1985

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Rama Khokha

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EFFECTS OF d-NORGESTREL ON LIPID METABOLISM IN THE RAT

by

Rama Khokha

Department of Biochemistry

Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

August, 1984

ABSTRACT

The progestin, d-norgestrel (d-Ng) has been widely administered to women in oral contraceptives. The present study was undertaken to examine the effects of d-Ng on lipid and lipoprotein metabolism and elucidate its mechanism of action using the rat as the experimental model.

d-Ng fed to female rats over a period of 18 days in conventional doses, significantly lowered the plasma total and very low density lipoprotein (VLDL)-triglycerides. The concentration of VLDL-phospholipids fell in proportion to the decrease in VLDL-triglycerides. In contrast, d-Ng significantly elevated the plasma total and low density lipoprotein (LDL)-cholesterol. Concurrently, LDL-phospholipids were also significantly elevated by d-Ng treatment.

The triglyceride synthesis was studied using isolated rat hepatocytes. d-Ng (0.1mM), in the presence of 0.1% dimethylsulfoxide concentration of the medium, significantly inhibited the incorporation of both [9, 10-^{3}H] palmitate and [U-^{14}C] glycerol into triglycerides synthesized by the hepatocytes (by 19-20%). Concurrently, d-Ng also inhibited (by 51-57%) the secretion of labelled triglycerides from isolated hepatocytes. The inhibition of triglyceride synthesis by d-Ng was dose-dependent.

The effect of d-Ng treatment on the rate limiting enzymes of triglyceride synthesis was examined. d-Ng significantly reduced the specific activity of hepatic glycerol phosphate acyltransferase in

the microsomes. However, the specific activity of hepatic mitochondrial glycerol phosphate acyltransferase was unchanged as compared to controls. In addition, d-Ng treatment also significantly reduced the specific activity of phosphatidic acid aqueous (PA_{aq}) dependent phosphatidic acid phosphatase specifically in the microsomes. The phosphatidic acid membrane bound (PA_{mb}) dependent phosphatidic acid phosphatase specific activity in microsomes as well as cytosol was not affected by d-Ng treatment. These results suggest that d-Ng acts by inhibiting hepatic microsomal glycerol phosphate acyltransferase and PA_{aq} dependent phosphatidic acid phosphatase specific activity, subsequently reducing the triglyceride synthesis and secretion by the liver. This explains, at least in part, the lower levels of plasma and VLDL triglycerides in the d-Ng treated rats.

Studies of VLDL and LDL turnover were carried out by examining the kinetics of labelled apolipoprotein-B of VLDL and LDL injected into d-Ng treated and control rats. Analysis of specific activity time curves showed that the fractional catabolic rate of VLDL-apolipoprotein B (human ^{125}I-VLDL apolipoprotein-B and rat ^{125}I-VLDL apolipoprotein-B) was markedly increased with d-Ng, in keeping with enhanced VLDL clearance in this group. This would explain the reduction in the pool size of VLDL-apolipoprotein B because the production rate was unaffected. The intermediate density lipoprotein (IDL)-apolipoprotein B clearance in treated rats was also increased above controls. The IDL and LDL-apolipoprotein B production

independent of VLDL-apolipoprotein B catabolism was observed in control as well as d-Ng treated rats. This however was similar in the two groups. Analysis of the specific activity time curves of LDL-apolipoprotein B (human ^{125}I-LDL and rat ^{131}I-LDL-apolipoprotein B) showed a lower fractional catabolic rate in d-Ng treated rats. This caused the markedly larger pool size of LDL-apolipoprotein B, since production rate was similar in the two groups. The primary effect of d-Ng on both the lipoproteins was on their efficiency of removal from the plasma. These divergent effects of d-Ng explain both its triglyceride-lowering and cholesterol-elevating effects in rats.

This investigation was supported by a Medical Research Council Studentship to Rama Khokha and a Medical Research Council Grant to Dr. B. Wolfe.

The author expresses her indebtedness.

ACKNOWLEDGEMENTS

I wish to express my sincerest appreciation to my supervisor, Dr. B. Wolfe for his continued guidance, interest and encouragement during the completion of this research and writing of this thesis. I am also grateful to the members of my advisory committee, Drs. R. Hobkirk and W. C. McMurray for their constructive criticism and valuable suggestions.

Special thanks are due to Dr. M.W. Huff, for his helpful advice, discussions and friendship. I am also most appreciative of the excellent technical assistance provided by Mr. B. Sutherland and Miss S. Koenig and Ms. S. Brock who ensured that equipment and supplies were readily available.

The experiments on phosphatidic acid phosphatase reported in Chapter 4 were done in collaboration with Mr. P. Walton and Dr. F. Possmayer of this department. The contribution of these investigators is greatly appreciated. I am extremely thankful to Dr. M. W. Khalil for his help in the use of HPLC for determining the medium concentrations of d-norgestrel reported in Chapter 3. I am also grateful to Dr. K.K.Carroll who generously donated the Lard and Fat Free diets.

I would also like to thank Mrs. E. Wolfe for her expert artwork and Miss D. Gingerich for typing the final version of this thesis.

Finally, I would like to acknowledge support of my family and friends, who gave me encouragement throughout the completion of this work.

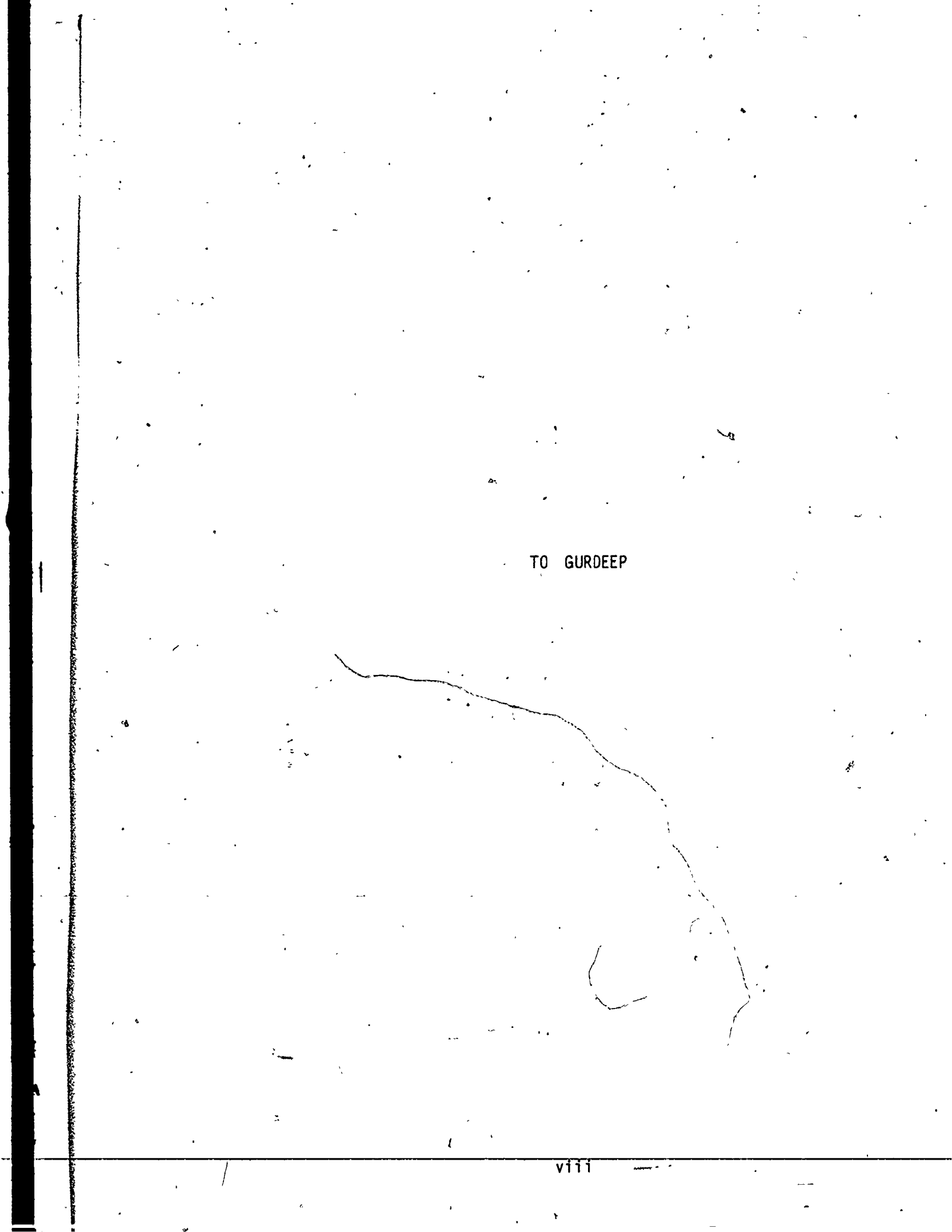
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

Ng	Norgestrel
TG	Triglyceride
CHOL	Cholesterol
CE	Cholesterol ester
PL	Phospholipid
VLDL	Very low density lipoprotein
LDL	Low density lipoprotein
IDL	Intermediate density lipoprotein
HDL	High density lipoprotein
CM	Chylomicrons
LCAT	Lecithin:cholesterol acyltransferase
LPL	Lipoprotein lipase
HTGL	Hepatic triglyceride lipase
GPAT	Glycerol phosphate acyltransferase
PA	Phosphatidic acid
FCR	Fractional catabolic rate
ACR	Absolute catabolic rate
PR	Production rate
PAPase	Phosphatidic acid phosphatase
SEM	Standard error of the mean
SD	Standard deviation
EDTA	Ethylenediaminetetraacetic acid

DMSO	Dimethylsulfoxide
cAMP	Adenosine 3', 5' cyclic monophosphate
cGMP	Guanosine 3', 5' cyclic monophosphate
kD	Kilo Daltons
mRNA	Messenger ribonucleic acid
apo	Apolipoprotein

CHAPTER 1

INTRODUCTION

1.1 Oral Contraceptives

Oral contraceptives are now recognized as the most effective means of preventing pregnancy and thus play a dominant role in family planning. Today, the pill is used world-wide by more than one hundred million women. Haberlandt first advocated the use of ovarian and placental hormones as a means of temporary hormonal sterilization in 1924. Later in 1955, Pincus demonstrated that ovulation was inhibited in women by orally administered progesterone. The combined effects of Pincus and the Searle company then led to the marketing of first oral contraceptive Enovid in 1962 (Pincus, 1956; Tyler, 1974; Diczfalusy, 1982).

Most oral contraceptive formulations are a combination of estrogen and progestin. While only two estrogens, ethinyl estradiol and its 3-methyl ester, mestranol have been used in commercial preparations, several different types of progestogens have been tried. The variety of progestogens fall into three main categories, i) pregnanes: chlormadinone acetate, megestrol and medroxyprogesterone, ii) estranes: norethynodrel, ethynodiol diacetate and lynestrol and iii) gonanes: norgestrel (Briggs, 1977). The three major contraceptive modes of action attributed to oral contraceptive preparations are inhibition of ovulation, alteration of cervical mucus and distortion of endometrium. There

are also other conjectured mechanisms such as changes in tubal motility and uterine contraction and possibly the elimination of capacitation. The inhibition of ovulation is mainly accomplished by the estrogen action on the hypothalamo-pituitary-ovarian axis which leads to the suppression of luteinizing hormone releasing factor (LHRF). This subsequently results in the failure of the midcycle surge of luteinizing hormone (LH) and ovulation. On the other hand, the progestogen is required for the control of the menstrual flow following oral contraceptive withdrawal. It also plays a major role in the alteration of cervical mucus, making it less penetrable to sperm (Edgren, 1969; Ulstein and Myklebust, 1982)

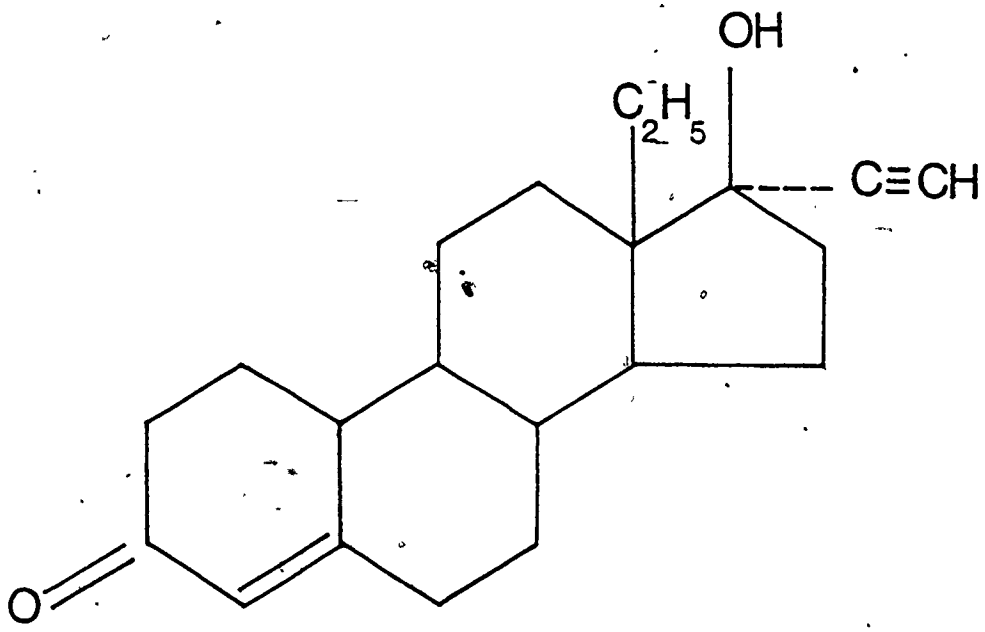
1.1.1. Norgestrel

Norgestrel, 13β-ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one (Ng) is considered today to be the most successful progestogen synthesized so far (Fig 1.1). It is a totally synthetic antiestrogenic compound, qualitatively similar to progesterone but much more potent when given by mouth (Edgren, 1969). The original compound was a racemic mixture composed of d and l isomers. Subsequent chemical separation has demonstrated that biological activity resides only in the d- isomer which is also known as levonorgestrel (Jones et al, 1979).

The bioavailability of orally administered norgestrel has been reported to be 80-90% in the women compared to only 9% in the rat (Dusterberg et al, 1981; Back et al, 1981). Disposition of

FIGURE 1.1

Structure of Norgestrel. A common synthetic gonane progestin used in the formulation of oral contraceptives.


OH
C2H5
C≡CH
O

norgestrel in the plasma follows a bi-exponential pattern in women. An initial rapid phase lasting up to 8 hr followed by a slower phase with a half-life between 8 to 24 hrs has been reported with peak plasma levels observed two hours after the ingestion (Gerhards et al, 1971; Victor et al, 1975). 3α5β-Tetrahydronorgestrel and 16β-hydroxynorgestrel are the major metabolites of dl-norgestrel degradation in the plasma. Besides these, at least 23 other metabolites are found in the urine present in either free form or as conjugates of sulfate and glucuronide. The glucuronide conjugates appeared to be predominantly derived from d isomer and sulfate conjugates from l-isomer (Sisenwine et al, 1975; Sisenwine et al, 1973; Littleton et al, 1968).

1.2. Lipoproteins

1.2.1 Structure and Function of Lipoproteins

Plasma lipoproteins are macromolecular complexes of lipids bound to a variety of polypeptides (the apolipoproteins), and serve to transport water-insoluble lipids in the blood. The five major classes of lipoproteins based on operational definitions such as electrophoretic mobility and rate of ultracentrifugal flotation in salt solutions are chylomicrons (CM), very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). Each class consists of a wide spectrum of particles varying in size. Table 1.1 summarizes the composition and properties of human plasma lipoproteins. The rat lipoproteins are similar to human

TABLE 1.1

CHARACTERISTICS OF HUMAN PLASMA LIPOPROTEINS

LIPOPROTEINS	ELECTROPHORETIC MOBILITY	PARTICLE SIZE nm	MOLECULAR WEIGHT kD	DENSITY gm.l-1	APOLIPOPROTEIN COMPONENTS MAJOR	MINOR
CM	Origin	75-120	4,000	0.93	apo A-I apo B-48 apo CI, CII, CIII	apo AII, proline rich protein apo E
VLDL	Prebeta	30-80	10-20	0.93-1.006	apo CI, CII, CIII apo B-100	apo E apo D
IDL	Slow Prebeta	25-35	5-10	1.006-1.019	apo B apo CI, CII, CIII	apo E
LDL	Beta	18-25	2	1.019-1.063	apo B	apo E
HDL	Alpha	5-12	0.18-0.36	1.063-1.021	apo AI, AII	apo CI, CII, CIII, apo E apo D

Adapted from Gotto, A.M., 1983 and Kostner, G.M., 1983

lipoproteins in many aspects. However, the fraction separated between the densities 1.040-1.063 contains both LDL and HDL although the former is predominant (Koga et al, 1969; Camejo, 1967).

Lipoprotein structure has been extensively studied by many investigators utilizing techniques such as nuclear magnetic resonance, differential scanning calorimetry, radioimmunoassay and with cross-linking reagents, specifically for HDL and LDL particles (Smith et al, 1978). In general, the major lipoproteins share a common structure that is well suited for transport of lipids in plasma. Lipoproteins are roughly spherical particles with an apolar core of triglyceride (TG) and cholesterol ester (CE), surrounded by a monolayer of specific apolipoproteins, unesterified cholesterol and polar head groups of phospholipids (PL). This monolayer allows the lipoprotein particle to remain miscible in the plasma. Lipoproteins are stabilized by noncovalent forces which allow exchange and transfer of their constituent lipids and apolipoproteins during intravascular metabolism (Jackson et al, 1976; Smith et al, 1978; Gotto, 1983).

The major triglyceride-carrying lipoproteins in the plasma are CM and VLDL. CM are responsible for the transport of exogenous triglycerides, whereas the major vehicle of endogenous triglycerides is VLDL. These carry the triglycerides to the peripheral tissues, providing these cells with an important source of energy. LDL constitutes about two thirds of the total plasma cholesterol in humans and functions to deliver cholesterol to extrahepatic tissues. On the other hand, HDL promotes cholesterol transport from

peripheral tissues to the liver.

More than twenty individual apolipoproteins have been recognized to date. The significance of apolipoproteins as the most probable determinants of lipoprotein structural stability has been established. Studies based on structures of apolipoprotein AI and AII suggest that a balanced competition between lipid-protein interactions and lipid-lipid interactions gives rise to a stable lipoprotein molecule (Smith et al, 1978). Besides this function, apolipoproteins also provide lipoproteins with recognition sites for cell surface receptors and co-factors for enzymes involved in the lipoprotein metabolism. Different lipoproteins have varying but overlapping distributions of these apolipoproteins (Table 1.1) (Owen and McIntyre, 1982; Kostner, 1983). The apolipoprotein B of CM is called B-48. It differs structurally and antigenically from the apolipoprotein of VLDL which is known as B-100 (Kane et al, 1980; Krishnaiah et al, 1980; Elovson et al, 1981). Thr rat liver synthesizes and secretes in VLDL substantial amounts of a protein with same apparent molecular weight as apo B-48, as well as apo B-100 (Elovson et al, 1981; Wu et al, 1981).

1.3 Lipoprotein Metabolism

1.3.1 Synthesis and Secretion

Plasma lipoproteins are the specialized products of only two cells; hepatic parenchymal cells and the absorptive cells of the small intestine. Nascent VLDL and nascent HDL containing endogenous triglygcerides originate from the liver, whereas the small intestine

is responsible for transport of exogenous fats mainly through CM and also VLDL to a small extent depending on the species. Secretion of the lipoprotein particle involves three steps: biosynthesis of protein and lipids, assembly of the requisite apolipoprotein and lipid constituents to form a lipoprotein particle and secretion of the mature particle into the blood. In general, the secretory pathway includes two cellular organelles, endoplasmic reticulum and Golgi apparatus (Alexander et al, 1976). The rough and smooth endoplasmic reticiulum are the sites of apolipoprotein synthesis (Bungenberg and Marsh, 1968) and glycerolipid production (Bell and Coleman, 1980) respectively, while the Golgi apparatus is the site of lipoprotein processing. Studies utilizing radioautographic and immunocytochemical techniques (Alexander et al, 1976; Stein and Stein, 1967) show that the TG-rich particle originates in smooth endoplasmic reticulum and receives apolipoproteins at the junction of the rough endoplasmic reticulum. These particles are transported to Golgi apparatus by specialized tubules where concentration and processing occurs in secretory vesicles. The vesicles then move to the sinusoidal surface and lipoproteins are secreted into the space of Disse by fusion of the vesicle membrane with the membrane of the hepatocyte. However, molecular details of the precise site of assembly, secretion and regulation of the lipoprotein secretion are still incompletely understood.

Apolipoprotein B-48 of CM and apolipoprotein B-100 of VLDL are synthesized in the intestine and liver respectively, whereas the majority of apolipoproteins C and E are acquired by transfer from

HDL as these lipoproteins enter the vascular system (Jackson et al, 1976; Roheim et al, 1976). Janero et al (1984) and Siuta-Mangano et al (1982) have demonstrated that the apolipoprotein B of VLDL is synthesized on membrane bound polysomes as a contiguous polypeptide chain and is not assembled post-translationally from smaller peptide precursors as suggested by Kuehl et al (1977) and Steele et al (1979). There is no doubt, however that posttranslational processing such as glycosylation gives rise to the distinct isoforms of some apolipoproteins, for instance apolipoproteins E (Zannis and Breslow, 1981).

Low density lipoproteins originate in the vascular system primarily by the action of lipoprotein lipase (LPL) on the triglyceride rich lipoproteins. Nascent HDL are secreted as discoidal molecules and differ from mature plasma HDL in apolipoprotein and cholesterol content (Hamilton et al, 1976).

1.3.2 Remodelling of Lipoproteins

Rapid modifications of the nascent plasma lipoproteins occur by physical transfer of lipid and apolipoprotein components, and by enzymatic action of LPL and lecithin:cholesterol acyltransferase (LCAT) (Glomset, 1962; Nestel et al, 1979). Such movements involve exchange or transfer of PL, apolipoproteins A, C and E, free cholesterol and CE between various lipoprotein classes either by specific transfer proteins or by equilibration process. In man, the transfer of CE from HDL to VLDL and TG from VLDL to HDL is known to be accomplished by a specific cholesterol ester transfer protein

(Pattnaik et al, 1978; Barter and Jones, 1979; Dobiasova, 1983) which was first discovered by Zilversmit et al (1975). This protein is not found in the rat (Barter and Lally, 1978). The enzymatic modification by LCAT involves esterification of HDL cholesterol thereby forming HDL_3, which may then be transformed to HDL_2 with the generation of more cholesterol (Schmitz et al, 1982; Daerr and Greten, 1982). LCAT activity along with LPL action results in a continuous cycle of cholesterol esterification and irreversible loss of triglycerides from the TG-rich lipoproteins. This subsequently results in the formation of LDL and mature HDL. Based on these observations Eisenberg and coworkers have suggested that both LDL and HDL are the metabolic products of the core and surface domains of TG-rich lipoproteins respectively (Eisenberg, 1980; Eisenberg et al, 1983).

1.3.3 Lipoprotein Catabolism:

The profile of a lipoprotein system is determined by the sum of three distinct processes. The first is the physical transfer and the exchange of lipid and apolipoprotein constituents discussed above. Activities of lipolytic enzymes constitute the second mode of action in the catabolism of lipoproteins (Nilsson-Ehle et al, 1980). Two lipolytic enzymes are released into the circulatory system by intravenous administration of heparin; LPL from adipose and muscle tissue, and HTGL from vascular endothelium of the liver (Kuusi et al, 1979b). LPL is associated with surface matrix of vascular endothelial cells through ionic interactions with cell

glycosaminoglycans. Extensive evidence indicates that LPL is the key enzyme responsible for the catabolism of TG-rich lipoproteins. It hydrolyses the sn 1 and sn 3 ester bonds of TG and sn 1 ester bond of PL and CE (Nillson-Ehle et al, 1980 and Quinn et al, 1982), and requires apolipoprotein CII for maximal activity (Kinnunen et al, 1977; Fitzharris et al, 1981; Vainio et al, 1983).

The conversion of small VLDL and IDL to LDL also occurs across the splanchnic bed by HTGL (Turner et al, 1981). This liver enzyme has both triglyceride hydrolase as well as phospholipase activity (Ehnholm et al, 1975; Jansen et al, 1980). The quantitative contribution of HTGL towards the catabolism of the TG-rich lipoprotein is not clearly defined. Although CM are a poor substrate for HTGL, VLDL-TG are efficiently removed by this enzyme (Goldberg et al, 1981). Nikkila and coworkers (Kuusi et al, 1979) have postulated a major role of HTGL in the conversion of HDL_2 to HDL_3 which has also been observed by other investigators. Nevertheless, the use of HTGL antiserum in the rat and cynomolgus monkeys raises both VLDL-TG and VLDL-PL levels implying its role in triglyceride hydrolysis (Grosser et al, 1981; Murase and Itakura, 1981; Goldberg et al, 1981;).

Half-time for the clearance of CM and VLDL from the plasma is 4-5 min and 1-3 hr respectively, following a meal. Catabolism of TG-rich lipoprotein constitutes a two step pathway involving TG removal by extrahepatic tissues and CE uptake in the liver. Apolipoprotein C II of these particles activates LPL resulting in the release of free fatty acids and monoglycerides which then enter

the adjacent muscle tissues and undergo either oxidation or re-esterification (Owen and McIntyre, 1982). As the TG core is depleted the CM and VLDL shrink with subsequent transfer of excess surface material, primarily PL, free cholesterol and apolipoprotein C to HDL (Patch et al, 1978). The resulting remnants, rich in CE and apolipoprotein B, re-enter the vascular system. CM remnants are taken up by the hepatic receptors and degraded in the lysosomes (Kita et al, 1982). In contrast, VLDL remnants called IDL are further processed and converted to IDL then to LDL. This difference in the fate of CM and VLDL remnant probably relates to the differences in the amount of apolipoprotein B relative to that of apolipoprotein E (Noel and Dupras, 1983) and to the apolipoprotein C content of the lipoprotein particle (Windler et al; 1980a). Furthermore Goldstein et al (1983b) have suggested that apolipoprotein B-100 interferes with recognition of apolipoprotein E by the chylomicron receptors. In healthy humans, most VLDL are converted to LDL (Eisenberg, 1976), whereas in the rat up to 90% of the IDL are cleared like the CM remnants (Faegerman et al, 1975; Eisenberg et al, 1976; Sigurdsson et al, 1975). The VLDL secreted by rat liver contains substantial amounts of a protein similar to apolipoprotein B-48, along with apolipoprotein B-100 (Van't Hooft, 1982) and the catabolism of apolipoprotein B-48 has been shown to exceed the catabolism of apolipoprotein B-100 (Wu et al, 1981; Sparks and Marsh, 1981).

The third process involved in lipoprotein metabolism is based on the presence of lipoprotein receptors on the cell membranes.

These serve to facilitate and control the uptake of cholesterol-containing lipoproteins, mainly LDL, into the cells. These receptors are subject to autoregulation and demonstrate a high degree of ligand specificity which arises from selective interaction between receptors and apolipoprotein on the surface of lipoproteins (Mahley and Innerarity, 1978). The liver is the major site in the body where receptor mediated catabolism occurs, at least in animals. Mahley and collegues have demonstrated the presence of two distinct type of lipoprotein receptors termed as apolipoprotein E and apolipoprotein BE. Apolipoprotein E receptors are limited to the liver and have a high affinity for apolipoprotein E containing lipoproteins including TG rich remnants, whereas apolipoprotein BE receptors are synonymous with LDL receptors (Mahley et al, 1981; Hui et al, 1981).

