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EFFECTS OF AGING AND NUTRITION ON PLASMA LIPOPROTEINS IN NONHUMAN PRIMATES

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EFFECTS OF AGING AND NUTRITION ON PLASMA LIPOPROTEINS IN NONHUMAN PRIMATES

by

Karen Kay Willcox

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APPROVAL BY THESIS DIRECTOR

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Date

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ABSTRACT

Sixty-six male and female pig-tailed macaques (Macaca nemestrina) were divided into three age groups and fed either a control diet or a high fat (40% of kcalories), high cholesterol (0.24 mg/kcal) diet. After three years, plasma lipid, protein and apolipoprotein concentrations of very low (VLDL), low (LDL) and high (HDL) density lipoproteins were determined. LDL cholesterol, protein and apoprotein B-100 levels were significantly elevated by the test diet. These elevations were confined to the young and adult groups, with little or no increases observed in the oldest animals. All test animals exhibited small but significant increases in HDL cholesterol, protein and apoprotein A-I levels. However, young males had lower HDL cholesterol and protein levels than young females, whereas this trend was not apparent in the adult group. M. nemestrina appears to be a suitable model for studying how sex, aging and nutrition interact to influence lipoprotein metabolism.

INTRODUCTION

Over 50% of all deaths in the United States each year can be attributed to atherosclerosis (Mahley 1981). Atherosclerosis is a disease process whereby lipid-laden plaques form on the interior arterial wall, eventually becoming so thick that blood flow is occluded (Ross 1979). Although a combination of genetic and environmental factors play a role in the development of atherosclerosis, compelling evidence obtained from animal studies as well as epidemiological surveys has revealed a positive correlation between plasma low density lipoprotein (LDL) concentrations and accelerated atherosclerosis, whereas the relationship with high density lipoproteins (HDL) is indirect (Gordon et al. 1977).

Factors such as diet, age and sex appear to play significant roles in determining relative concentrations of plasma lipoproteins. In humans, consumption of a high-cholesterol diet can elevate LDL cholesterol levels, presumably by triggering a cellular response which down-regulates LDL receptor synthesis so that excessive cholesterol accumulation is prevented (Goldstein and Brown 1982). Human epidemiological surveys have also indicated that LDL cholesterol levels are influenced by age, with levels increasing fairly linearly with increasing age (Miller (1984). This elevation may also result from modification of the LDL receptor. Studies with canine hepatic membranes and cultured human fibroblasts have revealed that LDL receptor activity

decreases with increasing age (Hui et al. 1981; Mahley et al. 1981; Lee et al. 1982). Sexual differences in lipoprotein profiles have also been well-documented. Females tend to have higher HDL levels than males (Nichols 1967; Castelli et al. 1977) and are more resistant than males to developing atherosclerosis (Anderson 1978).

Although many laboratory animals have been used in atherosclerosis research, nonhuman primates are the preferred animal model due to their close phylogenetic relationship to man (Blaton and Peeters 1976). The Macaque species in particular have been used extensively in this area because their lipoprotein metabolism is similar to that seen in man, and also because they develop naturally-occurring atherosclerotic lesions which are comparable to those observed in humans (Lapin et al. 1979). However, many studies of lipoprotein distribution in nonhuman primates have employed either standard laboratory diets (Lacko and Hazzard 1979) or diets with excessively high levels of cholesterol (Blaton and Peeters 1979). In order to extrapolate nonhuman primate data to humans, the animals should be fed a diet which closely resembles the human dietary situation.

Basically, the design of this project was to study the influence of sex, aging and nutrition on lipoprotein metabolism in the pig-tailed macaque, Macaca nemestrina. The dietary regimen employed resembles the typical American diet in fat, cholesterol, sodium and simple sugar content. Each age group was almost equally divided by sex and represented young, middle-aged and elderly animals. Data generated from this study will provide information not only on the roles of sex,

aging and nutrition on lipoprotein metabolism in M. nemestrina, but also on the suitability of this species as a nonhuman primate model for atherosclerosis research.

LITERATURE REVIEW

Lipoprotein Metabolism

Plasma lipoproteins are protein-lipid complexes that function to transport cholesterol, triglycerides and phospholipids through the blood to various tissues of the body. Lipoproteins can be separated from each other on the basis of their size, density or net surface charge into five major classes: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Although these classes are relatively discrete, all share a similar structure consisting of a non-polar lipid core surrounded by a polar monolayer of phospholipids and apolipoproteins (apoproteins) which serves to solubize the particle for transport (Havel et al. 1980).

There are separate pathways for the transport of exogenous and endogenous cholesterol. Exogenous cholesterol and fats are packaged into lipoproteins known as chylomicrons. They are synthesized by the intestine to transport dietary cholesterol and triglyceride from the mucosal surface to various cells of the body. Chylomicrons are therefore normally present only in the postprandial state (Mahley and Innerarity 1983). Upon entry into the plasma compartment, the triglyceride-rich chylomicron cores are hydrolyzed by lipoprotein lipases located on the surfaces of capillary endothelia. This hydrolysis releases monoglycerides and free fatty acids and results in the

formation of triglyceride-poor chylomicron remnant particles which are rapidly cleared from plasma by the liver.

