalanine	14.8	14.4	15.5	15.7
Proline	32.5	33.5	36.7	33.8
Serine	61.4	49.5	47.1	44.1
Threonine	29.2	25.4	24.3	23.4
Tyrosine	16.6	17.7	17.8	17.7
Valine	41.9	47.0	42.3	47.9

[a]Analyses were performed in the HDL fraction of d 1.110-1.250 g/ml. Values are presented as moles per 100,000 g of protein. Tryptophan analyses were not performed.

[b]HDL samples obtained from the dogs identified in parentheses were combined for analyses.

TABLE 11

EFFECT OF METHYLTESTOSTERONE ON CONCENTRATION OF SERUM PROTEIN FRACTIONS[a]

Protein Fractions	Control	Methyltestosterone	Difference[b]
Albumin	2.63±0.09	3.47±0.11	$p < 0.01$
α_1-Globulin	0.34±0.01	0.29±0.01	$p < 0.02$
α_2-Globulin	0.64±0.08	0.67±0.07	n.s.
β-Globulin	2.79±0.29	1.94±0.13	$p < 0.05$
γ-Globulin	0.68±0.09	0.73±0.07	n.s.
Total protein	7.08±0.24	7.10±0.15	n.s.

[a]Values (g/100 ml) for the four dogs are presented as mean ± standard error. The value used for each dog represented the average of at least 11 determinations in each period.

[b]Statistical significance of difference between values obtained during control and methyltestosterone periods (n.s. = not significant, i.e., $p \geq 0.05$).

Effect of Methyltestosterone on the Incorporation of ^{14}C-Lysine Into the Apolipoproteins of Serum HDL and LDL and Serum Albumin and on the Turnover of HDL-Apolipoprotein and Albumin

Additional studies were undertaken to evaluate the effect of methyltestosterone on the metabolism of apolipoprotein A, i.e., the apolipoprotein of HDL and the dominant apolipoprotein in canine plasma. These studies were performed using purified HDL of d 1.110-1.250 g/ml, inasmuch as results presented above indicated that this fraction contained the majority of apolipoprotein A but no apolipoprotein B or X. ^{14}C-lysine incorporation was also measured in LDL, a heterogeneous lipoprotein fraction containing apolipoproteins A, B and X. Similar studies were performed using albumin, which provided a reference for evaluating the effects of methyltestosterone on protein metabolism.

Incorporation Studies

The serum concentrations of LDL (d 1.019-1.063 g/ml) and HDL (d 1.110-1.250 g/ml) apolipoproteins and albumin during control and methyltestosterone periods are presented in Table 12. Methyltestosterone decreased the concentration of LDL and HDL apolipoproteins and increased albumin. The sum of the concentrations of these three proteins increased, however, due to the magnitude of the increase in albumin. Since the control and methyltestosterone periods represented steady states in which the pool sizes of these proteins differed significantly, results from the incorporation studies were calculated as total radioactivity (DPM per 100 ml of serum) by multiplying specific radioactivity values by the appropriate protein concentration obtained from Table 12.

Values representing the sum of total radioactivities incorporated

TABLE 12

CONCENTRATION OF LDL AND HDL APOLIPOPROTEINS AND ALBUMIN IN SERUM BEFORE AND DURING METHYLTESTOSTERONE TREATMENT

	Proteins[a]		
	LDL	HDL	Albumin
Dog 104			
Control	53	360	2,530
Methyltestosterone	6	181	3,520
Dog 181			
Control	31	316	2,760
Methyltestosterone	15	140	3,420
Dog 233			
Control	36	329	2,790
Methyltestosterone	4	119	3,740
Dog 236			
Control	43	390	2,440
Methyltestosterone	8	144	3,200

[a]Values are expressed as mg per 100 ml of serum.

into LDL and HDL apolipoproteins and albumin are presented in Figures 20-23. Maximal levels were attained within 8 hr after precursor injection. Results obtained for individual proteins were calculated as a percentage of the maximal radioactivity incorporated into the three proteins in each study. Results presented in Figures 20-23 demonstrate that the greatest proportion of radioactivity was incorporated into albumin and the least into LDL apolipoprotein. Methyltestosterone reduced the proportion in HDL and LDL apolipoproteins, while that in albumin was increased (Table 13).

Comparison of results obtained in individual studies was also facilitated by calculating the incorporation data for apolipoproteins relative to albumin. Results presented in Table 14 indicate that the HDL:albumin ratio was maximal at 0.5 hr after isotope injection, then declined to a level which remained relatively constant. A similar pattern was observed during control and methyltestosterone periods. Methyltestosterone reduced this ratio to less than 50% of control.

Turnover Studies

Radioactivity in serum and in fractions of d < 1.110 g/ml, d 1.110-1.250 g/ml (HDL) and d > 1.250 g/ml was measured at intervals up to 7 days. A significant portion of the radioactivity administered as ^{14}C-apolipoprotein A in HDL of d 1.110-1.250 g/ml was subsequently recovered in the lipoprotein fraction of d < 1.110 g/ml (control period) or in the latter fraction and the fraction of d > 1.250 g/ml (methyltestosterone period) (Table 15). The re-distribution of radioactivity appeared to be time-dependent, i.e., the decrement in radioactivity in the HDL fraction was associated with a progressive increment in radioactivity in other

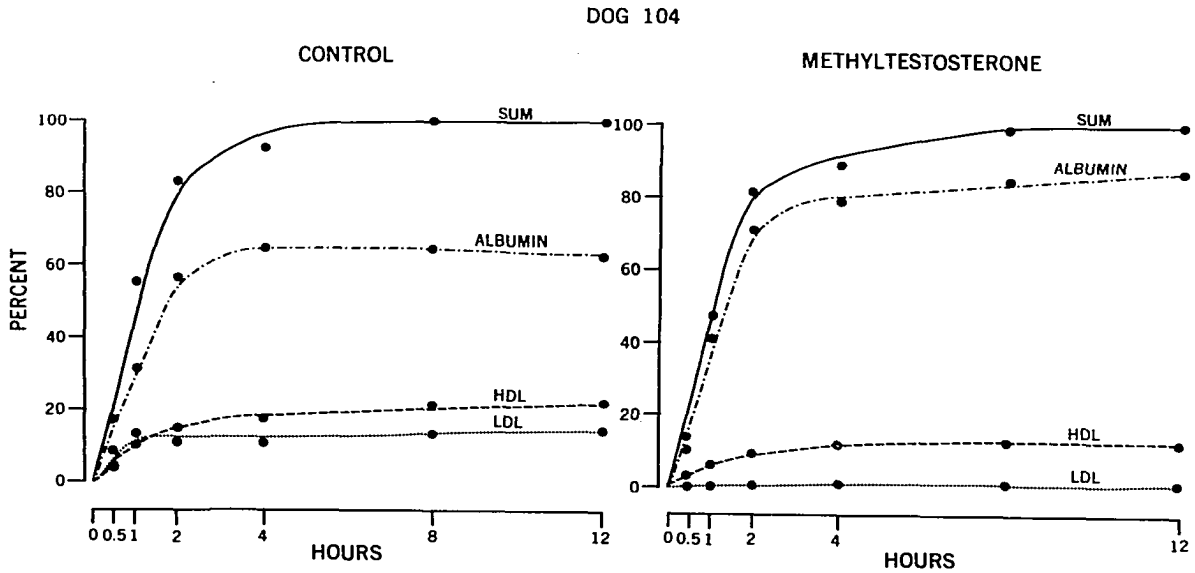


Fig. 20 - Time course and relative rate of ^{14}C-lysine incorporation into LDL and HDL apolipoproteins and albumin.

Abscissa: time in hr after precursor injection; ordinate: percent of the sum of maximal radioactivities incorporated into the three proteins.

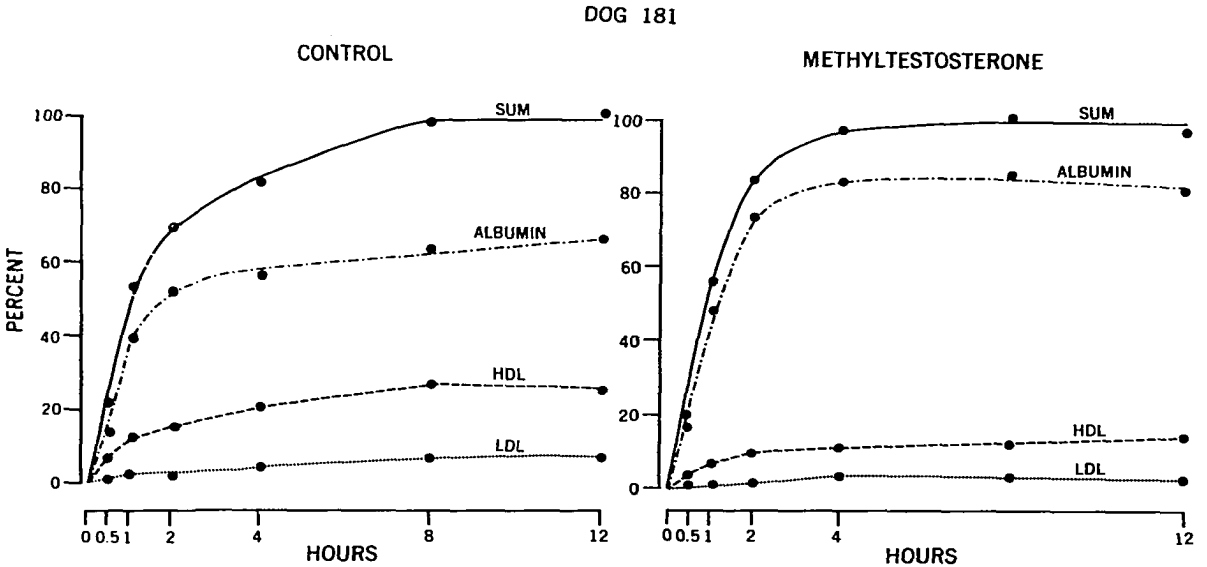


Fig. 21 - Time course and relative rate of ^{14}C-lysine incorporation into LDL and HDL apolipoproteins and albumin.

Abscissa: time in hr after precursor injection; ordinate: percent of the sum of maximal radioactivities incorporated into the three proteins.

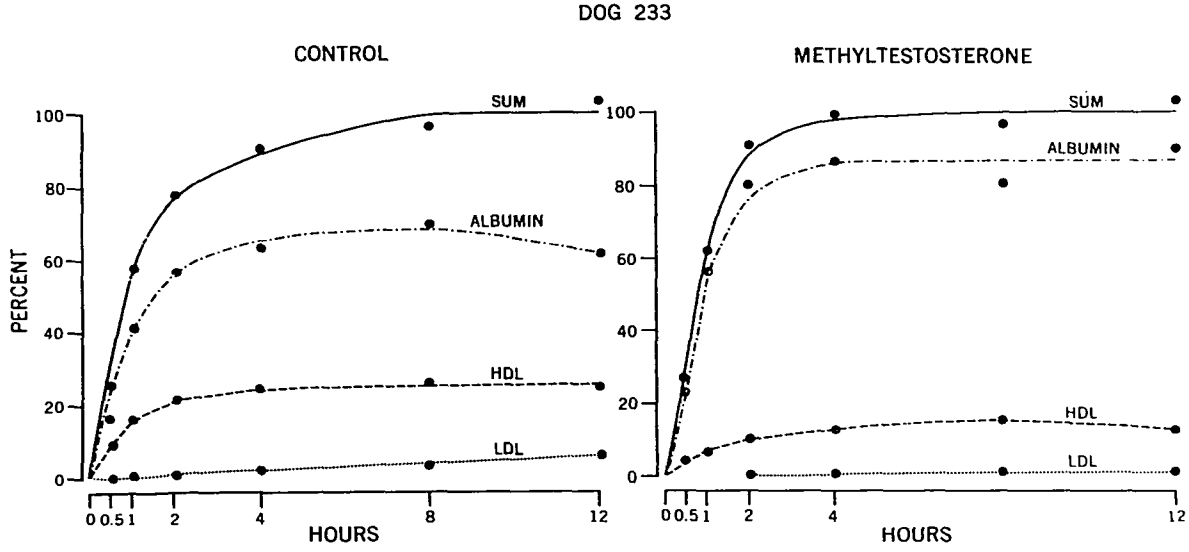


Fig. 22 - Time course and relative rate of ^{14}C-lysine incorporation into LDL and HDL apolipoproteins and albumin.

Abscissa: time in hr after precursor injection; ordinate: percent of the sum of maximal radioactivities incorporated into the three proteins.

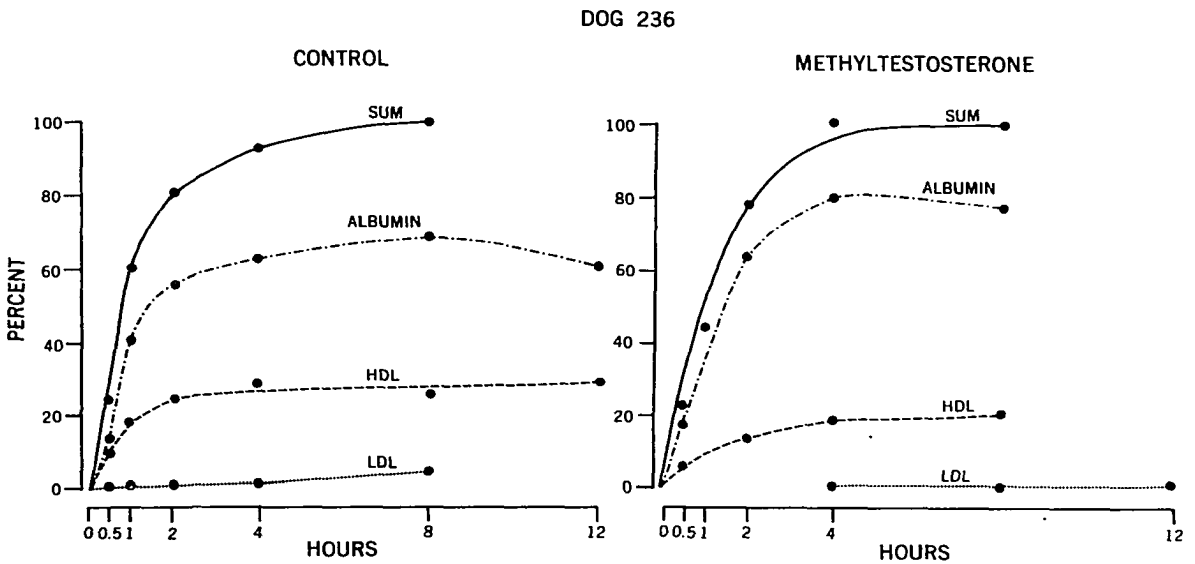


Fig. 23 - Time course and relative rate of ^{14}C-lysine incorporation into LDL and HDL apolipoproteins and albumin.

Abscissa: time in hr after precursor injection; ordinate: percent of the sum of maximal radioactivities incorporated into the three proteins.

TABLE 13

RATE OF INCORPORATION OF LYSINE-^{14}C INTO LDL AND HDL APOLIPOPROTEINS AND ALBUMIN BEFORE AND DURING METHYLTESTOSTERONE TREATMENT[a]

Proteins	Hours after Injection of Lysine-^{14}C		
	0.5	1.0	2.0
LDL			
Control	1.3±0.9	4.3±3.1	3.9±2.5
Methyltestosterone	0.2	0.4	0.6±0.4
Difference[b]	--	--	n.s.
HDL			
Control	7.9±1.4	14.4±2.0	19.1±2.2
Methyltestosterone	4.2±0.7	6.8±0.2	11.2±1.0
Difference	n.s.	$p < 0.001$	$p < 0.02$
Albumin			
Control	13.1±1.6	38.2±2.2	55.3±1.1
Methyltestosterone	17.0±2.7	47.8±3.2	72.3±3.3
Difference	n.s.	$p < 0.05$	$p < 0.01$
Sum of the above			
Control	22.3±1.9	56.9±1.5	78.2±3.1
Methyltestosterone	17.8±6.2	55.5±4.5	83.9±2.7
Difference	n.s.	n.s.	n.s.