The pioneer work of Goldstein and Brown has led to much of our understanding of the LDL catabolic pathway. LDL binds to the high affinity receptors in specific regions of plasma membrane called coated pits by recognition of apolipoprotein B 100 component of LDL. These pits invaginate into the cells and pinch off to form endocytic vesicles which then fuse with lysosomes and complete delivery of LDL-cholesterol. Exposure of the LDL particle to lysosomal enzymes results in the degradation of apolipoproteins and the hydrolysis of CE by acid lipases. The cholesterol thus liberated leaves lysosomes for use in cellular processes. This pathway coordinates the intracellular and extracellular sources of cholesterol and maintains a constant intracellular level of

cholesterol primarily by regulating the enzyme hydroxymethylglutaryl-CoA reductase. The mechanisms directly involved are incompletely understood (Goldstein and Brown, 1977; Brown et al, 1981). About two thirds of the LDL particles are known to be metabolised after binding to specific receptors located on the surface of liver and other body cells. In normal humans the remaining one-third of the LDL is metabolised by alternative receptor-independent mechanism (Goldstein and Brown, 1983a).

1.4 Metabolic Effects of Oral Contraceptive Steroids on lipids:

Of all the metabolic effects of oral contraceptives, the disturbance in lipid levels initially caused greatest concern primarily because of a possible association between altered lipid levels and atherogenesis (Stamler, 1967; Kannel et al, 1971; Bliss et al, 1972).

In premonopausal women, the endogenous sex hormone activity results in lower plasma levels of VLDL, LDL and apolipoprotein B, while levels of HDL, HDL_2 and apolipoprotein A are elevated compared to levels in men of similar age (DeGennes et al, 1983). After menopause the lipid and lipoprotein values change in direction towards male values and during sixth decade of life women may develop even higher LDL-CHOL levels than men. These differences in lipoprotein and apolipoprotein concentrations probably play a key role in protecting women against atherosclerosis development during the duration of ovarian activity. The relatively low incidence of coronary heart disease (CHD) and thrombotic stroke in premenopausal

women compared with age-matched men and increased incidence of these diseases after menopause has been well established (DeGennes et al, 1983; Kannel et al, 1976; Rifkind et al, 1979).

In 1966, Wynn et al (1966) first reported higher serum CHOL, TG, VLDL and LDL levels along with an increase in atherogenic index in women using combined oral contraceptives. Since then, varying metabolic effects of estrogens and progestins separately and in combination have been reported. Effects of estrogens are relatively better understood compared to those of progestins.

It is known that small amounts of synthetic and equine estrogens raise the plasma triglyceride levels (Stokes and Wynn, 1971, Glueck et al, 1975); rising estrogen potency is positively associated with plasma total and VLDL-TG levels (Wynn et al, 1982; Knopp et al, 1982). Kudzma et al (1979) have demonstrated marked quantitative and qualitative changes in plasma lipoproteins and apolipoproteins in estrogen-treated chicks. On the other hand, estrogens either increase or produce no change in plasma cholesterol levels, but usually an increase in the plasma HDL-CHOL: LDL-CHOL ratio has been observed (Furman et al 1967; Tikkanen et al, 1978). Wallace et al (1979) and Tikkanen et al (1981b) have reported that estrogen reduced the LDL-CHOL levels in postmenopausal women. Studies of Wallentine et al (1977) also suggested a HDL-CHOL elevating and LDL-CHOL reducing effect with estrogen use. Administration of the major human natural estrogen, 17 β-estradiol in large amounts, is also known to increase serum HDL and lower LDL lipoproteins in postmenopausal women (Wallentine et al, 1977,

Tikannen et al, 1979). In contrast to synthetic estrogens, estradiol valerate does not seem to alter the plasma triglyceride levels at least in oophorectomized women (Nielsen et al, 1977; Bostofte, 1978). It has been suggested that oral contraceptives formulated with natural estrogens induce less significant and qualitatively more benign changes in serum lipids and lipoproteins than synthetic estrogens (Bostofte et al, 1978; Wallentin and Larsson-Cohn, 1977).

Reports on the action of various progestogens are less clearly defined because their ultimate effect depends on the combined estrogenic, antiesterogenic, androgenic and progestational properties (Astwood, 1970). The administration of androgen leads to higher LDL-CHOL and lower HDL-CHOL levels with little change in the total plasma cholesterol concentration (DeGennes et al, 1983; Solyom, 1972). Some progestins, norgestrel and norethindrone acetate, exhibit such androgenic influences (Hirvonen et al, 1981; Silfverstolpe et al, 1979), whereas progesterone and pregnanes such as medroxyprogesterone have insubstantial effects on TG and CHOL levels (Silfverstolpe et al, 1982; Crona et al, 1983) in keeping with their much lower androgenic action. Most investigators have reported a decrease in HDL-CHOL (Larsson-Cohn et al, 1982; Bradley et al, 1978), particularly HDL_2-CHOL levels (Tikkanen et al, 1981a) with androgenic progestins. The users of combined oral contraceptives show HDL-CHOL values that are intermediate between those reported by the administration of estrogens and progestin alone, increasing with higher estrogen potency and decreasing with

progestin predominance of the preparation (Bradley et al, 1978; Knopp et al, 1982; Wynn et al, 1982). The HDL-CHOL-lowering ability of progestins is further supported by studies in which they were administered to estrogen-primed women (Goldzieher et al, 1978). This could partly arise from suppression of estrogen production by the ovaries during progestin treatment besides their androgenic property (Robertson et al, 1981). There is now a growing body of evidence which indicates that reduced HDL-CHOL concentration is a strong and independent risk factor for the development of atherosclerotic manifestations (Miller et al, 1977; Gordon et al, 1977; Carlson and Ericsson, 1975).

In contrast to the reduced HDL-CHOL levels, norgestrel-containing oral contraceptives lead to elevated VLDL-CHOL/TG and LDL-CHOL/TG ratios (Knopp et al, 1982). Silfverstolpe et al (1979) have reported higher LDL-CHOL and LDL-TG levels with medroxyprogesterone and levonorgestrel use respectively. Further, Oster et al (1982) have shown elevated LDL-apolipoprotein B levels in oral contraceptive users, the level rose consistently with longer duration of use, although, no effect on LDL-CHOL was observed.

Studies in postabsorptive women suggest that progestational compounds lower triglyceride levels both in normal and hypertriglyceridemic subjects (Fallat and Glueck, 1974). Cheng and Wolfe (1983) have reported a hypotriglyceridemic effect of the progestin, norethindrone acetate in female rats. Another report by Glueck et al (1969) also supports this finding by showing

consistently lower triglyceride levels in normal and hyperlipoproteinenic women during norethindrone administration. Reports on the influence of norgestrel-containing oral contraceptives on triglyceride levels are somewhat conflicting. Most investigators find that norgestrel lowers serum triglyceride levels (Nielsen et al, 1977; Robertson et al, 1981; Spellacy et at, 1974), although some have failed to confirm this finding (Foegh et al, 1980; Rossner et al, 1979; Roy et al, 1981; Pribicevic et al, 1979). This discrepancy likely relates to the type and dose of estrogen used in the formulations as estrogens tend to oppose the effects of progestins on plasma lipid and lipoprotein levels (Beck, 1973). Nevertheless, norgestrel use has been shown to be associated with a triglyceride lowering effect subsequently balancing the estrogen mediated increases during combined oral contraceptive use (Wynn et al, 1979).

The mechanisms underlying the altered lipid levels in contraceptive use are not clearly understood. Estrogens have been shown to increase hepatic triglyceride synthesis by a number of investigators (Kim and Kalkhoff, 1975; Weinstein et al, 1978; Glueck et al, 1975). This could be fueled by increased availability of glucose and free fatty acids observed in estrogen therapy or by a lower glucagon/insulin ratio (Beck et al, 1975; Javier et al, 1968). Induction of activity of the hepatic microsomal enzymes is also possible (Coleman et al, 1977). On the other hand, several studies have reported depressed post-heparin lipolytic activity, specifically reduced HTGL activity (Glad et al, 1978; Fallat and

Glueck, 1974; Applebaum et al, 1977). The latter changes are less likely to be associated with hypertriglyceridemia because it is difficult to establish a correlation between TG levels and HTGL activity (Applebaum et al, 1977; Reardon et al, 1982b; Tikkanen et al, 1981a).

By contrast to estrogens, progestins may act by inhibiting TG synthesis and secretion as demonstrated in the rat (Cheng and Wolfe, 1983) or in swine (Wolfe and Grace, 1979). Several studies have reported hyperinsulinemia and insulin resistance (Wynn and Nithyananthan, 1982) and even decrease in insulin receptors with the use of norgestrel (DePirro et al, 1981). However they failed to observe any changes in TG levels. On the other hand, Kekki et al, (1971) proposed that the progestational component of the oral contraceptive was responsible for increased triglyceride clearance, which was balanced by an increment in VLDL-TG synthesis by the estrogen component in normal women. Glueck et al, (1969; 1973) also attributed decreased TG level with norethindrone acetate administration to increased post-heparin lipolytic activity. Tikkanen et al, (1981a) have reported increased HTGL activity during norgestrel administration; this was however correlated negatively with HDL-CHOL levels. The effects of these hormones have recently been reviewed by Wolfe (1983).

1.5 Oral Contraceptives and Complications

This section is concerned with the complications ascribed to oral contraceptive use. One should bear in mind, however, the

interactive and synergistic nature of various risk factors for ischemic diseases. Adverse effects on the vascular system (atherogenesis and venous clotting) appear to be the most important unwanted reactions of current oral contraceptive usage. The combined multiple effects of multi-risk factors such as cigarette smoking, hypertension and obesity have been shown to be synergistic (Mann et al, 1975b).

Jordan (1961) was the first to suspect the thrombogenic effect of the pill, which was later confirmed by a large study undertaken by the British Medical Research Council (Inman and Vessey, 1968; Inman et al, 1970). Epidemiological studies report an excessive risk of cerebrovascular disease, venous thrombosis and pulmonary embolism among oral contraceptive users versus users of diaphragms and intrauterine devices (Vessey et al, 1976; Petitti et al, 1978). A recent report by the Royal College of General Practitioners (1981) also showed that the overall death rate of ever-users of oral contraceptives was 40% higher than controls, mainly due to diseases of the circulatory system. On the other hand, studies of Drill and Calhoun (1968) and Fuertes-de-la-haba (1973) have failed to confirm the above findings.

The estrogenic component is suspected to be responsible for the thrombogenic effect of the pill (Stolley et al, 1975). The Royal College of General Practitioners (1967), in a prospective study, reported an increase in the incidence of superficial thrombosis as the dose of estrogen increased. Further, the frequency of thrombosis was higher in women given diethylstilbestrol (Daniel et

al, 1967) and in men on estrogen treatment for myocardial infarction (Coronary heart project, 1973). Gow et al (1971) also reported high incidences of venous disease in oophorectomized women treated with ethinyl estradiol. The above mentioned studies were done with relatively larger doses of estrogens. Studies performed later (Stolley et al, 1975) supported the idea that lower dose of the estrogen component was associated with lower thromboembolic risk. However, studies of Bottiger et al (1980) failed to show a reduction in the frequency of arterial complications with the use of lower dose of estrogen.

Enhanced blood coagulability due to impairment of fibrinolytic activity may contribute to the increase in thromboembolism in oral contraceptive users. Oral contraceptives may act by depressing the fibrinolytic activator content below a critical level (Astedt, 1971). Coagulation factors, fibrinogen II, VII and X are higher in women using combined oral contraceptives but changes are more pronounced with most estrogenic preparations (Meade, 1982). On the other hand, change in coagulation has not been observed with use of gestagens (Nilsson and Kullander, 1967). However, the comprehensive review by Mammen (1982) suggests that the association between oral contraceptive use and hemostasis system is still not clear.

The use of oral contraceptive is also positively correlated to the occurance of myocardial infarction, particularly in women for whom the risk is increased because of other factors such as obesity, hypertension and diabetes (Mann and Inman, 1975; Shapiro et al, 1979). Most of prospective and case control studies investigating

the association between myocardial infarction and oral contraceptive use are consistent. They show a consistent risk ratio ranging from 3 to 5 fold for oral contraceptive users (review by Sartwell and Stolley, 1982).

It has been recognized that the high circulating level of HDL may have a protective effect against the development of atherosclerosis (Plunkett, 1982). There is much evidence that given alone progestins with androgenic influence e.g. norgestrel, and to a lesser extent norethisterone and norethindrone acetate, lower the HDL-cholesterol levels. This has raised some concern for oral contraceptive users (Bradley et al, 1978; Wingrave, 1982; Kay, 1982). The mean serum levels of HDL-cholesterol in oral contraceptives users show a striking inverse relationship to the rate of reporting of arterial diseases and to the progestogenic activity of the pill (Kay, 1982).

Hypertension occurs infrequently in women using oral contraceptives (McQueen, 1978). A recent study by the Royal College of General Practitioners (1977) has shown a correlation between the frequency of hypertension and the progestogen activity of oral contraceptive. Wingrave (1982) supported this view by showing that norethisterone and levonorgestrel led to higher incidence of hypertension. In contrast, the findings of Briggs and Briggs (1976) contradict the above view. They observed less striking changes in the vessel wall with 30 µg compared to 50 µg estrogen, implying a role for estrogen in hypertension.

The relationship between oral contraceptive use and cancer has been explored by many investigators with varying conclusions. Orey et al (1976) reported in a large follow-up study, that the use of oral contraceptive appeared to lower the risk of fibrocystic breast disease. Increasing progestogen content was protective of benign breast disease. However, the risk in preparous women was increased with oral contraceptive use (Harris et al, 1982). Fasal and Paffenbarger (1975) have suggested that the course of malignancy process may be accelerated if transformed cells are present during oral contraceptive use. Also, sequential oral contraceptives have been reported to be predispose towards endometrial carcinoma (Kelley et al, 1976; Silverberg and Makowski, 1975; Vessey et al, 1979). In general, the ultimate effect of the oral contraceptive pill depends on the type and dose of progestin and estrogen used in its formulation.

CHAPTER 2

EFFECTS OF D-NORGESTREL AND 17 β-ESTRADIOL ON PLASMA LIPID AND LIPOPROTEIN LEVELS IN THE RAT

2.1 INTRODUCTION

dl-Norgestrel (Ng) has been widely administered to women in the form of oral contraceptives (Tyler, 1974). Reports concerning the influence of dl-Ng containing medications on serum lipids are conflicting. This has been reviewed in section 1.4 which also summarizes the effects of estrogens.

The present studies were undertaken to examine the effects of d-Ng, and 17 β-estradiol separately on plasma lipid and lipoprotein levels in fed female rats *in vivo*.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Sprague Dawley rats (0, 250 g) were routinely purchased from Charles River Canada, Montreal. Rat chow was obtained from Ralston Purina Co., Minneapolis, MN. Wyeth Ltd., Toronto, Ont. supplied d-Norgestrel. 17 β-Estradiol was purchased from Sigma Chemical Co., St. Louis, MO. Absolute alcohol was supplied by Abbott Laboratories Ltd., Canada. Somnotol (sodium pentobarbital) was obtained from M.T.C. Pharmaceuticals, Hamilton, Canada. Precilip lipid standards for quality control were obtained from Boehringer-Mannheim, Dorval, Que.

2.2.2 Preparation of Animals

Female Sprague-Dawley rats (250-275g) were housed individually in metal cages with wire mesh bottoms, in an air-conditioned room with controlled temperature and lighting. All rats were fed rat chow pellets for 5-6 days after arrival, and were then transferred to the experimental diets. Each rat in the d-Ng group received an average daily dose of 1.4 µg d-Ng (4 µg/day .kg body wt $^{0.75}$, 2 weeks), a dose comparable to that used for human contraceptive purposes. The estradiol-treated rats were prepared by feeding 40 µg 17β-estradiol daily (106 µg/day .kg body wt $^{0.75}$, 18 days), a dose comparable to that used for the postmenopausal estrogen replacement therapy. The control groups received the placebo diets. The experimental diets were prepared by adding d-Ng or 17 β-estradiol, dissolved in absolute ethanol to one piece of rat chow; ethanol was then evaporated. The control diet was treated with an equal amount of ethanol which was likewise removed by evaporation before being fed.

2.2.3 Experimental Procedure

Experimental and placebo portions of the diet were first fed to each rat between 6:00 to 11:00 PM and the rats were observed until it was completely consumed. Thereafter, rat chow was available ad libitum to both groups. Water was available to rats at all times. The rats received a fat-free diet for 12 hr prior to sacrifice to eliminate chylomicrons from plasma. The fat-free diet was prepared in the lab. It was similar to that used by Hoehn and Carroll (1979)

and is shown in Table 2.1. The raw materials used for the fat-free diets were generously donated by Dr. K. K. Carroll, Department of Biochemistry, University of Western Ontario. The rats were venesected after administering a sodium pentobarbital injection (13 mg in 0.2 ml) intraperitoneally. 10 ml of blood was drawn by aortic puncture either using a heparinized syringe or it was collected in tubes contained EDTA (1mg/ml). The plasma was obtained by centrifugation for 20 min at 2000 rpm, 4°C.

2.2.4 Lipid Analysis

1 ml of plasma was transferred into 20 ml of chloroform:methanol (2:1, v/v, Folch et al, 1957) for lipid extraction. The lipoproteins were isolated from 2-3 ml plasma by sequential ultracentrifugation by the method of Havel et al (1956). Aliquots of the chloroform phase were aspirated from Folch mixture to determine cholesterol (Sperry and Webb, 1950) and phospholipids (Bartlett, 1959). Activated silicic acid (0.5 g) was added to the chloroform phase to obtain triglyceride extract. The tube was mixed and centrifuged at 1500 rpm for 5 min to sediment silicic acid with phospholipids adsorbed on its surface. The triglyceride content was assayed by the method of Carlson (1963). Precilip (control sera) was used as internal standard in all lipid assays. The lipid levels were corrected for recovery of losses incurred during ultracentrifugation.

TABLE 2.1

Composition of the Fat-Free Diet

Ingredients[a]	Percent
Casein (Vitamin free)	18.0
Dextrose	72.8
Celluflour	5.0
Salt Mixture[b]	4.0
Vitamin Mixture[c]	0.2

[a] The vitamins, salt mixture and "vitamin free" casein was obtained from ICN Life Sciences Group, Nutritional Biochemicals Division, Cleveland, Ohio. Dextrose mohohydrate was obtained from Teklad Test Diets, ARS/Sprague-Dawley Division of Mogul Corporation, Madison, WI, and Celluflour from Chicogo Dietitic Supply House, Chicago, II.

[b] Salt mixture as described by Bernhardt and Tomarelli (1966) provided 6.0g calcium, 1.8g potassium, 0.5g sodium, 0.4g magnesium, 5.0g phosporous, 0.5g chlorine, 0.33g sulfur, 0.025g iron, 0.012g zinc, 0.05g manganese, 0.005g copper and 150 mg iodine per Kg of diet.

[c] Composition: Thiamine Hcl, 10 mg; pridoxine - Hcl, 10 mg; biotin, 1.0 mg; riboflavin, 10 mg; nicotinic acid, 60 mg; choline bitartrate, 1800 mg; calcium pentothenate, 40 mg; folic acid 5 mg, vitamin B_{12}, 0.05 mg; ascobic acid, 75 mg; vitamin A, 65 mg (13000 IU) as ester concentrate; DL-α-tocopheryl acetate, 75 mg; vitamin D_3 (1220 IU), 3 mg; vitamin K_3, 1.0 mg per kg of diet. The composition was similar as described by Greenfield et al (1969). The water soluble vitamins were prepared and added to the diet as described by Carroll (1967).

2.2.5 Statistics.

Differences between the d-Ng and 17 β-estradiol groups were compared to their respective control groups and were evaluated according to Snedecor and Cochran (1967) using the unpaired two tailed 't' test . Variance is expressed as the standard error of the mean.

2.3 RESULTS

2.3.1. Effects of d-Norgestrel on Plasma Lipid and Lipoprotein Concentrations in vivo.

d-Ng administered for a period of 2 weeks at a dose of 4 μg/day.Kg body wt $^{0.75}$ showed a significant reduction in the plasma total TG concentration compared to controls. In contrast, the plasma total CHOL concentration was significantly elevated in the treated group. PL levels were unaffected by the d-Ng treatment (Table 2.2).

The lower mean plasma concentration of TG was attributable to the significantly lower mean concentration of VLDL-TG. The concentration of VLDL-PL fell in proportion to the decrease in VLDL-TG. In contrast, elevated CHOL levels were reflected in the significantly increased LDL-CHOL levels in the treated rats. Concurrently, LDL-PL were also significantly elevated by d-Ng treatment (Table 2.3). However, no significant differences were found between control and treated rats in the concentrations of CHOL and PL in HDL or its subfractions.

TABLE 2.2

EFFECT OF d-NORGESTREL ON THE CONCENTRATIONS OF PLASMA LIPIDS[a] IN VIVO IN RATS

GROUP	TOTAL		
	TRIGLYCERIDES	CHOLESTEROL	PHOSPHOLIPIDS
Control (n = 7)	34 ± 2	46 ± 2	62 ± 4
Norgestrel[c] (n = 7)	25 ± 3[b]	54 ± 4[b]	67 ± 6

[a] Mean ± SEM (mg/dl)

[b] Significantly different from control group, ($p<0.05$)

[c] Rats treated with d-norgestrel (4 µg/day kg body wt $^{0.75}$)

TABLE 2.3

EFFECT OF d-NORGESTREL ON THE CONCENTRATIONS[a] OF LIPIDS IN PLASMA LIPOPROTEIN FRACTIONS IN VIVO IN RATS

GROUP	TRIGLYCERIDES	CHOLESTEROL			PHOSPHOLIPIDS		
	VLDL	VLDL	LDL	HDL	VLDL	LDL	HDL
Control (n = 7)	25 ± 2	4 ± 1	11 ± 1	32 ± 2	7 ± 1	7 ± 1	48 ± 3
Norgestrel[c] (n = 7)	21 ± 1[b]	5 ± 1	15 ± 2[b]	35 ± 3	5 ± 2	10 ± 1[b]	52 ± 4

[a] Mean ± SEM (mg/dl)

[b] Significantly different from control group ($p<0.05$)

[c] Rats treated with d-norgestrel (4 µg/day kg body $wt^{0.75}$)

2.3.2 Effects of 17β-estradiol on plasma lipid and lipoprotein concentrations in vivo

Female Sprague-Dawley rats were treated daily with a dose of 106 μg 17β-estradiol/day.kg body $wt^{0.75}$ for 18 days. The plasma TG, CHOL and PL concentrations were not significantly different in the 17 β-estradiol treated rats compared to controls (Tables 2.4 and 2.5). Further, there were no significant changes in lipid concentrations of the different lipoprotein fractions between the two groups at the end of the experimental period. It is noteworthy that the two sets of controls differed in the plasma TG and PL concentrations (Tables 2.2 and 2.4), though the explanation is unclear.

2.4 DISCUSSION

The present study was done to outline the effects of d-Ng and 17 β-estradiol on lipid levels in the fed female rats. The results obtained demonstrate that a conventional dose of d-Ng given alone significantly lowers rat plasma triglycerides, while elevating plasma cholesterol levels significantly (Table 2.2). The reduction in plasma TG is attributable to a significant reduction of VLDL-TG (Table 2.3), compatible with the hypothesis that VLDL is the main carrier lipoprotein for TG in the vascular system. The parallel reductions of VLDL-PL and VLDL-TG (Table 2.3) suggest that the lowering of VLDL is attributable to the reduction of the number of VLDL particles rather than formation of abnormal VLDL particles. Therefore, d-Ng did not seem to alter the lipid composition of the VLDL particle.

TABLE 2.4

EFFECT OF 17 β-ESTRADIOL ON THE CONCENTRATIONS[a] OF PLASMA LIPIDS IN VIVO IN RATS

GROUP	TOTAL TRIGLYCERIDE	TOTAL CHOLESTEROL	TOTAL PHOSPHOLIPIDS
Control (n=8)	86 ± 10	46 ± 3	103 ± 7
17 β-Estradiol[b] (n=8)	70 ± 8	41 ± 2	88 ± 6

[a] Mean ± SEM (mg/dl)

[b] Rats treated with 17 β-estradiol (106 μg/day, kg body wt $^{0.75}$)

TABLE 2.5

EFFECT OF 17 β-ESTRADIOL ON THE CONCENTRATION[a] OF LIPIDS IN PLASMA LIPOPROTEIN FRACTIONS IN VIVO IN RATS

GROUP	TRIGLYCERIDES				CHOLESTEROL				PHOSPHOLIPIDS			
	VLDL	IDL	LDL	HDL	VLDL	IDL	LDL	HDL	VLDL	IDL	LDL	HDL
Control	70	7.8	4.5	3.6	5.7	1.3	5.6	33.7	10.4	1.2	2.8	89
(n=8)	±	±	±	±	±	±	±	±	±	±	±	±
	10	1.1	0.5	0.5	0.6	0.2	2.0	1.8	1.8	0.3	0.3	5.9
17 β-Estradiol[b]	57	5.9	4.1	2.7	4.1	1.1	4.4	31.7	8.3	0.8	1.9	77
(n=8)	±	±	±	±	±	±	±	±	±	±	±	±
	8	0.6	0.6	0.3	0.4	0.2	0.3	1.4	1.5	0.2	0.5	4.4

[a] Mean ± SEM (mg/dl)

[b] Rats treated with 17 β-estradiol (106 μ/day. kg body $wt^{0.75}$).

The hypotriglyceridemic effect of d-Ng is comparable to the effects reported for progestin norethindrone acetate in female rats (Cheng and Wolfe, 1983). d-Ng is known to be more potent than norethindrone acetate (Edgren, 1969) and it is noteworthy that a much smaller dose of d-Ng was required to produce a TG-lowering effect compared to the dose of norethindrone acetate (4 vs 100 µg/day. kg body $wt^{0.75}$). The present finding is consistent with the observations of Nielsen et al (1977), Robertson et al (1981) and Spellacy et al (1974) that Ng reduces serum TG in women. However, the findings of Rossner et al (1979) and Pribicevic et al (1979) differ from our results. This may be due to the fact that both the latter studies were performed with combined oral contraceptives. It is well known that estrogens counteract the effects of progestins on plasma lipid and lipoprotein parameters (Beck, 1973).

In contrast to the hypotriglyceridemic effect, d-Ng elevated plasma total and LDL CHOL levels (Tables 2.2 and 2.3). Knopp et al (1982) have also observed a LDL-CHOL-elevating effect by this progestin. Although, LDL is not the major cholesterol-carrying lipoprotein in the rat, changes in LDL-CHOL level occur in both propylthiouracil-induced hypothyroid rat and estrogen treatment of normal rats (Sykes et al, 1981; Davis and Roheim, 1978). A number of investigators have reported that Ng lowers the HDL-CHOL levels in women (Bradley et al, 1979; Wynn et al, 1982). However, in this study no effect of d-Ng on rat HDL-CHOL levels was observed.

d-Ng showed opposite effects on TG and CHOL concentrations in VLDL and LDL fractions (Table 2.3) respectively. These results are

comparable to the observations of Tamai et al (1979) who demonstrated reciprocal changes of LDL-CHOL occuring with reduction of VLDL-TG in type IV and type IIb hypertriglyceridemic patients treated with oxandrolone. Similar changes between concentrations of VLDL and LDL have also been reported following clofibrate administration (Wilson and Lees, 1972).

The measurements of serum lipids were made under fed state conditions using a fat-free diet which excluded the effect of chylomicrons from the circulation which would have confounded the interpretation of the results. Although lipogenesis from carbohydrate is likely to be enhanced by a fat-free diet (Antonis and Bersohn, 1961; Waddel and Fallon, 1973), the diet was same for both Ng-treated and control rats. Control of diet allowed one to ascertain the effect of the d-Ng alone.