Endogenous cholesterol transport begins with the formation of very low density lipoproteins (VLDL) by the liver. Like the chylomicrons, VLDL are triglyceride-rich particles containing small amounts of cholesteryl ester. However, VLDL are synthesized and secreted by the liver to redistribute lipids to extrahepatic tissues. The major surface apoproteins are apoprotein B-100 (apo B-100), apoprotein E (apo E), and apoprotein C (apo C). The catabolism of VLDL proceeds in a manner similar to that of chylomicrons. Endothelial lipoprotein lipase in the capillary beds of muscle and adipose tissue releases monoglycerides and fatty acids from the VLDL cores. During this hydrolysis, apo C leaves the VLDL and the resulting triglyceride-poor particles are converted to intermediate density lipoproteins (IDL). A portion of the IDL is rapidly cleared by the liver, with the remainder being depleted of residual triglycerides to leave cores of nearly pure cholesteryl ester. During the depletion stage apo-E dissociates from the particle. The cholesterol-enriched macromolecules that remain contain apo B-100 as their surface protein and are known as low-density lipoproteins (LDL), which are the major cholesterol-transporting lipoproteins. Approximately two-thirds of the LDL are metabolized after binding to specific receptors located on the liver and peripheral tissues. Alternate scavenger pathways exist for catabolism of the LDL that is not taken up by receptors (Goldstein et al. 1983).

High density lipoproteins (HDL) appear to be synthesized by both the liver and the intestine (Tall and Small 1978). In rats, it is hypothesized that the liver secretes discoidal, nascent HDL which contains mainly phospholipids and apoproteins E and A-I. This nascent HDL picks up free cholesterol from the surfaces of cell membranes and triglyceride-rich particles. Through the action of the enzyme lecithin-cholesterol acyltransferase (LCAT) the free cholesterol is esterified, enters the core and transforms the disc into mature, spherical HDL which is rich in cholesteryl ester (Glomset 1980). It is postulated that HDL is involved in reverse cholesterol transport, a process whereby the cholesteryl ester in HDL can either be taken up by the liver for excretion, or transferred to lipoprotein remnants which also deliver cholesterol to the liver (Mahley and Innerarity 1983).

Lipoprotein metabolism is regulated primarily through the action of lipoprotein receptors. These receptors are located in special regions of the cell membrane called coated pits; once the lipoprotein is bound, the coated pits have the ability to invaginate and form vesicles which ultimately fuse with lysosomes. Lysosomal enzymes then degrade the surface proteins into free amino acids and the cholesteryl ester core into free cholesterol which can be used by the cell for processes such as membrane synthesis or steroid hormone production. This type of clearance has been termed receptor-mediated endocytosis (Goldstein and Brown 1982).

There appears to be two different hepatic lipoprotein receptors which are under independent regulation. The first is the LDL receptor system which regulates endogenous cholesterol homeostasis. The LDL receptor protein has an apparent molecular weight of 160,000 and is localized in the liver, fibroblasts, smooth muscle, adrenal cortex, testes and ovaries (Mahley and Innerarity 1983). The LDL receptor recognizes two distinct apoproteins: apo B-100, the major surface protein in LDL, and also apo E which is a constituent of chylomicrons, VLDL, IDL and certain types of HDL. Approximately two-thirds of circulating LDL is cleared by this receptor, with the remainder being degraded through receptor-independent "scavenger" pathways of the reticuloendothelial system. Lipoproteins such as IDL that contain both apo B-100 and apo E have a higher affinity for the LDL receptor, suggesting that apo E is the preferential ligand (Brown and Goldstein 1983).

However, not all lipoproteins with apo E bind to the LDL receptor. For example, triglyceride-rich chylomicrons and VLDL both contain apo E but are unable to bind to this receptor. This inability has been attributed to the presence of the C-apoproteins, which function to activate lipoprotein lipase as well as to mask the receptor binding site on apo E (Brown and Goldstein 1983). Although chylomicron remnants can bind to the LDL receptor, most of these particles are cleared through a second receptor system which does not recognize apo B-100. Studies on the binding specificities of this remnant receptor (apo E receptor) have revealed that apo E alone mediates hepatic uptake of

remnants (Mahley et al. 1981). It is believed that apo B-100 somehow modifies the apo E binding site since lipoproteins such as IDL that contain both proteins are not cleared by this receptor (Brown and Goldstein 1983).