[a]Values are presented as mean ± standard error in the four dogs, of the percent of maximum protein radioactivity incorporated into each protein. The total radioactivity incorporated into the three protein components at 8 and 12 hr after lysine-^{14}C injection was averaged and assigned the value of 100%, i.e., the maximum protein radioactivity. The mean ± standard error of these values in the four dogs was 574±44 x 10^3 DPM per 100 ml of serum.

[b]Statistical significance of difference between values obtained before and during methyltestosterone treatment is indicated (n.s. = not significant, i.e., $p \geq 0.05$).

TABLE 13--Continued

4.0	8.0	12.0
4.7±2.2	7.0±2.3	9.5±2.7
1.4±0.7	1.6±0.6	1.5±0.2
n.s.	n.s.	$p < 0.05$
22.8±2.5	25.6±1.2	25.7±1.5
13.8±1.8	15.5±2.1	13.3±0.8
$p < 0.05$	$p < 0.01$	$p < 0.001$
62.3±1.9	66.3±1.2	66.1±2.7
82.4±1.6	82.3±2.1	86.0±2.4
$p < 0.001$	$p < 0.001$	$p < 0.01$
89.8±2.7	98.7±0.9	101.7±1.1
97.6±2.0	99.4±1.1	100.8±1.5
n.s.	n.s.	n.s.

TABLE 14

EFFECT OF METHYLTESTOSTERONE ON HDL:ALBUMIN RATIO OF RADIOACTIVITY INCORPORATED FROM ^{14}C-LYSINE

	Dog	Hours after Lysine-^{14}C Injection					
		0.5	1.0	2.0	4.0	8.0	12.0
Control	104	0.52	0.32	0.27	0.27	0.34	0.35
	181	0.47	0.31	0.30	0.37	0.43	0.39
	233	0.65	0.40	0.38	0.38	0.39	0.34
	236	0.72	0.46	0.43	0.45	0.38	0.48
	Mean ±S.E.	0.59 ±0.06	0.37 ±0.04	0.35 ±0.04	0.37 ±0.04	0.39 ±0.02	0.39 ±0.03
Methyltes-tosterone	104	0.29	0.16	0.15	0.15	0.15	0.14
	181	0.22	0.15	0.13	0.14	0.14	0.18
	233	0.18	0.12	0.13	0.15	0.19	0.14
	236	0.34	--	0.22	0.24	0.28	--
	Mean ±S.E.	0.26 ±0.04	0.14 ±0.01	0.16 ±0.12	0.17 ±0.02	0.19 ±0.03	0.15 ±0.01
Difference[a]		$p < 0.001$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.001$

[a]Statistical significance of difference between mean values for control and methyltestosterone periods is indicated (n.s. = not significant, i.e., $p \geq 0.05$).

TABLE 15

EFFECT OF METHYLTESTOSTERONE ON PERCENTAGE DISTRIBUTION OF RADIOACTIVITY RECOVERED IN LIPOPROTEIN FRACTIONS[a]

	Lipoprotein Fractions	Days after Injection of HDL(^{14}C-Apolipoprotein)								
		0.5	1.0	1.5	2.0	3.0	4.0	5.0	6.0	7.0
Control	d < 1.110	19	23	27	30	44	52	50	69	66
	d 1.110-1.250	81	77	73	70	56	48	50	31	34
	d > 1.250	0	0	0	0	0	0	0	0	0
Methyltes-tosterone	d < 1.110	0	2	0	0	2	9	17	23	20
	d 1.110-1.250	94	92	88	90	77	67	60	48	68
	d > 1.250	6	6	12	10	21	24	23	29	12

[a]Values, expressed as percent of total serum radioactivity recovered in individual fractions, are presented as the mean for the four dogs studied.

fractions. Individual variations were observed among the four dogs in respect to the proportion of total serum radioactivity recovered in the HDL fraction relative to that found in the d < 1.110 g/ml fraction. In studies during the control period in Dogs 233 and 236, the HDL fraction and the fraction of d < 1.110 g/ml each contained about 50% of the serum radioactivity at 7 days, whereas the HDL fractions contained less than 35% of total serum radioactivity in Dog 181 and less than 20% in Dog 104, i.e., the majority of serum radioactivity in Dogs 104 and 181 was present in the lipoprotein fraction of d < 1.110 g/ml. The appearance of radioactivity in the fraction of d < 1.110 g/ml was delayed during methyltestosterone administration.

Control values for the rate of disappearance of radioactivity from the HDL fraction (T/2 = 2.28±0.08 days) (Figures 24-27, Table 16) were less than in serum (T/2 = 4.74±0.97 days) (Figures 28-31, Table 17). Methyltestosterone treatment resulted in an increased half time of apolipoprotein A in the HDL fraction to a mean of 3.50±0.61 days, while the value in serum was decreased to a mean of 3.52±0.32 days. The control turnover rates for apolipoprotein A in the HDL fraction were similar in the four dogs (Table 16). When compared with these values, two of the control turnover rates for apolipoprotein A in serum were less and two were greater (Table 17). The lower values were observed in Dogs 104 and 181, in which 65 to 80% of the labeled apolipoprotein A was present in lipoproteins of d < 1.110 g/ml at 7 days, compared with approximately 50% in this fraction in Dogs 233 and 236. Turnover rates were significantly lowered by methyltestosterone, particularly for apolipoprotein A in the HDL fraction.

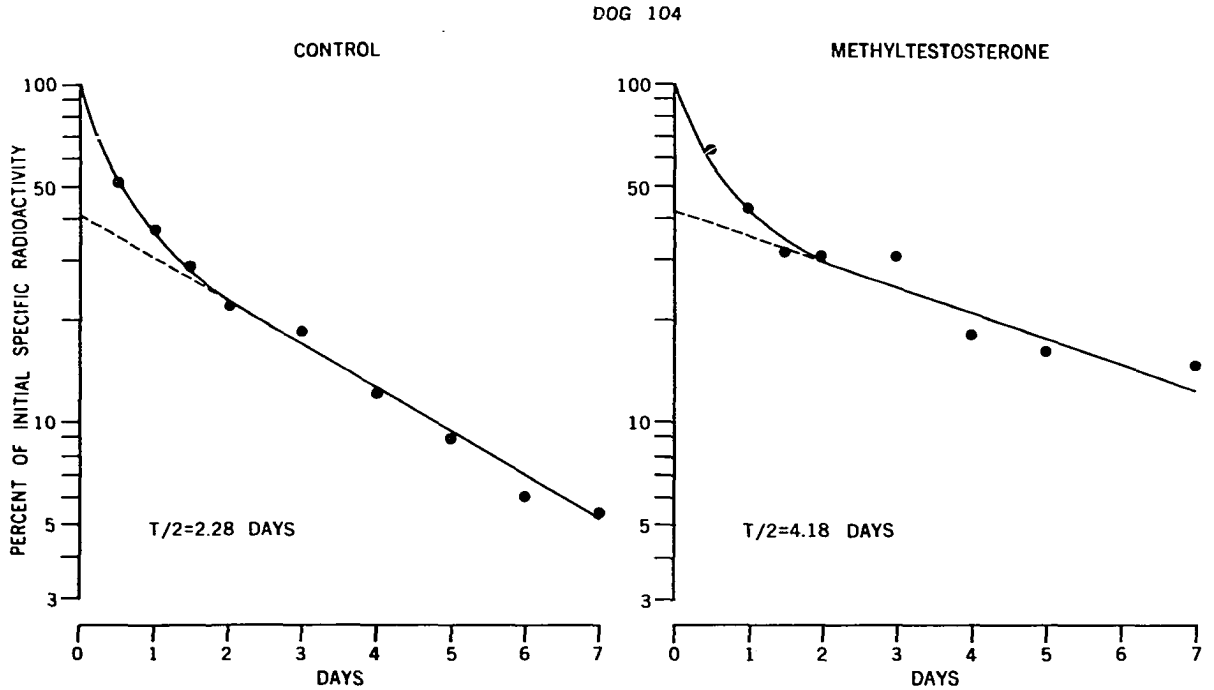


Fig. 24 - Disappearance of ^{14}C-apolipoprotein A from HDL (d 1.110-1.250 g/ml) fraction of serum.

Abscissa: time in days after injection of HDL containing ^{14}C-apolipoprotein (d 1.110-1.250 g/ml); ordinate: logarithm of the percent of initial specific radioactivity of apolipoprotein A. The initial specific radioactivity (specific radioactivity at zero time, in DPM per mg of protein) was calculated using the values for injected radioactivity and for the intravascular pool of apolipoprotein A in HDL (d 1.110-1.250 g/ml).

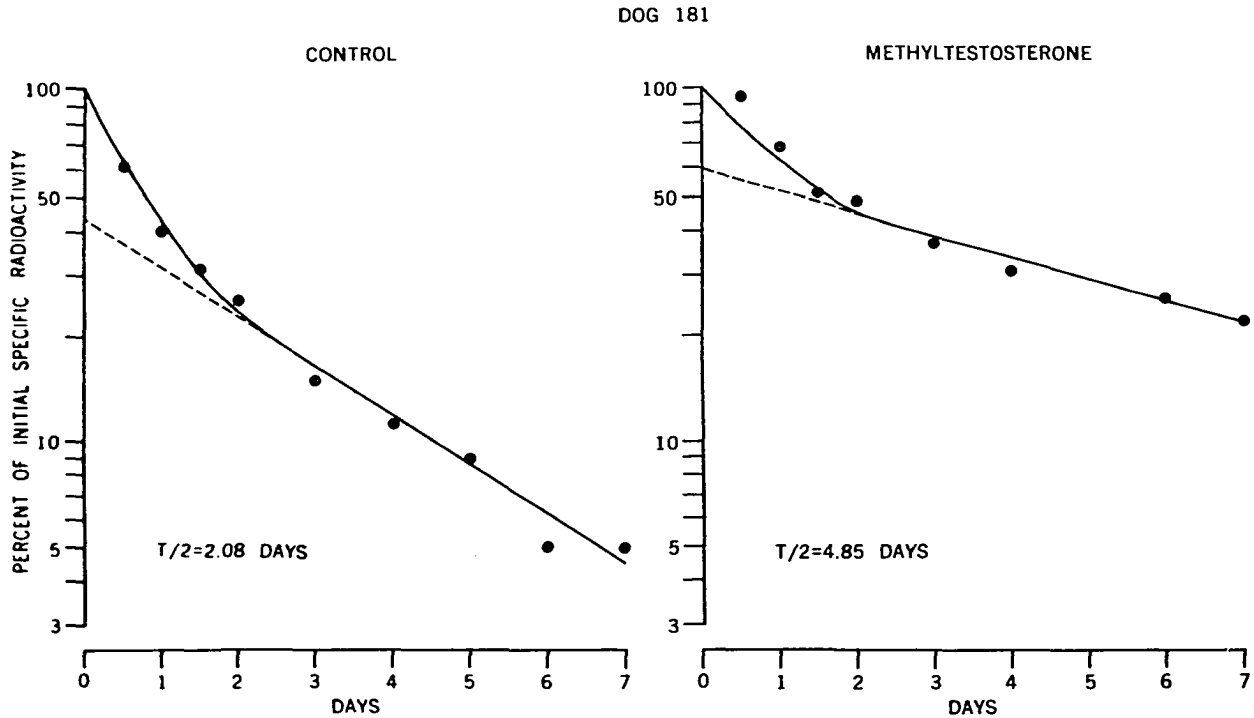


Fig. 25 - Disappearance of ^{14}C-apolipoprotein A from HDL (d 1.110-1.250 g/ml) fraction of serum.

Abscissa: time in days after injection of HDL containing ^{14}C-apolipoprotein (d 1.110-1.250 g/ml); ordinate: logarithm of the percent of initial specific radioactivity of apolipoprotein A. The initial specific radioactivity (specific radioactivity at zero time, in DPM per mg of protein) was calculated using the values for injected radioactivity and for the intravascular pool of apolipoprotein A in HDL (d 1.110-1.250 g/ml).

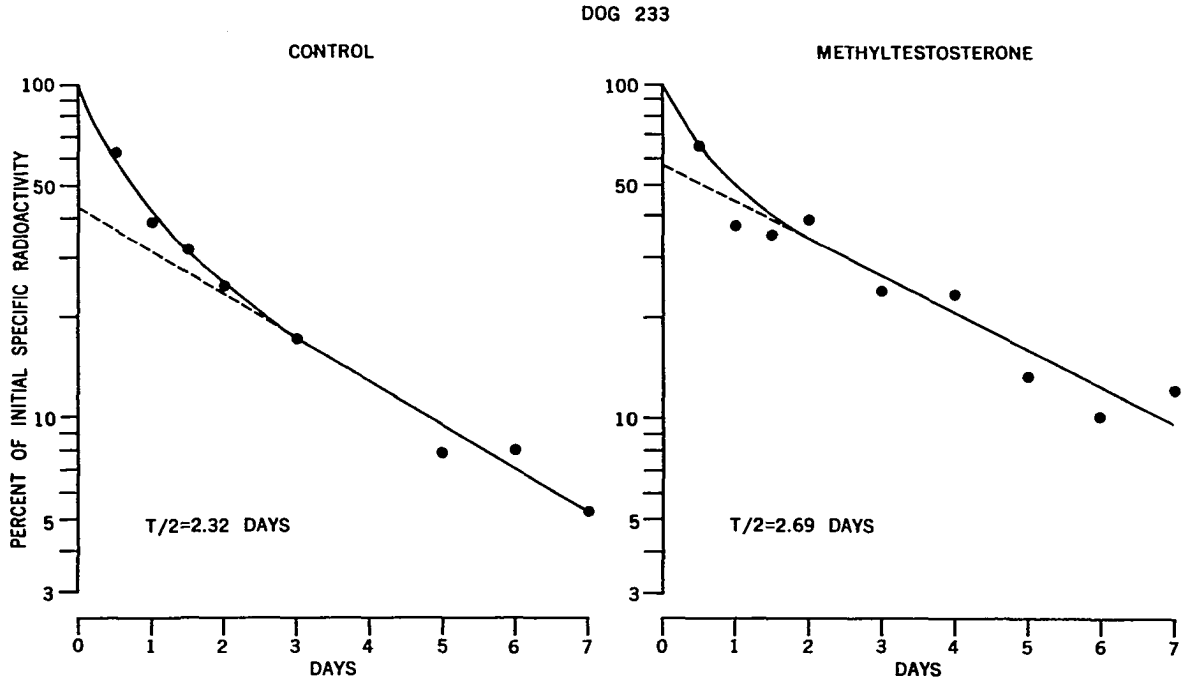


Fig. 26- Disappearance of ^{14}C-apolipoprotein A from HDL (d 1.110-1.250 g/ml) fraction of serum.

Abscissa: time in days after injection of HDL containing ^{14}C-apolipoprotein (d 1.110-1.250 g/ml); ordinate: logarithm of the percent of initial specific radioactivity of apolipoprotein A. The initial specific radioactivity (specific radioactivity at zero time, in DPM per mg of protein) was calculated using the values for injected radioactivity and for the intravascular pool of apolipoprotein A in HDL (d 1.110-1.250 g/ml).