The potential mechanisms responsible for changes in lipoprotein concentrations during d-Ng treatment could include lower hepatic lipid and lipoprotein synthesis and secretion and/or enhanced clearance of VLDL from plasma. The progestin norethindrone acetate is known to act by suppressing the hepatic VLDL synthesis and secretion in the rat (Cheng and Wolfe, 1983) and swine (Wolfe and Grace, 1979). Kim and Kalkhoff (1975) and Weinstein et al (1978) attributed the estrogen-induced hypertriglyceridemia to increased hepatic triglyceride synthesis. However, changes in postheparin lipolytic activity, specifically HTGL have also been observed with estrogen treatment (Fallat and Glueck, 1974; Applebaum et al, 1977). Tikkanen et al (1981a) have shown that HTGL is elevated in women administered Ng. However, the physiological significance of changes

in HTGL, are difficult to evaluate because it seems to be more closely related to HDL_2 levels rather than triglyceride levels (Tikkanen et al, 1981a; Reardon et al, 1982b). On the other hand, d-Ng may elevate LDL-CHOL by a direct effect on the LDL receptors impairing LDL uptake by the receptor-mediated endocytosis. Alternatively, higher LDL-CHOL could result from increased direct synthesis and/or increased input of LDL from the VLDL catabolic pathway.

17 β-Estradiol treatment did not produce any significant alterations in TG, CHOL and PL levels in plasma lipid and lipoprotein fractions (Tables 2.4 and 2.5), although a tendency towards LDL-CHOL reduction was observed. Synthetic estrogens in low doses are known to produce a hypertriglyceridemic effect in some species (Glueck et al 1975). However 17 β-estradiol does not increase triglycerides even in higher doses (Glueck et al, 1975; Gustafson et al, 1972; Nielsen et al, 1977). Our results were compatible with this finding. Furthermore, no changes in HDL-CHQL concentrations were observed, unlike the findings of Wallentin et al (1977).

CHAPTER 3

EFFECT OF d-NORGESTREL ON TRIGLYCERIDE SYNTHESIS AND SECRETION BY ISOLATED RAT HEPATOCYTES

3.1 INTRODUCTION

Treatment of female rats with conventional doses of d-Ng produced a hypotriglyceridemic and hypercholesterolemic effect as shown in the previous chapter. The reduction of TG in VLDL was mainly responsible for the lower plasma TG levels. However, the mode of action by which d-Ng exerts these effects is poorly understood. Possible mechanisms that could underline the altered lipid levels were dealt with in section 1.4. An important role was suspected for the d-Ng on the liver in triglyceride synthesis and secretion. This chapter describes further experiments that were designed to investigate the acute effect of d-Ng on liver lipid metabolism.

It was chosen to investigate this using suspensions of isolated rat hepatocytes to examine the incorporation of labelled precursors into triglcerides. In this chapter, evidence is presented which suggests that d-Ng suppresses the hepatic triglyceride synthesis in a dose-dependent manner. This mechanism could account at least for part of the lowering of plasma VLDL observed during d-Ng administration.

3.2 MATERIALS AND METHODS

3.2.1 Materials

[9, 10^{3}H(N)] Palmitic acid (12 Ci/mmol) and [U-^{14}C] glycerol (12 mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA. Collagenase (Type 1) was obtained from Millipore Corp., Mississauga, Ont. and Waymouth's medium (MB 752/1) was supplied by Gibco Canada, Burlington, Ont. Porcine albumin (fraction V), HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), TES (N-tris [hydroxymehthyl] methyl-2-aminoethane sulfonic acid) and TRICINE (N-tris [hydroxymethyl] methyl glycine) were purchased from Sigma Chemical Co., St. Louis, MO. Trypan blue was obtained from Matheson Coleman and Bell Manufacturing Chemists, Norwood, OH. Thymol blue, Methanol (HPLC grade) and chemical reagents of analytical grade were purchased from Fisher Scientific Co., Fair Lawn, NJ. Millipore filters 0.5 micron were purchased from Mellipore, Bedford, MA. SEP-PAK C_{18} cartridges were obtained from Waters Scientific, Canada. SP-Sephadex C_{25} was purchased from Pharmacia Ltd., Montreal, Que.

3.2.2 Preparation of Hepatocytes

Female Sprague-Dawley rats (200-250 g) maintained on rat chow were used for preparation of hepatocytes by the method of Seglen (1976). Glucose (10%, w/v) was added to the drinking water (to maintain a fed state) for 48 hr prior to sacrificing the rats. The rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (13mg in 0.2ml). The abdomen was opened by a U-shaped

nsverse incision and the intestines were displaced to the left side of the abdominal cavity. The portal vein was cannulated with polyethylene tubing (I.D. 0.044 and O.D. 0.065 inches) and subsequently another cut was made in the lower vena cava to permit perfusate efflux. In situ perfusion of the liver was performed at a rate of 50 ml/min with oxygen saturated Ca^{2+}-free buffer maintained at 37°C to wash out the blood. While perfusion with Ca^{2+}-free buffer continued, the liver was carefully removed from the carcass and placed on gauze sponges on top of a plastic dish with an outlet. The liver was then perfused with recirculating collagenase buffer at a rate of 50 ml/min; during this time a marked swelling of the liver was observed. This was followed by transfer of the liver to a Petri dish containing 75 ml of cold suspension buffer in which cells were liberated from the connective-vascular tissue after removal of the outer membrane of the liver. The cell suspension thus obtained was filtered through a cheese cloth to remove connective tissue debris.

Further purification of the parenchymal cells was accomplished by incubating the cell suspension in a gently shaking water bath at 37°C for 30 min, followed by cooling to 0°C on ice for 5 min. The cell suspension was again filtered through a cheese cloth, distributed equally between four round bottom test tubes and centrifuged four times at 400 rpm for 3 min. Each time the supernatant was discarded and the pellet resuspended in 120 ml of the washing buffer. The purified cell pellet was finally filtered and resuspended in a volume of Waymouth's medium to obtain a cell concentration of $0.4\text{-}0.6 \times 10^6$ cells/ml. Viability, determined by

the trypan blue dye exclusion test, ranged from 60-90% (mean 71±3%) and the final intact hepatocyte yield ranged from 0.1 to 1.0×10^8 cells per liver. The composition of various buffers used is shown in Table 3.1.

3.2.3 Incubation of Hepatocytes

Each set of control and experimental preparations were carried out with cells freshly isolated from the same rat liver. In a typical experiment, incubations were carried out in a shaking water bath at 37°C with hepatocytes suspended in Waymouth's medium in the presence or absence (control) of d-Ng. 25 µCi of [9, 10^3H] palmitate (complexed to porcine albumin, molar ratio approximately 1:1) and 25 µCi of [U-^{14}C] glycerol were subsequently added to the medium and incubations continued for a 60 min time study. 50 µM palmitic acid complexed to albumin was also added to the medium (both experimental and control) in some experiments. Three ml samples of the suspended cells were removed at 0, 30, and 60 min and centrifuged at 3500 rpm for 15 min at 5°C to sediment the cells. The pellet (suspended in 0.5 ml ice cold saline) and 1.0 ml of the supernatant, containing lipids secreted or released from hepatocytes, were each extracted with 10 ml chloroform: methanol (2:1, v/v) for determination of lipids.

3.2.4 Determination of Radioactivity

The chloroform layer of the chloroform:methanol extract of the lipids obtained from both the hepatocytes and the medium in which

TABLE 3.1

COMPOSITION OF BUFFERS USED FOR ISOLATION OF RAT HEPATOCYTES[a]

	Ca^{++} free Perfusion buffer	Collagenase buffer	Washing buffer	Suspension buffer
	.....mg/liter.....			
NaCl	8,300	3,900	8,300	4,000
KCl	500	500	500	400
$CaCl_2.2H_2O$	-	700	180	180
$MgCl_2.6H_2O$	-	-	-	130
KH_2PO_4	-	-	-	150
Na_2PO_4	-	-	-	100
HEPES	2,400	24,000	2,400	7,200
TES	-	-	-	6,900
Tricine	-	-	-	6,500
1M NaOH	5.5	66.0	5.5	52.5
Collagenase	-	500	-	-
pH	7.4	7.6	7.4	7.6

[a] Salt concentrations were given in milligrams per 1000 ml of final solution, and the concentration of NaOH (1M) as milliliters per 1000 ml of final solution.

they were incubated was treated with silicic acid to remove phospholipids. Labelled free fatty acids were removed by over-alkalinization (Borgstrom, 1952) from neutral lipids prior to determination of the ^{3}H and ^{14}C content of the neutral lipids (essentially triglycerides) of the hepatocytes and their supernatant. An aliquot of the phospholipid-free chloroform phase was evaporated to dryness under N_2 and 3 ml of heptane, 1 ml of thymol blue (0.01% in ethanol) and 200 µl of 0.02 N NaOH were added subsequently. The solution was bubbled with nitrogen for 30 sec and the heptane layer was transferred to counting vials. The lower phase was extracted twice with heptane to obtain residual triglycerides. The heptane was evaporated and the radioactive lipid was counted after the addition of 10 ml of scintillation fluid. The scintillation fluid consisted of toluene containing 0.4% 2,5-diphenyloxazole (PPO) and 0.1% 1,4 bis [2-5 (phenyloxazolyl)] benzene (POPOP) (W/V). Radioactivity was counted in a Nuclear Chicago Isocap 300 Liquid Scintillation Counter.

3.2.5 Lipid Analysis

The methods of estimation of CHOL, TG and PL concentrations were essentially the same as described in section 2.2.4.

3.2.6. Determination of Triglyceride Distribution Among Different Density Fractions

The hepatocyte cell suspensions were incubated for 60 min with 25 µCi of [9, 10^{3}H] palmitate + unlabelled palmitate (50 µM,

complexed to porcine albumin in a 1:1 molar ratio) and 25 µCi of [U-^{14}C] glycerol. The aliquots were obtained at 0 and 60 min and centrifuged at 3500 rpm, 4^{o}C for 15 min to sediment the cells. Four aliquots of the supernatant were separated and the densities adjusted to 1.006, 1.019, 1.063 and 1.21 g/ml using a concentrated salt solution. These fractions were mixed with human serum (2:1, v/v) prior to ultracentrifugation (Havel, 1956) and lipoproteins were isolated. Individual lipoprotein fractions were extracted in Folch's mixture, and radioactivity was determined in triglycerides as described above.

3.2.7 Determination of d-Norgestrel Concentrations by HPLC

Waymouth's medium was incubated with 0.1 mM d-Ng in the presence and absence of rat hepatocytes at 0.1% and 1% DMSO levels. Samples were obtained at 0, 30 and 60 min and centrifuged at 4^{o}C, 3500 rpm for 15 min. The separated supernatant was centrifuged once again to ensure sedimentation of the suspended d-Ng. The final supernatant was frozen in dry ice-acetone until it was further processed. d-Ng concentrations were estimated by the method of Khalil and Lawson (1983). The samples were loaded on a prewashed (10 ml methanol followed by 10 ml water) SEP-PAK C_{18} cartridge attached to a 10 ml syringe. The container was rinsed twice and washings loaded on SEP-PAK. The cartridge was rinsed with 5 ml methanol three times to collect the concentrated steroid in a total volume of 15 ml. The methanol fraction was evaporated, reconstituted to 2 ml with 72% methanol and applied to a SP-25 Sephadex column (40 X 6 mm) to remove

basic impurities. Prior to use, Sephadex was converted to its H^+ form by successive washing with 0.5 M HCl in 72% methanol and H_2O until neutral and was suspended in 72% methanol. The column was eluted with 2.5 ml methanol portions and eluant was filtered through a 0.5 micron millipore filter. An aliquot (200µl) was injected into HPLC. 200 Microgram of progesterone was added initially to the samples to monitor the recovery of the steroid.

3.2.8 High Pressure Liquid Chromatography (HPLC) System

All separations were performed on HPLC with Waters Scientific, Canada HPLC. This method has been described by Khalil and Lawson (1983). C_{18} Radial Pak cartridges (10 cm long, 5 mm I.D., 10 µ particle size) with an RCM-100 radial compression module and a 5 µ RESOLVE spherical C_{18} column were used. Mobile phase consisted of 70% methanol (sonicated for 20 min). Flow rate was 1 ml per min. Peak heights, peak areas and retention times were measured by a data module.

3.2.9 Statistics

Differences between groups were evaluated (Snedecor and Cochran, 1967) using the paired, two-tailed 't' test. Variance is expressed as the standard error of the mean.

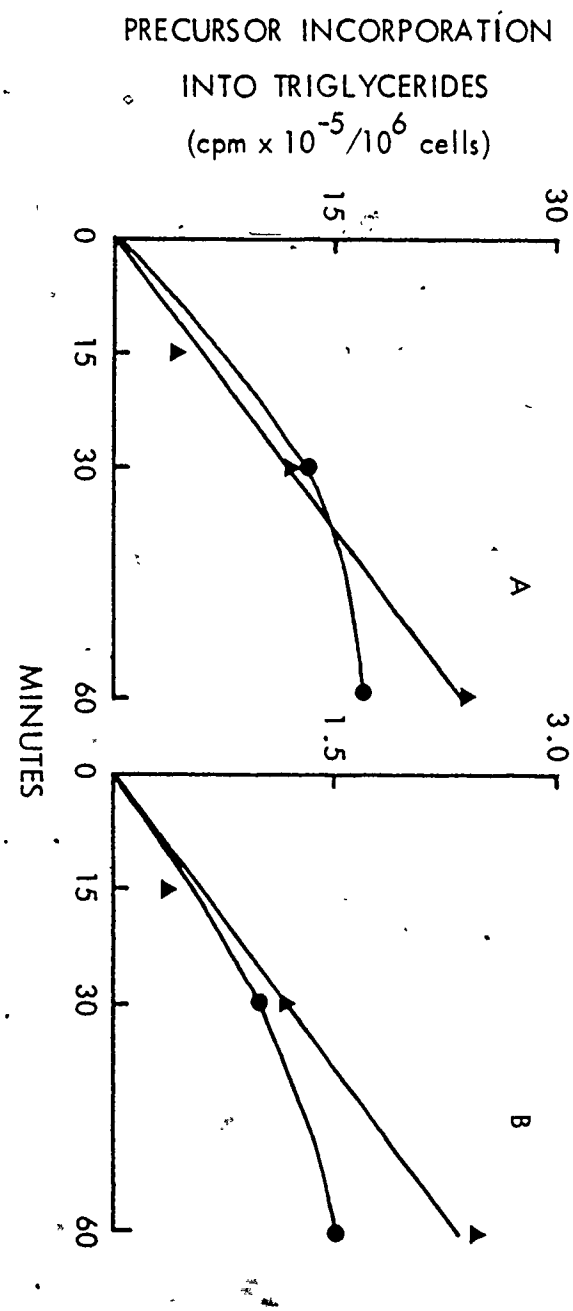
3.3 Results

3.3.1 Time Course of the Incorporation of Labelled Precursors into Hepatocyte Triglycerides

The time-course of the incorporation of both labelled palmitate

FIGURE 3.1

Time Course of incorporation of [9, 10-^{3}H] palmitate (A) and [U-^{14}C] glycerol (B) in the presence (▲—▲) or absence (●—●) of added unlabelled palmitate (50 µM) into triglycerides synthesized by hepatocytes. Each point represents mean of two experiments.


PRECURSOR INCORPORATION
INTO TRIGLYCERIDES
(cpm x $10^{-5}/10^{6}$ cells)
0
15
30
60
MINUTES
15
30
A
1.5
3.0
0
15
30
60
B

and glycerol into hepatocyte triglycerides was curvilinear, up to 60 min, in the absence of added palmitate, whereas with the addition of 50 μM unlabelled palmitate, it was linear (Fig. 3.1). The presence of unlabelled palmitate also resulted in change of incorporation of glycerol from curvilinear to linear in spite of no changes in medium glycerol concentrations.

3.3.2 Characteristics of Triglycerides Released by Hepatocytes

The release of labelled triglycerides into the medium was slow for the first 30 min, but increased three-fold in the next 30 min. As expected, the observed pattern is compatible with a precursor-product relationship between triglycerides synthesized and the triglycerides released into the medium by the hepatocytes. Over the 60 min period of incubation with labelled precursors the release of labelled triglycerides into the medium accounted for about 1 to 2% of the triglycerides synthesized by the hepatocytes. Most of the labelled triglycerides released into the medium were associated with the density fraction $d<1.006$ corresponding to VLDL, with only minimal amounts in $d>1.006$ (Table 3.2).

3.3.3 Effect of d-Norgestrel (in saline suspension) on Triglycerides Synthesized by Isolated Hepatocytes

As illustrated in Figure 3.2 d-Ng (0.05mM) suspended in saline inconsistently reduced the incorporation of both, [9, 10-^{3}H] palmitate and [U-^{14}C] glycerol, into hepatocyte triglycerides. d-Ng remained suspended in the medium because of its insolubility in

TABLE 3.2

PERCENT DISTRIBUTION OF LABELLED TRIGLYCERIDES
AMONG LIPOPROTEIN FRACTIONS OF THE INCUBATION MEDIUM

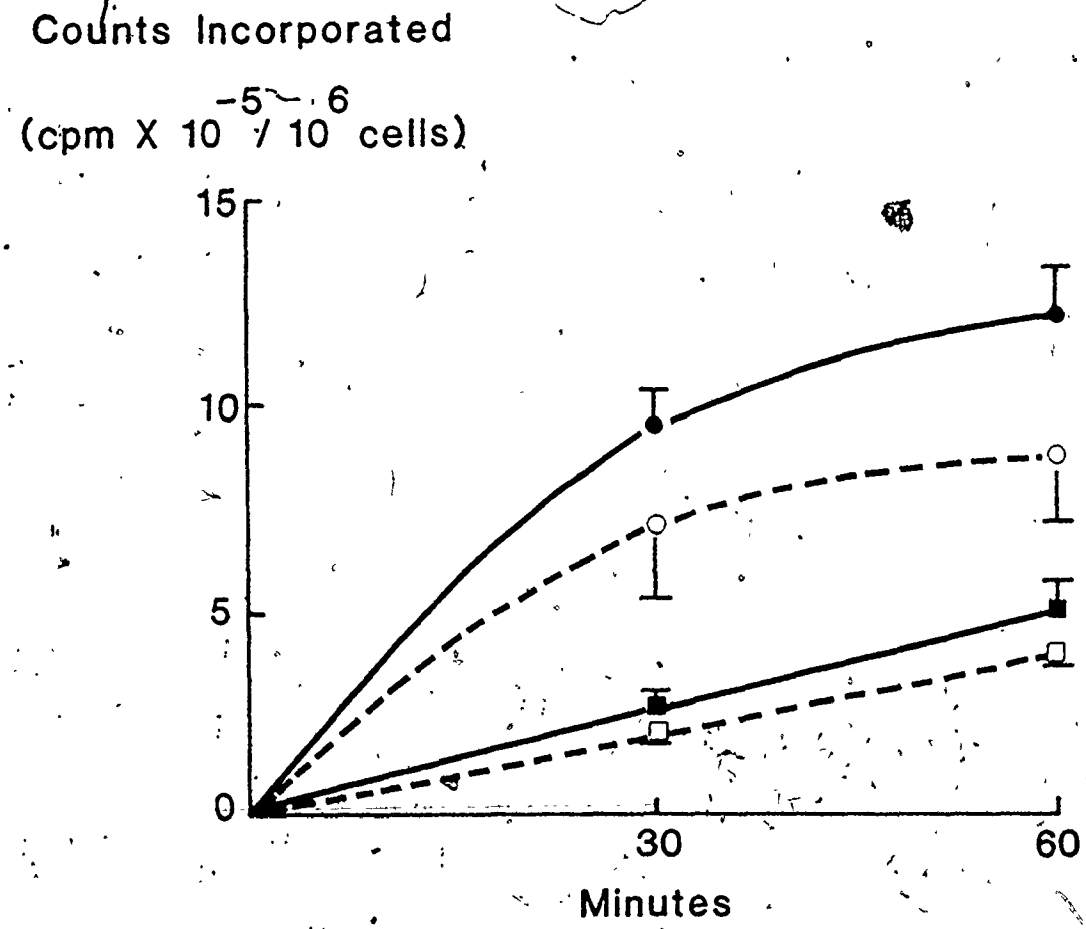
	DENSITY FRACTIONS[a]			
TRIGLYCERIDES	1.006	1.006-1.019	1.019-1.063	1.063
[^{3}H]	89 ± 5	6 ± 3	5 ± 3	N.D.[b]
[^{14}C]	95 ± 2	3 ± 2	2 ± 1	N. D.

[a] (g/ml)

[b] N.D. = not detected

FIGURE 3.2

Time course of incorporation of [9, 10 ^{3}H] palamite (circles) and [U ^{14}C] glycerol (squares) in the prescence (- - - -) or absence (——) of 0.05 mM d-norgestrel (suspended in saline) into triglycerides synthesized by hepatocytes. Each value represents Mean ± SEM for 3 paired experiments.


Counts Incorporated
(cpm X 10^-5 / 10^6 cells)
15
10
5
0
30
60
Minutes

aqueous medium. Therefore, for further experiments, d-Ng was dissolved in dimethylsufoxide (DMSO) to facilitate its dispersion; a similar amount of DMSO was present in the medium of the controls.

3.3.4 Effect of d-Norgestrel (dissolved in DMSO) on Triglycerides Synthesized by Isolated Hepatocytes

The incorporation of labelled precursors into hepatocyte triglycerides was not affected by 0.1% DMSO when compared to controls containing no DMSO. Incubation of rat liver cells with d-Ng (0.1 mM) in the presence of a 0.1% DMSO in the medium, resulted in significant inhibition of the incorporation of both [9, 10 ^{3}H] palmitate (Fig. 3.3) and [U-^{14}C] glycerol (Fig 3.4) into triglycerides synthesized by hepatocytes ($p<0.05$) in five paired experiments. The inhibitory effect of d-Ng reached a plateau within 30 min of incubation. At that point in time, the percent inhibition of the incorporation of labelled palmitate averaged 20 ± 3% and that of glycerol 19 ± 2%. Both values are significantly lower than control ($p<0.05$), though similar to each other.

3.3.5 Effect of d-Norgestrel (dissolved in DMSO) on Triglycerides Released by Isolated Hepatocytes

Dimethylsulfoxide (0.1%) did not affect the incorporation of labelled precursors into triglycerides released by the hepatocytes. At a concentration of 0.1 mM, d-Ng (in the presence of 0.1% DMSO) significantly diminished the release of [^{3}H] (Fig. 3.5) and [^{14}C] (Fig 3.6) triglycerides from isolated hepatocytes into the medium in

FIGURE 3.3

Time course of incorporation of [9, 10 ^{3}H] palmitate in the presence (○---○) or absence (●——●) of d-norgestrel (0.1 mM) into triglycerides synthesized by hepatocytes. DMSO contentration of the medium was 0.1%. Experimental values were significantly lower than control at 30 and 60 min ($p<0.05$). Each value represents Mean ± SEM for 5 paired experiments.

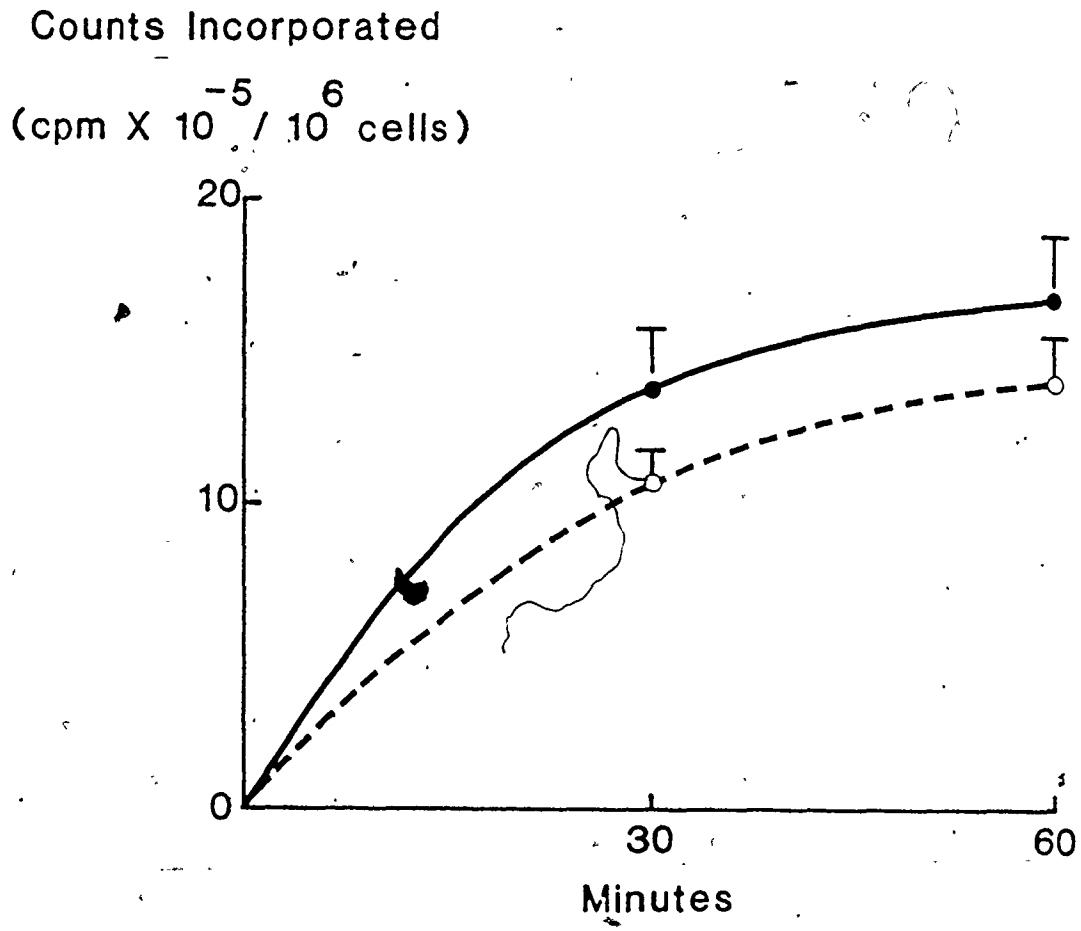

Counts Incorporated
(cpm X 10^-5 / 10^6 cells)
20
10
0
30
60
Minutes

FIGURE 3.4

Time course of incorporation of [U-^{14}C] glycerol in the presence (⊙---○) or absence (●——●) of d-norgestrel (0.1 mM) into triglycerides synthesized by hepatocytes. DMSO concentration of the medium was 0.1%. Experimental values were significantly lower than controls at 30 and 60 min ($p<0.05$). Each value represents Mean ± SEM for 5 paired experiments.