The LDL and apo E receptors are under separate mechanisms of regulation. Synthesis of the LDL receptor is directly controlled by the cholesterol level in the cell. Whenever cholesterol needs are met, LDL receptor synthesis is downregulated. Conversely, an increased demand for cholesterol results in a rise in the number of receptors produced (Brown and Goldstein 1983). The mechanism whereby apo E receptors are regulated is less well-defined. Hui et al. (1981) performed binding studies with both types of receptors in dogs and found apo E receptor activity to be unchanged by metabolic conditions that regulate the LDL receptor; plasma levels of chylomicron remnants remained constant even under situations which enhanced or suppressed LDL receptor synthesis.

Lipoproteins and Atherosclerosis

Over one-half of all deaths in the United States each year can be attributed to atherosclerosis. In approximately 60% of these cases, death results from occlusion of the coronary arteries which leads to myocardial infarction (Mahley 1981). Atherosclerosis begins with an injury to the endothelial lining of the arterial wall. Platelets and macrophages migrate and adhere to the injured area, releasing substances which cause proliferation of the subendothelial smooth muscle and

connective tissue layers. Atherosclerotic plaques form when plasma constituents such as LDL enter the arterial wall and deposit cholesteryl ester into and around the lesion. Eventually the plaque-induced thickening of the arterial wall increases until blood flow is completely occluded (Ross 1979).

No single factor appears to cause atherosclerosis; rather, results from the population surveys indicate that a combination of genetic and environmental risk factors can predispose certain individuals to develop the disease. The most well-defined risk factors include hyperlipidemia, hypertension, cigarette smoking and diabetes mellitus. The risk factors appear to be additive in that patients who exhibit more than one are prone to developing heart disease with greater frequency (Kannel et al. 1971).

Although the composition of the atherosclerotic plaque is very complex, compelling evidence has shown that cholesterol deposition is chiefly responsible for the virulence of the lesion (Walton 1975). This evidence has been compiled through animal studies as well as through human population surveys. Wissler (1973) reported that full-blown atherosclerosis leading to myocardial infarction can be produced in nearly every type of lab animal simply by feeding a diet which elevates plasma cholesterol levels. Using monkeys as a model, Ross and Harker (1976) observed that a mechanically-induced injury to the arterial endothelium resulted in atherosclerotic plaques only when the animals were consuming a cholesterol-enriched diet. Conversely, these researchers noted a regression and eventual disappearance of the injury in animals fed a low-cholesterol diet.

The results from animal studies seem to be applicable to humans. Kannel et al. (1971) found a positive correlation between an individual's plasma cholesterol level and his risk for developing coronary heart disease. In countries such as Japan where mean plasma cholesterol is typically under 175 mg/dl, the death rate from coronary heart disease is ten times lower than that in the United States where mean values are reported to be 225 mg/dl (Connor and Connor 1972).

Elevated plasma cholesterol levels seem to play a prominent role in promoting the formation of atherosclerotic plaques. However, the way in which cholesterol is transported and by which type of lipoprotein may be more important than the absolute level of total plasma cholesterol (Mahley 1981). Lipoproteins are a metabolically diverse group of macromolecules that may have entirely opposite effects on whether cholesterol is deposited into the arterial wall. In a study using rhesus monkeys, Armstrong et al. (1974) fed the animals either a cholesterol-free diet or a diet supplemented with low levels of cholesterol for 18 months. At the end of the test period, total plasma cholesterol levels between the two groups were not significantly different. However, examination of the arterial walls revealed that the cholesterol group had much more severe atherosclerosis than the control group, although plasma cholesterol levels were identical.

Apparently cholesterol-enriched diets alter lipoprotein metabolism in a way that enhances the formation of atherosclerotic plaques. Mahley (1978) examined the effects of cholesterol feeding on lipoprotein levels in swine and monkeys. Both species exhibited similar changes

in lipoprotein profiles that were characterized by elevations in LDL, reductions in typical HDL, and the appearance of a larger, less dense form of HDL referred to as HDL_c (Mahley 1978). The subscript c indicates that the lipoprotein is cholesterol-induced; it is thought to arise from typical HDL which picks up cholesterol from peripheral tissues and delivers it to the liver for excretion. The presence of apo E on the HDL_c allows for hepatic recognition and uptake (Mahley 1981). Similar changes in lipoproteins were noted in humans fed four to six eggs per day for a month. While typical HDL decreased, LDL and HDL_c levels were elevated by the cholesterol-enriched diet (Mahley et al. 1978).

Of these three observed changes, elevation in LDL appears to play the most important role in the development of atherosclerosis. Immunofluorescent studies of atherosclerotic plaques have revealed that the cholesteryl-ester deposition is largely LDL-derived (Anderson 1979). Additionally, epidemiological studies have shown a strong positive correlation between LDL levels and risk of coronary heart disease, whereas the relationship with HDL is indirect (Gordon et al. 1977; Kannel et al. 1979; Knuiman et al. 1982). Additional evidence implicating elevated LDL in atherogenesis has come from the study of the human genetic disorder familial hypercholesterolemia. Individuals with this disease are incapable of synthesizing normal LDL receptors due to a mutation in the LDL receptor gene. As a result, plasma LDL levels in homozygotes are six to eight times higher than normal and fulminant atherosclerosis leading to myocardial infarction commonly

occurs in early childhood. The absence of other risk factors in these individuals has led to the hypothesis that their coronary heart disease results directly from elevated LDL levels (Goldstein and Brown 1982).