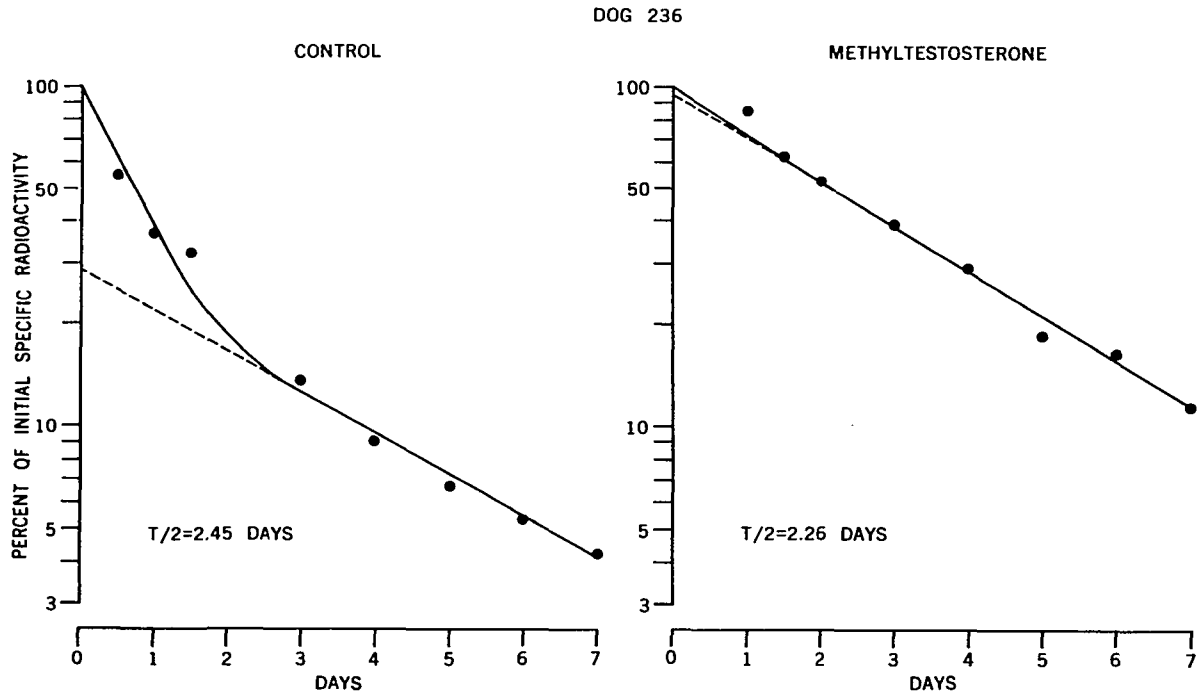


Fig. 27 - Disappearance of ^{14}C-apolipoprotein A from HDL (d 1.110-1.250 g/ml) fraction of serum.

Abscissa: time in days after injection of HDL containing ^{14}C-apolipoprotein (d 1.110-1.250 g/ml); ordinate: logarithm of the percent of initial specific radioactivity of apolipoprotein A. The initial specific radioactivity (specific radioactivity at zero time, in DPM per mg of protein) was calculated using the values for injected radioactivity and for the intravascular pool of apolipoprotein A in HDL (d 1.110-1.250 g/ml).

TABLE 16

EFFECT OF METHYLTESTOSTERONE ON HALF TIME, TURNOVER RATE AND TOTAL EXCHANGEABLE POOL OF APOLIPOPROTEIN A IN HDL (d 1.110-1.250 g/ml)

	Dog	Serum Concentration (mg/ml)	Half Time T/2 (day)	Turnover Rate (mg/kg/day)	Turnover Rate % of Control	Total Exchangeable Space (ml/kg)	Total Exchangeable Pool (mg/kg)
Control	104	3.18	2.28	76.6	100.0	77.7	247.0
	181	2.91	2.08	67.1	100.0	69.9	203.4
	233	3.02	2.32	63.5	100.0	70.1	211.8
	236	1.81	2.45	70.3	100.0	138.6	250.9
	Mean	2.73	2.28	69.4	100.0	89.1	228.3
	±S.E.	±0.31	±0.08	±2.8	±0.0	±16.6	±12.1
Methyltes-tosterone	104	0.97	4.18	13.6	17.8	82.5	80.1
	181	0.96	4.85	9.8	14.6	73.1	70.1
	233	0.60	2.69	11.9	18.7	76.1	45.7
	236	0.83	2.26	14.9	21.2	57.8	48.0
	Mean	0.84	3.50	12.6	18.1	72.4	61.0
	±S.E.	±0.09	±0.61	±1.1	±1.4	±5.2	±8.4
Difference[a]		$p < 0.01$	n.s.	$p < 0.001$	$p < 0.001$	n.s.	$p < 0.001$

[a]Statistical significance of difference between mean values for control and methyltestosterone periods is indicated (n.s. = not significant, i.e., $p \geq 0.05$).

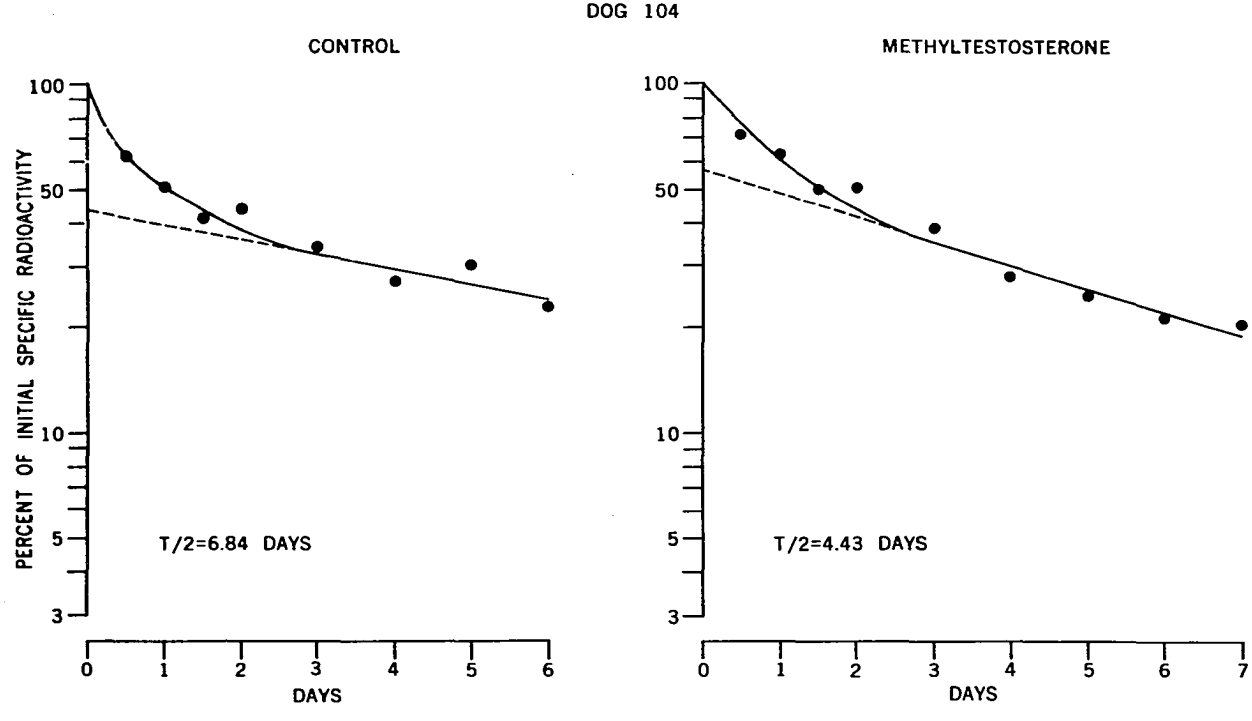


Fig. 28 - Disappearance of ^{14}C-apolipoprotein A from serum.

Abscissa: time in days after injection of HDL containing ^{14}C-apolipoprotein (d 1.110-1.250 g/ml); ordinate: logarithm of the percent of initial serum radioactivity.

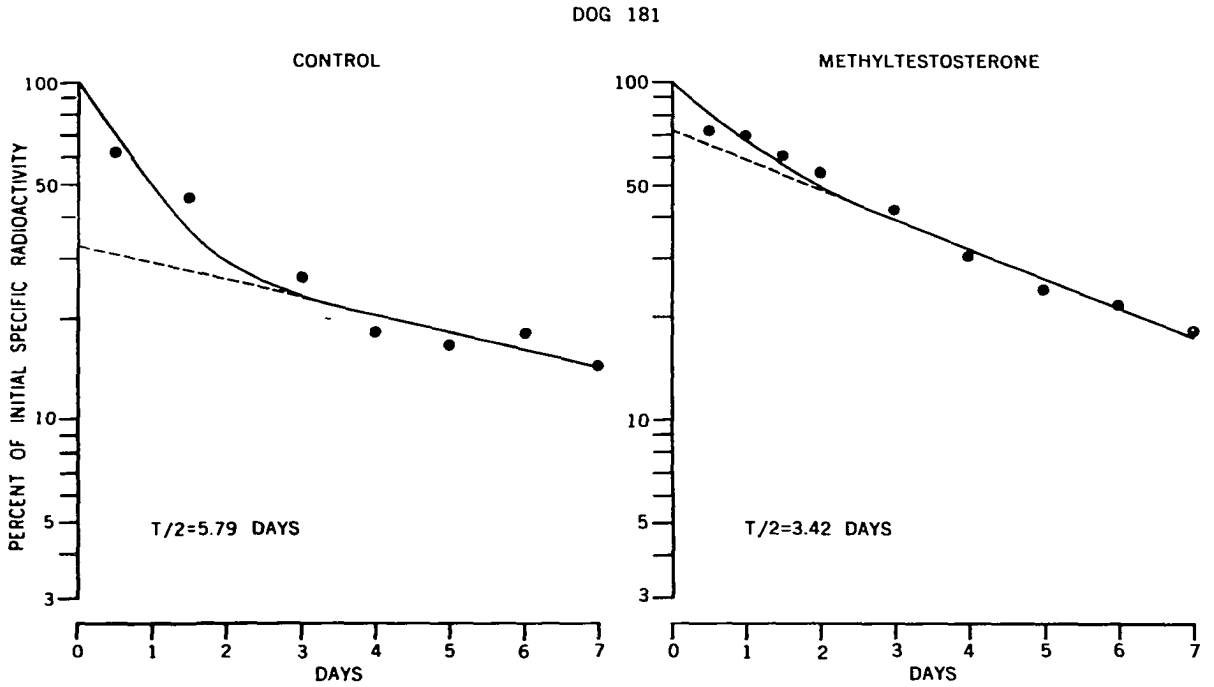


Fig. 29 - Disappearance of ^{14}C-apolipoprotein A from serum.

Abscissa: time in days after injection of HDL containing ^{14}C-apolipoprotein (d 1.110-1.250 g/ml); ordinate: logarithm of the percent of initial serum radioactivity.

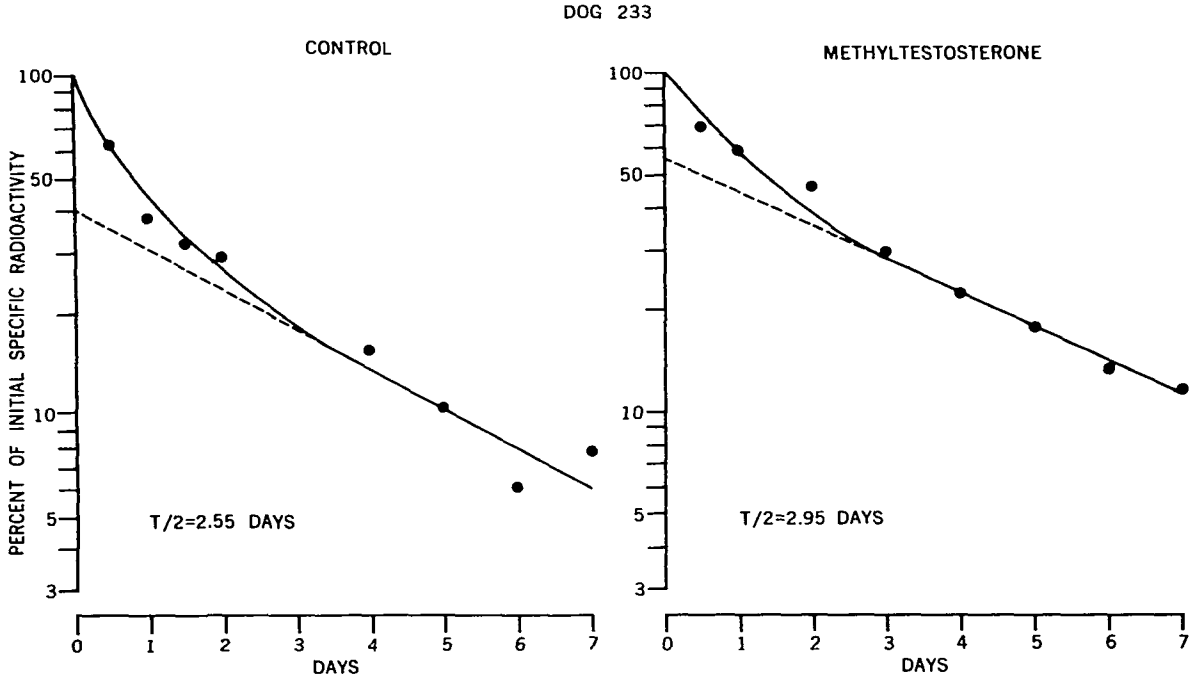


Fig. 30 - Disappearance of ^{14}C-apolipoprotein A from serum.

Abscissa: time in days after injection of HDL containing ^{14}C-apolipoprotein (d 1.110-1.250 g/ml); ordinate: logarithm of the percent of initial serum radioactivity.

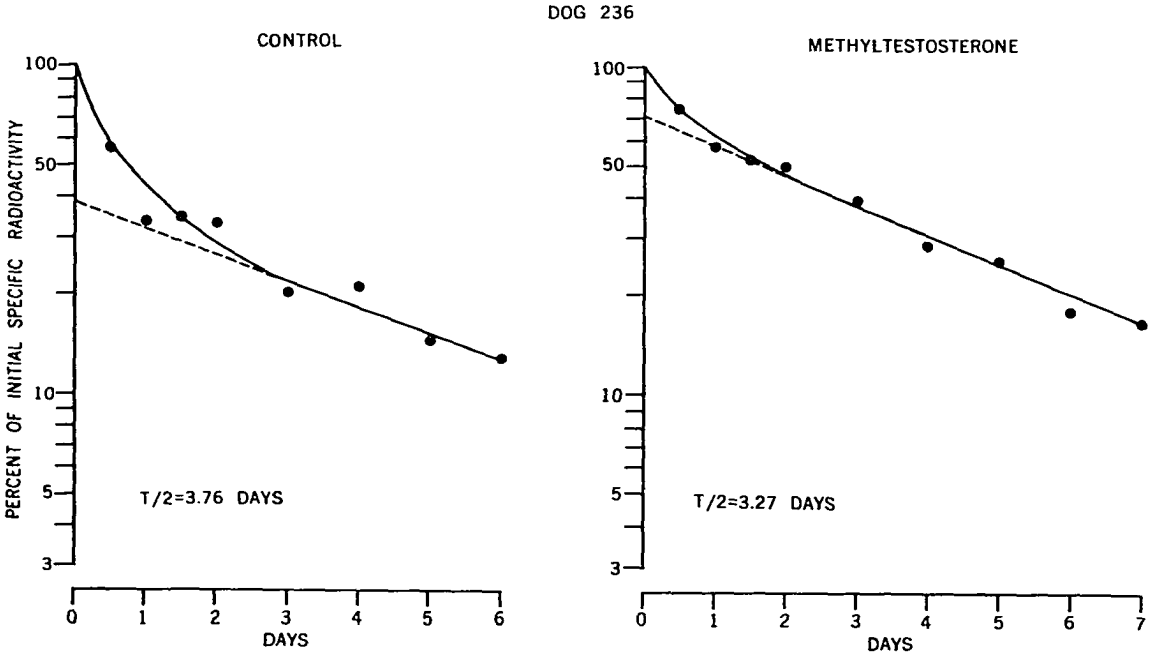


Fig. 31 - Disappearance of ^{14}C-apolipoprotein A from serum.

Abscissa: time in days after injection of HDL containing ^{14}C-apolipoprotein (d 1.110-1.250 g/ml); ordinate: logarithm of the percent of initial serum radioactivity.