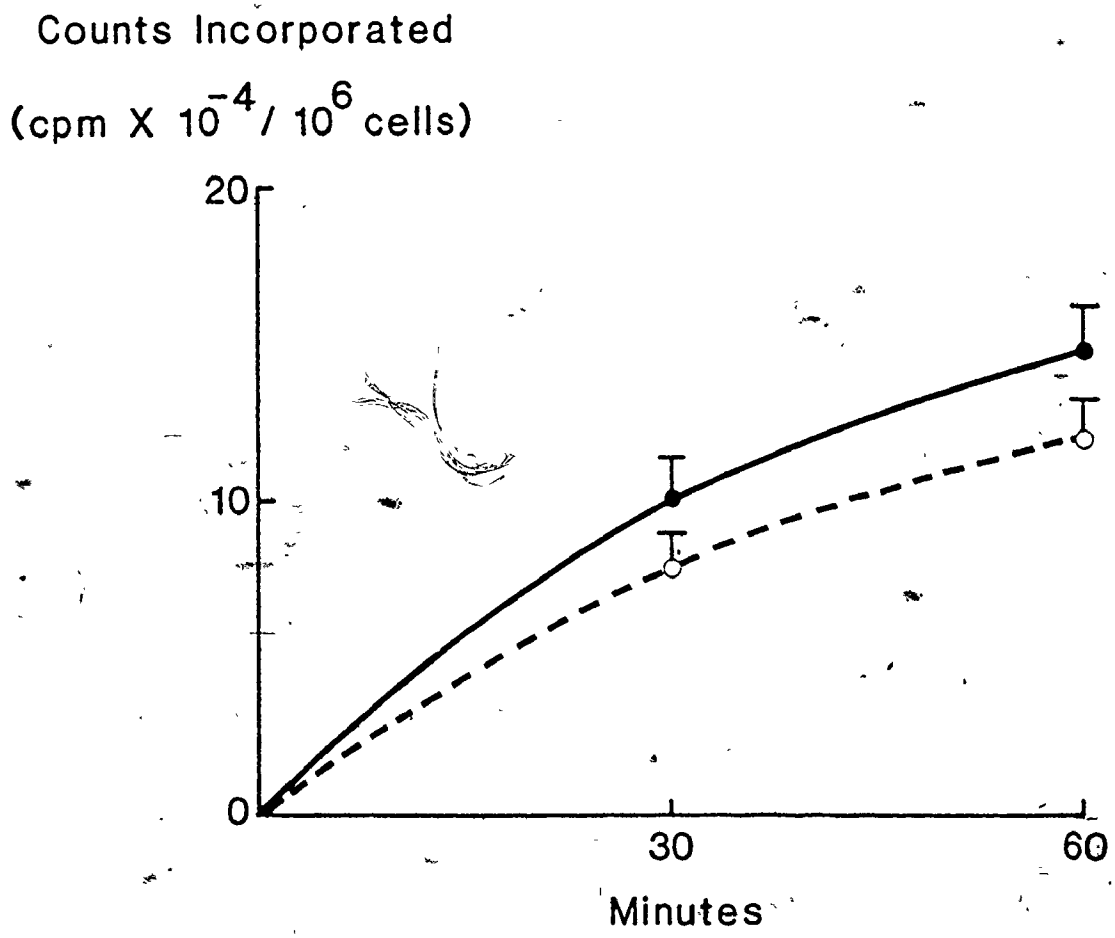
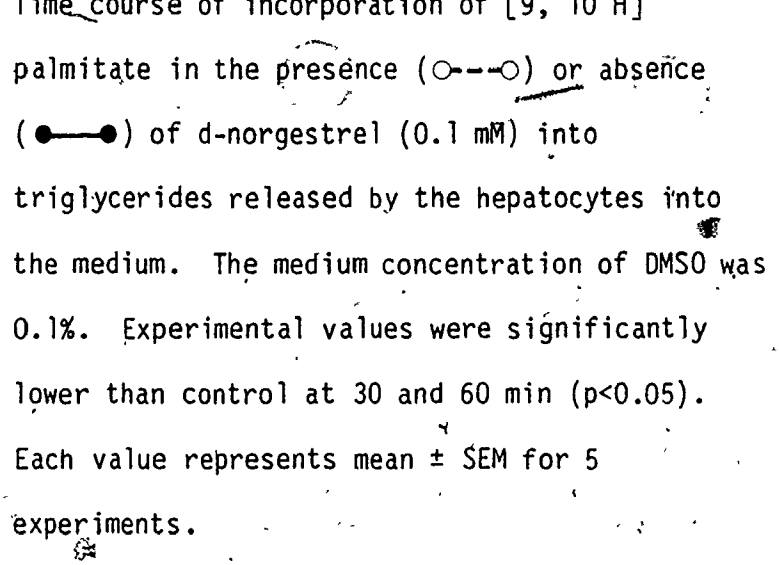

Counts Incorporated
(cpm X 10^{-4} / 10^{6} cells)
20
10
0
30
60
Minutes

FIGURE 3.5



Time course of incorporation of [9, 10^3H] palmitate in the presence (○---○) or absence (●——●) of d-norgestrel (0.1 mM) into triglycerides released by the hepatocytes into the medium. The medium concentration of DMSO was 0.1%. Experimental values were significantly lower than control at 30 and 60 min ($p<0.05$). Each value represents mean ± SEM for 5 experiments.

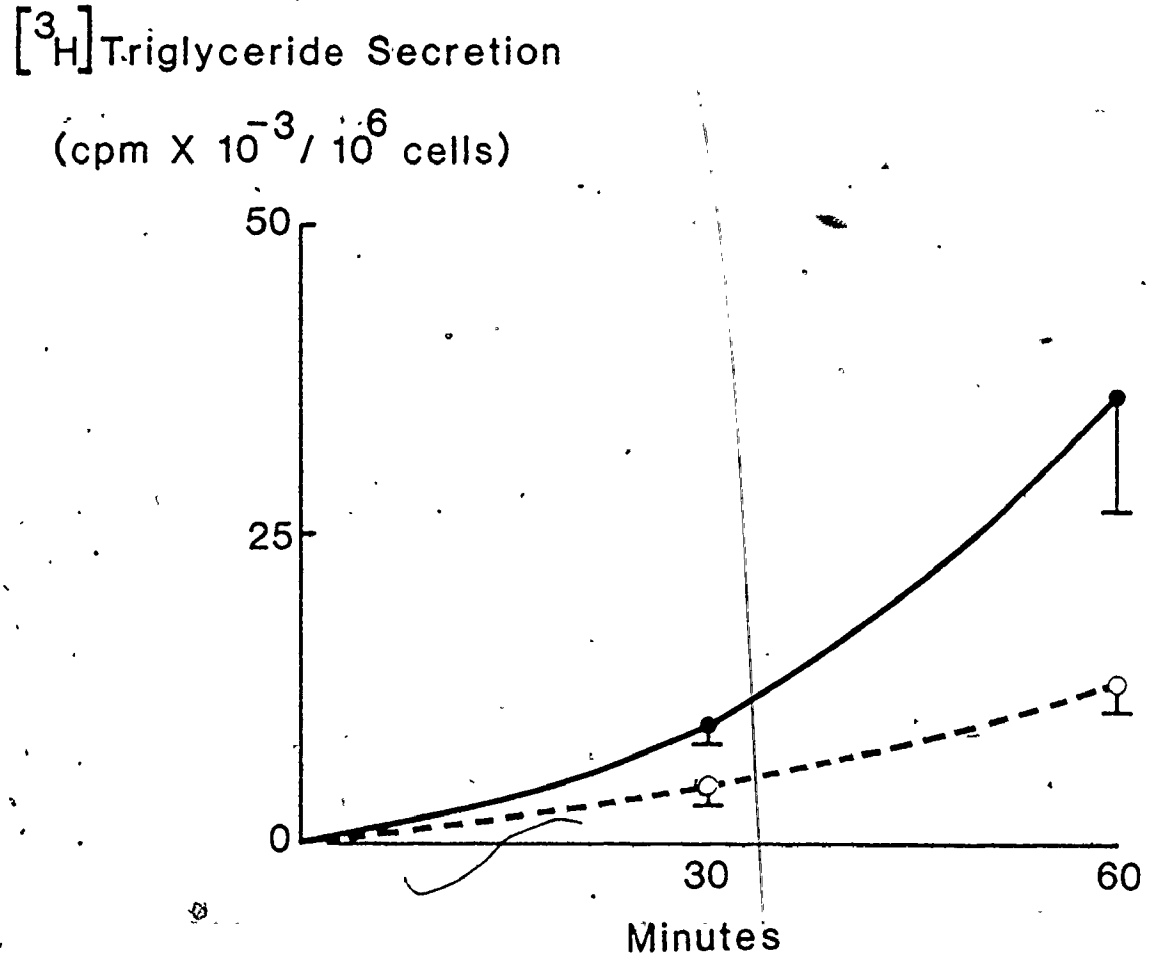
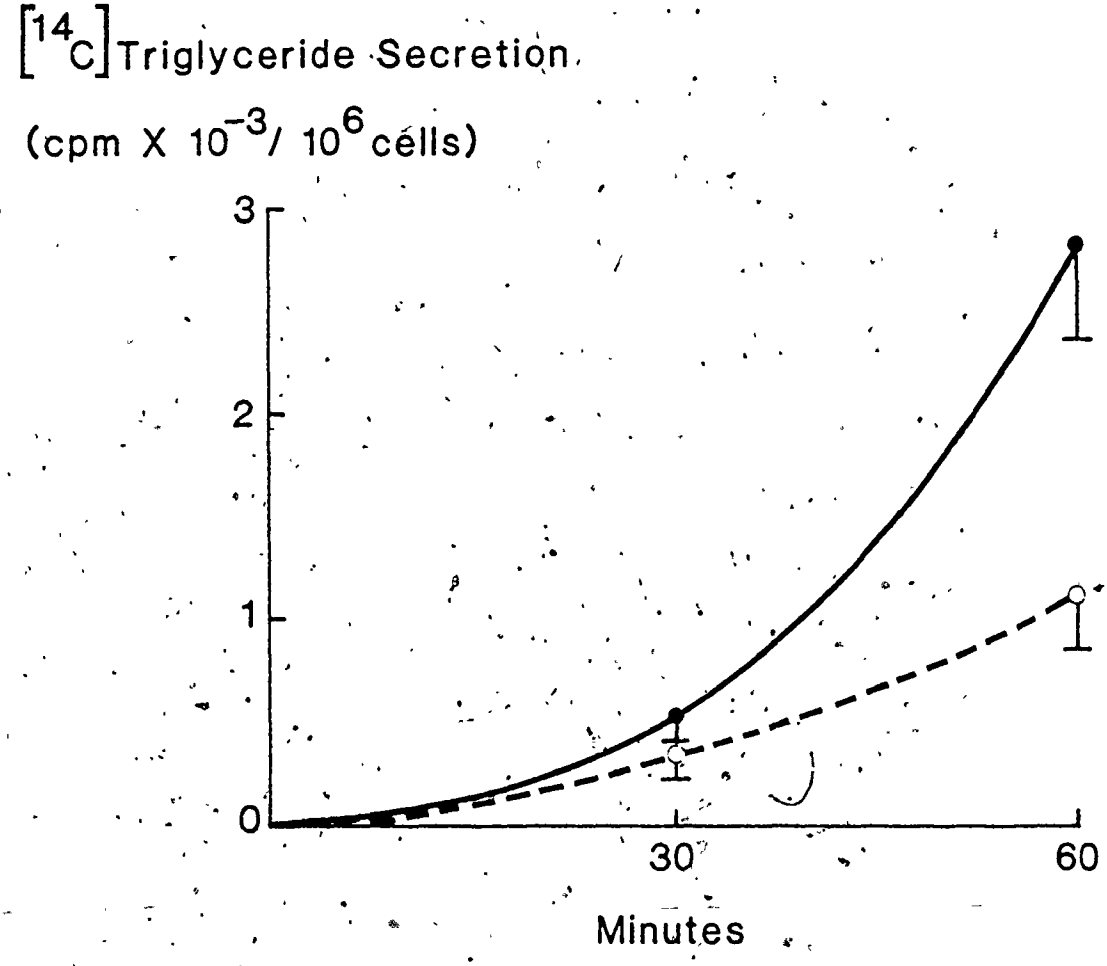

[3H] Triglyceride Secretion
(cpm X 10-3 / 10^6 cells)
50
25
0
30
60
Minutes

FIGURE 3.6

Time course of incorporation of [U-^{14}C] glycerol in the presence (○---○) or absence (●——●) of d-norgestrel (0.1mM) into triglycerides released by the hepatocytes into the medium. DMSO concentration in the medium was 0.1%. Experimental values were significantly lower than controls at 30 and 60 min ($p<0.05$). Each value represents mean ± SEM for 5 experiments.


[14C]Triglyceride Secretion
(cpm X 10-3/ 106 cells)
3
2
1
0
30
60
Minutes

five paired experiments ($p<0.05$). Maximum inhibition of the incorporations of both labelled palmitate and glycerol into triglycerides released into the medium was observed at 60 min (51 ± 11% and 54 ± 13%, respectively, $p<0.05$).

3.3.6 Effect of Higher Levels of DMSO on the Triglycerides Synthesized by Isolated Hepatocytes

d-Ng (0.1 mM) initially dissolved in 0.1% DMSO, partially precipitated upon addition to the medium. Therefore, progressively higher amounts of DMSO were used to suspend d-Ng (0.1 mM) in the medium. The formation of triglycerides by hepatocytes at different DMSO levels (0.1%, 0.25%, 0.5% and 1.0%) were compared to their respective controls i.e. hepatocytes incubated without DMSO . It had no effect on the triglyceride formation by the hepatocytes. Mean values for the incorporation of palmitate as well as glycerol into triglycerides synthesized by hepatocytes in the presence of DMSO were not significantly different ($p>0.2$) from their respective controls at any of the levels of DMSO that were tested. These results are summarized in Table 3.3.

3.3.7 Effect of Higher Levels of DMSO on the Hepatocyte Response to d-Norgestrel

Studies of incorporation of labelled precursors into triglycerides synthesized by hepatocytes were done with 0.1 mM d-Ng in presence of progressively higher DMSO concentrations (0.1%, 0.25%, 0.5%, 1.0%) in the medium. The results were compared to their

TABLE 3.3

THE FORMATION OF HEPATOCYTE TRIGLYCERIDES AT DIFFERENT DIMETHYLSULFOXIDE CONCENTRATIONS

	0.1%DMSO	CONTROL	0.25%DMSO	CONTROL	0.5%DMSO	CONTROL	1.0%DMSO	CONTROL
$[^{3}H]$ TG[a]	16.8	15.4	19.8	17.9	20.2	17.9	18.2	16.2
	±	±	±	±	±	±	±	±
	1.9	2.4	1.6	0.8	1.5	0.8	1.8	0.7
$[^{14}C]$ TG[b]	14.8	14.3	16.7	16.1	15.7	16.1	16.7	14.7
	±	±	±	±	±	±	±	±
	1.5	1.8	0.3	0.6	0.8	0.6	1.0	0.5

[a] Values expressed as cpm x $10^{-5}/10^{6}$ cells.

[b] Values expressed as cpm x $10^{-4}/10^{6}$ cells.

respective controls i.e. hepatocytes incubated without d-Ng but the same amount of DMSO (0.1%, 0.25%, 0.5%, 1.0%). The inhibition by d-Ng of the incorporation of labelled palmitate in triglyceride synthesized by hepatocytes was twofold higher (Fig. 3.7) at 1.0% compared to 0.1% DMSO concentration of the medium (39 ± 0.8 vs 20 ± 3 %). Likewise, the inhibition by d-Ng of the incorporation of labelled glycerol into triglycerides synthesized by hepatocytes increased (Fig. 3.8) from 19 ± 2% to 42± 2%, when DMSO concentration in the medium increased from 0.1% to 1.0%.

3.3.8 Concentrations of d-Norgestrel in the Medium as Determined by HPLC at Different DMSO Levels

The concentrations of d-Ng were determined in the incubation medium free of hepatocytes at two different levels of DMSO (0.1% and 1.0%). The results summarized in Table 3.4 show that d-Ng concentration was highest at 0 min which declined at 30 min and remained same for the next 30 min. This indicated that a part of d-Ng initially dissolved, precipitated from the medium. The concentration of d-Ng was consistently higher, at all times, in the presence of 1% DMSO level when compared to 0.1% DMSO. Although, the amount of d-Ng initially added was similar (0.1 mM) for both incubations (Table 3.4). Higher levels of DMSO increased the solubility of d-Ng by 44%.

3.3.9 Uptake of d-Norgestrel by Isolated Hepatocytes examined by HPLC

d-Ng concentrations were also determined in the medium incubated

FIGURE 3.7

The percent inhibition by d-norgestrel (0.1 mM) of the incorporation of [9, 10 ^{3}H] palmitate into triglycerides synthesized by hepatocytes in presence of increasing medium concentrations of DMSO (0.1% to 1%). Each bar represents mean inhibition ± SEM of 3 paired experiments.

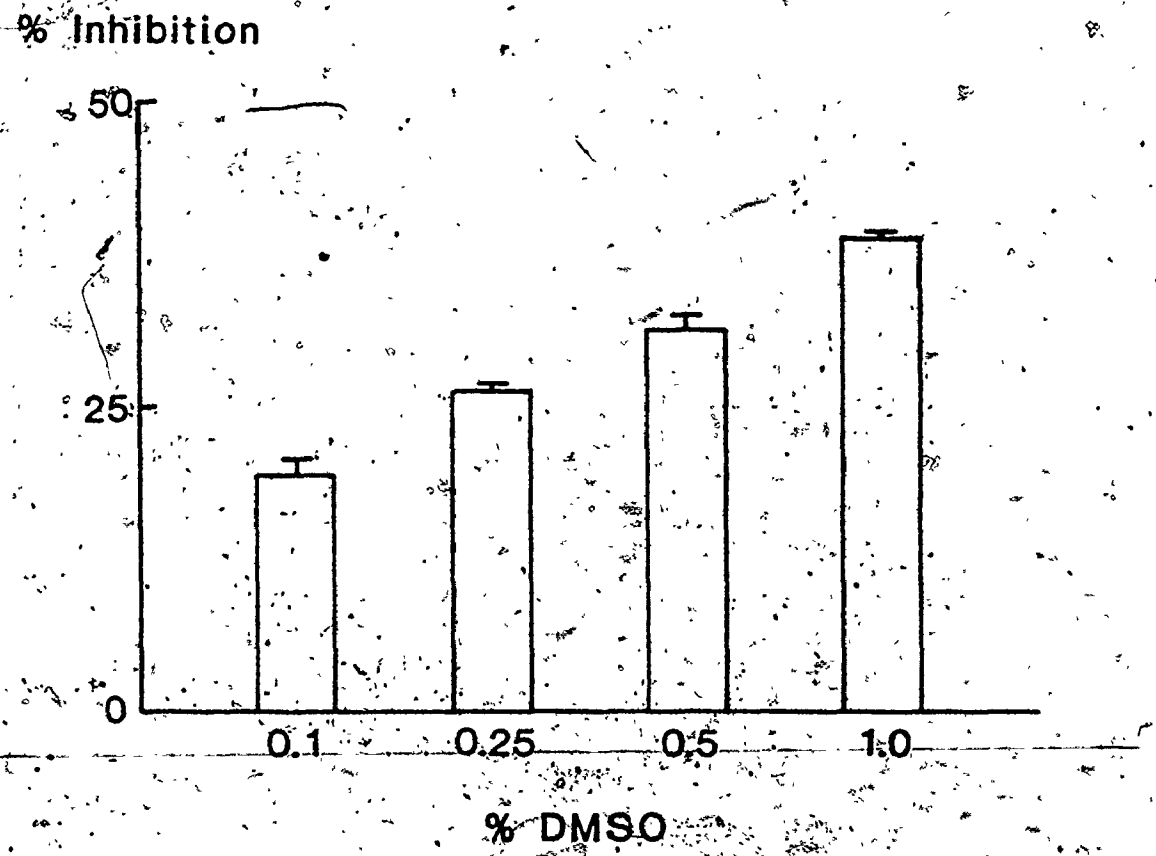

% Inhibition
50
25
0
0.1
0.25
0.5
1.0
% DMSO

FIGURE 3.8

The percent inhibition by d-norgestrel (0.1 mM) of the incorporation of [U-^{14}C] glycerol into triglycerides synthesized by hepatocytes in presence of increasing medium concentrations of DMSO (0.1% to 1%). Each bar represents mean inhibition ± SEM of 3 paired experiments.

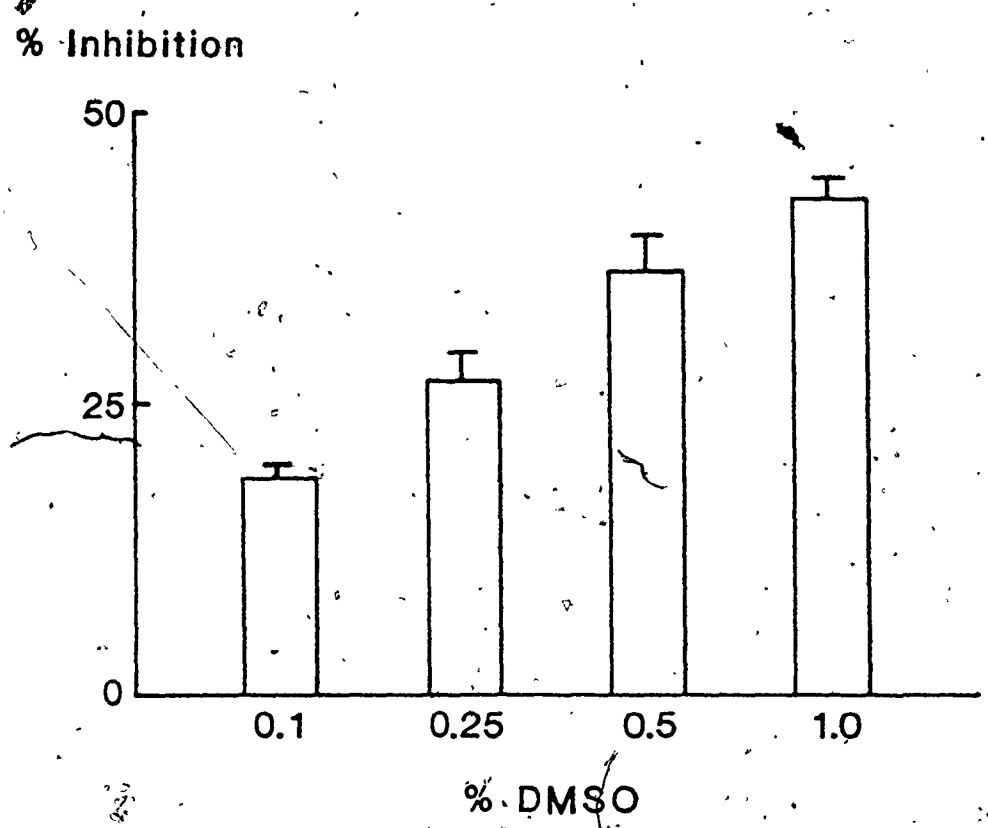

% Inhibition
50
25
0
0.1
0.25
0.5
1.0
% DMSO

TABLE 3.4

MEDIUM CONCENTRATIONS[a] OF d-NORGESTREL

AT DIFFERENT DMSO LEVELS IN ABSENCE OF HEPATOCYTES

Time	DMSO	
min	0.1%	1.0%
0	10.3 ± 2.0	22.4 ± 6.4
30	6.3 ± 1.3	9.1 ± 0.9
60	5.9 ± 1.8	8.6 ± 1.2

[a] Values expressed as μM (Mean ± SEM).

TABLE 3.5

MEDIUM CONCENTRATIONS[a] OF d-NORGESTREL

AT DIFFERENT DMSO LEVELS IN PRESENCE OF HEPATOCYTES

Time	DMSO	
min	0.1%	1.0%
0	32.7 ± 1.3	49.7 ± 0.3
30	5.0 ± 2.1	16.2 ± 2.2
60	0.2 ± 0.1	2.1 ± 1.6

[a] Values expressed as µM (Mean ± SEM)

with hepatocytes at two different levels of DMSO (0.1% and 1%). Again, higher DMSO levels resulted in higher soluble concentrations of d-Ng when the same amount (0.1 mM) was initially added to the medium (Table 3.5). In the presence of hepatocytes, there was a gradual decline of d-Ng in the medium which decreased to minimal levels at 60 min (Fig 3.9). At 0.1% DMSO concentration, the uptake of d-Ng by the hepatocytes was 0.05 nmole compared to 0.14 nmole at 1% DMSO level. The medium concentration of d-Ng was found to be higher in the presence than in the absence of hepatocytes (compare Tables 3.4 with 3.5) at 0 min.

3.4 DISCUSSION

This chapter describes the studies done to elucidate the mechanisms underlying lowered triglyceride levels observed in d-Ng. treated rats. Suspensions of isolated hepatocytes were used to study the effects of d-Ng on triglyceride synthesis by examining the incorporation of labelled precursors into triglycerides. In contrast to the *in vivo* studies which were done over an extended time period with a dose of d-Ng comparable to the conventional contraceptive doses given to women, the *in vitro* studies with isolated hepatocytes employed much higher doses. This permitted the study of acute effects of d-Ng on triglyceride synthesis.

Although solubilization of the d-Ng posed a problem in the *in vitro* studies, DMSO was found to be a useful solvent for suspending d-Ng. Higher DMSO concentrations in the medium allowed greater solubility of d-Ng. This was proven with HPLC determinations. The medium concentrations of d-Ng rose by 52% (32.7 vs 49.7 μM,

FIGURE 3.9

Uptake of d-norgestrel by isolated rat hepatocytes as a function of time. Retention times for d-norgestrel (a) and progesterone (b) were 9.78 to 9.8 min and 14.27 to 14.33 min respectively.

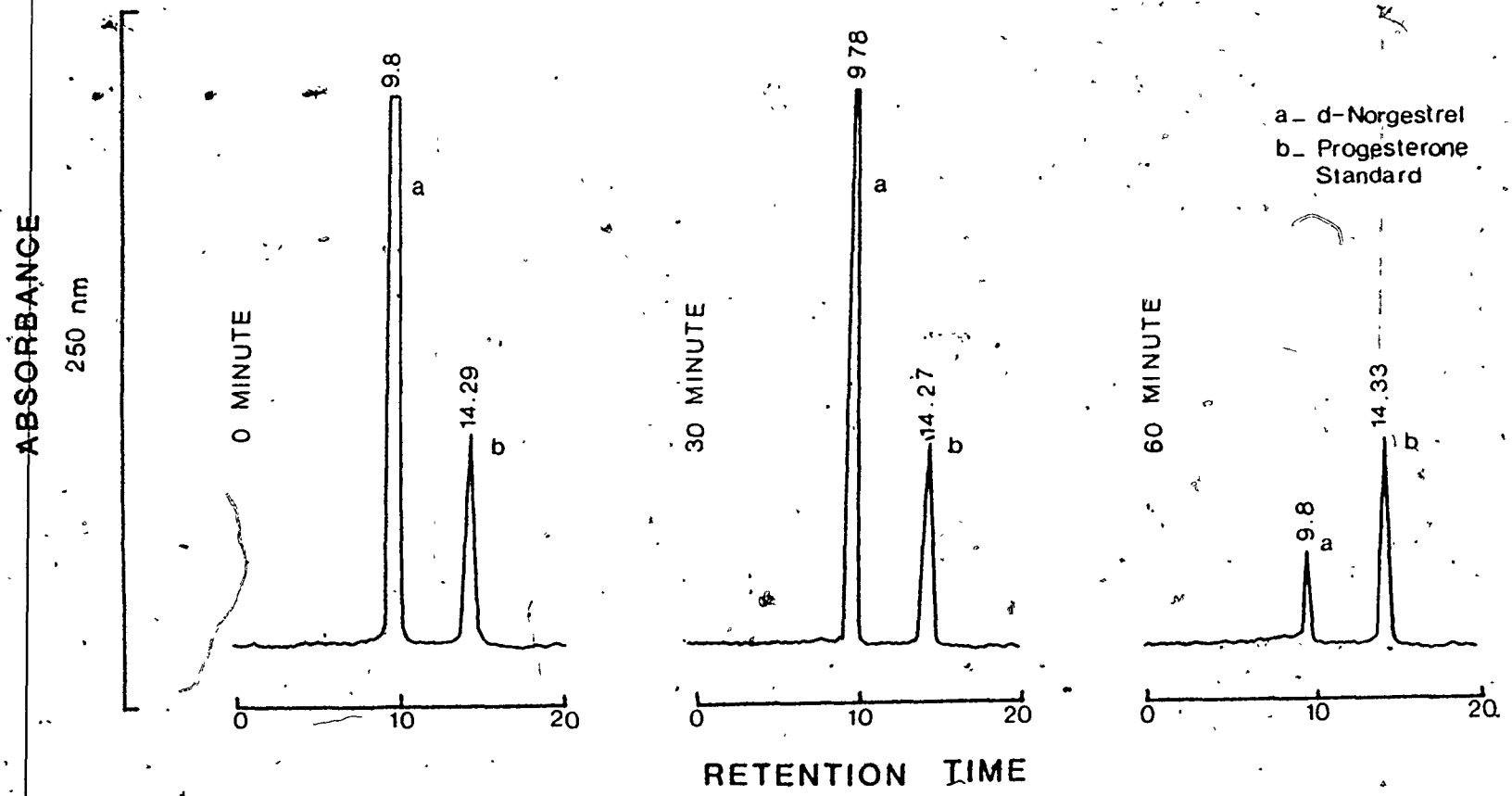

ABSORBANCE
250 nm
0 MINUTE
9.8
a
14.29
b
30 MINUTE
9.78
a
14.27
b
60 MINUTE
9.8
a
14.33
b
a_ d-Norgestrel
b_ Progesterone Standard
0
10
20
RETENTION TIME

Table 3.5) when DMSO level was increased from 0.1% to 1%. Furthermore, a higher amount of d-Ng stayed in solution at 1% compared to 0.1% DMSO (16.2 vs 5.0 µM, Table 3.5) thereby making more d-Ng available for uptake by the hepatocytes. At 1% DMSO level 14.1 µM d-Ng was taken up by the hepatocytes compared to only 4.8 µM at 0.1% DMSO. In the concentrations employed, DMSO alone had no effect either on triglycerides synthesized by the hepatocytes or their release by the hepatocytes (Table 3.3). This contrasts with a previous report by Bell et al (1982) that a higher concentration of DMSO (5%) produces a 7-fold increase in the incorporation of [^{14}C] acetate into triglycerides of surviving rat liver slices. It is noteworthy that Bell et al (1982) used 5 to 50 times greater concentrations of DMSO compared to the present study.