Factors Which Affect Lipoprotein Metabolism

Diet

Dietary cholesterol has been shown to increase total plasma cholesterol levels, with the elevation confined primarily to the LDL fraction in animals such as swine, monkeys and humans (Mahley 1978; Mahley et al. 1978). This rise in plasma LDL can be attributed to the stringent regulation of cholesterol homeostasis observed at the cellular level. When cholesterol-carrying lipoproteins such as LDL are taken up by the cell and enzymatically hydrolyzed, the free cholesterol generated enters the cytoplasm and elicits three major intracellular regulatory responses. The first is activation of the enzyme acyl CoA-cholesterol transferase (ACAT), which functions to esterify free cholesterol for storage inside the cell. The second is suppression of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), which catalyzes the formation of mevalonate from the six-carbon intermediate HMG-CoA. Since this reaction is the rate-limiting step in cholesterol biosynthesis, endogenous cholesterol production is decreased. The third and most significant cellular response to free cholesterol is the downregulation of LDL receptor synthesis to prevent excessive intracellular cholesterol accumulation (Brown and Goldstein 1978). This feedback response directly affects plasma LDL levels. Reduced receptor

synthesis results in decreased cellular uptake and degradation of both IDL and LDL. A greater proportion of IDL is converted to LDL, which in turn is catabolized at a decreased rate. As a result plasma LDL levels rise (Goldstein and Brown 1982).

Although expression of the LDL receptor is influenced by many factors, studies have shown that a high-cholesterol diet reduces receptor synthesis, presumably by saturating tissues with cholesterol so that downregulation occurs (Brown and Goldstein 1983). Mahley et al. (1981) compared binding of ^{125}I-labeled LDL to hepatic membranes of dogs fed either a control diet or a diet supplemented with 5% cholesterol for one month. While control animals bound measurable amounts of ^{125}I-LDL, receptor binding in the cholesterol-fed animals was completely absent. Kovanen et al. (1981) reported similar findings for rabbits fed a 2% cholesterol diet. After a month of treatment, uptake of labeled lipoprotein by hepatic membranes fell by 95% with the number of LDL receptors decreasing by 60%. Receptor suppression induced by cholesterol-feeding also occurs in humans. Mistry et al. (1981) examined ^{125}I-LDL binding to lymphocytes isolated from healthy adults receiving 1500 mg cholesterol daily in the form of egg yolk. A two-week treatment period produced a 74% reduction in LDL uptake by lymphocytes as compared to baseline values.

In addition to direct experimentation, epidemiological studies support the evidence that a high cholesterol intake results in elevated LDL levels and accelerated atherosclerosis. Connor and Connor (1972) compared daily cholesterol consumption to deaths resulting from

atherosclerosis and heart disease in 25 countries. In populations such as the United States and Canada, where cholesterol intake averaged 600 mg/day, the death rate approached 700 per 100,000 males aged 55 to 59. Conversely, the average intake in Japan was less than 150 mg/day and the death rate from coronary heart disease was only 125 per 100,000. In a similar study Keys (1975) found a positive correlation between cholesterol intake and the incidence of myocardial infarction in 40 to 59-year old men from seven countries.

Population surveys have also revealed a lower risk of atherosclerosis associated with a low-cholesterol diet. Death from heart disease is virtually unknown in the Tarahumara Indians of Mexico, whose daily consumption of cholesterol is reported to be only 71 mg/day (Groom 1971). Over a four year period Connor et al. (1978) surveyed plasma lipids and lipoproteins in 523 Tarahumaras aged 5 to 70 years. Total plasma cholesterol averaged 125 mg/dl, with LDL cholesterol only 87 mg/dl; as compared to U.S. values these figures represent a reduction of 40 and 60% respectively. Further evidence associating a high-cholesterol, Western diet with heart disease has been shown in studies of Japanese men living in Japan, Hawaii and California. Gordon (1957, 1967) first described a gradient in mortality from heart disease in men of these three cultures, with rates highest in California and lowest in Japan. Kato et al. (1973) and Nichaman et al. (1975) expanded these findings by examining dietary cholesterol intake and plasma cholesterol levels respectively within these three populations. The results showed a strong correlation between the Western diet, elevated plasma

cholesterol, and the incidence of heart disease. Japanese men in California exhibited higher cholesterol intake, higher plasma cholesterol, and a greater frequency of heart disease than the other two groups. Japanese men in Hawaii had values intermediate to the other two populations, with men in Japan the lowest in all three measurements.