TABLE 17

EFFECT OF METHYLTESTOSTERONE ON HALF TIME, TURNOVER RATE AND EXCHANGEABLE POOLS OF APOLIPOPROTEIN A IN SERUM

	Dog	Serum Concentration[a] (mg/ml)	Half Time T/2 (day)	Turnover Rate (mg/kg/day)	Turnover Rate % of Control
Control	104	4.88	6.84	36.6	100.0
	181	3.88	5.79	43.1	100.0
	233	4.51	2.55	89.7	100.0
	236	4.73	3.76	92.9	100.0
	Mean	4.50	4.74	65.6	100.0
	±S.E.	±0.22	±0.97	±14.9	±0.0
Methyltes-tosterone	104	2.53	4.43	30.7	83.9
	181	1.84	3.42	22.3	51.7
	233	1.70	2.95	30.8	34.3
	236	2.15	3.27	36.0	38.8
	Mean	2.06	3.52	30.0	52.2
	±S.E.	±0.18	±0.32	±2.8	±11.2
Difference[b]		$p < 0.001$	n.s.	n.s.	$p < 0.01$

[a]These values were approximated as the apolipoprotein concentration in the HDL fraction of $d > 1.063$ g/ml.

[b]Statistical significance of the difference between mean values for control and methyltestosterone periods is indicated (n.s. = not significant, i.e., $p \geq 0.05$).

TABLE 17--Continued

Total Exchangeable		Intravascular		Percent Partition	
Space (ml/kg)	Pool (mg/kg)	Space (ml/kg)	Pool (mg/kg)	Intra-vascular	Extra-vascular
73.5	358.6	32.0	156.1	43.5	56.5
92.6	359.3	30.7	119.1	33.2	66.8
73.1	329.7	29.7	133.9	40.6	59.4
106.2	502.5	40.4	191.1	38.0	62.0
86.4 ±8.0	387.5 ±38.9	33.2 ±2.4	150.1 ±15.7	38.8 ±2.2	61.2 ±2.2
77.3	195.5	43.5	110.1	56.3	43.7
59.7	109.8	43.3	79.7	72.5	27.5
77.0	130.9	44.2	75.1	57.4	42.6
79.2	170.2	56.2	120.4	71.0	29.0
73.3 ±4.6	151.6 ±19.3	46.8 ±3.1	96.3 ±11.2	64.3 ±4.3	35.7 ±4.3
n.s.	$p < 0.001$	$p < 0.02$	$p < 0.05$	$p < 0.01$	$p < 0.01$

Values for total exchangeable pool of apolipoprotein A in the HDL fraction (Table 16) are less than those for apolipoprotein A in whole serum (Table 17). This discrepancy is attributable to differences in serum concentrations, i.e., the amount of apolipoprotein A in the HDL fraction represents only a portion of that in serum. However, values for total exchangeable space of apolipoprotein A, calculated by two methods, gave similar results. Methyltestosterone reduced the mean total exchangeable pool for apolipoprotein A to less than 50% of control. This change reflects a greater loss from the extravascular pool than from the intravascular pool (Table 17).

Measurements of albumin radioactivity were performed for 7 days following injection of purified ^{14}C-albumin. Radioactivity disappeared from the plasma during the control period with a rate representing a mean half time of 16.26±2.84 days (Figures 32-35, Table 18). This value was reduced to 8.23±0.78 days during methyltestosterone administration. Furthermore, methyltestosterone increased the total exchangeable pool for albumin by almost 50%. These changes in albumin metabolism are reflected in an almost three-fold increase in turnover rate during methyltestosterone treatment. Results presented in Table 18 also indicate that the increase in total exchangeable albumin produced by methyltestosterone represented equivalent increments in the intravascular and extravascular pools.

Measurements of the intravascular space (plasma volume) using HDL containing ^{14}C-apolipoprotein (Table 17) gave somewhat lower values than were obtained with ^{14}C-albumin (Table 18). These values were increased by approximately 30% during methyltestosterone administration.

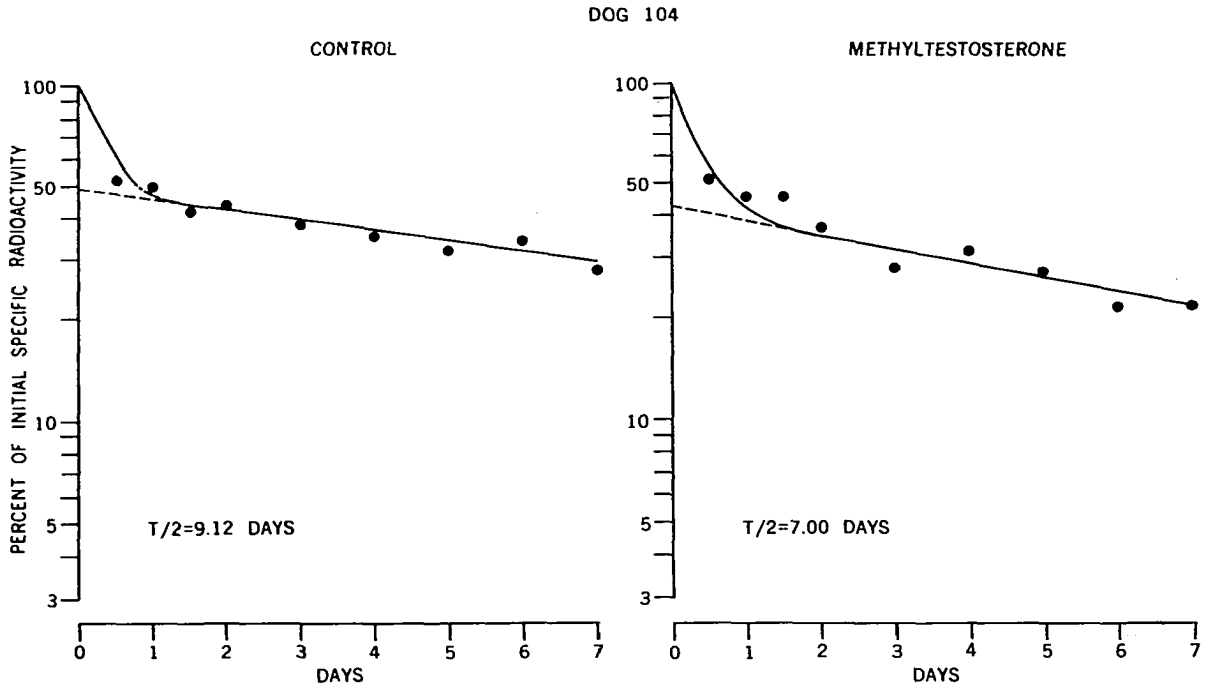


Fig. 32 - Disappearance of ^{14}C-albumin from serum.

Abscissa: time in days after injection of ^{14}C-albumin; ordinate: logarithm of the percent of initial specific radioactivity of albumin. The initial specific radioactivity (specific radioactivity at zero time, as DPM per mg of protein) was calculated using values for injected radioactivity and for the intravascular pool of albumin.

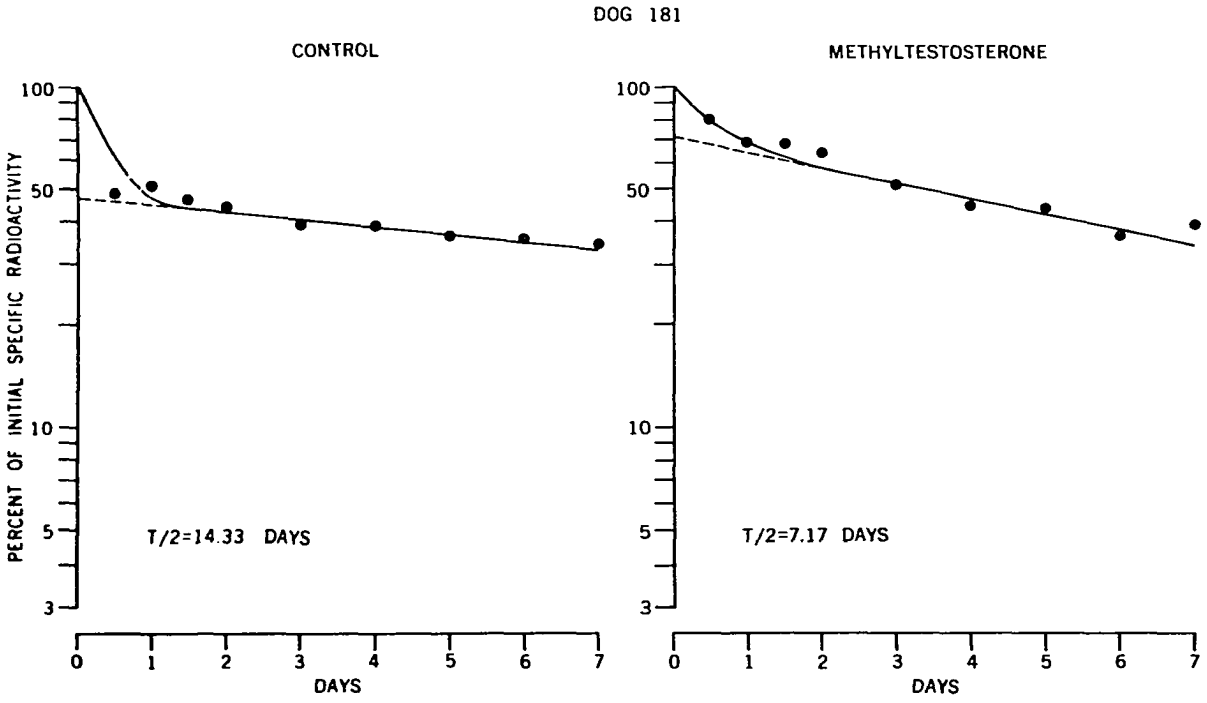


Fig. 33 - Disappearance of ^{14}C-albumin from serum.

Abscissa: time in days after injection of ^{14}C-albumin; ordinate: logarithm of the percent of initial specific radioactivity of albumin. The initial specific radioactivity (specific radioactivity at zero time, as DPM per mg of protein) was calculated using values for injected radioactivity and for the intravascular pool of albumin.

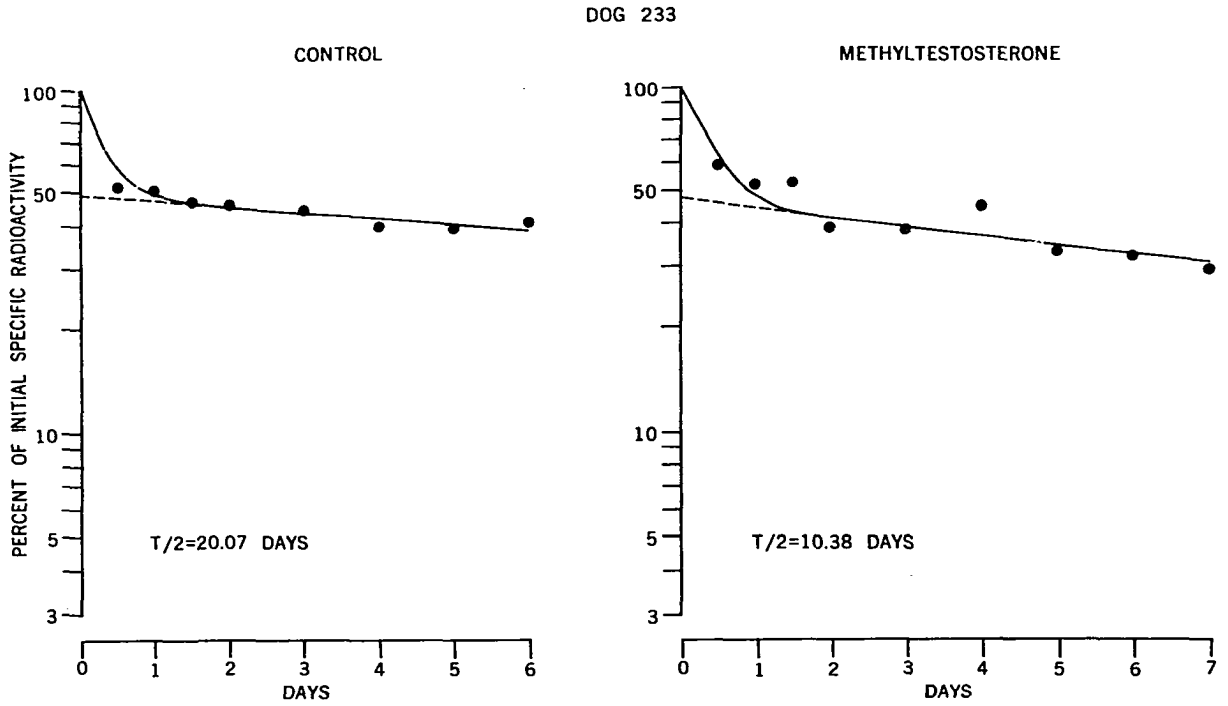


Fig. 34 - Disappearance of ^{14}C-albumin from serum.

Abscissa: time in days after injection of ^{14}C-albumin; ordinate: logarithm of the percent of initial specific radioactivity of albumin. The initial specific radioactivity (special radioactivity at zero time, as DPM per mg of protein) was calculated using values for injected radioactivity and for the intravascular pool of albumin.

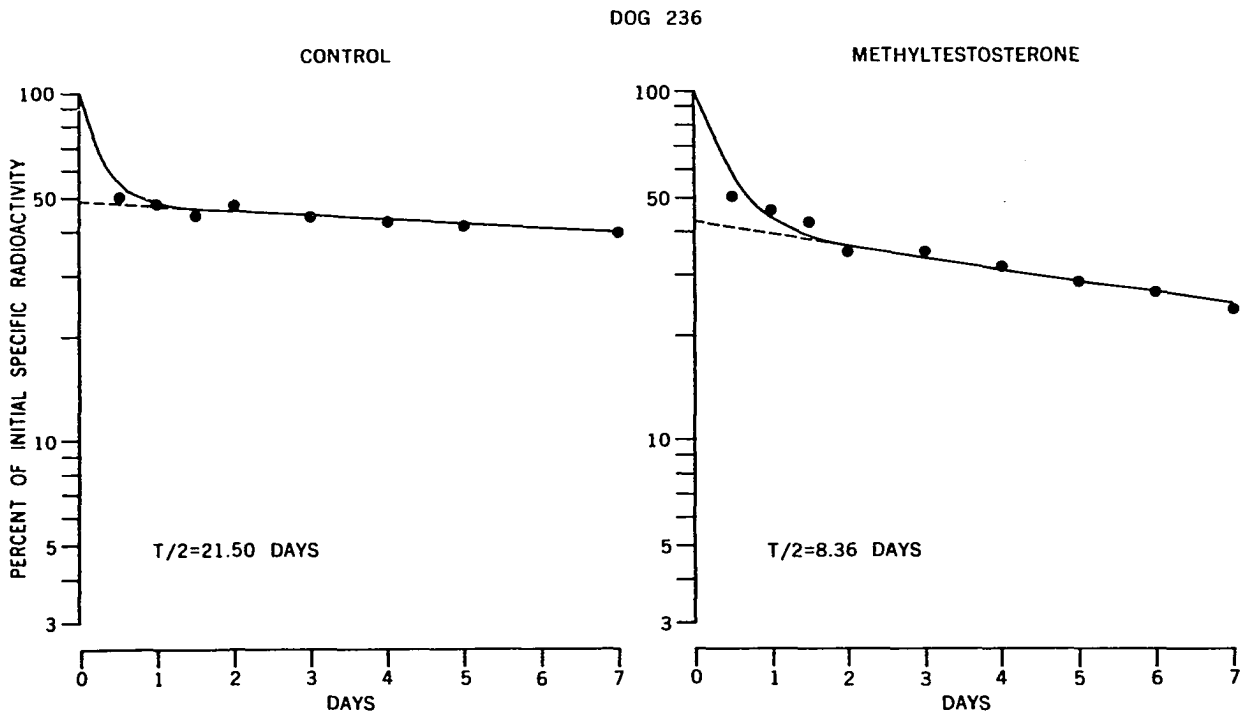


Fig. 35 - Disappearance of ^{14}C-albumin from serum.