At a concentration of 0.1 mM, d-Ng significantly inhibited the incorporation of both precursors ([9, 10^{3}H] palmitate and [U-^{14}C] glycerol) into triglycerides synthesized by the hepatocytes as well as into triglycerides released by the hepatocytes (Fig 3.3 and 3.4). There was no significant difference in the percent inhibition between palmitate and glycerol. The extent of inhibition was influenced by the amount of DMSO used to suspend d-Ng. The inhibition of palmitate incorporation by d-Ng increased from 20% at 0.1% DMSO concentration to 39% at 1% DMSO concentration in the medium. Likewise, the inhibition of glycerol by d-Ng incorporation also increased stepwise from 19% at 0.1% DMSO level to 42% at 1% DMSO (Fig. 3.7 and 3.8). These findings suggested that d-Ng inhibited triglyceride synthesis in a dose-dependent manner; with successively higher concentrations of

DMSO, higher amounts of d-Ng dissolved in the incubation medium thereby producing a greater inhibition of TG synthesis by the hepatocytes.

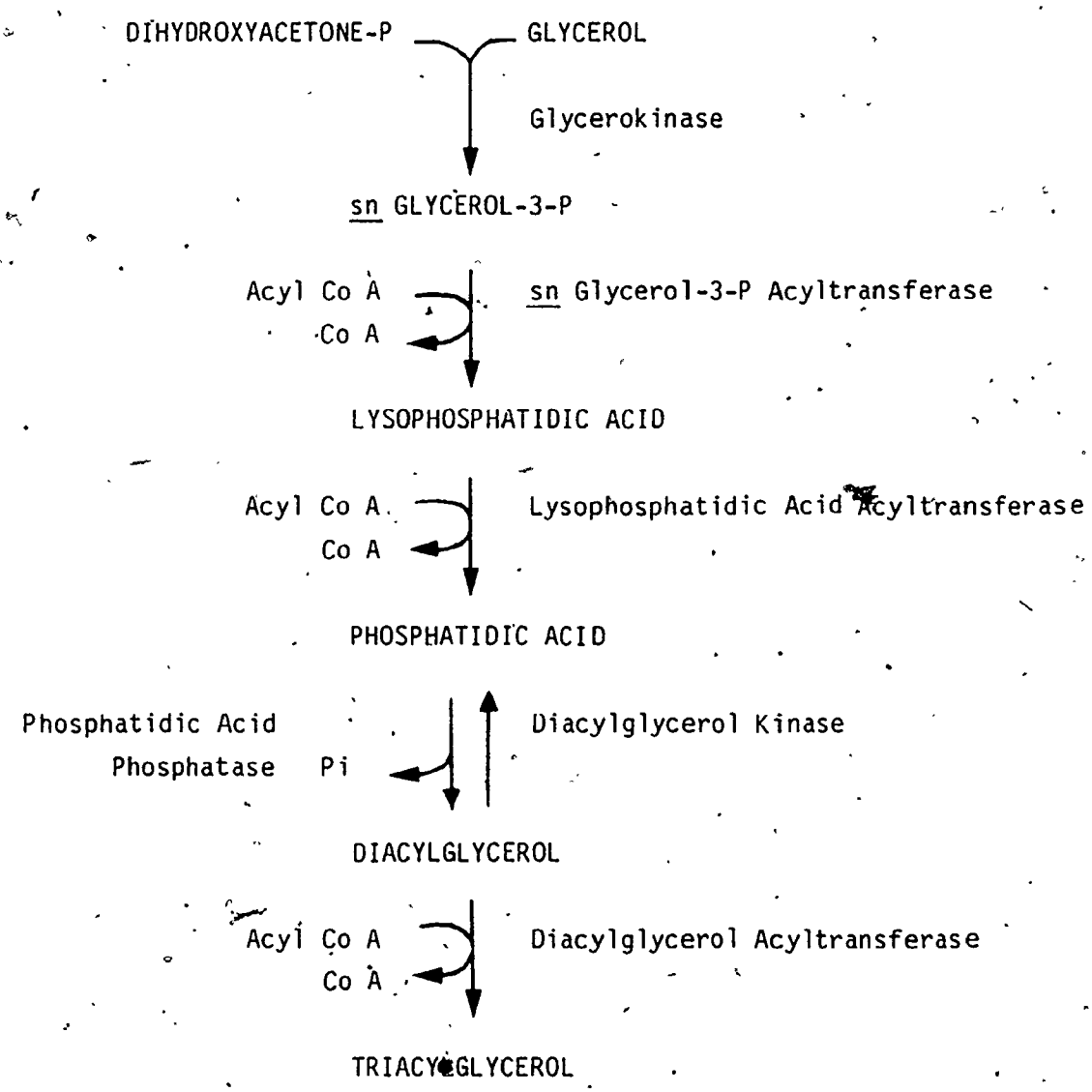
The inhibition by d-Ng of triglyceride release from hepatocytes could be accounted for solely by the reduction of hepatocyte triglyceride synthesis. However, because triglyceride release was inhibited by d-Ng to a greater extent (51-54%) than hepatocyte triglyceride synthesis (19-20%) it is conceivable that d-Ng might have additional effects on the release of VLDL from hepatocytes. The present results suggest that (i) liver may be an important site of the hypolipidemic action of d-Ng and (ii) that inhibition of hepatic triglyceride synthesis and release can account at least for part of the hypolipidemic effect of d-Ng observed in vivo in fed rats.

The pathway of hepatic glycerolipid biosynthesis is shown in Figure 3.10. Because the incorporation of both substrates into triglycerides was inhibited to a similar extent by d-Ng, it is attractive to suggest that the inhibition may be at the level of either glycerol-3-phosphate acyltransferase, phosphatidic acid phosphatase and/or diacylaglycerol acyltransferase, the three potentially rate-limiting enzymes shared by these substrates on their pathway to triglycerides. The effect of d-Ng on these enzymes will be discussed in more detail in Chapter 4.

The present findings that d-Ng inhibits triglyceride synthesis and release are in agreement with reports of Cheng and Wolfe (1983) who demonstrated a similar inhibition of triglyceride synthesis and release by the progestin norethindrone acetate in isolated rat

Figure 3.10

PATHWAY OF TRIACYLGLYCEROL FORMATION IN LIVER



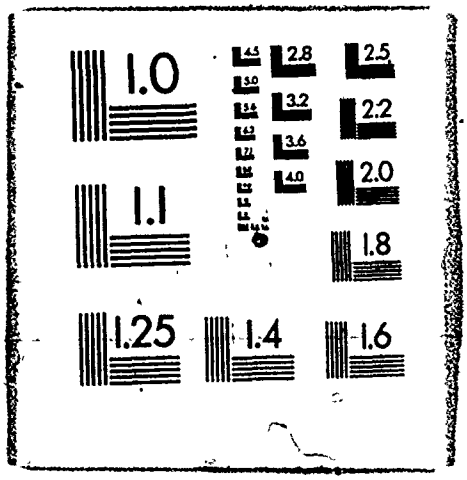
hepatocytes. Wolfe and Grace (1979) have shown reduced hepatic triglyceride secretion in miniature swine treated with norethindrone acetate. In contrast to progestins, estrogens have been shown to increase triglyceride production leading to hypertriglyceridemia (Kim and Kalkhoff, 1975; Glueck et al, 1974 and Weinstein et al, 1978).

Because the inhibitory effect of d-Ng occurred so rapidly, the inhibitory action in vitro of d-Ng on hepatocyte triglyceride synthesis and release may have occurred by a mechanism which is different from that generally known to account for steroid action. Steroids are generally thought to act by binding to receptors in the cytosol which translocate to the nucleus, bind to DNA and then mediate their action by inducing transcription of specific mRNA (Gorski and Ganon, 1976; Jensen and DeSombre, 1973; Muldoon, 1980). However, recent studies of King and Green (1984) and Welshons et al (1984) have challenged the widely accepted belief that unoccupied steroid receptors are cytosolic. By the use of monoclonal antibodies and cytochalasin B-induced enucleation, they demonstrated that unoccupied estrogen receptors are exclusively located in the nucleus.

In addition to the known action of progesterone via cytosolic receptors (Murayama et al, 1980; Mulvihill and Palmiter, 1980), it has been reported to alter cAMP levels and adenylate cyclase activity by binding to membrane receptors (Schorderet-Slatkine et al, 1982 ; Maller and Sadler, 1981). The effect was immediate and persistent. Kuehl et al (1974) have also reported significant increases in cGMP levels with reductions in cAMP levels in the uterine tissue of

estrogen treated rats. It is plausible that d-Ng could act by altering cAMP levels by a mechanism similar to progesterone or 17 β-estradiol. It is not yet known whether there are membrane receptors for d-Ng. Although d-Ng is a 19-nortestosterone derivative and has some androgenic activity the cytosolic receptors for d-Ng have been shown to be competitively displaced by progesterone, but only weakly by testosterone (Uniyal et al, 1977). Additional explanations for the rapid effect of d-norgestrel could include 1) the phosphorylation-dephosphorylation mechanism regulating the enzymes of glycerolipid pathway and/or 2) selective translation of mRNA independent of nucleus events (Vydelingum, 1982). Haagsman et al (1982) have proposed that glucagon exerts the inhibitory action on hepatic triglyceride synthesis by regulating the enzyme diacylglycerol acyltransferase by a phosphorylation-dephosphorylation mechanism. Glycerol-3-phosphate acyltransferase of Escherichia coli is also reported to be stimulated by ATP through interaction with adenylate cyclase (Rock, C.O. et al, 1981).

2



Chapter 4

EFFECTS OF d-NORGESTREL TREATMENT ON HEPATIC GLYCEROL PHOSPHATE ACYLTRANSFERASE AND PHOSPHATIDIC ACID PHOSPHATASE ACTIVITIES IN THE RAT

4.1 INTRODUCTION

The observation that d-Ng inhibits the incorporation of labelled precursors into triglycerides synthesized by isolated rat hepatocytes suggested a possible suppressive effect of d-Ng on the enzymes involved in the triglyceride synthetic pathway. This chapter describes the studies undertaken to examine the effects of d-Ng treatment on hepatic glycerol phosphate acyltransferase (GPAT) and phosphatidic acid phosphatase (PAPase) activities in the rat.

Although the pathway of glycerolipid biosynthesis was described in eucaryotes about 20 years ago by Kennedy and co-workers (1961), little is known about the precise routes of production and regulation. Glycerol phosphate acyltransferase (EC 2.3.1.15) primarily found in microsomes and outer mitochondrial membrane catalyzes the first committed step for production of glycerolipids and is a possible control site of this process. In 3T3-L1 cell lines as well as rat liver microsomes, the specific activity of GPAT is reported to be much lower than the specific activities of fatty acid CoA ligase, lysophosphatidic acid, phosphatidic acid phosphatase and diacylglcerol acyltransferase (Coleman et al, 1978; Lloyd-Davies and Bridley, 1975; Lloyd-Davies and Brindley, 1973).

Studies in adipose tissue and liver (Sooranna and Saggerson, 1976a and 1976b; Bates and Saggerson, 1977a and 1977b) indicate the possibility of acute hormonal regulation of GPAT activity. Coleman et al. (1977) also reported 2.5 to 3.6 fold increase in GPAT activity along with similar increases in fatty acid CoA ligase and diacylglycerol acyltransferase in livers from estrogenized chicks. GPAT activity in the rat liver is also known to be decreased in fasting and fat feeding and increased in carbohydrate feeding and chronic ethanol consumption which was accompanied by proliferation of endoplasmic reticulum (Bremmer et al, 1976; Joly et al, 1973).

The reaction catalyzed by phosphatidic acid phosphatase (EC 3.1.3.4) occurs at an important branch point in glycerolipid synthesis. There are several lines of evidence which support a role of this enzyme in the regulation of glycerolipid and phospholipid pathways in the liver and adipose tissue (van den Bosch et al, 1972; Fallon et al, 1977; Bridley et al, 1979; Brindley and Sturton, 1982). PAPase activity is found in microsomal and cytosol fractions of the liver. Two operationally-distinct enzyme activities have been detected corresponding to the form of substrate available (Casola and Possmayer, 1980b), referred to as phosphatidic acid aqueous (PA_{aq})-dependent and phosphatidic acid membrane bound (PA_{mb})-dependent PAPase.

4.2 MATERIALS and METHODS

4.2.1 Materials

L-[U-^{14}C] glycerol-3-phosphate (171 mCi/mmol) was purchased

from Amersham, Oakville, Ont. Palmitoyl coenzyme A, dithiothreitol, fatty Acid-Free Albumin, L-α-glycerophosphate, Trizma [Tris (Hydroxymethyl) aminomethane], N-ethylmaleimide and trichloroacetic acid, cytochrome 'c', Triton X-100, glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADP) and its reduced form (NADPH) and rotenone were obtained from Sigma Chemical Co., St. Louis, MO. All the other chemicals were purchased from Fisher Scientific Co., Fair Lawn, N.J.

4.2.2 Preparation of Subcellular Fractions

Rats were anesthetised by the administration of sodium pentobarbital. The subcellular fractions were prepared essentially as described by Bates and Saggerson (1979). The entire liver was first perfused with normal saline for 1-2 min to wash out the blood. The livers were removed, weighed after blotting and cut into small pieces in ice cold buffer (0.25 M Sucrose, 10mM Tris-Cl pH 7.4, 1 mM EDTA). This was followed by two washes with the same buffer. Liver was homogenized in 30 ml buffer by using a motor-driven Teflon pestle in a glass homogenizing tube (clearance 0.1-0.15 mm). Each homogenate was centrifuged at 620xg for 10 min at 4°C in a Sorvall RC2-B refrigerated centrifuge with a Sorvall type SS-34 rotor. The resulting supernatant was centrifuged at 7250xg for 10 min to yield a mitochondrial pellet. The pellet was washed twice with 30 ml of the buffer by resuspension and centrifugation at 9200xg for 10 min each time. Microsomal fraction was obtained by centrifuging post-mitochondrial supernatant at 105000xg for 60 min in a Beckman model L3-50 ultracentrifuge with a type 50 Ti rotor. The high speed supernatant called cytosol was

saved. The microsomal pellet was resuspended and centrifuged again at 105,000xg for 60 min at 4°C using the same buffer. Mitochondria and microsomes were resuspended in a buffer containing 0.25M Sucrose, 10mM Tris-Cl (pH 7.4), 1mM EDTA and 1mM dithiothreitol (DTT) at approximate protein concentrations of 5-6mg/ml. Cytosol, mitochondria and microsomes were frozen in dry ice-acetone and stored at -70°C. Protein concentrations were determined by the method of Lowry et al (1951) using bovine serum albumin as standard. Samples were thawed only once prior to estimation.

4.2.3 Enzyme Assay (GPAT) EC 2.3.1.15

Glycerol phosphate acyltransferase was assayed in a total volume of 1.0 ml, at 30°C for 3 min in the presence of 65 µM palmitoyl CoA, 120 mM KCl, 50mM Tris-Cl buffer (pH 7.4), 0.7mM DTT, 1.5µCi L[U-^{14}C] glycerol-3-phosphate and 2.5mM unlabelled L-α-glycerophosphate. 1.75 mg and 6 mg of fatty acid-poor albumin was included respectively for assaying microsomal and mitochondrial activities as described by Bates and Saggerson (1977a). The reaction was initiated with 0.1 ml of the subcellular fraction containing 0.25-0.3 mg protein. Reactions were terminated by the addition of 2 ml of water-saturated butanol. Radioactivity incorporated into butanol-soluble products was determined as described by Daae and Bremer (1970) after washing the butanol phase twice with two volumes of butanol-saturated water. One unit of enzyme activity represented the conversion of 1 µmol substrate to products per min at 30°C.

4.2.4 Isolation of Reaction Products by Thin Layer Chromatography

Samples were taken for total radioactivity measurement from the butanol extract. An aliquot of the butanol phase was evaporated to dryness under a stream of N_2. The residue was dissolved in chloroform:methanol (1:1, v/v) and chromatographed on silica gel G 60 treated plates with 0.25 M oxalate. The formation of the products TG, diglyceride, monoglyceride, lysophosphatidic acid and phosphatidic acid were determined by developing the plates with petroleum ether-acetone-formic acid (76:24:0.2, v/v/v). The radioactive bands in the chromatograms were localized by scraping 1 cm bands of the silica into counting vials followed by scintillation fluid and were identified by comparison with authentic standards.

4.2.5 Radioactivity Measurements

Samples of the butanol phase were pipetted directly into counting vials containing 10 ml of the scintillation fluid (5g PPO; 0.05g dimethyl POPOP; 385 ml xylol; 385 ml dioxane; 230 ml ethanol and 80g naphthalene). Bands from the thin-layer chromatograms were scraped off into vials containing scintillation fluid. The radioactivity was measured in a Nuclear Chicago Isocap 300 Liquid Scintillation Counter.

4.2.6 Enzyme Assay (PAPase) EC 3.1.3.4

Enzyme assays were same as described by Casola and Possmayer (1981a). The PA_{mb}-dependent PAPase activities were assayed in an incubation system with 100 µl final volume containing 50 mM

Tricine buffer pH 7.4, 150 µM [^{32}P] PA_{mb} (as lipid vesicles), EDTA 1 µM, and 50-200 µg cytosol or microsomal protein at 37°C. The reaction was terminated after 20 min by adding 1.5 ml chloroform: methanol (5:4) and 0.5 ml of 1N HCl. The radioacivity in the aqueous phase was measured in Aquasol. The assays were performed in the presence and absence of 6 mM $MgCl_2$. The Mg^{2+}-dependent component served as a measure of PA_{mb}-dependent PAPase activity (Walter and Possmaymer, 1984). One unit of PA_{mb}-dependent PAPase activity was defined as the activity which will hydrolyse 1 nmol of [^{32}P] PAmb/min at 37°C.

PA_{aq}-dependent PAPase activity was assayed using ^{32}P-labelled Pa_{aq} mixed with phosphatidic acid and converted to liposomes as described by Casola and Possmayer (1981a). The reaction was incubated at 37°C and contained 50 mM Tricine buffer pH 6.8, 1 mM [^{32}P] PA_{aq} and 50-200 µg cytosol or microsomal protein in a total volume of 100µl. The reaction was terminated after 20 min and the radioactivity in the samples was measured as described above. One unit of PA_{aq}-dependent PAPase was defined as the activity which will hydrolyse 1 nmol of [^{32}P] PA_{aq}/minute at 37°C.

4.2.7 Assays for Marker Enzymes

Cytochrome 'c' oxidase (EC 1.9.3.1) served as the marker enzyme for mitochondria and was assayed according to Applemans et al (1955). 0.1 ml of organelle preparation (25-50µg protein) was mixed with 0.1 ml of 0.3% triton X-100 for 30 to 45 sec. 2.7 ml of 50 mM phosphate buffer (pH 7.5) and 0.45 mM reduced cytochrome 'c'

were subsequently added for a final volume of 3 ml. The change in absorbance at 550 nm was recorded over 1 to 2 min by using a Schimadzu UV-250 spectrophotometer. The rate of change in absorbance at 550 nm was calculated from the linear portion of the slope and concentration of cytochrome 'c' oxidized was calculated as follows

$$\frac{A_{550\ nm}\ .min^{-1}}{29.5}$$

where 29.5 is the εmM of cytochrome 'c' obtained from the manufacturer.

Glucose-6-phosphatase (EC 3.1.3.9) and NADPH cytochrome 'c' reductase (EC 1.6.2.3) used as marker enzymes for microsomes were assayed as described by Rip et al (1981) and Omura et al (1967) respectively. Glucose-6-phosphatase was assayed in a final volume of 3 ml containing 3.6 mM imidazole-HCl, pH 6.5, 2 mM glucose-6-phosphate, 4 mM $MgCl_2$ and 25-50 µg organelle protein at 37°C. Reaction was terminated after 20 min by adding cold perchloric acid to a final concentration of 5%. Inorganic phosphate liberated was calculated from the standard curve after measuring the absorbance of each sample at 660 nm. Cytochrome c reductase was assayed in 3 ml reaction mixture consisting of 0.1 ml enzyme (25-50 µg protein), 0.1 ml 50 mM sodium cyanide, 0.2 ml 0.45 mM cytochrome 'c' and 2.5 ml 50 mM phosphate buffer (pH 7.5). The reaction was started with the addition of 0.1 ml of NADPH and reduction of cytochrome 'c' at 550 nm was measured. Activity was calculated as described above. Cytochrome c reductase and

cytochrome 'c' oxidase assays were performed at room temperature.

Lactate dehydrogenase (EC 1.1.1.27) served as the marker enzyme for cytosol and was assayed by the method of Possmayer et al (1973). The incubation mixture contained phosphate buffer 100 mM, pH 7.5 0.25 mM NADH, 0.02 ml of a saturated ethanolic solution of rotenone, Triton X-100 (0.01%, v/v) and 25-50µg of organelle preparation. The decrease in absorbance at 340 nm produced by the addition of sodium pyruvate 0.2 mM at room temperature was recorded for 1 to 2 min. The initial linear portion of the slope was used to calculate the reaction rate.

4.2.8 Statistics

Differences between the enzyme activities of d-Ng treated and control groups were evaluated according to Snedecor and Cochran (1967) using the unpaired two-tailed 't' test. Variance is expressed as the standard error of the mean.

4.3 RESULTS

4.3.1 Incubation Conditions for Optimal Incorporation of sn [^{14}C] Glycerol-3-Phosphate

A detailed examination of factors such as substrate concentration, temperature, time and protein concentration is illustrated in Fig 4.1 and Fig 4.2 for microsomal and mitrochrondrial fractions respectively. The arrows indicate the conditions used in the standard procedure. Enzyme activity as a function of protein concentration proved to be linear up to 0.5 mg

Figure 4.1

Glycerol phosphate acylation by rat liver microsomes as a function of time (A) and concentrations of protein (B), palmitoyl CoA (C) and glycerol phosphate (D).

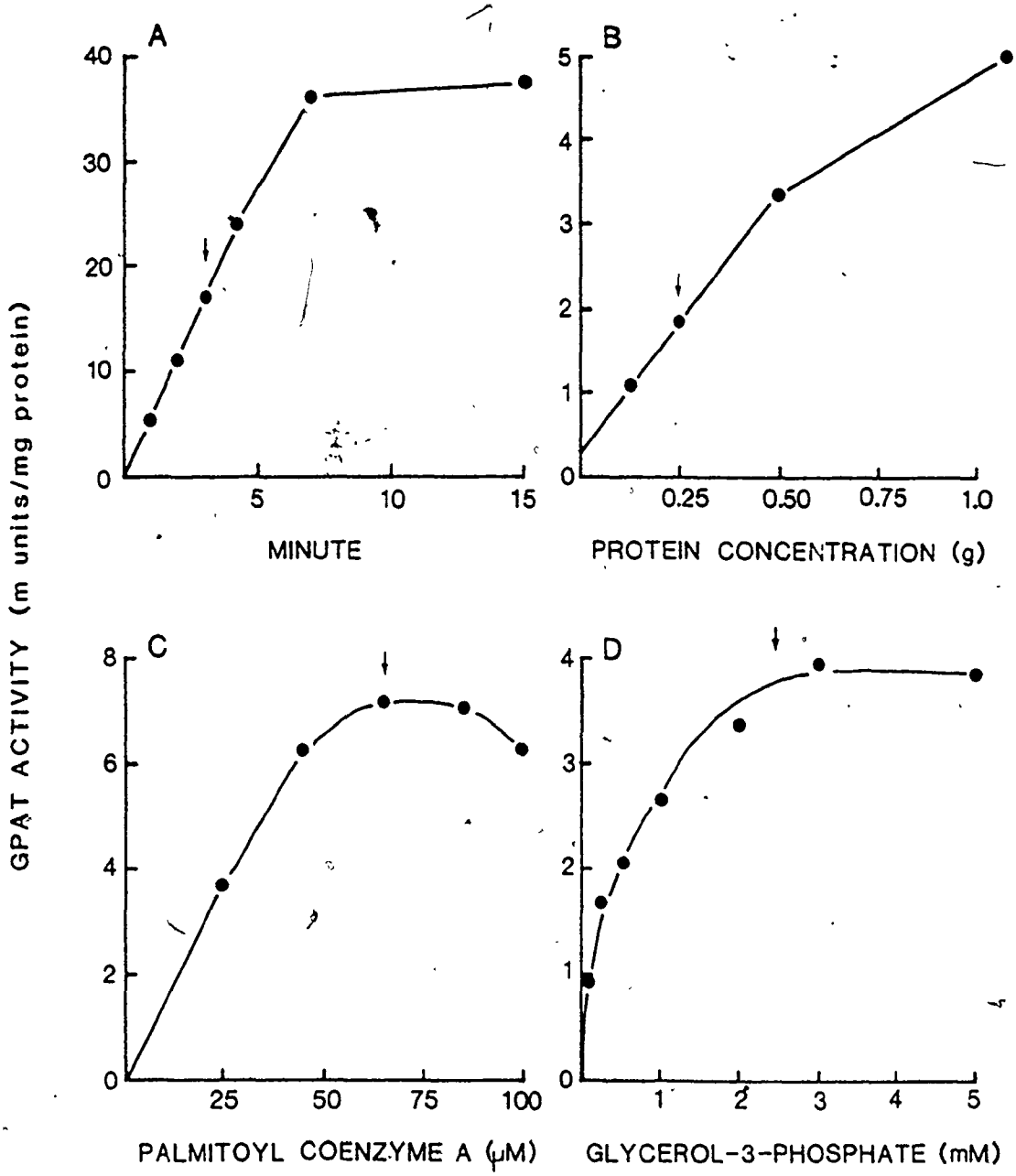
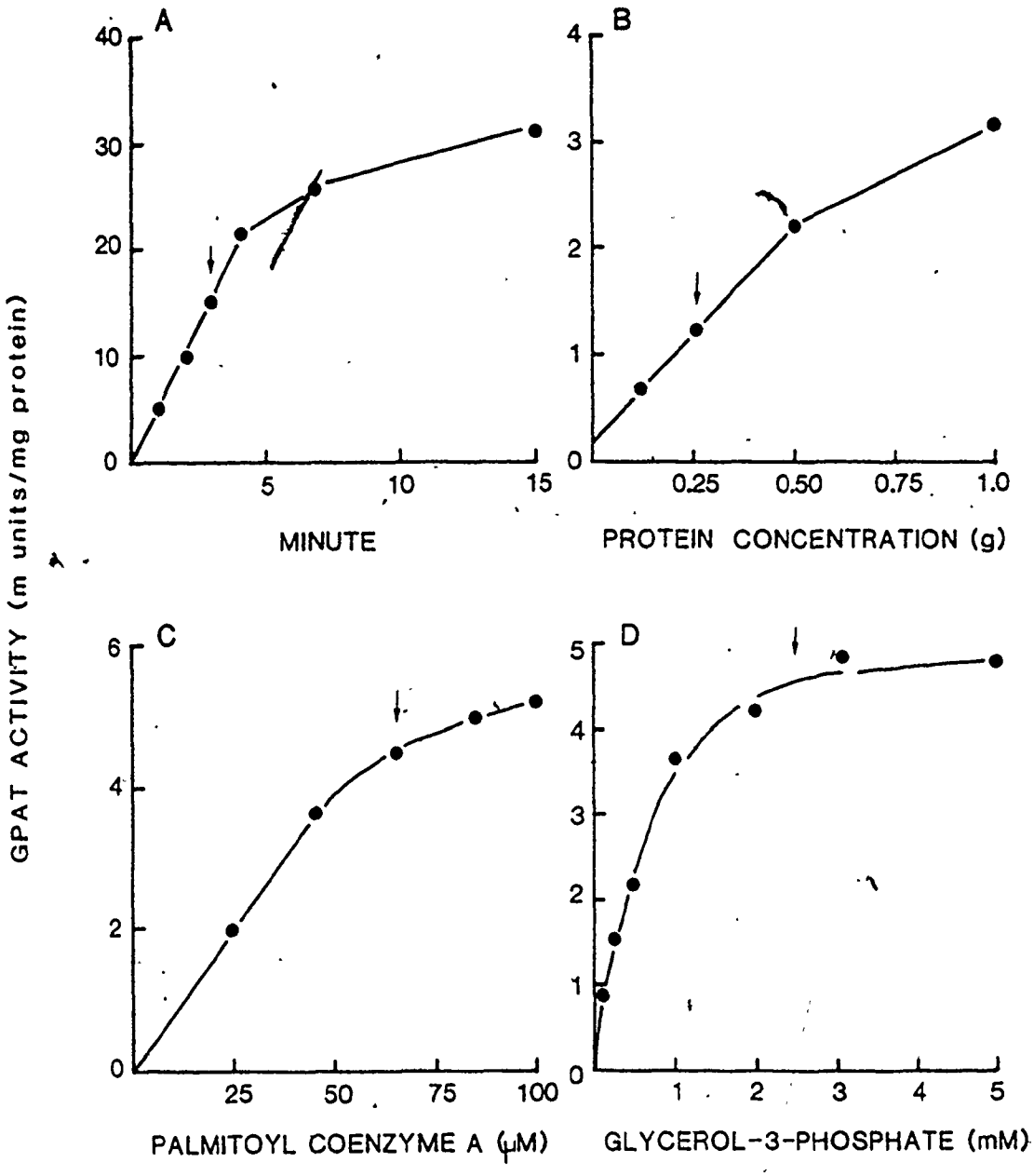

A
B
C
D
GPAT ACTIVITY (m units/mg protein)
MINUTE
PROTEIN CONCENTRATION (g)
PALMITOYL COENZYME A (μM)
GLYCEROL-3-PHOSPHATE (mM)
0
10
20
30
40
5
10
15
1
2
3
4
0.25
0.50
0.75
1.0
6
8
25
50
75
100

Figure 4.2

Glycerol phosphate acylation by rat liver mitochondria as a function time (A) and concentrations of protein (B), palmitoyl Co A (C) and glycerol phosphate (D).