Age

Evidence for age-related changes in plasma cholesterol was first presented by Keys et al. (1950), who surveyed mean concentrations in 2056 healthy Americans. While males ranged in age from 17 to 78 years, females were restricted in age to between 17 and 30 years. Similar trends were observed in both sexes up to age 30, with plasma cholesterol increasing by approximately 2 mg/dl each year. After age 30, males exhibited a steady increase which leveled off at about 55 to 60 years, eventually declining in very old age. Smaller-scale studies performed by Kornerup (1950) in Denmark and Tanner (1951) in England indicated that the rise in plasma cholesterol associated with aging was not restricted to populations in the United States.

The change in total plasma cholesterol is due primarily to increases in the LDL fraction. Miller (1984) reported an average annual increase of 1.4 mg/dl in LDL cholesterol in humans ranging from 20 to 70 years. Results from the Framingham Study revealed that LDL cholesterol rose by 57% in women and 29% in men as age increased from 24 to 64 years (Abbott et al. 1983). Similarly, Rifkind et al. (1979) observed a steady increase in LDL cholesterol up to the age of 70 in

over 7000 males and females screened from 14 different populations; beyond 70 years there was a slight but significant decline in LDL cholesterol in both sexes. Hershcopf et al. (1982) confirmed the findings of cross-sectional studies in the Baltimore Longitudinal Study of Aging, a fourteen-year survey of plasma cholesterol in 1011 males aged 17-102.

Researchers have attempted to elucidate the biochemical mechanism responsible for the age-related increase in LDL cholesterol that has been identified by epidemiological studies. One aspect of LDL metabolism studied extensively with regard to aging is receptor activity. Mahley et al. (1981) and Hui et al. (1981) found the expression of the LDL receptor in dogs to be highly dependent on age. In vitro binding of ^{125}I-LDL to canine hepatic membranes decreased linearly with increasing age. Similar results were observed by Mahley et al. (1981) for liver membranes isolated from swine and humans. Additionally, in vivo turnover studies revealed that canine LDL were cleared from the plasma more rapidly in immature dogs than in adults (Mahley et al. 1981). Lee et al. (1982) examined LDL catabolism by aging human diploid fibroblasts in culture, and found that binding, internalization and degradation of ^{125}I-LDL decreased with increasing cell division while receptor affinity for ^{125}I-LDL remained unchanged. Although genetic, hormonal and environmental factors all affect LDL receptor expression, these binding and turnover studies indicate that the elevation in LDL cholesterol associated with aging may be due, at least in part, to decreased LDL degradation following a suppression of receptor synthesis.

Sex

It has long been recognized that Caucasian females in the reproductively active years are more resistant to atherosclerosis than males in the same age group (Anderson 1978). Part of this protection has been attributed to relative concentrations of circulating plasma lipoproteins. As early as 1963, Barclay and co-workers established that HDL concentrations were significantly higher in women than men. Nichols (1967) compared HDL levels between females aged 16 to 49 years and males aged 17 to 65 and found markedly higher concentrations in females at all ages. Similar male-female differences were described in the Cooperative Lipoprotein Phenotyping Study which was restricted to age groups 40 years and older (Castelli et al. 1977). Given the inverse relationship between HDL and the risk of developing atherosclerosis and coronary heart disease (Gordon et al. 1977), the possibility exists that higher HDL levels contribute to the so-called "female protection" against developing atherosclerosis (Rudel and Pitts 1978).

Higher HDL levels in females are not apparent until the onset of sexual maturation. In the Lipid Research Clinics Prevalence Study, Rifkind et al. (1979) reported that up to age 13, males had slightly higher HDL cholesterol levels than females. During adolescence, however, both sexes experienced a drop in total plasma cholesterol. In females this drop was primarily in LDL cholesterol, whereas in males HDL cholesterol levels were reduced. Hence, at all ages beyond 13 years, females exhibited higher HDL cholesterol concentrations. Additionally, the magnitude of the difference appeared to increase with

age, reaching 30% by age 45. Similar decreases in total cholesterol during adolescence were reported for American (Morrison et al. 1979) and English (Orchard et al. 1980) schoolchildren; males typically showed reductions in HDL cholesterol while the drop in females was confined to the LDL fraction.

Sex hormones are thought to be responsible for the observed variation between gender. Furman et al. (1958) first determined that estrogens elevated plasma HDL while androgens had the opposite effect. Krauss et al. (1979) showed higher HDL levels in menopausal estrogen users as compared to menopausal women of comparable age not receiving estrogen therapy. In keeping with this hypothesis, the drop in HDL cholesterol observed in adolescent males has been attributed to increased androgen secretion (Morrison et al. 1979). Although sex hormones undoubtedly affect lipoprotein metabolism, the biochemical mechanism responsible for the observed changes is not fully understood (Miller and Miller, 1984).