Abscissa: time in days after injection of ^{14}C-albumin; ordinate: logarithm of the percent of initial specific radioactivity of albumin. The initial specific radioactivity (specific radioactivity at zero time, as DPM per mg of protein) was calculated using values for injected radioactivity and for the intravascular pool of albumin.

TABLE 18

EFFECT OF METHYLTESTOSTERONE ON HALF TIME, TURNOVER RATE AND EXCHANGEABLE POOLS OF ALBUMIN

	Dog	Serum Concentration (mg/ml)	Half Time T/2 (day)	Turnover Rate (mg/kg/day)	Turnover Rate % of Control
Control	104	29.0	9.12	249.8	100.0
	181	28.1	14.33	136.8	100.0
	233	28.1	20.07	113.2	100.0
	236	28.0	21.50	109.7	100.0
	Mean	28.3	16.26	152.4	100.0
	±S.E.	±0.2	±2.84	±33.0	±0.0
Methyltes-tosterone	104	33.5	7.00	610.3	244.3
	181	32.9	7.17	332.5	243.1
	233	32.2	10.38	285.4	252.1
	236	28.5	8.36	374.8	341.7
	Mean	31.8	8.23	400.8	270.3
	±S.E.	±1.1	±0.78	±72.2	±23.9
Difference[a]		$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.001$

[a]Statistical significance of difference between mean values for control and methyltestosterone periods is indicated (n.s. = not significant, i.e., $p \geq 0.05$).

TABLE 18--Continued

Total Exchangeable		Intravascular		Percent Partition	
Space (ml/kg)	Pool (mg/kg)	Space (ml/kg)	Pool (mg/kg)	Intra-vascular	Extra-vascular
113.3	3286.9	55.3	1603.4	48.8	51.2
101.5	2851.4	49.1	1351.4	48.4	51.6
115.1	3234.3	57.0	1601.1	49.5	50.5
122.4	3428.1	59.7	1672.2	48.8	51.2
113.1 ±4.3	3200.2 ±123.2	55.3 ±2.2	1557.0 ±70.5	48.9 ±0.2	51.1 ±0.2
184.0	6164.7	79.3	2657.2	43.1	56.9
104.2	3428.1	73.4	2414.2	70.4	29.6
132.3	4259.4	63.6	2047.8	48.1	51.9
158.5	4516.4	68.0	1938.4	42.9	57.1
144.8 ±17.1	4592.2 ±573.3	71.1 ±3.4	2264.4 ±165.8	51.1 ±6.5	48.9 ±6.5
n.s.	n.s.	$p < 0.01$	$p < 0.01$	n.s.	n.s.

CHAPTER V

DISCUSSION

Apolipoprotein and Lipid Composition of Serum Lipoprotein Fractions

Results obtained in the present study and those reported previously (12, 104, 105, 109, 153) indicate that the HDL are the principal lipoproteins in dog plasma. Three antigenic components, designated A_1, A_2 and A_3 were demonstrated consistently in purified HDL fractions. The A_1- and A_2-components had similar immunoelectrophoretic behavior and distribution among the lipoprotein fractions, suggesting the possibility that these two reactions may reflect antigenic components of apolipoprotein sub-units having analogous polypeptide structures. The immunoelectrophoretic behavior and distribution among the lipoprotein fractions served to differentiate HDL containing the A3-component from those in which the A_1- and A_2-components were found. These observations support the conclusion that at least two groups of lipoproteins, which differ in their immunologic characteristics and hydrated densities are present in HDL of canine plasma. Furthermore, in HDL the proportion of the A_3-reacting component, relative to the A_1- and A_2-components, appears to increase with increasing hydrated density and decreasing particle size (later elution in gel filtration). Composition analysis of the purified lipoproteins in HDL subfractions indicates that the ratio of phospholipid

to protein decreases as the density of the subfraction increases. Although these observations may reflect differences in the lipid binding properties of the apolipoproteins present in these two groups of lipoproteins, additional studies of HDL subfractions will be required to evaluate the relationship between the quantitative distribution of these antigenic components and lipids in the spectrum of HDL.

The electrophoretic mobility of canine HDL and the water solubility of the apolipoprotein preparation obtained after total delipidization of this fraction are similar to characteristics described for human HDL (25). Scanu and Page (205) and Furman and co-workers (52) reported studies in the dog and man, respectively, in which HDL apolipoprotein labeled with ^{131}I was injected and found to be metabolized at a similar rate to native HDL containing labeled apolipoprotein. This observation suggests that HDL apolipoprotein is not significantly altered by removal of lipids. This conclusion is further supported by results obtained in the present study in the dog, as well as by Scanu and co-workers (206) in man, demonstrating that delipidization produced no change in the immunochemical characteristics of HDL apolipoprotein. Rodbell and Fredrickson (18) reported that the N-terminal amino acids of delipidized canine serum HDL and LDL were similar to those of human serum, i.e., aspartic acid in HDL and glutamic acid in LDL. However, the polypeptide fingerprint patterns of hydrolysates of canine HDL protein were different from those obtained with human HDL protein. In the present study differences in immunochemical characteristics and amino acid composition were detected between canine and human apolipoprotein A. The present results of the amino acid analysis of purified HDL of d 1.110-1.250 g/ml are gen-

erally similar to those reported for dog serum HDL by Wathen and Levy (207). Minor discrepancies between these results may be due to differences in the methods of isolation, purification and analysis, as well as in the segment of HDL utilized for study. Canine HDL apolipoprotein contains less histidine, lysine, methionine, phenylalanine, threonine and tyrosine than human (208). Nevertheless, the appearance of the immunoelectrophoretic and disc electrophoretic patterns of apolipoprotein A as well as the quantitative chemical composition of HDL from canine serum are similar to those reported for human samples (23, 38, 209-211). Thus, the present study suggests that apolipoprotein A in the dog resembles that in man in respect to its heterogeneity and complexity, but differs in specific components and/or chemical composition.

The nature of the plasma component which exhibited the A_X-reaction when reacted with anti-HDL or anti-VLDL serum is only partially discernible from the present study. This antigenic component was demonstrated consistently, but in very small quantities, in purified VLDL and in serum samples from which lipoproteins of $d < 1.250$ g/ml had been removed. In addition, the A_X-reaction was observed in unpurified, but not purified, HDL preparations, although antibodies which produced this reaction were elicited in rabbits following the administration of some purified HDL preparations. These observations suggest the possibility that the protein or other component responsible for this reaction is highly antigenic. Evidence presented herein that the A_X-component disappears from the HDL fraction during purification and is recovered in the infranatant portion during the washing procedure may indicate that this component is interacting with HDL in a relatively reversible manner, possibly by simple ad-

sorption to the lipoproteins. A similar explanation may account for the presence of the A_X-component in the VLDL fraction. Lewis and Page (212) have described complexes containing immunoglobulin and lipoproteins in subjects with lipemia. A second possibility is that the A_X-component reflects a specific antibody to lipoproteins similar to the auto-antibodies described in human subjects by Beaumont and Lorenzelli (213). Evidence favoring this conclusion is provided by the immunoelectrophoretic behavior of the A_X-component, i.e., its migration to the γ-globulin region, and the demonstration that antibodies to this component are present in anti-canine γ-globulin serum. Additional evidence favoring the conclusion that the A_X-component is a γ-globulin is provided by the elution of this component in the fractions containing γ-globulins when whole serum or lipoprotein-free serum was subjected to Sephadex gel filtration. A third possibility is that the A_X-component represents a distinct apolipoprotein, i.e., this component is capable of binding lipids. Additional assessment of these alternatives and of the significance of the A_X-component in respect to canine plasma lipoproteins must await further investigation.

Purified LDL contained primarily apolipoprotein B, although variable amounts of lipoproteins containing A- and X-components were found in several preparations. This finding is similar to that reported by Lee and Alaupovic (35) for human LDL fractions. Analysis of this lipoprotein fraction indicated a high ratio of phospholipid to protein, due primarily to relatively less protein and greater proportions of cholesterol and triglyceride than were found in the HDL fractions. Antigenic similarity (B-reaction) between canine and human LDL was observed in the present study and by Storiko and Fisher (113).

Immunochemical analysis demonstrated that purified VLDL contained two components, i.e., A_X and B, which were also found in higher density fractions. In addition, a third component designated X was observed consistently in this fraction. Results from these immunochemical analyses suggest that the VLDL fraction contains a group of lipoproteins in which the apolipoprotein is unique. A similar thesis concerning VLDL in human plasma has been advocated by Gustafson and co-workers (21, 22), who first recognized the presence of lipoproteins containing a unique apolipoprotein and proposed its designation as apolipoprotein C. The possibility exists that the X-component in canine VLDL corresponds to the apolipoprotein C found in man. The immunoreaction between anti-canine VLDL serum and human chyle VLDL is identical with the C-reaction observed when anti-human VLDL serum and human chyle VLDL are reacted. Electrophoretic analysis of partially delipidized lipoproteins from canine serum in 7% polyacrylamide gel demonstrated protein bands which are similar to those found by McConathy and Alaupovic (32) in partially delipidized VLDL from human chyle. Moreover, the distribution in canine serum of lipoproteins containing the X-component is similar to that in human plasma of lipoproteins containing apolipoprotein C (32, 35).

VLDL contained primarily triglycerides, with relatively small proportions of phospholipids and cholesterol. However, the protein content of serum VLDL was higher in dogs than has been observed in man (46, 214, 215). Yokoyama and Zilversmit (216) found that dog lymph chylomicrons less than 140 mμ in diameter contained almost 10% protein, whereas the protein content of larger chylomicrons was less. Few, if any, large chylomicrons may have been present in the fraction of $d < 1.006$ g/ml iso-

lated in the present study, inasmuch as the serum samples for composition analysis were obtained from dogs which were being maintained on a low-fat diet and had been fasted for at least 20 hr.

Results described herein provide evidence that the HDL, LDL and VLDL fractions in canine plasma have relatively distinct immunologic characteristics. Nevertheless, the frequency with which lipoproteins containing an apolipoprotein characteristic of one density range were found in purified fractions isolated at an adjacent density, e.g. the demonstration of the apolipoprotein containing the A-component in the LDL fraction and the apolipoprotein containing the B-component in the lower density HDL sub-fractions, indicates that in the dog, as in man (23, 217), the use of conventional density boundaries for classifying lipoproteins has limited applicability. A classification system based on apolipoproteins, as advocated by Alaupovic (23, 38) for human serum lipoproteins, is favored by the present findings in the dog.

Lewis and co-workers (12) have observed that "the lipoprotein concentration, determined at density 1.063, of healthy mongrel dog sera varied greatly from animal to animal". Therefore, these authors suggested that "changes must be evaluated by using the animal's own pattern as control". In the present study substantial individual variations were found not only in serum lipid levels and lipid distribution, but also in the apolipoprotein composition of lipoprotein fractions, particularly those of $d < 1.110$ g/ml. This variability may well be the underlying reason for the conclusions presented by these investigators.

Effect of Methyltestosterone on the Apolipoprotein and Lipid Composition of Serum Lipoprotein Fractions and on Serum Proteins

The reduction in serum lipids observed in the present as well as previous studies (153, 155) in dogs given methyltestosterone is associated with an equivalent reduction in apolipoprotein concentration. Changes in the distribution of apolipoproteins and lipids among the lipoprotein fractions, as well as alterations in the composition of individual lipoprotein fractions, were also observed. Methyltestosterone diminished the proportion of total serum apolipoproteins, cholesterol and phospholipid and increased the proportion of triglyceride present in lower density lipoproteins, i.e., those of $d < 1.063$ g/ml. Methyltestosterone also increased the triglyceride content in all fractions except the HDL fraction of d 1.110-1.250 g/ml and increased the ratio of phospholipid to protein in all fractions except that of $d < 1.019$ g/ml. This increase in phospholipid relative to protein is consistent with a decrease in lipoproteins containing HDL apolipoproteins, inasmuch as the latter lipoproteins have a much lower phospholipid:protein value than lipoproteins containing apolipoproteins B and X. Immunochemical analysis of the apolipoprotein changes produced by methyltestosterone demonstrated a marked diminution of apolipoprotein A in the lower density fractions, without a comparable decrease in apolipoproteins containing the B- and X-components. These observations suggest that methyltestosterone may produce a re-distribution of triglyceride and other serum lipids primarily through a selective effect on HDL apolipoproteins. A significant diminution of apolipoprotein A might result in a greater proportion of plasma triglyceride being transported in triglyceride-rich, lower density lipoproteins, especially in the dog in

which this apolipoprotein may comprise as much as 90% of total apolipoproteins. Further evidence supporting the conclusion that methyltestosterone reduced the concentration of apolipoprotein A was obtained from immunochemical and quantitative chemical analyses of the HDL fractions. Although no qualitative changes were demonstrated by immunochemical analyses, marked diminution in the quantity of all HDL components was observed. The amino acid composition of apolipoprotein A was not altered appreciably by methyltestosterone, providing further support for the conclusion that methyltestosterone produces a quantitative, rather than a qualitative change in the HDL apolipoproteins.

Methyltestosterone produced a significant reduction in all density classes of lipoproteins. This observation might be interpreted as indicating that HDL and LDL apolipoproteins, as well as other components, were affected similarly by methyltestosterone. Additional results obtained in this study, however, fail to support this conclusion. The diminution in LDL comprised a proportionately greater reduction in lipoproteins containing apolipoprotein A than in those containing apolipoproteins B or X. These results indicate that methyltestosterone alters primarily lipoproteins containing apolipoprotein A. Moreover, these observations emphasize that criteria in addition to hydrated densities and conventional lipid analyses are necessary to permit meaningful evaluation of the response to agents which alter lipoprotein metabolism.

Methyltestosterone produced a statistically significant increment in albumin concentration and decrement in α_1- and β-globulins, without altering total protein levels in serum. Although α_2-globulin concentration was not changed in the present study, an increase has been observed in

other studies in our laboratory. Little information is available concerning the effect of methyltestosterone on albumin and other serum protein fractions. In patients given testosterone, no changes were observed in serum total protein concentration (140). Norethandrolone administration to human subjects increased the concentration of the pre-albumin fraction which binds thyroxine (143). In addition, it has been suggested that this agent (143) or methyltestosterone (142) diminished the concentration of the α_1-globulin which binds thyroxine. Natural androgens and synthetic anabolic steroids have been found to increase the α_2-globulin concentration (144). These observations suggest that the serum protein response in dogs given methyltestosterone resembles changes observed in human subjects given this or related agents. Nevertheless, clinical investigations in which a number of anabolic steroids were used demonstrated no consistent effects on serum total protein concentration or individual protein fractions (218). Attempts to relate structure of these agents to their pharmacological activity have generally proved unsuccessful.

Effect of Methyltestosterone on the Incorporation of ^{14}C-Lysine into the Apolipoproteins of Serum HDL and LDL and Serum Albumin and on the Turnover of HDL Apolipoprotein and Albumin

The metabolic steady state established by prolonged administration of methyltestosterone was characterized by a significant decrease in the concentration of apolipoprotein A and an increase in albumin concentration in serum. The incorporation and turnover studies provide information concerning the metabolic status of these proteins during methyltestosterone treatment. Results from these studies may permit inferences concerning the possibility that changes in the rates of apolipoprotein

synthesis or degradation may represent the primary metabolic response to methyltestosterone.

In the metabolic steady state, the rate of synthesis of a metabolite equals the rate of degradation. A new steady state, e.g. that characterized by diminished concentration of a protein, can be produced by any of the following:

1. an increase in the space of distribution,
2. an increase in the rate of degradation, and
3. a decrease in the rate of synthesis.

If methyltestosterone were to alter only one of these variables, the relationship between the total exchangeable pool and the half time of apolipoprotein A in response to the changes listed above would be as follows:

1. $Pool_{(C)} = Pool_{(MT)}$; $T/2_{(C)} = T/2_{(MT)}$
2. $Pool_{(C)} > Pool_{(MT)}$; $T/2_{(C)} > T/2_{(MT)}$
3. $Pool_{(C)} > Pool_{(MT)}$; $T/2_{(C)} \leqq T/2_{(MT)}$

Inverse relationships to those listed above would characterize a new steady state in which the pool size was increased, i.e., that observed with albumin during methyltestosterone administration.