A
B
C
D
GPAT ACTIVITY (m units/mg protein)
MINUTE
PROTEIN CONCENTRATION (g)
PALMITOYL COENZYME A (μM)
GLYCEROL-3-PHOSPHATE (mM)

of microsomal and mitochondrial protein. Assays performed over this protein range responded hyperbolically to increasing concentrations of glycerol-3-phosphate reaching a plateau between 1 to 2 mM. The reaction was stimulated by increasing palmitoyl coenzyme A concentration up to 65 to 85μM. For both assays, the incorporation of radioactivity into products was linear up to 4 min. The optimal temperature was observed to be 30°C.

4.3.2 Chromatographic Identification of the Reaction Products

Fig 4.3 shows the reaction products formed with the microsomal preparation. Phosphatidic acid was the main product (73%), lysophosphatidic acid constituted 18% and mono-, di- and triglycerides were 3, 4 and 3 percent respectively. With mitrochondria the chief product was lysophophatidic acid (52%) (Fig. 4.4). Other mitochondrial products included phosphatidic acid (31%) and mono-, di- and triglycerides accounted for 1, 5 and 10 percent respectively. The ratio between the reaction products formed did not differ between control and experimental groups.

4.3.3 Effect of N-Ethylmaleimide on Glycerol Phosphate Acyltransferase Activity

The sensitivity of microsomal and mitochondrial GPAT activity to N-ethylmaleimide was examined by adding it to a concentration of 2 mM to the standard assay mixture. N-Ethylmaleimide selectively inhibits the microsomal GPAT actvity (Haldar and Pullman, 1975). Results showed a distinct difference between the GPAT from the two

Figure 4.3

Glycerol phosphate acylation products formed by rat liver microsomes. The abscissa shows the sequential number of counting vials into which bands of silica acid were scraped. 1 represents the origin and 18 the solvent front.

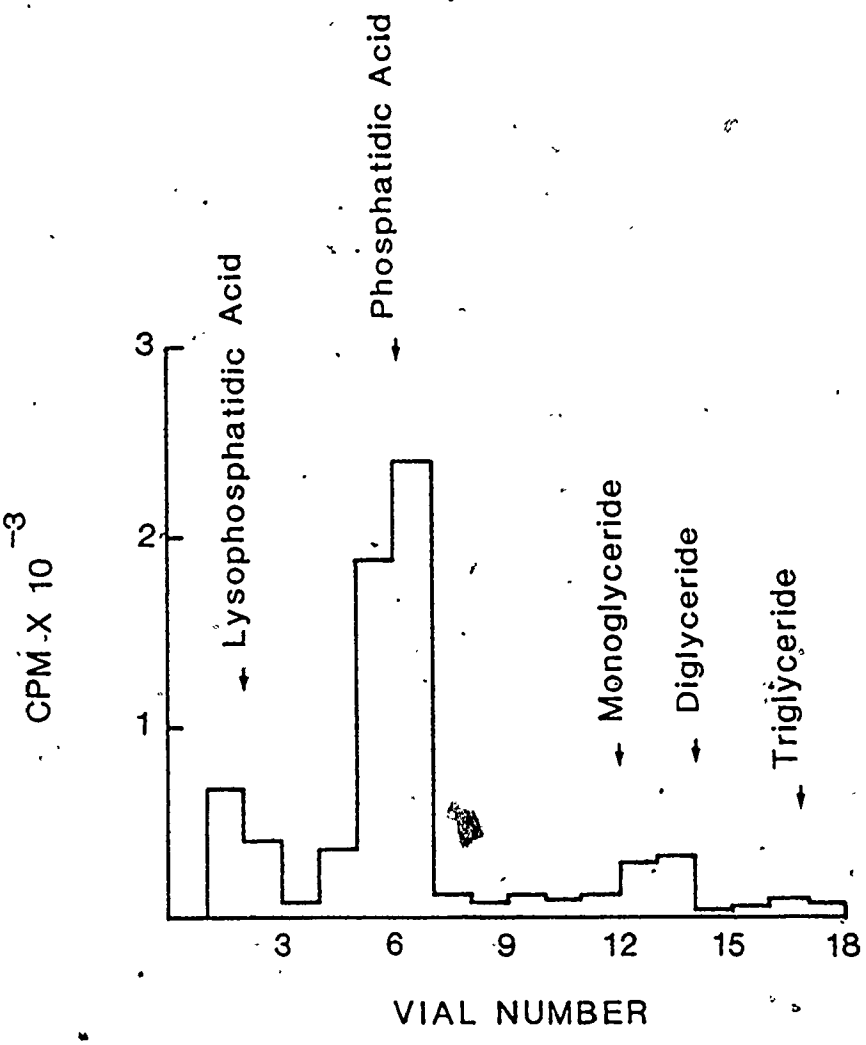

Lysophosphatidic Acid
Phosphatidic Acid
Monoglyceride
Diglyceride
Triglyceride
CPM X 10 -3
1
2
3
3
6
9
12
15
18
VIAL NUMBER

Figure 4.4

Glycerol phosphate acylation products formed by rat liver mitochondria. The abscissa shows the sequential number of counting vials into which bands of silica acid were scraped. 1 represents the origin and 18 the solvent front.

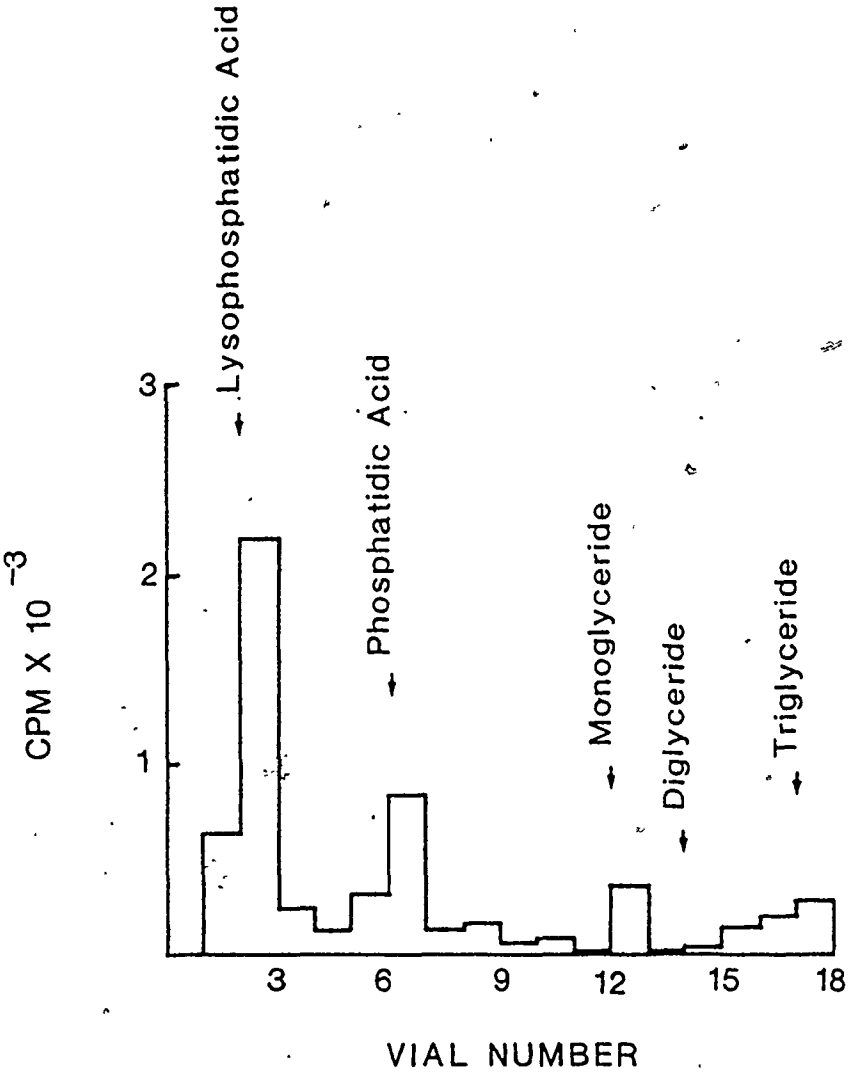
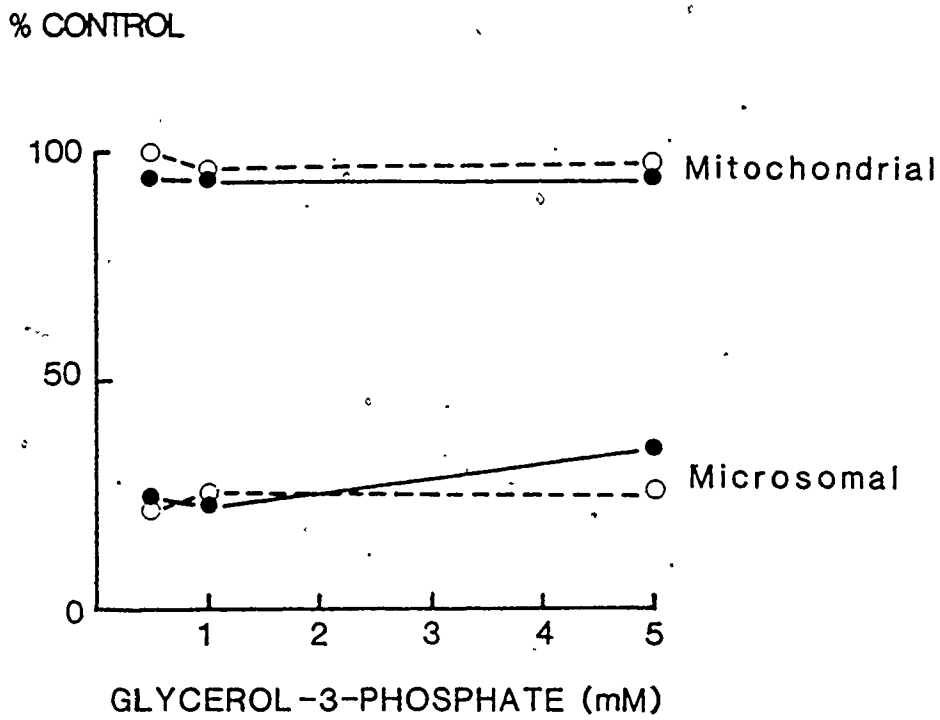

Lysophosphatidic Acid
Phosphatidic Acid
Monoglyceride
Diglyceride
Triglyceride
CPM X 10 -3
3
2
1
3
6
9
12
15
18
VIAL NUMBER

Figure 4.5

Glycerol phosphate acylation products formed by rat liver mitochondria and microsomes in control (●——●) and d-norgestrel treated rats (○---○) in the presence of 2mM N-ethylmaleimide.


% CONTROL
100
50
0
1
2
3
4
5
Mitochondrial
Microsomal
GLYCEROL-3-PHOSPHATE (mM)

fractions (Fig 4.5). Microsomal GPAT obtained was severely inhibited by more than 75% whereas mitochondrial activity was not affected by the presence of 2 mM N-ethylmaleimide. The enzymes in control and experiment groups showed a similar response towards N-ethylmaleimide sensitivities.

4.3.4 Effect of d-Norgestrel Treatment on Glycerol Phosphate Acyltransferase Activity

Table 4.1 shows that d-Ng (4µg/day. kg body $wt^{0.75}$) administered to female rats for a period of 18 days significantly decreased GPAT specific activity per mg of microsomal protein ($p<0.05$). The average inhibition was 26% as compared to control. However, mitochondrial GPAT specific activity was not affected by d-Ng treatment.

4.3.5 Effect of d-Norgestrel Treatment on Phosphatidic Acid Phosphatase Activity.

d-Ng treatment significantly reduced the PA_{aq} dependent PAPase specific activity by an average of 20% in the microsomes, whereas, the specific activity of PA_{aq}-dependent PAPase in the cytosol was increased by 19% in cytosol as compared to the control group (Table 4.2). However the increase in cytosol was not statistically significant. The PA_{mb}-dependent PAPase specific activity in microsomal as well as cytosol fractions was not affected by d-Ng treatment.

TABLE 4.1

EFFECT OF d-NORGESTREL ON

GLYCEROL PHOSPHATE ACYLTRANSFERASE SPECIFIC ACTIVITY[a]

GROUP	MICROSOMES	MITOCHONDRIA
Control (n=6)	7.0 ± 0.5	4.3 ± 0.3
Norgestrel[b] (n=6)	5.1 ± 0.3[c]	4.0 ± 0.1

[a] Values expressed as nmoles/min.mg protein (mean ± SEM)

[b] Rats treated with d-norgestrel (4 µg/kg. body $wt^{0.75}$)

[c] Significantly different from control ($p<0.05$)

TABLE 4.2

EFFECT OF d-NORGESTREL ON

PHOSPHATIDIC ACID PHOSPHATASE SPECIFIC ACTIVITY[a]

GROUP	MICROSOMES		CYTOSOL	
	PA_{aq}-dependent	PA_{mb}-dependent	PA_{aq}-dependent	PA_{mb}-dependent
Control (n=6)	9.1 ± 0.4	2.9 ± 0.13	1.1 ± 0.08	1.68 ± 0.13
Norgestrel[b] (n=6)	7.3 ± 0.31[c]	2.6 ± 0.11	1.3 ± 0.07	1.61 ± 0.10

[a] Values expressed as nmoles/min. mg protein

[b] Rats treated with d-norgestrel (4µg/day.kg body $wt^{0.75}$)

[c] Significantly different from control ($p<0.05$)

4.3.6 Purity of Subcellular Fractions

The purity of each of the isolated subcellular fractions separated is shown in Fig 4.6. The fractions were well separated and mutual contamination of the different subfractions was minimal as assessed by the activity of marker enzymes .

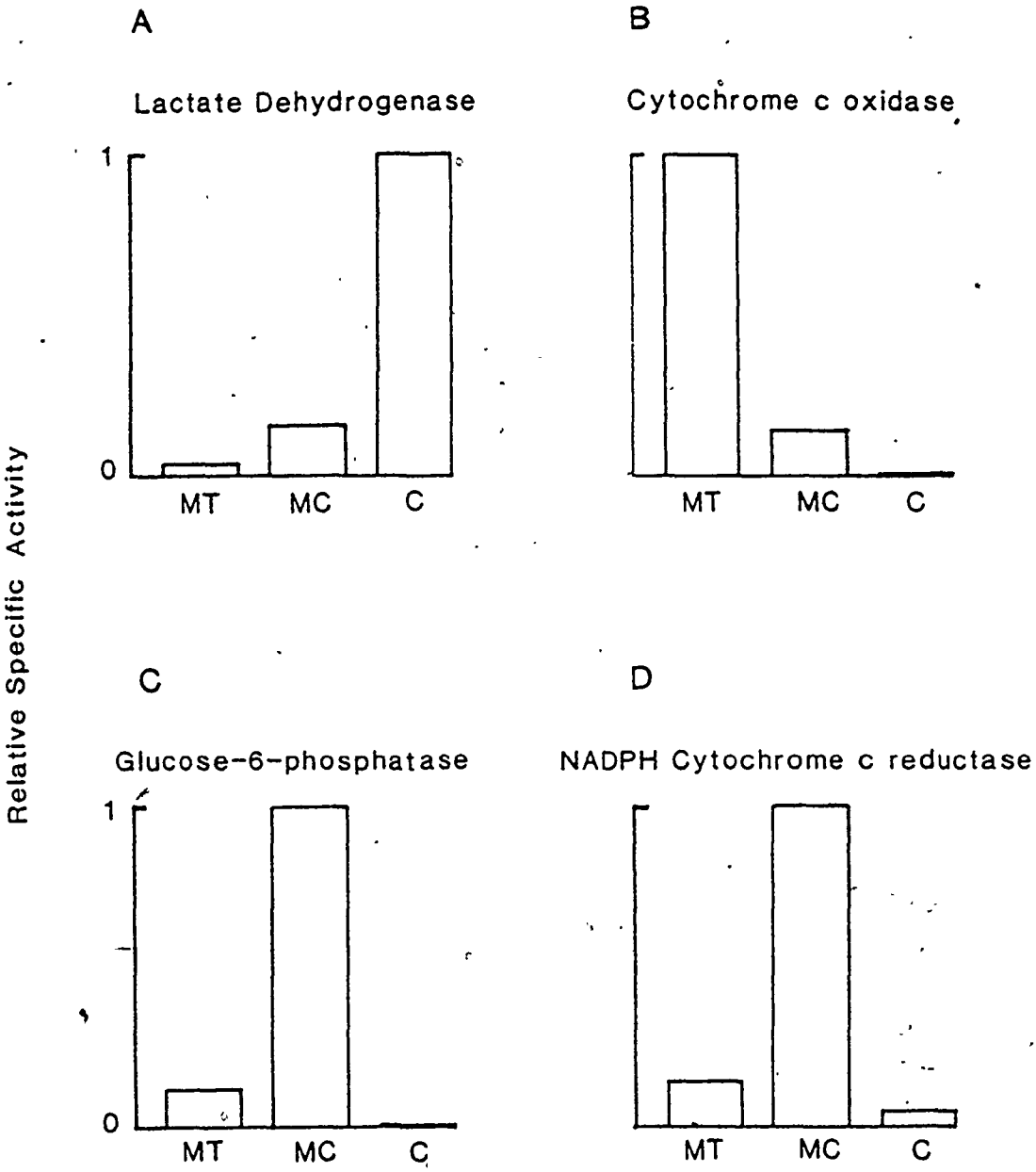
4.4 DISCUSSION

The aim of the present study was to further clarify the mode of action of d-Ng. Hepatic glycerol phosphate acyltransferase and phospatidic acid phosphatase activities were assayed under optimal incubation conditions in subcellular fractions (mitochondria, microsomes and cytosol) isolated from d-Ng treated and control rats.

The marked decrease by d-Ng in the specific activity of GPAT in liver microsomes is reported here for the first time. The effect was specific to the microsomal GPAT since the specific activity of mitochondrial GPAT was similar in the control and d-Ng treated rats (Table 4.1). The quantitative contributions of these two enzymes for cellular phosphatidic acid synthesis is not clearly defined (Bell and Coleman, 1980). The specific activity of GPAT in microsomes and mitochondria is reported to be similar in the liver, whereas in other organs the microsomal enzyme is at least 10 times more active than mitochondrial GPAT (Haldar et al, 1979). Microsomal GPAT specific activity has been shown to be increased 30-fold in mouse 3T3-L1 fibroblasts during differentiation into adipocytes (Coleman et al, 1978). Strong evidence indicates that

Figure 4.6

The marker enzymes lactate dehydrogenase (A), cytochrome 'c' oxidase (B), cytochrome 'c' reductase (C) and glucose-6-phosphatase (D) in the subcellular fractions mitochondria (MT), microsomes (MC) and cytosol (C).


A
Lactate Dehydrogenase
B
Cytochrome c oxidase
Relative Specific Activity
1
0
MT
MC
C
C
Glucose-6-phosphatase
D
NADPH Cytochrome c reductase

the biosynthesis of TG, phosphatidyl choline and phosphatidyl ethanolamine occurs assymetrically on the cytoplasmic surface of endoplasmic reticulum (Bell and Coleman, 1980), whereas, mitochondria lack the terminal enzymes for production of these lipids (van Golde et al, 1974; Jelsema and Morre, 1978). Therefore, the utilization of mitochondrially-derived intermediates would require movement to endoplasmic reticulum. Whether such movement occurs remains to be established (van Golde et al, 1974). The present data support the hypothesis that microsomal GPAT may be the priciple enzyme in glycerolipid formation.

Mitochondrial GPAT is reported to be mainly responsible for regulating the positional specificity of fatty acids seen in naturally-occuring glycerolipids (Stern et al, 1978). However, there are many reports that show a selective regulation of mitochondrial GPAT by insulin administration. Furthermore, mitochondrial GPAT is also reported to be decreased in diabetes, starvation, after adrenalectomy and after anti-insulin serum treatment shown to be associated with a decrease in triglyceride synthesis (Bates and Saggerson, et al, 1979). Microsomal GPAT activity was reported to be unaffected by any of these treatments (Bates and Saggerson, 1977a; 1977b). This differed from the present findings that d-Ng selectively inhibited microsomal GPAT specific activity (Table 4.1).

In addition to the synthesis of phosphatidic acid from glycerophosphate, dihydroxyacetone phosphate can also act as an acyl acceptor ultimately leading to the formation of phosphatidic acid.

This step is catalyzed by the enzyme dihydroxyacetone phosphate acyltransferase present in peroxisomes. The relative contribution of this enzyme to glycerolipid formation is not clearly established. Although, many in vitro studies have implicated an important role for this enzyme in glycerolipid formation (reviewed by Brindley and Sturton, 1982), peroxisomes like mitochondria lack the terminal enzymes of the glycerolipid synthesis and the physiological significance of dihydroxyacetone phosphate acyltransferase is still obscure. The present study did not attempt to examine the effect of d-Ng on this enzyme.

The main product obtained after esterification of glycerolipid by microsomal fraction was phosphatidic acid, whereas mitochondria produced mainly lysophosphatidic acid (Fig 4.3 and 4.4). These results are in agreement with those reported by Daae and Bremmer (1970) and Lloyd-Davies and Brindley (1975).

d-Ng treatment also decreased PA_{aq}-dependent PAPase specific activity in microsomal fractions (Table 4.2). The function of PA_{aq}-dependent PAPase activity is not clearly understood, although, it has been shown to be increased in rabbit lung during fetal development after induction of pulmonary maturation by glucocorticoids (Casola and Possmayer, 1981a; Rooney et al, 1979). Also in the rat and rabbit an increase in PA_{aq}-dependent PAPase activity in microsomal fractions parallels the increase in phosphatidyl choline content (Casola and Possmayer, 1981b). This observation leads one to speculate on its potential role in controlling the production of phosphatidyl choline for pulmonary surfactant.

The physiological significance of this enzyme towards TG formation is not clearly established. It is noteworthy that the specific activity of microsomal PA_{aq}-dependent PAPase is much higher than the specific activities of cytosolic PA_{aq}-dependent PAPase and PA_{mb}-dependent PAPase in the microsomes and cytosol (Table 4.2).

The specific activity of PA_{aq}-dependent PAPase was slightly (not significant) increased in the cytosol fractions in d-Ng treated rats (Table 4.2). Soluble PAPase activity has been suggested to have an important role in the glycerolipid synthesis (Whiting et al, 1978). The increase observed in the present study may not be significant because of two reasons. Firstly, an isolated increase in PA_{aq}-dependent PAPase was observed, whereas PA_{mb}-dependent PAPase remained unchanged by d-Ng treatment. Secondly, the specific activity of PA_{aq}-dependent PAPase was about eight fold higher in the microsomes compared to the cytosol (Table 4.2).

In contrast to PA_{aq}-dependent PAPase activity, PA_{mb}-dependent PAPase activities in microsomes and cytosol were unaffected by d-Ng treatment. Fallon et al (1977) have suggested that Pa_{mb}-dependent PAPase activity is more closely related to glycerolipid formation in liver and adipose tissue (Fallon et al, 1977). Cheng and Saggerson (1977a; 1977b) have shown a noradrenaline dose-dependent decrease selectively in PA_{mb}-dependent PAPase activity in adipocytes which was restored by insulin administration (1977a and 1977b). Furthermore, recent studies by (Possmayer and Walter, 1983) have demonstrated that washing microsomes and cytosol with high salt solution resulted in a

decrease in PA_{mb}-dependent PAPase activity and was accompanied by a loss of the ability to produce glycerolipids.

The above findings suggest that d-Ng acts by inhibiting specifically the lipogenic enzymes (GPAT and PA_{aq}-dependent PAPase specific activity) in the microsomes. This subsequently reduces triglyceride synthesis and secretion by liver resulting in lower plasma and VLDL triglyceride levels in d-Ng treated rats.

CHAPTER 5

EFFECTS OF d-NORGESTREL ON THE TURNOVER OF RAT VERY LOW DENSITY AND LOW DENSITY APOLIPOPROTEIN B

5.1 INTRODUCTION

The previous studies in Chapter 2 showed that d-Ng treatment results in lower plasma TG and higher plasma CHOL concentrations. The effects of d-Ng on hepatic TG synthesis, secretion and activities of enzymes GPAT and PAPase were discussed in Chapters 3 and 4 . Alternatively, d-Ng could also reduce serum triglycerides by increasing the clearance of VLDL from plasma. On the other hand, possible mechanisms underlying the cholesterol-elevating effect could include a) higher input of LDL via catabolism of VLDL (Eisenberg, 1976), b) greater direct synthesis of LDL independent of VLDL catabolism (Fidge and Poulis, 1978) and/or c) impaired LDL receptor function (Brown et al, 1981).