Animal Models for Atherosclerosis Research

Old World Monkeys

Due to the limitations in studying atherosclerosis in man, animals have become increasingly important as models for atherosclerosis research. The most common animal model, the laboratory rat, is unsatisfactory due to its extreme resistance to atherosclerosis (Mahley 1978). Although intimal plaques can be induced in both rabbits and pigeons, neither species is suitable since the lesions are remarkably

dissimilar to those seen in man (Stills and Clarkson 1979). As a result, three criteria have been established for the selection of appropriate models: 1) circulating plasma lipoproteins must be nearly identical biochemically and physiologically to human lipoproteins, 2) the animal must be susceptible to developing hypercholesterolemia resembling human hypercholesterolemia, and 3) the arterial lesions produced experimentally should be similar to those seen in humans (Blaton and Peeters 1976).

Nonhuman primates, especially the Old World monkeys, are the preferred models for atherosclerosis research due to their close phylogenetic relationship to man (Blaton and Peeters 1976). The Macaque species in particular have been used extensively to study the relationship between diet, plasma lipoproteins and atherogenesis (Chapman 1980). These animals exhibit lipoproteins similar in structure to human lipoproteins (Chapman 1980), are susceptible to dietary-induced hypercholesterolemia (Blaton and Peeters 1976), and develop naturally-occurring atherosclerotic lesions comparable to those seen in man (Lapin et al. 1979). In most chow-fed Old World monkeys HDL carries 40 to 50% of total plasma cholesterol (Srinivasan et al. 1974; Bullock et al. 1975). Although this level is 10 to 20% higher than that observed in man, consumption of a cholesterol-enriched diet will typically shift lipoprotein profiles in Old World monkeys to closely resemble those in humans (Rudel and Lofland 1976).

The most extensively studied Old World species is the rhesus monkey Macaca mulatta. Baseline lipoprotein levels for newly-imported

animals have been determined (Eggen et al. 1982), as have been the distributions under various atherogenic diets. Srinivasan et al. (1976) fed rhesus monkeys a 0.5% cholesterol diet (1.8 mg/kcal) for three weeks and observed a rise in mean plasma cholesterol from 147 to 241 mg/dl. Further studies by Nelson and Morris (1977) revealed that the elevation in plasma cholesterol was due primarily to increases in the LDL fraction. During the control period LDL and HDL cholesterol values were 56 and 92 mg/dl respectively. However, after a month of a 0.5% cholesterol diet, LDL cholesterol rose to 213 mg/dl while HDL cholesterol did not change significantly. Blaton and Peeters (1976) fed a 3.3% cholesterol diet to rhesus monkeys for six months and reported total plasma cholesterol values in excess of 600 mg/dl; electrophoretic separation revealed a marked increase in β-migrating lipoproteins (LDL). Another cholesterol-feeding experiment designed to more closely approximate the typical North American diet (0.3 mg cholesterol/kcal) produced elevations in LDL cholesterol comparable to those in humans consuming a Western diet (Ershow et al. 1981).

Although atherosclerotic lesions have been observed in free-ranging rhesus monkeys (Chawla et al. 1967), the degree of intimal thickening increases significantly with an atherogenic diet. Taylor (1965) summarized the early work on the characterization of the dietary-induced lesions in rhesus monkeys. Plaques developed first in the aorta, with gradual involvement of the carotid and coronary arteries. Lesions became increasingly complex with continued hypercholesterolemia; vascularization, calcification and thrombosis of the arteries were

common after several years of consuming a cholesterol-enriched diet. Additionally, atherosclerosis culminating in myocardial infarction has been observed, showing a similar progression of the disease in humans and rhesus monkeys (Stills and Clarkson 1979).

A number of other Old World monkeys have been used for atherosclerosis research. Macaca fascicularis has a lipoprotein distribution quite similar to M. mulatta and is also susceptible to dietary-induced hypercholesterolemia (Corey et al. 1974; Corey and Hayes 1974). Analysis of lipoproteins following various levels of cholesterol-enrichment revealed an elevation primarily in LDL cholesterol in this species (Rudel and Pitts 1978; Klein and Rudel 1983; Srinivasan et al. 1984). Other characteristics which make M. fascicularis suitable for atherosclerosis research is the development of lesions which worsen with hypercholesterolemia, and the fact that females of this species share with human females a certain degree of protection against atherosclerosis during the reproductively active years (Stills and Clarkson 1979). Dietary-induced hypercholesterolemia has also been produced in the African green monkey Cercopithecus aethiops, accompanied by elevations in LDL concentration, size and apo B-100 content (Johnson et al. 1983). The increase in LDL molecular weight was due largely to cholesteryl ester enrichment of the core and was highly correlated with the severity of coronary artery atherosclerosis (Rudel and Bullock 1981). Although formation of experimentally-induced lesions in baboons (Papio cynocephalus) has been attempted, this species is fairly resistant to hypercholesterolemia even after prolonged periods of

cholesterol feeding (Stills and Clarkson 1979). Blaton and Peeters (1976) found HDL to be the major cholesterol-carrier in baboons fed an atherogenic diet, and histological examination of the arteries after 1.5 to 3 years of treatment revealed only slight aortic lesions.