The observation that methyltestosterone reduced the pool size but not the half time of apolipoprotein A favors the conclusion that a decreased synthesis rather than an accelerated degradation was responsible for the diminished apolipoprotein A concentration in plasma. Similarly, since the half time of albumin was less while the albumin pool was enlarged significantly, it appears that methyltestosterone stimulated the synthesis of albumin rather than inhibiting the catabolism of this protein. Thus, results obtained in the turnover studies are consistent with

the amino acid incorporation studies and indicate that apolipoprotein A synthesis is decreased and albumin synthesis increased by methyltestosterone.

The time course of the incorporation of labeled lysine into apolipoprotein A and albumin was similar before and during methyltestosterone treatment. Approximately 50% of the maximum radioactivity incorporated into each protein was reached within 1 hr. However, there was a significant difference in the amount of radioactivity incorporated into apolipoprotein A and albumin before and during methyltestosterone treatment, suggesting an opposite change in the rate of synthesis of these two proteins. This is reflected in the significant decrease in the HDL: albumin protein radioactivity ratios during methyltestosterone treatment, as compared with the control state.

Calculations based on the fractional turnover times of these proteins indicate that less than 5% of circulating protein is degraded during the first few hours after ^{14}C-lysine injection. Therefore, any effect of catabolism on the specific radioactivities measured in the incorporation study must be negligible. Apolipoprotein A and albumin may not equilibrate at an equal rate with the extravascular pool during the 12-hr period of this study. Results obtained from the turnover studies suggest that albumin may equilibrate more rapidly than apolipoprotein A. If this conclusion were correct, the values presented for HDL:albumin radioactivity ratios would be higher than true values, and the effect of methyltestosterone on this ratio underestimated. The presumption that these proteins are synthesized from the same amino acid pool in liver eliminates any problem concerning the effect of possible differences in

the intracellular pool size of precursor before and during treatment.

Studies by Wilcox and co-workers (89) of the incorporation of labeled lysine into serum apolipoproteins in the isolated perfused rat liver and by Fried and co-workers (219) in five species of higher animals other than the dog suggest that LDL apolipoproteins are synthesized and degraded more rapidly than are those in HDL. Results obtained in the present study indicate that specific radioactivities differ significantly among the three proteins. Highest values were observed in LDL and lowest in albumin. Results from incorporation studies in the pig, rabbit and chicken are similar to those we obtained in the dog, in so far as HDL apolipoprotein and albumin are concerned. However, the rapid decline in LDL radioactivity which has been found in other animals was not observed in the dog. The presence in canine LDL of apolipoprotein A, which apparently has a longer turnover time than apolipoprotein B, may be a factor in the slower LDL turnover in this species. This possibility is supported by the observation that a proportionately greater incorporation into LDL apolipoproteins occurred in those dogs, especially Dog 104, in which apolipoprotein A represented a greater portion of the protein moiety than in others. Methyltestosterone treatment appears to eliminate these differences by reducing apolipoprotein A in LDL. During methyltestosterone treatment the specific radioactivity of albumin was unchanged or lower and that of the apolipoproteins higher than in the control state, due to changes in the pool sizes.

Little information is available concerning the regulation of apolipoprotein synthesis, although the importance of unimpaired protein synthesis for normal lipid transport is well established (64, 65, 89, 98, 99,

220). The effect of several hormones on synthesis of LDL apolipoprotein has been studied in the rooster by Florsheim and co-workers (221), using labeled methionine and tyrosine. Results reported by these investigators demonstrated that the time course of incorporation, as well as the relative amounts of radioactivity incorporated into LDL apolipoprotein, albumin and 'other proteins', were similar to those obtained in the present study. Estrogens produced a marked increase in LDL apolipoprotein and cholesterol concentrations in plasma and also increased more than ten-fold the total radioactivity incorporated into the apolipoprotein. L-triiodothyronine depressed somewhat the lipoprotein levels but produced a marked increase in apolipoprotein specific radioactivity. Cortisone had little effect on either lipoprotein levels or specific radioactivity of LDL apolipoprotein. Interpretation of these results is complicated by the failure of these investigators to evaluate the possibility that hormone-'nduced changes in intravascular:extravascular distribution, which have been observed (222, 223) may be a factor in their study. Nevertheless, the magnitude of increase in incorporation during estrogen treatment leaves little question that apolipoprotein synthesis is increased. This response is the converse of that obtained in the present study with methyltestosterone.

Methyltestosterone produced an increase in albumin synthesis and a decrease in apolipoprotein synthesis. The possibility might be considered that a reciprocal relationship exists in the metabolism of these proteins, i.e., that an acceleration in the rate of albumin synthesis in liver is associated with a reciprocal attenuation of the rate of apolipoprotein synthesis and vice versa. In nephrosis, hypoalbuminemia is asso-

ciated with hyperlipoproteinemia, representing the converse state to that produced by methyltestosterone. Hepatic synthesis of LDL (224) and HDL (86) apolipoproteins is accelerated in this disorder and probably accounts for the hyperlipoproteinemia. Moreover, albumin synthesis is also enhanced (225-228), but the rate of synthesis is inadequate to replace the large quantities of albumin which are lost through the defective glomeruli. The response to dextran is another instance in which apolipoprotein and albumin synthesis are not related reciprocally. Dextran produces hypoalbuminemia without lipemia. This response is associated with a decrease in albumin synthesis (229). Furthermore, in experimental nephrosis (230) and in cortisone-induced lipemia (231), dextran has a lipid lowering effect which apparently reflects a re-distribution of lipoproteins to the extravascular pool, rather than any change in apolipoprotein synthesis (232). These observations suggest that the rate of albumin synthesis is not necessarily related to the plasma concentration of albumin and that apolipoprotein and albumin synthesis are not reciprocally related.

The possible role of an androgen-induced fat mobilization (168) in respect to the changes in albumin synthesis produced by methyltestosterone should be considered, inasmuch as increased free fatty acid influx into the liver could increase synthesis of albumin for transporting free fatty acids in plasma. If free fatty acid influx into the liver were increased, an accelerated hepatic synthesis of triglycerides should be evident through increased serum triglyceride levels (233). Furthermore, changes in free fatty acid influx were found by Ruderman and co-workers (102) to alter the synthesis of VLDL apolipoproteins without influencing

other apolipoproteins or other serum proteins.

Methyltestosterone might also influence lipoprotein metabolism through androgen-like effects on thyroid function. Administration of methyltestosterone or norethandrolone to human subjects produced a fall in thyroxine-binding capacity of the thyroxine-binding globulin and increased the fractional turnover rate of thyroxine (142, 143). On the other hand, the association of serum lipid abnormalities with thyroid dysfunction is well recognized. In hypothyroid subjects, LDL are increased while in thyrotoxicosis LDL and HDL are decreased (234-237). These changes probably reflect more generalized alterations in protein metabolism, since the metabolism of LDL apolipoprotein (238), albumin (239) and γ-globulin (240) were affected in a similar manner by alteration in thyroid activity. Hence, it appears unlikely that methyltestosterone is mediated through the thyroid.

Half time values for the apolipoprotein of canine serum HDL (d 1.063-1.210 g/ml) of approximately 3.5 days have been reported by Scanu and Page (78, 205). Similar values were obtained for intact lipoprotein or the lipid-free protein moiety both in the dog (205) and in man (52, 59). These values compare favorably with those obtained in the present study. However, no data are available concerning the time sequence of the distribution of apolipoprotein radioactivity among various lipoprotein classes. Results from the present study suggest a continuous transfer at a relatively constant rate from high density (d 1.110-1.250 g/ml) to lower density (d < 1.110 g/ml) lipoprotein fraction. Individual transfer rates were of similar magnitude to the amount of apolipoprotein A in fraction of d < 1.110 g/ml, i.e., Dog 104 > Dog 181 > Dogs 233 and 236.

These individual variations were apparently reflected also in the half times obtained for disappearance of radioactivity from serum, inasmuch as the turnover of apolipoprotein A within the fraction of d 1.110-1.250 g/ml was practically the same in each dog. Furthermore, the significant reduction of apolipoprotein A in the lower density fractions during methyltestosterone administration was accompanied by a barely detectable transfer of radioactivity into this fraction and by shorter overall half time values in those dogs in which the greatest transfer was observed in the control state, i.e., Dogs 104 and 181. Although these observations suggest that a transfer of apolipoprotein A from high to lower density lipoproteins occurs, they do not favor the conclusion that an exchange takes place among different apolipoproteins, i.e., apolipoprotein A does not exchange with apolipoprotein B. A similar conclusion has been advocated by Scanu and Page (205). The lower turnover rates for apolipoprotein A in serum, when compared with those in HDL (d 1.110-1.250 g/ml), suggest the rate of removal of apolipoprotein A is much slower in lower density lipoproteins. The possibility exists that the transfer of radioactivity observed here represents only a continuous equilibration of apolipoprotein A within the lipoprotein spectrum, or alternatively that this represents a metabolic sequence through which at least part of the apolipoprotein A from HDL normally passes before being removed from the circulation. Additional studies will be required to evaluate these and other possible explanations for these observations.

Methyltestosterone produced a much greater reduction in the total exchangeable pool for apolipoprotein A in HDL (d 1.110-1.250 g/ml) and serum than in the half time for this protein. The reduction in the ex-

changeable pool of apolipoprotein A was much greater than predicted from changes in the intravascular pool alone, due to a significant shift of apolipoprotein A from the extravascular to the intravascular compartment.

Individual components of lipoproteins have been shown to turn over with significantly different rates. Scanu and Page (78) obtained the following half time values in canine serum: cholesterol, approximately 8 days; phospholipids, 36 hr; triglycerides, 10 min; LDL apolipoprotein (d 1.006-1.063 g/ml), 2.3 days; and HDL apolipoprotein (d 1.063-1.210 g/ml), 3.5 days. The observation that serum lipid levels decreased to a similar extent as the apolipoprotein concentrations suggests that their metabolism may have been altered similarly by methyltestosterone, possibly through the decreased availability of the transport protein.

The intravascular and extravascular spaces for albumin were increased to an equivalent extent during methyltestosterone treatment. Consequently, the increase in the total exchangeable pool of albumin was much greater than appeared from the serum concentration. Inasmuch as the half time also decreased, these results indicate that the production rate for albumin was much greater during methyltestosterone treatment than in the control state. Methyltestosterone thus appears to produce an increased rate of albumin synthesis, i.e., the accumulation of albumin is not due to a decreased rate of degradation. The decreased half time during methyltestosterone treatment is possibly due to a metabolic adjustment to the increased rate of synthesis caused by methyltestosterone. Control values for the half time and turnover rate of albumin obtained in this study are similar to those observed in humans (241).

The increase in intravascular space demonstrated in response to methyltestosterone using either protein is in agreement with previous observations (142) and it is possibly due to the fact that androgens cause positive balances of nitrogen, phosphorus and water (242). It seems likely, however, that the increased albumin production and albumin pool described here could also be a factor in increasing these spaces.

The extrapolation method of Sterling (202) used in this study for estimating total pool size and turnover rate was applied initially to studies of the metabolism of ^{131}I-labeled human serum albumin. A number of other analytical methods have been advocated for protein turnover studies (243). The Sterling method is an "approximation method", rather than one in which "specific models" are based on multi-exponential analysis of plasma curves (244). It has been stated that the Sterling method tends to over-estimate the total pool and the turnover rate, due to the fact that the final slope of the plasma curve is altered by the presence at this time of a higher specific radioactivity in the extravascular pool than in the intravascular pool. Nevertheless, results obtained by this method have provided acceptable values in studies with human albumin in normal subjects when different methods of analysis were compared (241). Furthermore, the basic assumption of the Sterling procedure, i.e., that uniform specific radioactivity is attained throughout the intravascular and extravascular compartments after an equilibration period, was supported by direct determination of extravascular albumin in the rat. In this latter study Sellers and co-workers (245) also showed that among the different methods of analysis the extrapolation method of Sterling gave the closest values to those obtained with direct quantitative immunochemical

determinations of the extravascular albumin pool.

The general assumption made in plasma protein turnover studies is that the newly synthesized protein enters the intravascular compartment before mixing with the extravascular compartment. This assumption has been recently substantiated by the findings of Smallwood and coworkers (246) in the dog for albumin and fibrinogen synthesized in the liver. In the present study the observation that the HDL:albumin radioactivity ratios were higher at 0.5 hr after precursor injection than at later intervals suggests that the newly synthesized apolipoprotein A is released into the circulation more rapidly than albumin. Therefore, apolipoprotein A must enter the intravascular compartment directly.

The cholestatic properties of C_{17}-alkylated steroids in human subjects are well known. The possibility that the changes in protein and lipid metabolism described herein are secondary to methyltestosterone-induced hepatotoxicity seems most unlikely, however, inasmuch as considerable evidence is available which indicates that the canine liver lacks the sensitivity to this agent which characterizes the response in man. The relative insensitivity in the canine response may be due to a more rapid excretion of methyltestosterone in the dog, when compared with man (159, 183). A similar accelerated excretion has been observed with testosterone (186). Tests of liver function were normal in the present and previous (153, 156) studies in this laboratory. In addition, electron microscopic and other morphological evaluations demonstrated no clear-cut changes in liver from dogs treated with methyltestosterone.

Results obtained in the present study indicate that methyltestosterone produces opposite effects on the synthesis of two proteins formed

in the liver. The possibility exists that this agent may prove useful in studies of the regulation of apolipoprotein and other protein synthesis in the liver.

It can be concluded that methyltestosterone significantly reduces the production of the principal apolipoprotein in canine serum lipoproteins. This should result in diminished production in the cell and/or transport from the cell of lipid components of the lipoproteins (76). In fact, decreased cholesterol synthesis during methyltestosterone treatment was observed by Furman and co-workers (156) following ^{14}C-acetate incorporation in the dog. This observed decrease in serum cholesterol formation, however, does not explain the concomitant methyltestosterone-induced decrease in phospholipids and triglycerides in serum. On the other hand, an impairment of apolipoprotein synthesis, such as demonstrated in the present study, provides a mechanism whereby androgens may lower all serum lipid levels.

CHAPTER VI

SUMMARY

1. Lipoprotein fractions were isolated from canine serum by sequential preparative ultracentrifugation and purified. Immunochemical and quantitative chemical analyses were performed to characterize their apolipoprotein and lipid composition.

2. High density (d 1.063-1.210 g/ml) and very high density (d 1.210-1.250 g/ml) lipoproteins contain most of the cholesterol and phospholipid present in canine serum and at least two immunologically distinct apolipoprotein components (A_1-A_2 and A_3). Relatively more protein and phospholipid are present in these fractions than in lower density lipoproteins and the amounts of cholesterol and triglyceride are relatively less. As the hydrated density of high density lipoprotein subfractions increased, the ratio of phospholipid to protein decreased and proportionately greater amounts of the lipoprotein characterized by the A_3-antigenic component, relative to A_1 and A_2, were found. Differences in immunochemical characteristics and amino acid composition suggest that the apolipoprotein A of canine serum is distinct in specific components and/or chemical composition from the human.

3. Low density lipoproteins (d 1.006-1.063 g/ml) contain primarily apolipoprotein B which is also demonstrable in very low density lipoproteins (d < 1.006 g/ml). In addition, the very low density lipoprotein

fraction contains apolipoprotein X, which may correspond with the apolipoprotein C found in human plasma.

4. Immunochemical analyses also demonstrated the presence of very small quantities of an unusual protein component in purified very low density lipoproteins, in unpurified but not in purified high density lipoproteins and in the serum sample from which lipoproteins of $d < 1.250$ g/ml had been removed. This component migrated on immunoelectrophoresis as a γ-globulin and also reacted with anti-canine γ-globulin serum. These observations suggest the possibility that this protein is a specific autoantibody to canine lipoproteins, although alternative explanations are possible.