Studies of apolipoprotein B provide insight into lipoprotein turnover because it stays with the VLDL particle during its sequential delipidation leading to IDL and finally ending with the formation of LDL. Apolipoprotein B also gives a measure of LDL turnover as it is the major apolipoprotein of LDL and mediates the uptake of this particle by the receptors.

The present chapter describes the studies undertaken to investigate VLDL and LDL turnover by examining the apolipoprotein B kinetics of VLDL, IDL and LDL after injecting isotopically labelled

lipoproteins in d-Ng treated and control rats. Studies were initiated using lipoproteins isolated from human sources and were concluded finally using homologous lipoproteins obtained from donor rats. The influence of d-Ng on the interrelationship between different apolipoprotein B-containing lipoproteins is also discussed.

5.2 MATERIALS and METHODS

5.2.1 Materials

Potassium Iodide, Sodium Iodide, Sodium Iodate and EDTA (Ethylenediaminetetraacetic acid) were purchased from Fisher Chemical Co., Fair Lawn, NJ. $Na^{125}I$ was obtained from Amersham, Oakville, Ont., and $Na^{131}I$ was obtained from either Amersham or Frosst, Kirkland, Que. Sephadex G-50 was obtained from Pharmacia Fine Chemicals, Uppasala, Sweden. Glycine was purchased from BDH Chemicals, Toronto, Ont., Millipore Corporation, Mississauga, Ont. supplied the millipore filters. PM10 membranes were purchased from Amicon Corporation, Lexington, MA. Isopropanol was obtained from J.T. Baker Chemical Co., Phillipsburg, NJ. Lard (5%) and fat-free diets were kindly donated by Dr. K. K. Carroll, Department of Biochemistry, University of Western Ontario.

5.2.2 Preparation of Very Low Density and Low Density Lipoproteins

Lipoproteins for injection were prepared from plasma obtained either from human volunteers (12 hr fasting) or from female Sprague-Dawley donor rats (250 g, n = 44). The donor rats were maintained on a synthetic diet containing 5% lard for 10 days prior to use.

This was done to enhance the labelling of rat VLDL-protein by decreasing the polyunsaturated:saturated fatty acid ratio in the plasma lipids, leading to less incorporation of the label in the lipid moiety of the lipoproteins as suggested by Fidge and Poulis (1975). However, a fat-free diet was fed for 12 hr prior to sacrifice to eliminate chylomicrons from plasma.

Blood was collected by aortic puncture from the donor rats and pooled into tubes containing EDTA (1 mg/ml). The plasma was separated and subjected to ultracentrifugation to isolate VLDL ($d<1.006$) in a Beckman 60 Ti rotor (16 hr, 40,000 rpm, 15°C). The VLDL was washed twice through sterile saline in a 50 Ti rotor (16 hr, 40,000 rpm, 15° C). The LDL (d 1.02-1.05) was then isolated from the plasma infranatant remaining after separation of VLDL in a 60 Ti rotor by successive ultracentrifugations at densities 1.02 and 1.05 (24 hr, 40,000 rpm, 15°C) and was washed once, through sterile saline solution of d = 1.05 in a 50 Ti rotor (24 hr, 40,000 rpm, 15°C). The narrow density range (d 1.02-1.05) was chosen to obtain a LDL fraction free of any contamination by VLDL or HDL.

5.2.3 Iodination Procedure

The VLDL and LDL were radiolabelled by the method of McFarlane (1958) as modified by Fidge and Poulis (1974). Briefly, the VLDL and LDL obtained were equilibrated to 0.4 M glycine-NaOH buffer pH 10 and radioiodinated with ^{125}I and ^{131}I, respectively. Free iodine was then removed by passage through a small Sephadex G-50

column equilibrated to saline. Isotopically labelled lipoproteins were sterilized by filtration through 0.45 micron Millipore filter. The percentage of radioisotope ^{125}I bound to the VLDL protein was 93%, 4% was associated with lipid and 3% remained unbound. The ^{131}I bound to the LDL protein was 94%, less than 5% was associated with lipid and 1% remained unbound. The amount of label associated with apolipoprotein B in VLDL and LDL determined by isopropanol precipitation (Holmquist and Carlson, 1977) was 27% and 69%, respectively.

5.2.4. Lipoprotein Kinetic Study

Two different protocols were designed to study the turnover of labelled lipoproteins in d-Ng-treated and control rats. Protocol I was designed simply to assess qualitatively any effect of d-Ng on lipoprotein clearance, whereas protocol II was designed to yield more specific quantitative information from the radioactivity time curves.

5.2.4.1 Protocol I

d-Ng treated (n=4) and control (n=4) rats of Spraque-Dawley strain (0, 250 g) were prepared as described in section 2.2.2. 0.16 g KI/l was added to drinking water for two days prior to and after the administration of labelled lipoproteins.

The animals were anesthetized with ether and 8 µCi of ^{125}I-LDL in 0.25 ml saline was administered intravenously via foot

vein. At designated times thereafter (0.167, 0.33, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 18, 24, 26, 28, 30, 32, and 50 hr) 50-70 µl blood was collected in heparinized microhematocrit tubes by cutting the tip of the tail. Plasma was separated by centrifugation in a microhematocrit centrifuge for 5 min. 20 Microliters of plasma was mixed with 980 µl Kreb's Ringer bicarbonate buffer containing 5% bovine serum albumin and the radioactivity in the samples was measured for total ^{125}I activity. One ml of 10% trichloroacetic acid (TCA) was subsequently added, samples were vortexed and one ml of the supernatant was measured for the radioactivity in TCA soluble fraction. Total ^{125}I activity minus TCA-soluble activity gave the ^{125}I activity associated with protein fraction and was taken to represent intact ^{125}I-LDL.

5.2.4.2 Protocol II

Female Spraque-Dawley rats (250g), twenty-two in each group (control and experimental) were treated with d-Ng and placebo diets as described in section 2.2.2. Potassium iodide was added to the drinking water as described above.

Each recipient rat received 8 µCi of ^{125}I-VLDL alone (Study I) or in combination with 2 µCi of ^{131}I-LDL (double label study) in 0.5 ml normal saline intravenously via a foot vein. At 5 min post injection, a 50-70 µl sample of blood was collected from the tail vein into a heparinized microhematocrit tube to monitor the amount of radioactivity injected. At each subsequent time point

(0.17, 0.33, 0.5, 1, 2, 4, 6, 12, 18, 24 and 50 hr), two rats from each of the d-Ng and control groups were anesthetized with ether and 10 ml of blood was drawn by aortic puncture into tubes containing EDTA (1 mg/ml). Plasma was pooled and lipoprotein fractions VLDL (d 1.006), IDL (d 1.006-1.019) and LDL (d 1.019-1.063) were isolated at appropriate salt densities by the method of Havel et al (1956).

All lipoprotein fractions were counted and concentrated by ultrafiltration using amicon PM10 membranes. The loss of radioactivity was proportional to loss of protein during ultrafiltration The apolipoprotein B was isolated by isopropanol precipitation (Holmquist and Carlson, 1977) and its specific activity determined. Briefly, 250 µl of the lipoprotein (approximately 1 mg protein/ml) was extracted with an equal volume of isopropanol and the apolipoprotein B pelleted by centrifugation. The pellet was washed with 250 µl of isopropanol:saline (1:1, v/v). Lipids were then extracted with 1.0 ml of methanol:chloroform:diethyl ether (2:3:5, v/v/v) and finally washed with diethyl ether alone. The apolipoprotein B pellet was dried and dissolved in 0.1 N NaOH and radioassayed for ^{125}I and ^{131}I. The protein content of the sample was determined by the method of Lowry et al (1951) to determine the specific activity.

5.2.5 Calculation of Model Parameters

The LDL and apolipoptotein B specific activity time curves were plotted semilogarithemically as a function of time. The curves were

subjected to a computer programme (Provencher, 1976) for the analysis of multicomponent exponential decay data based on a nonlinear least squares method (Bard, 1974). This provided the constants of exponential rate of fall for each kinetically distinguishable pool.

5.2.5.1 Protocol I

Further calculations were based on the method described by Matthew (1957). The curves best conformed to a two pool model.

1. Fractional Catabolic Rate (FCR) = $\dfrac{1}{\frac{C_A}{\alpha} + \frac{C_B}{\beta}}$

2. Absolute Catabolic Rate (ACR) = Plasma level of Apo B X FCR

3. Half life ($t_{1/2}$) = $0.693/\alpha$ or β

where C_A = Time zero intercept of slow phase

C_B = Time zero intercept of rapid phase

α = Slope of slow phase

β = Slope of rapid phase

5.2.5.2 Protocol II

Calculations were based on the method described by Goodman and Noble for two pool model (1968) or Goodman et al (1973) for three pool model.

	Two Pool	Three Pool
1. Pool Size (M_A) =	$\frac{R}{C_A + C_B}$	$\frac{R}{C_A + C_B + C_C}$
2. FCR =	$\frac{-\alpha C_A M_A - \beta C_B M_A}{R}$	$\frac{-\alpha C_A M_A - \beta C_B M_A - \gamma C_C M_A}{R}$
3. Production rate =	$\frac{R\alpha\beta}{\alpha C_B + \beta C_A}$	$\frac{R}{\frac{C_A}{\alpha} + \frac{C_B}{\beta} + \frac{C_C}{\gamma}}$

Where R = Amount of isotope injected into pool A

C_A = Time zero intercept of slow phase

C_B = Time zero intercept of intermediate phase

C_C = Time zero intercept of rapid phase

α = Slope of slow phase

β = Slope of intermediate phase

γ = Slope of rapid phase pecific activity time

5.2.6 Lipid Analysis

Plasma lipids were extracted and TG and CHOL concentrations were assayed by methods previously described in section 2.2.4. Variations in body weight and plasma lipids were assessed using the

T-test for unpaired samples Snedecor and Cochran (1967). Variance is expressed as the standard error of the mean.

5.3. RESULTS

5.3.1 Turnover of Human ^{125}I-VLDL Apolipoprotein B in d-Norgestrel-Treated Rats

The apolipoprotein B specific activity for VLDL was plotted on a semilogarithemic scale over 6 hr following injection of human ^{125}I-VLDL (Protocol II, study I) as shown in Fig. 5.1. The VLDL-apolipoprotein B decay curve best resolved into two exponential functions. The clearance of VLDL-apolipoprotein B was enhanced in d-Ng treated rats compared to controls. The kinetics of VLDL turnover calculated from ^{125}I-VLDL-apolipoprotein B decay curve are shown in Table 5.1. The higher initial specific activity of VLDL-apolipoprotein B in d-Ng treated rats reflected the smaller pool of apolipoprotein B (29 vs 75 µg) versus controls. The FCR was markedly higher (5.88 vs 4.14 h^{-1}) and values of $t_{1/2}$ were smaller in the treated rats. However, the production rates were similar in both groups (117 vs 119 µg/hr) compared to controls.

5.3.2 Turnover of Human ^{125}I-LDL in d-Norgestrel-Treated Rats

Figure 5.2 shows the disappearance of radioactive LDL from plasma, after the injection of human ^{125}I-LDL into rats, as a function of time (Protocol I). All values were normaliz00 using the initial concentration of radioactivity in the plasma as 100%. The clearance of LDL was markedly delayed in d-Ng-treated rats versus

FIGURE 5.1

Specific radioactivity of VLDL-apolipoprotein B following intravenous injection of ^{125}I-labelled human VLDL into control (C) and d-norgestrel-treated (E) rats. Each point represents the value obtained from the mean of two experiments, each obtained from pooled plasma of two rats.

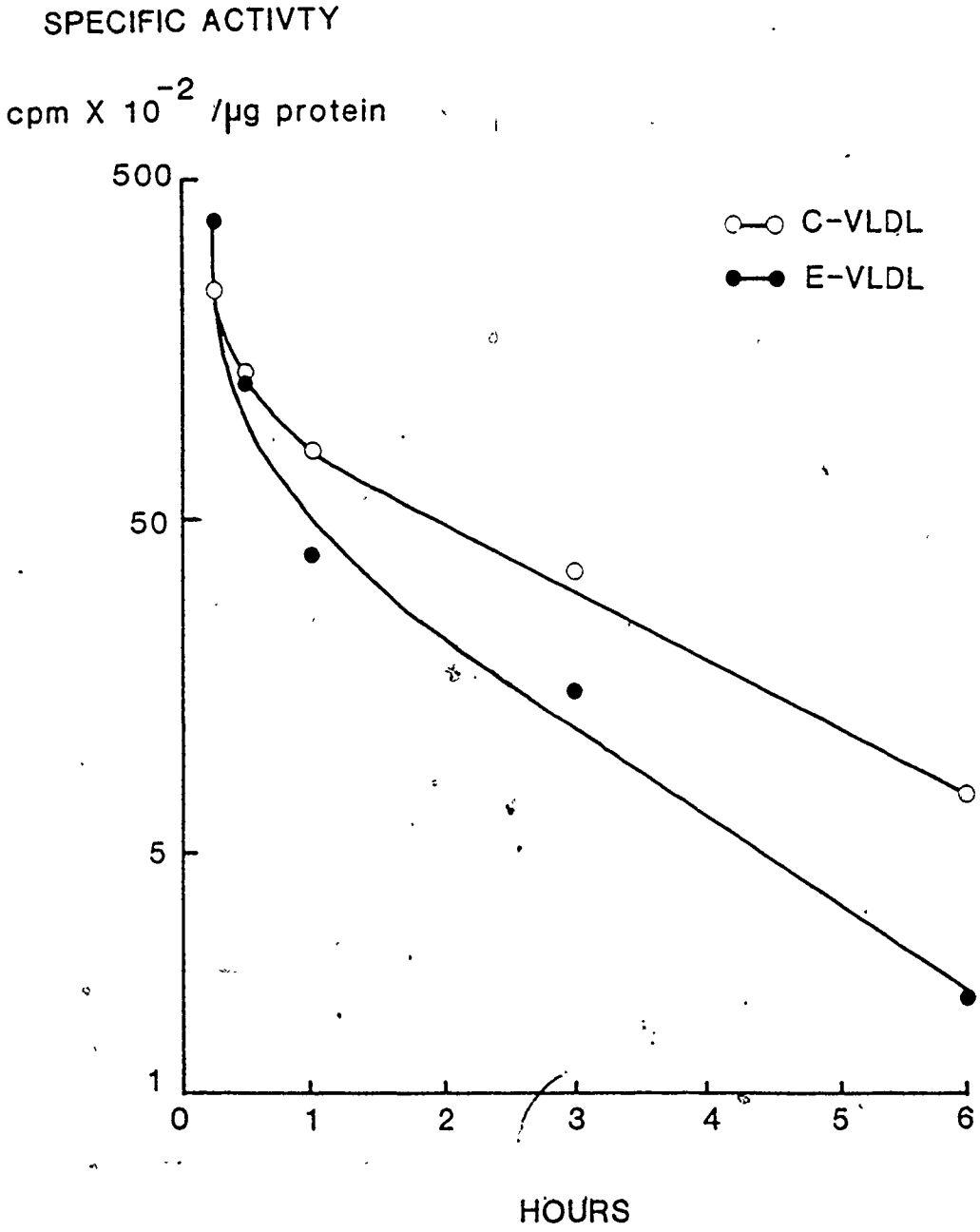

SPECIFIC ACTIVTY
cpm X 10^-2 /µg protein
500
50
5
1
C-VLDL
E-VLDL
0
1
2
3
4
5
6
HOURS

TABLE 5.1

KINETIC PARAMETERS OF
VLDL (S_f 20-400) APOLIPOPROTEIN B TURNOVER
IN d-NORGESTREL-TREATED RATS

Group	Half-Life of Phase		FCR[b]	VLDL-apo B	
	I	II		POOL SIZE	PR
	hr		h^{-1}	µg	µg/hr
Control	0.13	1.50	4.14	75	119
Norgestrel[a]	0.11	1.13	5.88	29	117

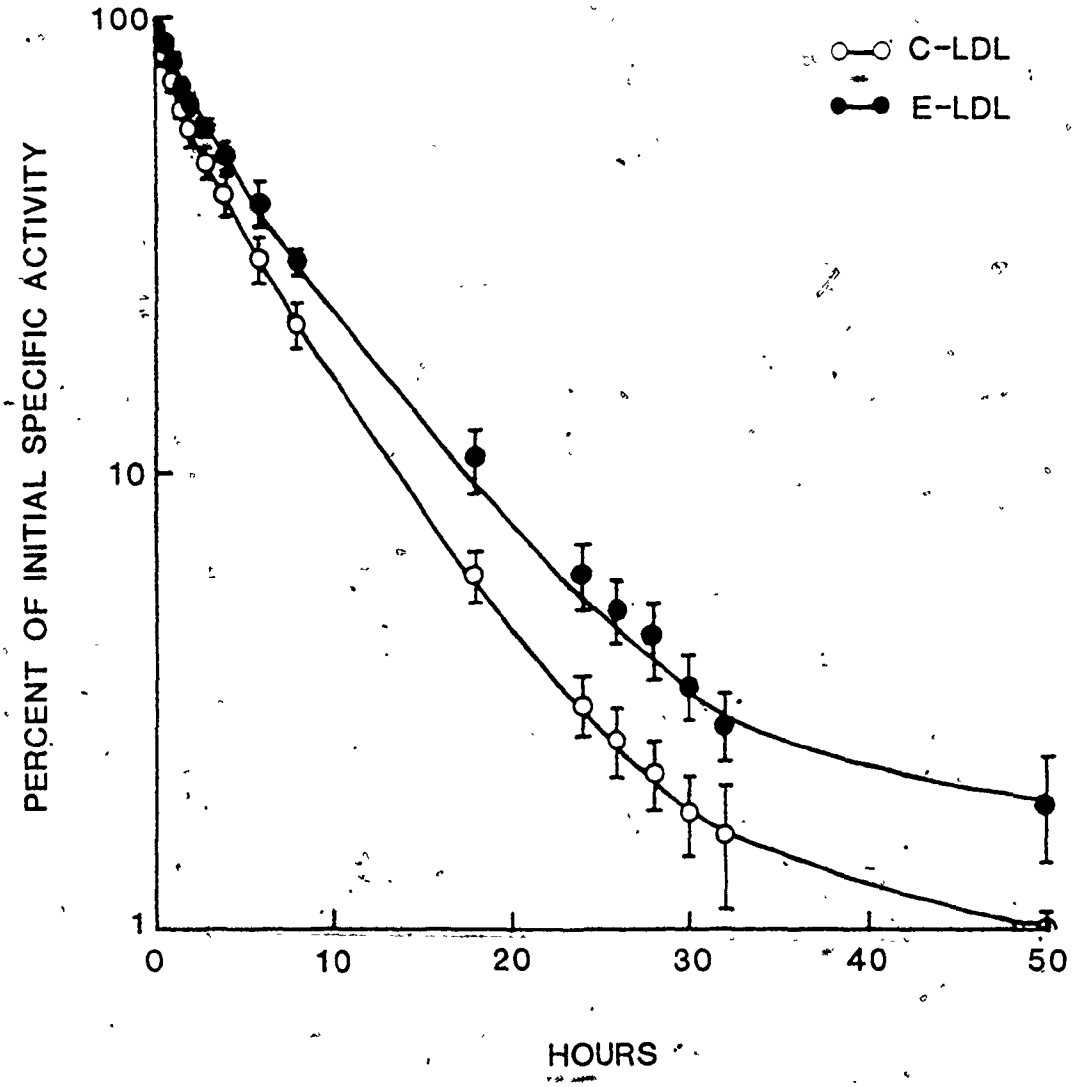
[a] Dose was 4 µg d-norgestrel/day .kg body $wt^{0.75}$ for 18 days

[b] Fractional catabolic rate

[c] Production rate

FIGURE 5.2

The plasma clearance of ^{125}I-labelled human LDL in control (C) and d-norgestrel-treated (E) rats. Each value represents the Mean ± SEM of 4 experiments.


C-LDL
E-LDL
PERCENT OF INITIAL SPECIFIC ACTIVITY
100
10
1
0
10
20
30
40
50
HOURS

controls. The plasma die-away curves of ^{125}I-LDL were multiexpontial and the decay curve best conformed to a two pool model. Table 5.2 shows the kinetic parameters of LDL clearance. FCR was significantly lower and the half-life of the slow phase significantly higher in the treated rats a compared to controls. Absolute catabolic rate was not affected by d-Ng treatment.

5.3.3 Turnover of Rat ^{125}I-VLDL Apolipoprotein B in d-Norgestrel-Treated Rats

The values for specific activity of ^{125}I apolipoprotein B in VLDL, IDL and LDL over 50 hr following injection of ^{125}I-VLDL were plotted on a semilogrithemic scale (Fig. 5.3). VLDL-apolipoprotein B clearance was enhanced in the treated versus control rats. The kinetics of VLDL turnover calculated from ^{125}I-VLDL-apolipoprotein B decay curve are shown in Table 5.3. The higher initial specific activity of VLDL-apolipoprotein B in d-Ng-treated rats as compared to controls reflected the smaller pool of apolipoprotein B. The fractional catabolic rate of VLDL-apolipoprotein B was markedly increased (4.21 vs 2.26 hr^{-1}). However, the production rate was similar in the two groups (157 vs 141 µg/hr).

5.3.4 Precursor-Product Relationship Between Different Lipoproteins

For the purpose of clarity, the values for specific activity of ^{125}I-apolipoprotein B in VLDL, IDL and LDL over 50 hr were plotted on a linear scale as a function of time on log scale to study the precursor-product relationships between the different lipoprotein

TABLE 5.2

KINETIC PARAMETERS[a] OF
^{125}I-LABELLED HUMAN LDL CLEARANCE
IN d-NORGESTREL-TREATED RATS

GROUP	Half life of phase I	Half life of phase II	FRC[c]	LDL-apo B POOL SIZE	ACR[d]
	hr		hr^{-1}	µg	µg/hr
Control	0.68±	5.4±	0.19±	950±	181±
(n=4)	0.22	0.50	0.02	30	14.5
Norgestrel[a]	0.97±	6.6±	0.14± [c]	1114± [c]	155±
(n=4)	0.30	0.7	0.01	107	21.0

[a] Values expressed as mean ± SEM

[b] Dose was 4 µg d-norgestres/day.kg body $wt^{0.75}$ for 18 days

[c] Fractional catabolic rate

[d] Absolute catabolic rate

[c] Significantly different from control ($p<0.05$)

FIGURE 5.3

Specific radioactivity of apolipoprotein B of VLDL, IDL and LDL following intravenous injection of ^{125}I-labelled rat VLDL in control (C) and d-norgestrel-treated (E) rats. Each point represents the value obtained from the pooled plasma of two rats.

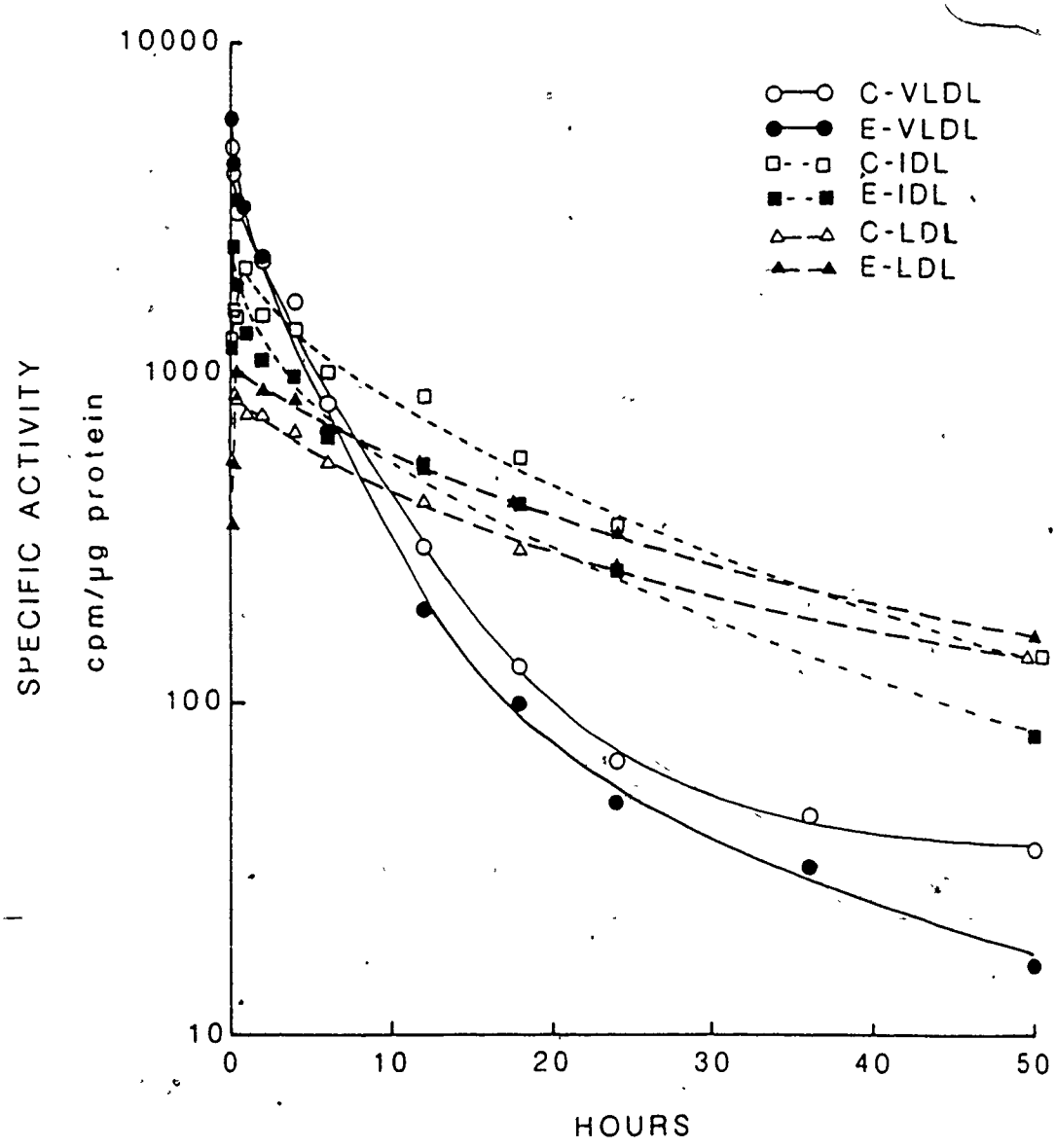

10000
1000
100
10
SPECIFIC ACTIVITY
cpm/µg protein
0
10
20
30
40
50
HOURS
C-VLDL
E-VLDL
C-IDL
E-IDL
C-LDL
E-LDL

TABLE 5.3

KINETIC PARAMETERS OF
RAT ^{125}I-VLDL-APOLIPOPROTEIN B TURNOVER
IN d-NORGESTREL-TREATED RATS

	Half-life of phase				VLDL-apo B	
Group	I	II	III	FCR[b]	Pool Size	PR[c]
		hr		h^{-1}	µg	µg/hr
Control	0.1	3.0	27	2.3	424	141
Norgestrel[a]	0.1	2.5	16	4.2	271	157

[a] Dose was 4 µg d-norgestrel/day/kg body $wt^{0.75}$ for 18 days.

[b] Fractional catabolic rate

[c] Production rate

fractions as illustrated in Fig 5.4. Faster clearance of VLDL-apolipoprotein B in d-Ng-treated rats was accompanied by a rapid rise of IDL-apolipoprotein B specific activity which peaked before the IDL-apolipoprotein B specific activity curve for controls. The IDL-apolipoprotein B specific activity curve declined faster in the treated versus control rats. In both the groups, the IDL-apolipoprotein B and LDL-apolipoprotein B specific activity time curves reached maximal values well before intersecting VLDL-apolipoprotein B curves indicating direct input of these lipoproteins independent of VLDL decay.