Macaca nemestrina

The pig-tailed macaque, Macaca nemestrina, has also been used in atherosclerosis research. Baseline lipoprotein distributions for newly-imported animals have been determined (Blakley et al. 1973). As seen in other macaques, HDL typically transports 40 to 50% of total plasma cholesterol in chow-fed animals (Rudel and Lofland 1976). However, cholesterol feeding results in lipoprotein patterns similar to those seen in humans consuming a Western diet. Rudel et al. (unpublished) fed six M. nemestrina 1.0 mg cholesterol/kcal for two years and observed a 20-fold increase in LDL cholesterol while HDL cholesterol remained unchanged. Conflicting results were reported by Kushwaha et al. (1982) after feeding animals 400 mg/day cholesteral for nine months. While total cholesterol levels increased fourfold, HDL cholesterol was reduced by 60% in the test animals.

M. nemestrina has also been used as a model for human aging research. McMahan et al. (1980) looked at the interactions between age, sex and a diet approximating that of a typical North American (0.3 mg cholesterol/kcal). In general, the test diet produced elevations in LDL cholesterol that appeared to increase over the small age range studied. Sexual differences were also observed in that males

had significantly more LDL cholesterol than females of comparable ages. HDL cholesterol increased with increasing age in both sexes consuming the test diet. In a small cross-sectional study, Lacko and Hazzard (1979) observed increased HDL cholesterol and decreased LDL cholesterol with increasing age in female M. nemestrina. Additionally, histological examinations have revealed spontaneous lesions of the coronary arteries that appeared more frequently in older animals; dietary-induced hypercholesterolemia resulted in the formation of atherosclerotic plaques similar to those seen in humans (Baba et al. 1979).

MATERIALS AND METHODS

Animals and Diets

A colony of pig-tailed macaques (Macaca nemestrina) established at the University of Washington Regional Primate Research Center, Medical Lake, Washington, was used in this study. A total of 66 animals were divided into three age groups: 19 young (3 to 4 years old), 25 adult (8 to 14 years old), and 22 old (18 years and older). Each age group was almost equally divided by sex. For six months prior to beginning the study, all animals were fed a semi-purified control diet; thereafter, half of each age group was fed either the test or control diet for three years (Table 1). The control diet was stringent in fat (20% of kcalories), cholesterol (0.04 mg/kcal), simple sugars and sodium. The test diet, designed to mimic the "typical American diet," was high in fat (41% of kcalories), cholesterol (0.24 mg/kcal), sodium and simple sugars. Fiber, protein, vitamin and mineral contents were identical in both diets. Diets were prepared at the Primate Research Center although proximal analysis was performed at the University of Arizona for total kcalories, protein, carbohydrate, fat, moisture, fiber and ash. The respective diets were provided ad libitum and all care, feeding and sample collection were performed at the Primate Research Center. After three years of dietary treatment, fasting (24 hour) blood samples were drawn and plasma was immediately

Table 1. Control and Test Diet Composition.

Ingredient	Control (g/kg)	Test (g/kg)
Lard	31.4	64.0
Corn Oil	25.6	48.6
Safflower Oil	11.8	24.0
Applesauce	100.4	204.3
Refined Sugar	79.6	162.2
Wheat Flour	223.7	90.0
Dextrin	230.1	19.0
Oat Hulls	175.2	196.4
Dry Egg Yolk	6.4	46.3
Lactalbumin	79.6	89.6
NaCl	5.5	22.3
Hegsted Mix	31.0	31.5
Vitamin Mix	2.0	2.1
kcals/kg	3560	3600
Protein (% kcals)	13%	13%
Carbohydrate (% kcals)	67%	46%
Fat (% kcals)	20%	41%
Cholesterol (mg/kcal)	0.04	0.24
Fiber	6.3%	6.3%

removed by centrifugation. A total of 10 ml of plasma was obtained for each animal. Plasma volumes were determined by the modified Evans blue dye (T-1824) dilution method described by Greenleaf et al. (1979). Samples (8 to 10 per week) were packed in ice and shipped by overnight mail on the day of collection to the University of Arizona for processing. As this study was double-blind, samples were identified by a five or six digit code number. Information regarding each animal's age, sex and dietary regimen was sent by the Primate Research Center to the University of Arizona upon completion of plasma lipoprotein analysis.

Biochemical Procedures

Plasma Lipoprotein Separation

Plasma lipoproteins were separated and purified by the method described by Rudel et al. (1974). Upon arrival each 10 ml plasma sample was adjusted to d1.221 by adding solid KBr (0.3512 g/ml plasma). Plasma was placed in an ultracentrifuge tube and overlayered with 10 ml of a d1.221 buffered solution (Scanu and Granada 1966). Tubes were centrifuged in a type 30 rotor for 24 hours at 18° C at 100,000 x g (28,000 rpm) in a Beckman model L ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). The top 2 ml containing the lipoproteins were removed and applied to an agarose chromatography column.