5. Lipoproteins containing an antigenic component characteristic of one density range were often found in purified, i.e., albumin-free lipoproteins isolated in an adjacent density range, supporting the conclusion that in the dog, as in man, a lipoprotein classification system based solely on hydrated densities has limited applicability.

6. Methyltestosterone reduced the apolipoprotein, cholesterol, phospholipid and triglyceride concentration in each fraction. Less of the total apolipoprotein, cholesterol and phospholipid and a greater proportion of the triglyceride in serum lipoproteins was found in the fraction of $d < 1.063$ g/ml during methyltestosterone administration. Methyltestosterone diminished the protein content of all lipoprotein fractions and increased the phospholipid to protein ratio in each fraction except that of $d < 1.019$ g/ml.

7. Immunochemical analyses demonstrated that methyltestosterone markedly reduced the intensity of the A-reactions, i.e., the antigenic

components characteristic of the principal apolipoproteins in high density lipoproteins. These apolipoproteins are also found in purified low density lipoprotein fractions isolated from control serum, but are markedly diminished during methyltestosterone treatment. Methyltestosterone produced no change in the amino acid composition and immunochemical characteristics of apolipoprotein A. Evidence is presented which favors the conclusion that methyltestosterone selectively reduces the concentration of apolipoproteins which contain the A-antigenic components.

8. Methyltestosterone increased albumin concentration and decreased α_1- and β-globulins significantly, without altering total protein concentration in plasma. No evidence of hepatotoxicity was observed in response to methyltestosterone.

9. Incorporation of ^{14}C-lysine into the protein moiety of high density lipoproteins (d 1.110-1.250 g/ml) and albumin was studied in metabolic steady states before and during methyltestosterone administration. The proportion of radioactivity incorporated into apolipoprotein A was significantly decreased while that incorporated into albumin was increased, suggesting a selective and opposite change in the rate of synthesis of these proteins in response to methyltestosterone.

10. High density lipoproteins (d 1.110-1.250 g/ml) containing ^{14}C-apolipoprotein A and ^{14}C-albumin were obtained from a donor dog given ^{14}C-lysine and used to evaluate the turnover rate and half time of these proteins before and during methyltestosterone administration. Methyltestosterone markedly decreased the total exchangeable pool and turnover rate of apolipoprotein A without significantly altering half time. The albumin pool and turnover rate increased significantly and half time decreased.

11. Results obtained in the incorporation and turnover studies indicate that methyltestosterone significantly and conversely affects the production of two proteins synthesized in the liver, i.e., apolipoprotein A synthesis decreases and albumin synthesis increases. These results are consistent with the conclusion that methyltestosterone exerts a primary effect on the synthesis, rather than on the degradation, of these proteins. Reduction in the synthesis of apolipoprotein A, which may comprise as much as 90% of total apolipoproteins in canine serum, may explain the diminished concentrations in all serum lipids and lipoprotein fractions produced by methyltestosterone.

BIBLIOGRAPHY

1. Macheboeuf, M., Bull. Soc. Chim. Biol., 11, 268 (1929).

2. Blix, G., Tiselius, A., and Svensson, H., J. Biol. Chem., 137, 485 (1941).

3. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., J. Am. Chem. Soc., 68, 459 (1946).

4. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., Jr., J. Am. Chem. Soc., 71, 541 (1949).

5. Gurd, F. R. N., Oncley, J. L., Edsall, J. T., and Cohn, E. J., Discussions Faraday Soc., 6, 70 (1949).

6. McFarlane, A. S., Biochem. J., 29, 660 (1935).

7. Pedersen, K. O., J. Phys. Chem., 51, 156 (1947).

8. Gofman, J. W., Lindgren, F. T., and Elliott, H. A., J. Biol. Chem., 179, 973 (1949).

9. Gofman, J. W., Lindgren, F., Elliott, H., Montz, W., Hewitt, J., Strisower, B., Herring, V. and Lyon, T. P., Science, 111, 166 (1950).

10. Lindgren, F. T., Elliott, H. A., and Gofman, J. W., J. Phys. Chem., 55, 80 (1951).

11. de Lalla, O. F., and Gofman, J. W., Methods Biochem. Analy., 1, 459 (1954).

12. Lewis, L. A., Green, A. A., and Page, I. H., Am. J. Physiol., 171, 391 (1952).

13. Kunkel, H. G., Federation Proc., 9, 193 (1950).

14. Levine, L., Kauffman, D. L., and Brown, R. K., J. Exptl. Med., 102, 105 (1955).

15. Avigan, J., Redfield, R., and Steinberg, D., Biochim. Biophys. Acta, 20, 557 (1956).

16. Shore, B., Arch. Biochem. Biophys., 71, 1 (1957).

17. Rodbell, M., Science, 127, 701 (1958).

18. Rodbell, M., and Fredrickson, D. S., J. Biol. Chem., 234, 562 (1959).

19. Shore, V., and Shore, B., Biochem. Biophys. Res. Commun., 9, 455 (1962).

20. Bobbit, J. L., and Levy, R. S., Biochemistry, 4, 1282 (1965).

21. Gustafson, A., Alaupovic, P., and Furman, R. H., Biochim. Biophys. Acta, 84, 767 (1964).

22. Gustafson, A., Alaupovic, P., and Furman, R. H., Biochemistry, 5, 632 (1966).

23. Alaupovic, P., Progr. Biochem. Pharmacol., 4, 91 (1968).

24. Gofman, J. W., and Tandy, R. K., in Atherosclerotic Vascular Disease, Brest, A. N., and Moyer, J. H., ed., Appleton-Century-Crofts, New York, 1967, p. 162.

25. Scanu, A., Lewis, L. A., and Bumpus, F. M., Arch. Biochem. Biophys., 74, 390 (1958).

26. Fisher, W. R., and Gurin, S., Science, 143, 362 (1964).

27. Oncley, J. L., in Proc. of an International Symposium on Lipid Transport, Meng, H. C., ed., C. C. Thomas, Springfield, Ill., 1964, p. 70.

28. Ewing, A. M., Freeman, N. K., and Lindgren, F. T., Advan. Lipid Res., 3, 25 (1965).

29. Cook, W. H., and Martin, W. G., Can. J. Biochem. Physiol., 40, 1273 (1962).

30. Forte, G. M., Nichols, A. V., and Glaeser, R. M., Chem. Phys. Lipids, 2, 396 (1968).

31. Jones, A. L., and Price, J. M., J. Histochem. Cytochem., 16, 366 (1968).

32. McConathy, W. J., and Alaupovic, P., Circulation, 40 (Suppl. III), 17 (1969).

33. Brown, W. V., Levy, R. I., and Fredrickson, D. S., J. Biol. Chem., 244, 5687 (1969).

34. Brown, W. V., Levy, R. I., and Fredrickson, D. S., Biochim. Biophys. Acta, 280, 573 (1970).

35. Lee, D. M., and Alaupovic, P., Biochemistry, 9, 2244 (1970).

36. Shore, B., and Shore, V., Biochemistry, 7, 2773 (1968).

37. Scanu, A., Reader, W., and Edelstein, C., Biochim. Biophys. Acta, 160, 32 (1968).

38. Alaupovic, P., Lee, D. M., and McConathy, W. J., Circulation, 40 (Suppl. III), 2 (1969).

39. Utermann, G., and Wiegandt, H., Humangenetik, 8, 39 (1969).

40. Gotto, A. M., Levy, R. I., Birnbaumer, M. E., and Fredrickson, D. S., Clin. Res., 16, 551 (1968).

41. Dearborn, G. D., and Wetlaufer, D. B., Proc. Natl. Acad. Sci. U. S., 62, 179 (1969).

42. Scanu, A. M., Biochim. Biophys. Acta, 200, 570 (1969).

43. Gotto, A. M., and Shore, B., Nature, 224, 69 (1969).

44. Sodhi, H. S., and Gould, R. G., J. Biol. Chem., 242, 1205 (1967).

45. Scanu, A., J. Biol. Chem., 242, 711 (1967).

46. Hatch, F. T, and Lees, R. S., Advan. Lipid Res., 6, 1 (1968).

47. Nichols, A. V., and Smith, L., J. Lipid Res., 6, 206 (1965).

48. Hagerman, J. S., and Gould, R. G., Proc. Soc. Exptl. Biol. Med., 78, 329 (1951).

49. Havel, R. J., Felts, J. M., and Van Duyne, C. M., J. Lipid Res., 3, 297 (1962).

50. Roheim, P. S., Haft, D. E., Gidez, L. I., White, A., and Eder, H. A., J. Clin. Invest., 42, 1277 (1963).

51. Mahley, R. W., Bersot, T. P., LeQuire, V. S., Levy, R. I., Windmueller, H. G., and Brown, W. V., Science, 168, 380 (1970).

52. Furman, R. H., Sanbar, S. S., Alaupovic, P., Bradford, R. H., and Howard, R. P., J. Lab. Clin. Med., 63, 193 (1964).

53. Korn, E. D., J. Biol. Chem., 215, 15 (1955).

54. Glomset, J. A., J. Lipid Res., 9, 155 (1968).

55. Nichols, A. V., Strisower, E. H., Lindgren, F. T., Adamson, G. L., and Coggiola, E. L., Clin. Chim. Acta, 20, 277 (1968).

56. Furman, R. H., Howard, R. P., and Alaupovic, P., Metabolism, 11 879 (1962).

57. Glomset, J. A., Janssen, E. T., Kennedy, R., and Dobbins, J., J. Lipid Res., 7, 639 (1966).

58. Goodman, D. S., J. Clin. Invest., 43, 2026 (1964).

59. Gitlin, D., Cornwell, D. G., Nakasato, D., Oncley, J. L., Hughes, W. L., Jr., and Janeway C. A., J. Clin. Invest., 37, 172 (1958).

60. Havel, R. J., and Gordon, R. S., Jr., J. Clin. Invest., 39, 1777 (1960).

61. Strisower, E. H., J. Atherosclerosis Res., 3, 445 (1963).

62. Kolb, F. O., de Lalla, O. F., and Gofman, J. W., Metabolism, 4, 310 (1955).

63. Fredrickson, D. S., Levy, R. I., and Lees, R. S., New Engl. J. Med., 276, 94 (1967).

64. Sabesin, S. M., and Isselbacher, K. J., Science, 147, 1149 (1965).

65. Windmueller, H. G., and Levy, R. I., J. Biol. Chem., 243, 4878 (1968).

66. Gould, R. G., Am. J. Med., 11, 209 (1951).

67. Bruckdorfer, K. R., and Green, C., Biochem. J., 104, 270 (1967).

68. Reed, C. F., J. Clin. Invest., 47, 749 (1968).

69. Sakagami, T., Minari, O., and Orii, T., Biochim. Biophys. Acta, 98, 111 (1965).

70. Mulder, E., and Van Deenen, L. L. M., Biochim. Biophys. Acta, 106, 348 (1965).

71. Graham, J. M., and Green, C., Biochem. J., 103, 16C (1967).

72. Oncley, J. L., Gurd, F. R. N., and Melin, M., J. Am. Chem. Soc., 72, 458 (1950).

73. Krinsky, N. I., Cornwell, D. G., and Oncley, J. L., Arch. Biochem. Biophys., 73, 233 (1958).

74. Trams, E. G., Comp. Biochem. Physiol., 28, 1177 (1969).

75. Walton, K. W., Campbell, D. A., and Tonks, E. L., Clin. Sci., 29, 199 (1965).

76. Trams, E. G., and Brown, E. A., J. Theoret. Biol., 12, 311 (1966).

77. Mueller, P., and Rudin, D. O., J. Theoret. Biol., 4, 268 (1963).

78. Scanu, A., and Page, I. H., J. Clin. Invest., 41, 495 (1962).

79. Omura, T., Siekevitz, P., and Palade, G. E., J. Biol. Chem., 242, 2389 (1967).

80. Miller, L. L., Bly, C. G., Watson, M. L., and Bale, W. F., J. Exptl. Med., 94, 431 (1951).

81. Rodbell, M., Fredrickson, D. S., and Ono, K., J. Biol. Chem., 234, 567 (1959).

82. Isselbacher, K. J., and Budz, D. M., Nature, 200, 364 (1963).

83. Hatch, F. T., Aso, Y., Hagopian, L. M., and Rubenstein, J. J., J. Biol. Chem., 241, 1655 (1966).

84. Roheim, P. S., Gidez, L. I., and Eder, H. A., J. Clin. Invest., 45, 297 (1966).

85. Marsh, J. B., and Whereat, A. F., J. Biol. Chem., 234, 3196 (1959).

86. Radding, C. M., and Steinberg, D., J. Clin. Invest., 39, 1560 (1960).

87. Avigan, J., Eder, H. A., and Steinberg, D., Proc. Soc. Exptl. Biol. Med., 95, 429 (1957).

88. Radding, C. M., Bragdon, J. H., and Steinberg, D., Biochim. Biophys. Acta, 30, 443 (1958).

89. Wilcox, H. G., Fried, M., and Heimberg, M., Biochim. Biophys. Acta, 106, 598 (1965).

90. Gitlin, D., Cornwell, D. G., Nakasato, D., Oncley, J. L., Hughes, W. L., Jr., and Janeway, C. A., J. Clin. Invest., 36, 172 (1957).

91. Marsh, J. B., J. Biol. Chem., 238, 1752 (1963).

92. Bungenberg de Jong, J. J., and Marsh, J. B., J. Biol. Chem., 243, 192, (1968).

93. Eylar, E. H., J. Theoret. Biol., 10, 89 (1966).

94. Jones, A. L., Ruderman, N. B., and Herrera, M. G., J. Lipid. Res., 8, 429 (1967).

95. Hamilton, R. L., Regen, D. M., Gray, M. E., and LeQuire, V. S., Lab. Invest., 16, 305 (1967).

96. Robinson, D. S., and Harris, P. M., Biochem. J., 80, 361 (1961).

97. Robinson, D. S., and Seakins, A., Biochim. Biophys. Acta, 62, 163 (1962).

98. Seakins, A., and Robinson, D. S., Biochem. J., 86, 401 (1963).

99. Windmueller, H. G., and Levy, R. I., J. Biol. Chem., 242, 2246 (1967).

100. Faloona, G. R., Stewart, B. N., and Fried, M., Biochemistry, 7, 720 (1968).

101. Trams, E. G., Brown, E. A., and Lauter, C. J., Lipids, 1, 309 (1966).

102. Ruderman, N. B., Richards, K. C., Valles de Bourges, V., and Jones, A. L., J. Lipid Res., 9, 613 (1968).

103. Eder, H. A., Roheim, P. S., and Switzer, S., Trans. Assoc. Am. Physicians, 77, 259 (1964).

104. Hillyard, L. A., Entenman, C., Feinberg, H., and Chaikoff, I. L., J. Biol. Chem., 214, 79 (1955).

105. Havel, R. J., Eder, H. A., and Bragdon, J. H., J. Clin. Invest., 34, 1345, (1955).

106. Evans, L., J. Dairy Sci., 47, 46 (1964).

107. Evans, L., Patton, S., and McCarthy, R. D., J. Dairy Sci., 44, 475 (1961).

108. Puppione, D. L., Univ. of Calif. Radiation Laboratory Report, UCRL-18821, 1969.

109. Kirkeby, K., Scand. J. Clin. Lab. Invest., 18, 437 (1966).

110. Wilcox, H. G., and Heimberg, M., J. Lipid Res., 11, 7 (1970).

111. Gergely, J., Protides Biol. Fluids, Proc. Colloq., 8, 138 (1961).

112. Walton, K. W., and Darke, S. J., Protides Biol. Fluids, Proc. Colloq., 10, 146 (1962).

113. Storiko, K., and Fisher, G. B., Protides Biol. Fluids, Proc. Colloq., 13, 291 (1965).

114. Janado, M., Martin, W. G., and Cook, W. H., Can. J. Biochem., 44, 1201 (1966).

115. Kalab, M., and Martin, W. G., J. Chromatog., 35, 230 (1968).

116. Cox, A. C., and Tanford, C., J. Biol. Chem., 243, 3083 (1968).

117. Camejo, G., Biochemistry, 6, 3228 (1967).

118. Narayan, K. A., Dudacek, W. E., and Kummerow, F. A., Biochim. Biophys. Acta, 125, 581 (1966).

119. Koga, S., Horwitz, D. L., and Scanu, A. M., J. Lipid Res., 10, 577 (1969).

120. Nichols, A. V., Proc. Natl. Acad. Sci. U. S., 64, 1128 (1969).

121. Alaupovic, P., Sanbar, S. S., Furman, R. H., Sullivan, M. L., and Walraven, S. L., Biochemistry, 5, 4044 (1966).

122. Emmens, C. W., and Parkes, A. S., J. Endocrinol., 1, 323 (1939).

123. Foss, G. L., Lancet, I, 502 (1939).

124. Biskins, G. R., and Mark, J., Bull. Johns Hopkins Hosp., 65, 212 (1959).

125. Simonson, E., Kearns, W. M., and Enzer, N., Endocrinology, 28, 506 (1941).

126. Samuels, L. T., Henschel, A. F., and Keys, A., J. Clin. Endocrinol., 2, 649 (1942).

127. Reifenstein, E. C., Jr., Forbes, A. P., Albright, F., Donaldson, E., and Carroll, E., J. Clin. Invest., 24, 416 (1945).