5.3.5 Turnover of Rat ^{131}I-LDL Apolipoprotein B in d-Norgestrel-Treated Rats

Fig. 5.5 and 5.6 show, respectively, the LDL-apolipoprotein B absolute specific activity curves and the LDL-apolipoprotein B relative specific activities expressed as percent of the highest initial value (10 min post injection) following the injection of ^{131}I-LDL in treated and control rats. Clearance of LDL-apolipoprotein B was definitely delayed in the d-Ng-treated rats compared to controls. The kinetic parameters of LDL-apolipoprotein B turnover (Table 5.4), calculated from data in Fig. 5.5, indicated a longer half-life and lower fractional catabolic rate (1.49 vs 4.71 hr^{-1}) with a markedly larger pool size (840 vs 414 µg) of apolipoprotein B in the treated versus control rats. However, the production rate of apolipoprotein B was similar in both groups (127 vs 121 µg/hr).

FIGURE 5.4

Precursor-Product relationship of different apolipoprotein B containing lipoproteins. Specific radioactivity of apolipoprotein B of VLDL, IDL and LDL following intravenous injection of ^{125}I-labelled rat VLDL into controls (C) and d-norgestrel-treated (E) rats. Each point represents the value obtained from pooled plasma of two rats.

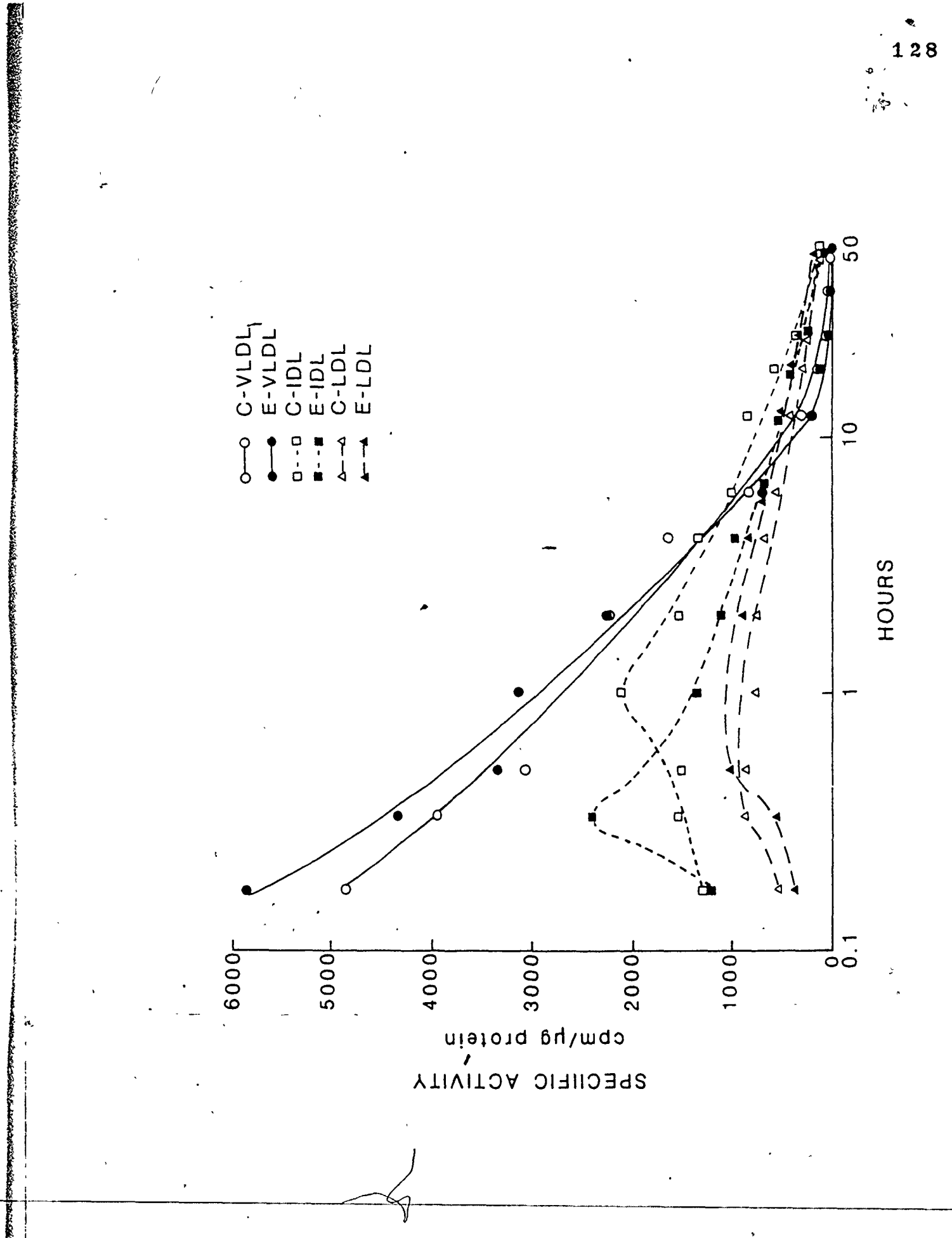

C-VLDL
E-VLDL
C-IDL
E-IDL
C-LDL
E-LDL
SPECIFIC ACTIVITY
cpm/µg protein
6000
5000
4000
3000
2000
1000
0
0.1
1
10
50
HOURS

FIGURE 5.5

Specific radioactivity of LDL-apolipoprotein B following intravenous injection of ^{131}I-labelled rat LDL into controls (C) and d-norgestrel-treated (E) rats. Each point represents the value obtained from the pooled plasma of two rats.

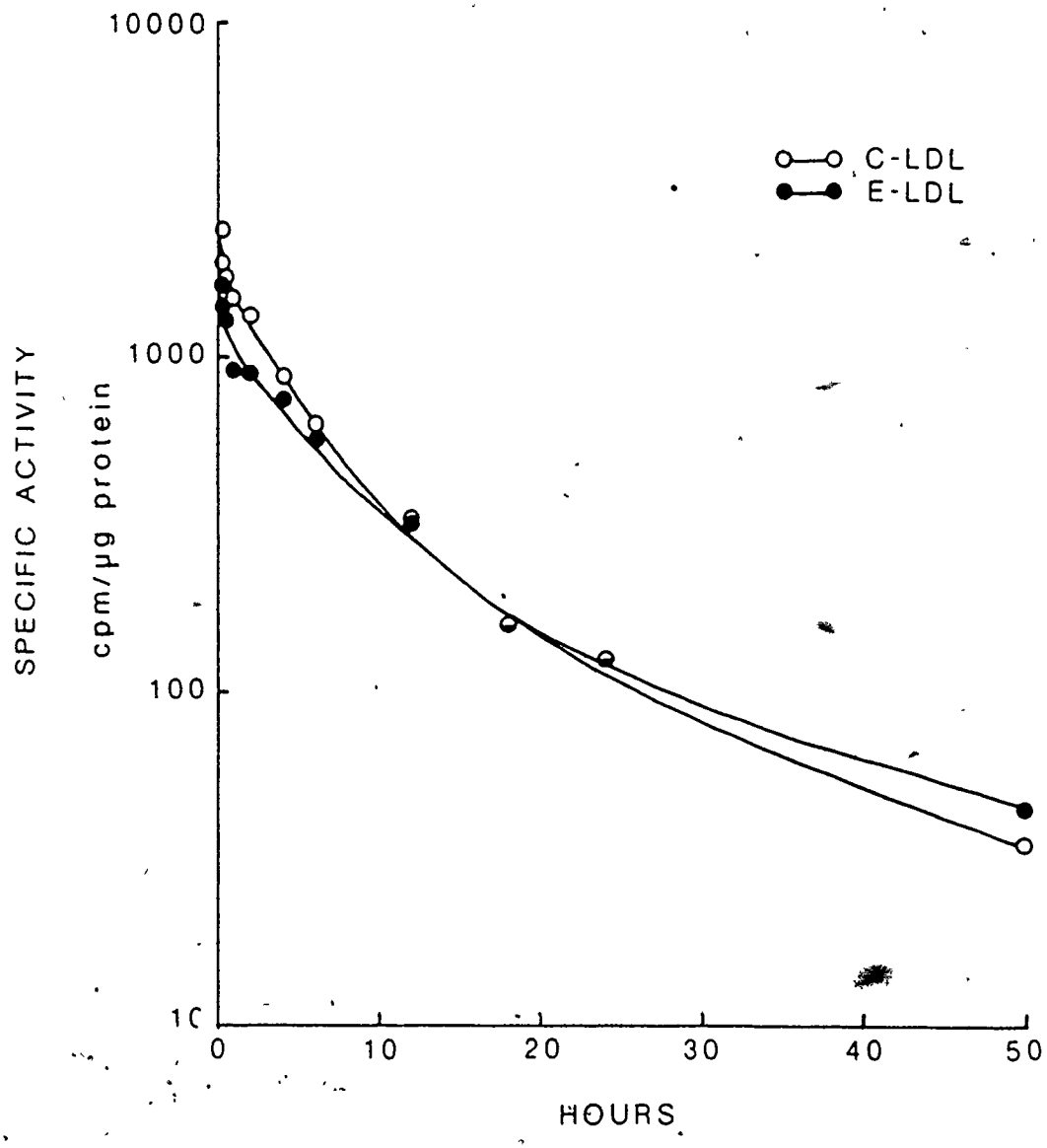

10000
1000
100
10
SPECIFIC ACTIVITY
cpm/µg protein
C-LDL
E-LDL
0
10
20
30
40
50
HOURS

FIGURE 5.6

Specific radioactivity of LDL-apolipoprotein B expressed as percent of the initial highest specific activity (10 min post injection), following intravenous injection of ^{131}I-labelled rat LDL. Each point represents the value obtained from pooled plasma of two rats.

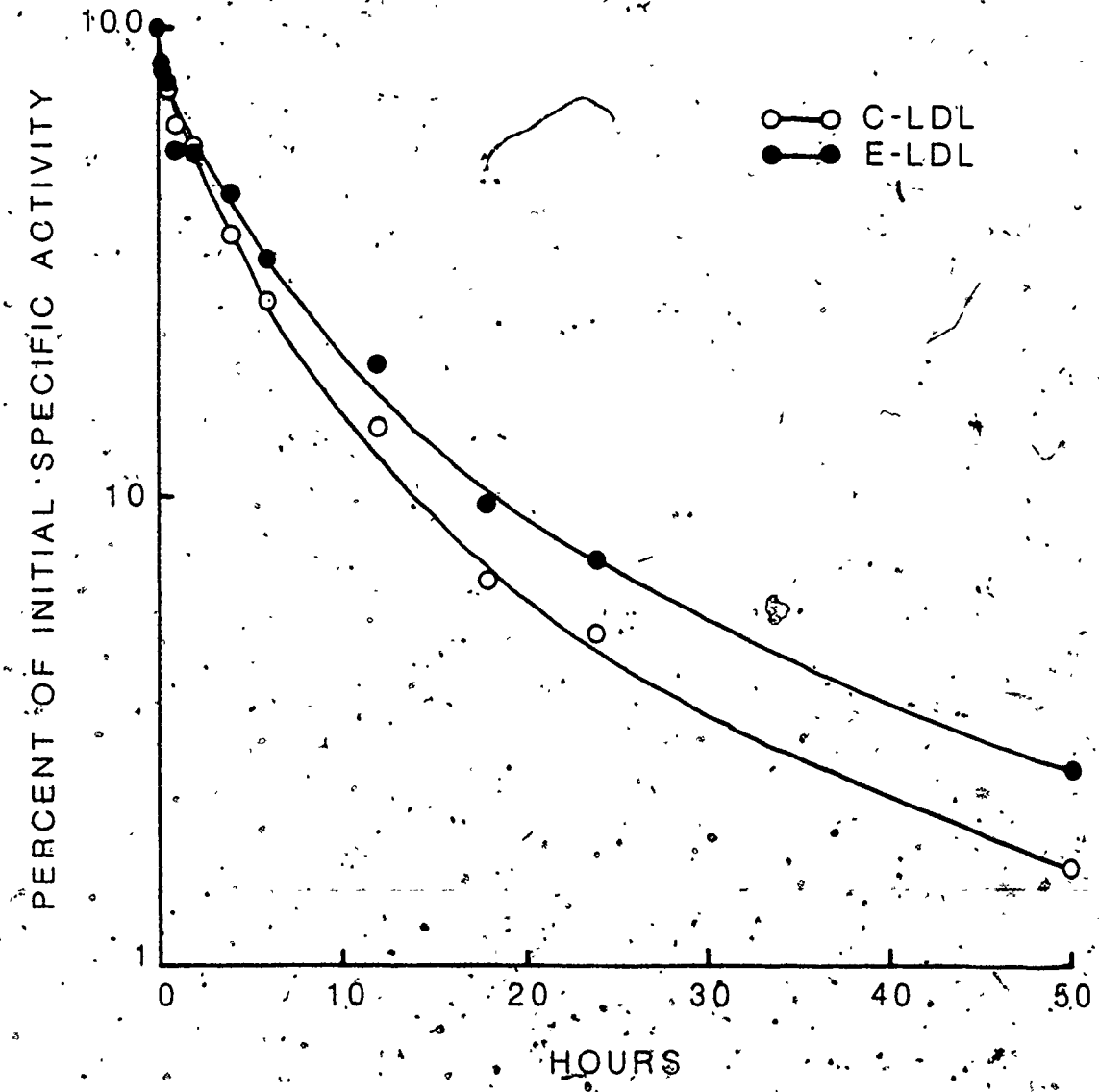

100
10
1
PERCENT OF INITIAL SPECIFIC ACTIVITY
C-LDL
E-LDL
0
10
20
30
40
50
HOURS

TABLE 5.4

KINETIC PARAMETERS OF
RAT ^{131}I-LDL-APOLIPOPROTEIN 'B' TURNOVER
IN d-NORGESTREL-TREATED RATS

	Half-life of phase			LDL-apo B		
Group	I	II	III	FCR[b]	Pool Size	PR[c]
	hr			h^{-1}	µg	µg/hr
Control	0.08	2.7	14	4.7	414	121
Norgestrel[a]	0.21	4.2	17	1.5	840	127

[a] Dose was 4 µg d-norgestrel/day/kg body $wt^{0.75}$ for 18 days.

[b] Fractional catabolic rate

[c] Production rate

5.4 DISCUSSION

The present studies provided new insight into the mechanisms underlying altered lipid levels which occur during treatment with d-Ng. The studies of lipoprotein turnover were performed with labelled lipoproteins prepared from human plasma as well as with homologous lipoproteins obtained from the same specie.

The curves obtained following administration of labelled lipoproteins were curvilinear and could be resolved into two or three single exponential conponents in each group. This has been interpreted to represent either (i) a rapid equilibration phase with one or more extravascular pools and longer phases of irreversible decay or (ii) a heterogenous population of lipoprotein particles with different catabolic rates. The specific radioactivity time curves of rat VLDL-apolipoprotein B and rat LDL-apolipoprotein B best conformed to a three pool model as it provided a significantly better fit than the two pool model (Goodman et al, 1973). Marcel et al (1978) have reported a three exponential system for LDL catabolic pathway in swine. It is noteworthy however that the interpretation of the results by two pool or three pool model did not change the ultimate observation that d-Ng affects the apolipoprotein B catabolism of VLDL and LDL.

The significantly lower mean plasma triglyceride level in the treated group versus controls (49 ± 5 vs 66 ± 7 mg/dl, $p<0.05$) was consistent with the lower VLDL-apolipoprotein B pool. The

decreased pool size was related mainly to an increased fractional catabolic rate of VLDL-apolipoprotein B, since the production rate was essentially unchanged by d-Ng (Table 5.4). The half-life of the third phase was markedly increased compared to controls, however, d-Ng did not alter the configuration of the decay curves.

In contrast to increased fractional catabolic rate of VLDL-apolipoprotein B, d-Ng treatment lowered the fractional catabolic rate of LDL-apolipoprotein B. This was reflected in the longer half-lives of all the three phases of LDL-apolipoprotein B. This consequently resulted in an increased pool size of LDL-apolipoprotein B (Table 5.4). In agreement with this finding, plasma CHOL concentration was significantly higher (64 ± 2 vs 54 ± 3 mg/dl, $p<0.05$) in treated versus control rats. The production rate of LDL-apolipoprotein B was not affected by d-Ng treatment. The primary effect of d-Ng on lipoprotein metabolism appeared to be on the apolipoprotein B catabolism in both VLDL and LDL.

The turnover of VLDL-apolipoprotein B after injection of labelled VLDL isolated from the human plasma compared well qualitatively with the results obtained following administration of labelled rat VLDL. However, the results were not comparable in absolute terms. Human VLDL-apolipoprotein B cleared much faster (approximately 1.5 to 2 fold) compared to rat VLDL-apolipoprotein B. Within 6 hours of VLDL administration only 3% human apolipoprotein B remained in the plasma compared to 17% of rat VLDL-apolipoprotein B. This suggests that human VLDL-apolipoprotein is not completely analogous to the rat VLDL-apolipoprotein B. It is

also known that rat VLDL contains apolipoprotein B-48 along with apolipoprotein B-100 and the injection of human VLDL which contains only apolipoprotein B-100 may not equilibrate with rat VLDL apolipoprotein B in a homologous manner.

The catabolism of VLDL is a stepwise process involving initially the formation of remnants which may then be either catabolized to form LDL or directly removed from circulation without the formation of LDL (Reardon et al, 1978; Barter and Nestel, 1972). This latter pathway is especially important in the rat. Eisenberg and Rachmilewitz (1973a, 1973b) have shown that in rats the major part of plasma VLDL-apolipoprotein B removed from the circulation is degraded, presumably in liver, without conversion to LDL. Although, the ultimate effects of d-Ng on VLDL receptor interaction can not de ascertained from the present studies, one possible mechanism underlying increased catabolism of VLDL and IDL could include increased uptake of these lipoproteins by hepatic receptors (Brown et al, 1981). Two distinct lipoprotein receptors, apolipoprotein E and apolipoprotein B E, are known to exist which mediate the uptake of CM remnants and LDL respectively (Mahley et al, 1981; Hui et al, 1081; Sherrill et al, 1980).

Secondly, increased enzymatic activity of lipoprotein lipase and/or hepatic triglyceride lipase, primarily responsible for the conversion of VLDL to IDL and to LDL could also account for enhanced catabolism of VLDL and IDL observed in the present experiments with d-Ng. Hepatic triglyceride lipase has been shown to be elevated by norgestrel treatment in humans (Tikkanen et al, 1981a) and depressed

by estrogen treatment (Appelbaum et al, 1977). Human hepatic triglyceride lipase has been shown to have a high affinity for IDL (Nicoll et al, 1977) and therefore may play a role in its catabolism. Murase and Itkura (1981) and Goldberg et al (1981) have suggested that blocking hepatic triglyceride lipase in rats or monkeys may impair the catabolism of IDL. However, it has been suggested that in man, lipoprotein lipase is the rate-limiting enzyme (Reardon et al, 1982).

Comparison of the specific activity time curves of VLDL, IDL and LDL (Fig 5.4) allowed an examination of the precursor-product relationship between these fractions as previously discussed by Zilversmit (1943). LDL-apolipoprotein B specific activity time curves reached maximal values well before intersecting IDL-apolipoprotein B or VLDL-apolipoprotein B decay curves indicating the presence of LDL-apolipoprotein B production independent of VLDL-apolipoprotein B catabolism. This has previously been demonstrated by Fidge and Poulis (1978). Nevertheless, the direct input of LDL into plasma did not seem to be affected by d-Ng treatment because the fraction of LDL-apolipoprotein B derived from catabolism of VLDL-apolipoprotein B (calculated from the ratio of ^{125}I-apolipoprotein B specific activity of LDL to VLDL when LDL specific activity reached maximal value, Reardon et al, 1982a) was similar in the two groups (0.25 vs 0.24). The total apolipoprotein B synthesis was also unchanged by d-Ng treatment.

The increased size of the LDL-apolipoprotein B pool in d-Ng

treated rats Table 5.4) appeared to be related to impaired removal of the LDL-apolipoprotein B because the fractional catabolic rate was decreased whereas the production rate was unchanged. A possible mechanism to account for delayed metabolic degradation includes impaired LDL degradation by receptor-dependent or receptor-independent processes in d-Ng treated rats. Sykes et al (1981) have reported decreased receptor dependent as well as receptor independent function in propylthiouracil-induced hypothyroid rats which led to increased LDL-CHOL concentrations. Large pharmacological doses of ethinyl estradiol, which drastically lower plasma LDL, have been shown to increase the number of hepatic receptors for LDL in the rat (Davis and Roheim, 1978; Windler et al, 1980). d-Ng, although fed in physiological doses could act by a converse mechanism through reducing receptor activity in d-Ng-treated rats.

d-Ng had reciprocal effects on the clearance of VLDL-apolipoprotein B and LDL-apolipoprotein B. The catabolism of VLDL-apolipoprotein B was enhanced whereas that of LDL-apolipoprotein B was delayed. This is plausible since the Watanabe heritable hyperlipidemic (WHHL) rabbit model has made clear that the receptors for apolipoprotein B-48 containing lipoproteins differ from those containing apolipoprotein B-100 (Goldstein et al, 1983). Despite the deficiency of hepatic LDL receptors in WHHL rabbits, the CM and their remnants that contain apolipoprotein B-48 are cleared rapidly and normally from the circulation. However, a selective elevation of all the apolipoprotein B-100 containing

lipoproteins (VLDL, IDL and LDL) occurs. The rat differs from rabbit as the rat VLDL also contains a substantial amount of apolipoprotein B-48 which may mediate the uptake of some of the VLDL by the chylomicron remnant (apolipoprotein E) receptors. Studies of Sparks and coworkers have demonstrated that the hepatic catabolism of a subpopulation of TG-rich lipoproteins containing B_L (B-48) is faster and differs from lipoproteins containing B_H (B-100)(Sparks and Marsh, 1981; Sparks et al, 1983). Furthermore, Cooper et al (1982) have suggested that VLDL remnants share the same hepatic removal mechanisms in the rat. On the other hand, LDL would be mainly cleared by the hepatic and extrahepatic LDL (apolipoprotein B E) receptors since LDL has no affinity for the E receptors. Therefore it could be postulated that d-Ng increased the activity or number of apolipoprotein E receptors that recognize VLDL remnants and decreased the activity or number of LDL (apolipoprotein B E) receptors.

These divergent effects of d-Ng on apolipoprotein B metabolism in VLDL, IDL and LDL explained and were consistent with the triglyceride-lowering and cholesterol-elevating effects observed during d-Ng administration.

CHAPTER 6

EPILOGUE

The objectives of the present investigation were to examine the effects of d-Ng on the lipid levels in the rat and to further our understanding of the mode of its action. This was important because only a few reports exist on the separate effects of progestin and estrogen on lipid levels of the treated subjects.

Initial experiments were designed to test the effects of d-Ng and 17 β-estradiol separately on lipid and lipoprotein levels in the rat. The results showed that d-Ng fed to female rats in low doses exhibited hypotriglyceridimic and hypercholesterolemic effects. The hypotriglycerdemic effect was attributed to a pronounced and proportional reduction of TG and PL components of the VLDL. In contrast to its triglyceride-lowering effect, d-Ng significantly elevated the plasma total and LDL-CHOL levels. Concurrently, LDL-PL also rose significantly, in proportion to the rise of LDL-CHOL.

Treatment of rats with therapeutic dose of 17 β-estradiol did not change plasma lipid or lipoprotein levels significantly. Nevertheless, a trend towards reduction of plasma total CHOL and LDL-CHOL was observed.

Experiments were undertaken to study the acute effects of d-Ng on triglyceride synthesis and secretion utilizing isolated rat hepatocyte

suspensions. The incorporation of labelled precursors, [9, 10-^{3}H] palmitate and [U-^{14}C] glycerol into hepatocyte triglycerides and triglycerides released into the medium was studied in the presence or absence of d-Ng (0.1 mM). d-Ng significantly reduced the incorporation of both precursors into hepatocytic triglycerides. The percent inhibition ranged from 19-20% in a 60 min time study. The incorporation of precursors into triglycerides released by hepatocytes (mainly in density fraction less than 1.006 corresponding to VLDL) was also reduced (51-54%) by d-Ng.

Dimethylsulfoxide was used to disperse d-norgestrel in the acqueous medium. In the concentrations employed (0.1% to 1.0%) dimethylsulfoxide had no effects of its own either on triglycerides synthesized or triglycerides released by the hepatocytes. d-Ng inhibited the TG formation in a dose-dependent manner. With increasing medium DMSO levels the soluble concentration of d-Ng increased progressively resulting in progressively greater inhibition of TG synthesis by d-Ng. It can be postulated that d-Ng-induced inhibition of hepatic TG synthesis accounts for the reduction of TG secretion and lowering of plasma VLDL-TG levels.

Studies were extended to determine the mode of action of d-Ng by examining its effects on the potential rate-limiting enzymes of the glycerolipid pathway. The activities of hepatic lipogenic enzymes, GPAT and PAPase were examined in different subcellular fractions isolated from d-Ng-treated and control rats. The specific activity of hepatic microsomal GPAT was significantly reduced by d-Ng, whereas that of mitochondrial GPAT remained unaffected.

The hepatic microsomal PA_{aq} dependent PAPase specific activity was also significantly inhibited by d-Ng treatment. On the other hand, d-Ng had no effect on the PA_{mb} dependent PAPase specific activity either in microsomes or cytosol. These results suggested that d-Ng acts by suppression of hepatic lipogenic enzymes in the microsomal fraction leading to reduced esterification of free fatty acids thereby resulting in lower triglyceride production and secretion by the liver. The interference with the assembly of TG molecule would in turn inhibit the formation of VLDL. This explained in part the lower plasma and VLDL triglyceride levels observed in d-Ng treated rats.

The second approach to study the mechanism of d-Ng action included the examination of VLDL and LDL apolipoprotein B metabolism in d-Ng-treated and untreated control rats following injection of labelled lipoprotein particles. Studies were initiated by injecting labelled lipoproteins obtained from human sources and the results suggested that clearance of VLDL-apolipoprotein B was enhanced in d-Ng-treated rats. In contrast, LDL particle clearance was significantly delayed by d-Ng treatment of the rats as compared to untreated controls.

Further studies were done with homologous lipoproteins obtained from donor rats. The results confirmed the previous observations that faster VLDL-apolipoprotein B clearance in the treated rats resulted in a smaller pool size of VLDL-apolipoprotein B since the production rates were similar in d-Ng versus control rats. In contrast to higher clearance of VLDL-apolipoprotein B, the LDL-apolipoprotein B

fractional catabolic rate was lower in d-Ng treated rats. This caused the markedly larger pool size of LDL-apolipoprotein B. The production rates of LDL-apolipoprotein B from both i) VLDL -apolipoprotein B catabolism and (ii) direct input independent of VLDL catabolism remained unaltered by the d-Ng treatment.

The primary effect of d-Ng on lipoprotein metabolism was on apolipoprotein B catabolism. d-Ng had reciprocal effects; it enhanced VLDL-apolipoprotein B clearance whereas LDL-apolipoprotein B clearance was reduced. These studies provided new insight into the mechanisms that lead to the altered lipid levels during d-Ng treatment of the rat.

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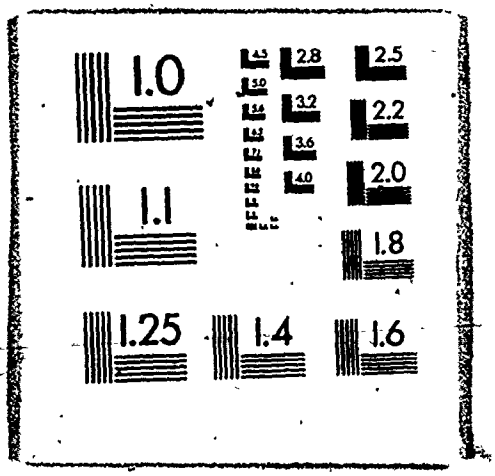

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