128. Levedahl, B. H., and Samuels, L. T., J. Biol. Chem., 186, 857 (1950).

129. Partridge, J. W., Boling, L., De Wind, L., Margen, S., and Kinsell, L. W., J. Clin. Endocrinol., 13, 189 (1953).

130. Novak, A., Am. J. Physiol., 191, 306 (1957).

131. Frieden, E. H., Laby, M. R., Bates, F., and Layman, N. W., Endocrinology, 60, 290 (1957).

132. Bernelli-Zazzera, A., Bassi, M., Comolli, R., and Lucchelli, P., Nature, 182, 663 (1958).

133. Torizuka, K., Hamamoto, K., Koshiyama, K., Iwai, K., Takayama, H., Miyake, T., Metabolism, 12, 11 (1963).

134. Kassenaar, A., Kouwenhoven, A., and Querido, A., Acta Endocrinol., 39, 223 (1962).

135. Kochakian, C. D., and Harrison, D. G., Endocrinology, 70, 99 (1962).

136. Kochakian, C. D., Tanaka, R., and Hill, J., Am. J. Physiol., 201, 1068 (1961).

137. Wilson, J. D., and Loeb, P. M., in Developmental and Metabolic Control Mechanisms and Neoplasia, Williams and Wilkins Co., Baltimore, Md., 1965, p. 375.

138. Wilson, J. D., Bruchovsky, N., and Chatfield, J. N., in Progress in Endocrinology, Excerpta Medica Fndn., Amsterdam, The Netherlands, 1969, p. 17.

139. Russ, E. M., Eder, H. A., and Barr, D. P., Am. J. Med., 19, 4 (1955).

140. Sachs, B. A., Danielson, E., and Weston, R. E., J. Clin. Endocrinol., 16, 1388 (1956).

141. Studnitz, W. V., and Nyman, M., J. Clin. Endocrinol., 17, 910 (1957).

142. Federman, D. D., Robbins, J., and Rall, J. E., J. Clin. Invest., 37, 1024, 1958.

143. Ingbar, S. H., in Progress in Endocrinology, Excerpta Medica Fndn., Amsterdam, The Netherlands, 1969, p. 1200.

144. Furman, R. H., Alaupovic, P., and Howard, R. P., Progr. Biochem. Pharmacol., 2, 215 (1967).

145. Furman, R. H., Alaupovic, P., Bradford, R. H., and Howard, R. P., Progr. Biochem. Pharmacol., 4, 334 (1968).

146. Furman, R. H., and Howard, R. P., Ann. Internal Med., 47, 969 (1957).

147. Furman, R. H., Howard, R. P., Norcia, L. N., and Keaty, E. C., Am. J. Med., 24, 80 (1958).

148. Lewis, L. A., Masson, G. M. C., and Page, I. H., Proc. Soc. Exptl. Biol. Med., 82, 684 (1953).

149. Howard, R. P., and Furman, R. H., Ann. Internal Med., 56, 668 (1962).

150. Furman, R. H., and Howard, R. P., Metabolism, 11, 76 (1962).

151. Furman, R. H., Howard, R. P., and Alaupovic, P., J. Lab. Clin. Med., 60, 876 (1962).

152. Robinson, R. W., Le Beau, R. J., and Cohen, W. D., J. Clin. Endocrinol. Metab., 24, 708 (1964).

153. Furman, R. H., Norcia, L. N., Robinson, C. W., Jr., and Gonzales, I. E., Am. J. Physiol., 191, 561 (1957).

154. Mosbach, E. H., and Abell, L. L., Circulation, 22, 658 (1960).

155. Abell, L. L., Mosbach, E. H., and Kendall, F. E., Circulation Res., 10, 846 (1962).

156. Furman, R. H., Robinson, C. W., Jr., Bradford, R. H., Alaupovic, P., and Norcia, L. N., Proc. Soc. Exptl. Biol. Med., 113, 789 (1963).

157. Abell, L. L., and Mosbach, E. H., J. Lipid Res., 9, 98 (1968).

158. Dorfman, R. I., and Shipley, R. A., Androgens, John Wiley and Sons, Inc., New York, 1956, p. 117.

159. Mosbach, E. H., Shefer, S., and Abell, L. L., J. Lipid Res., 9, 93 (1968).

160. Fillios, L. C., and Mann, G. V., Circulation Res., 4, 406 (1956).

161. Nakamura, K., Toyota, A., Masuda, Y., and Nakamura, H., J. Atheroscler. Res., 7, 783 (1967).

162. Abell, L. L., and Mosbach, E. H., J. Lipid Res., 3, 88 (1962).

163. Nakamura, K., Masuda, Y., and Nakamura, H., J. Atherosclerosis Res., 7, 253 (1967).

164. Nakamura, K., Nakamura, H., Masuda, Y., and O'Saki, Y., Naunyn-Schmiedebergs Arch. Pharmak., 264, 327 (1969).

165. Kochakian, C. D., and Stettner, C. E., Am. J. Physiol., 155, 255 (1948).

166. Korner, A., and Young, F. G., J. Endocrinol., 13, 78 (1955).

167. Coxon, R. V., Korenchevsky, V., and Lawrence, A., J. Pathol. Bacteriol., 72, 613 (1956).

168. Laron, Z., and Kowadlo-Silbergeld, A., Acta Endocrinol., 45, 427 (1964).

169. Gerhards, E., Nieuweboer, B., and Richter, E., Arzneimittel-Forsch., 19, 765 (1969).

170. Jailer, J. W., Am. J. Physiol., 130, 503 (1940).

171. Kinsell, L. W., Hertz, S., and Reifenstein, E. C., Jr., J. Clin. Invest., 23, 880 (1944).

172. Wilkins, L., Fleischmann, W., and Howard, J. E., Bull. Johns Hopkins Hosp., 69, 493 (1941).

173. Samuels, L. T., Sellers, D. M., and MacCauley, G. J., J. Clin. Endocrinol., 6, 655 (1946).

174. Hoagland, C. L., Gilder, H., and Shank, R. E., J. Exptl. Med., 81, 423 (1945).

175. Werner, S. C., Am. J. Med., 3, 52 (1947).

176. deLorimier, A. A., Gordan, G. S., Lowe, R. C., and Carbone, J. V., Arch. Internal. Med., 116, 289 (1965).

177. Kory, R. C., Bradley, M. H., Watson, R. N., Callahan, R., and Peters, B. J., Am. J. Med., 26, 238 (1959).

178. Schaffner, F., Popper, H., and Chesrow, E., Am. J. Med., 26, 249 (1959).

179. Schaffner, F., Popper, H., and Perez, V., J. Lab. Clin. Med., 56, 623 (1960).

180. Glober, G. A., and Wilkerson, J. A., J. Am. Med. Assoc., 204, 168 (1968).

181. Dorfman, R. I., Ann. Rev. Biochem., 26, 523 (1957).

182. Segaloff, A., Gabbard, R. B., Carriere, B. T., and Rongone, E. L., Steroids Suppl., 1, 149 (1965).

183. Quincey, R. V., and Gray, C. H., J. Endocrinol., 37, 37 (1967).

184. Horton, R., Shinsako, J., and Forsham, P. H., Acta Endocrinol., 48, 446 (1965).

185. Camacho, A. M., and Migeon, C. J., J. Clin. Invest., 43, 1083 (1964).

186. Chapdelaine, A., J. Clin. Invest., 48, 2063 (1969).

187. Folch, J., Lees, M., and Sloane Stanley, G. H., J. Biol. Chem., 226, 497 (1957).

188. Sperry, W. M., and Webb, M., J. Biol. Chem., 187, 97 (1950).

189. Fiske, C. H., and Subbarow, Y., J. Biol. Chem., 66, 375 (1925).

190. Youngburg, G. E., and Youngburg, M. V., J. Lab. Clin. Med., 16, 158 (1930).

191. Van Handel, E., and Zilversmit, D. B., J. Lab. Clin. Med., 50, 152 (1957).

192. Kessler, G., and Lederer, H., in Automation in Analytical Chemistry, Skeggs, L. T., Jr., ed., Mediad, Inc., New York, 1966, p. 341.

193. Weichselbaum, T. E., Am. J. Clin. Pathol. (Tech. Sec. 16), 10, 40 (1946).

194. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

195. Gustafson, A., Biochim. Biophys. Acta, 84, 223, 1964.

196. Ouchterlony, O., Acta Pathol. Microbiol. Scand., 32 231 (1953).

197. Scheidegger, J. J., Intern. Arch. Allergy Appl. Immunol., 7, 103 (1955).

198. Schwert, G. W., J. Am. Chem. Soc., 79, 139 (1957).

199. Gordon, C. F., and Wolfe, A. L., Anal. Chem., 32, 574 (1960).

200. Campbell, R. M., Cuthbertson, D. P., Matthews, C. M. E., and McFarlane, A. S., Int. J. Appl. Radiat., 1, 66 (1956).

201. Bancroft, H., in Introduction to Biostatistics, Hoeber-Harper, New York, 1957, p. 163.

202. Sterling, K., J. Clin. Invest., 30, 1228 (1951).

203. Rosenthal, S. M., and White, E. C., J. Am. Med. Assoc., 84, 1112 (1925).

204. Kingsley, G. R., Getchell, G., and Schaffert, R. R., in Standard Methods of Clinical Chemistry, Vol. I., Reiner, M., ed., Academic Press, New York, 1953.

205. Scanu, A., and Page, I. H., J. Lipid Res., 2, 161 (1961).

206. Scanu, A., Lewis, L. A., and Page, I. H., J. Exptl. Med., 108, 185 (1958).

207. Wathen, J. D., and Levy, R. S., Biochemistry, 5, 1099 (1966).

208. Levy, R. S., Lynch, A. C., McGee, E. D., and Mehl, J. W., J. Lipid Res., 8, 463 (1967).

209. Fredrickson, D. S., Levy, R. I., and Lees, R. S., New Engl. J. Med., 276, 34 (1967).

210. Scanu, A., J. Lipid Res., 7, 295 (1966).

211. Scanu, A., and Granda, J. L., Biochemistry, 5, 446 (1966).

212. Lewis, L. A., and Page, I. H., Am. J. Med., 38, 286 (1965).

213. Beaumont, J. -L., and Lorenzelli, L., Ann. Biol. Clin., 25, 655 (1967).

214. Gustafson, A., Alaupovic, P., and Furman, R. H., Biochemistry, 4, 596 (1965).

215. Skipski, V. P., Barclay, M., Barclay, R. K., Fetzer, V. A., Good, J. J., and Archibald, F. M., Biochem. J., 104, 340 (1967).

216. Yokoyama, A., and Zilversmit, D. B., J. Lipid Res., 6, 241 (1965).

217. Nichols, A. V., Advan. Biol. Med. Phys., 11, 110 (1967).

218. Kruskemper, H. -L., Anabolic Steroids, Academic Press, New York, 1968.

219. Fried, M., Wilcox, H. G., Faloona, G. R., Eoff, S. P., Hoffman, M. S., and Zimmerman, D., Comp. Biochem. Physiol., 25, 651 (1968).

220. Isselbacher, K. J., Scheig, R., Plotkin, G. R., and Caulfield, J. B., Medicine, 43, 347 (1964).

221. Florsheim, W. H., Faircloth, M. A., Graff, D., Austin, N. S., and Velcoff, S. M., Metabolism, 12, 598 (1963).

222. Rothschild, M. A., Bauman, A., Yalow, R. S., and Berson, S. A., J. Clin. Invest., 36, 422 (1957).

223. Rothschild, M. A., Schreiber, S. S., Oratz, M., and McGee, H. L., J. Clin. Invest., 37, 1229 (1958).

224. Marsh, J. B., and Drabkin, D. L., Metabolism, 9, 946 (1960).

225. Drabkin, D. L., and Marsh, J. B., J. Biol. Chem., 212, 623 (1955).

226. Marsh, J. B., and Drabkin, D. L., J. Biol. Chem., 230, 1073 (1958).

227. Braun, G. A., Marsh, J. B., Drabkin, D. L., Biochem. Biophys. Res. Commun., 8, 28 (1962).

228. Katz, J., Bonorris, G., Okuyama, S., and Sellers, A. L., Am. J. Physiol., 212, 1255 (1967).

229. Rothschild, M. A., Oratz, M., Wimer, E., and Schreiber, S. S., J. Clin. Invest., 40, 545 (1961).

230. Allen, J. C., Baxter, J. H., and Goodman, H. C., J. Clin. Invest., 40, 499 (1961).

231. Rothschild, M. A., Oratz, M., Wimer, E., and Schreiber, S. S., Proc. Soc. Exptl. Biol. Med., 104, 478 (1960).

232. Whitehead, T. P., Dykes, P. W., Gloster, J., and Harris, P., Clin. Sci., 38, 233 (1970).

233. Carlson, L. A., Boberg, J., and Hogstedt, B., in Handbook of Physiology, Section 5, Renold, A. E., and Cahill, G. F., Jr., ed., American Physiological Society, Washington, D. C., 1965, p. 625.

234. Gofman, J. W., Robin, L., McGinley, J. P., and Jones, H. B., Am. J. Med., 17, 514 (1954).

235. Jones, R. J., Cohen, L., and Corbus, H., Am. J. Med., 19, 71 (1955).

236. Furman, R. H., Howard, R. P., Lakshmi, K., and Norcia, L. N., Am. J. Clin. Nutr., 9, 73 (1961).

237. Cornwell, D. G., Kruger, F. A., Hamwi, G. J., and Brown, J. B., Am. J. Clin. Nutr., 9, 24 (1961).

238. Walton, K. W., Scott, P. J., Dykes, P. W., and Davies, J. W. L., Clin. Sci., 29, 217 (1965).

239. Schwartz, E., J. Lab. Clin. Med., 45, 340 (1955).

240. Farthing, C. P., Gerwing, J., and Shewell, J., J. Endocrinol., 21, 83 (1960).

241. Takeda, Y., and Reeve, E. B., J. Lab. Clin. Med., 61, 183 (1963).

242. Reifenstein, E. C., Jr., Albright, F., and Wells, S. L., J. Clin. Endocrinol., 5, 367 (1945).

243. Donato, L., J. Nucl. Med., 1, 3 (1966).

244. Matthews, C. M. E., Phys. Med. Biol., 2, 36 (1957).

245. Sellers, A. L., Katz, J., Bonorris, G., and Okuyama, S., J. Lab. Clin. Med., 68, 177 (1966).

246. Smallwood, R. A., Jones, E. A., Craigie, A., Raia, S., and Rosenoer, V. M., Clin. Sci., 35, 35 (1968).