Columns with an internal diameter of 2.5 cm and a bed height of 90 cm were packed with sepharose CL-4B (Pharmacia Inc., Piscataway, NJ) and maintained in a cold room at 4° C. Less than 75 mg of

lipoprotein cholesterol in a 2 ml volume were applied to each column and eluted at a flow rate of 21 ml/hr with 0.15 M NaCl, 0.01% EDTA, 0.02% Na azide, pH 7.4. Fractions of the eluate were collected by an LKB model 2111 fraction collector (LKB Instruments, Inc., Rockville, MD) timed at 12 min/fraction. The protein component was monitored at 280 nm. Three separate regions of lipoproteins were eluted and collected: region 1, very-low density lipoproteins (VLDL, fractions 40-63); region 2, low density lipoproteins (LDL, fractions 64-79); region 3, high density lipoproteins (HDL, fractions 80-92). Proper identification of the three regions was validated by calibrating the columns with VLDL, LDL and HDL that were separated from plasma by sequential ultracentrifugation as described by Havel et al. (1955). The plasma densities used to separate the lipoproteins were d 1.006 for VLDL, d 1.063 for LDL, and d 1.220 for HDL. Fractions from the VLDL, LDL and HDL regions were concentrated in a 2 ml volume by ultrafiltration using YM 30 filters in Amicon stirred-cells (Amicon Corp., Danvers, MD) and stored in 0.05 M NaCl, 0.01% EDTA, 0.02% Na azide, pH 7.4. One ml of each concentrated sample was used for protein determination, after which the protease inhibitor phenylmethylsulfonyl flouride (PMSF, Calbiochem-Behring, LaJolla, CA) was added to a concentration of 100μM. Samples were then stored at 4° C until apolipoprotein analysis was performed. The other half of the concentrate was stored at 0° C for cholesterol and triglyceride determinations.

Plasma Protein Determination

The protein concentration of VLDL, LDL and HDL were measured colorimetrically using the method of Lowry et al. (1951). A standard curve was constructed using bovine serum albumin (BSA, A4378, crystalline and lyophilized, Sigma Chemical Co., St. Louis, MO) as the standard protein. Known quantities of BSA (0, 25, 60, 100, 150 and 250 µg/ml) were used to construct the standard curve. Plasma protein in solution reacted with alkaline copper solution to form products which quantitatively reduced a phosphotungstic-phosphomolybdic reagent. The blue color complex was read against a blank at 750 nm in a double-beam Beckman model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

Plasma Cholesterol Determination

Lipoprotein cholesterol levels were measured colorimetrically by the method of Roeschlau et al. (1974) using an enzyme kit purchased from Boehringer Mannheim (Indianapolis, IN). A standard curve was constructed using certified cholesterol standards of 50, 100, 125, 150 and 200 mg/dl (Boehringer Mannheim). Cholesteryl esters in the samples were quantitatively cleaved to produce free cholesterol, which was then oxidized to cholest-4-en-3-one. Equivalent hydrogen peroxide was produced in the reaction which, in the presence of catalase, converted methanol to formaldehyde. Formaldehyde formed a yellow complex, 3, 5-diacetyl-1, 4-dihydrolutidine, through the Hantzsch reaction (Nash 1953). The yellow color was read against a blank at 520 nm in a

double-beam Beckman model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

Plasma Triglyceride Determination

Lipoprotein triglycerides were measured colorimetrically by the method of Bucolo and David (1973) using an enzymatic kit purchased from Boehringer Mannheim (Indianapolis, IN). A certified triglyceride standard (129 mg/dl, Boehringer Mannheim) was run with each set of samples. Triglycerides were hydrolyzed to free glycerol and fatty acids by an esterase/lipase solution. The free glycerol liberated in the reaction was phosphorylated and oxidized, generating NADH quantitatively. The NADH reduced MTT (3-(4, 5-Dimethyl Thiazolyl-2)-2, 5 Diphenyl Tetrazolium Bromide) to MTT·H, a colored complex. MTT·H was read against a blank at 560 nm using a double-beam Beckman model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

LDL and HDL Apolipoprotein Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) was used to analyze the apolipoprotein composition of LDL and HDL. For HDL, a gradient slab gel of 7.5 to 20% acrylamide with a 4.5% stacking gel was used to separate the apoproteins. A slightly modified gradient slab (4.5 to 20% acrylamide with a 4.5% stacking gel) was employed for LDL to facilitate migration of apo B-100 into the gel. Protein concentrations in both LDL and HDL had been determined prior to electrophoresis. Approximately 25 µg of protein were applied to the gel and run on a

Hoefer model SE 400 vertical slab unit (Hoefer Scientific Instruments, San Francisco, CA). The gels were run at a constant current of 20 mA for approximately 4 hours or until the front was within 1 cm of the bottom edge. Approximately 30 µg of SDS-PAGE low molecular weight standard (#27074, MW range 10,000-100,000, Biorad Laboratories, Richmond, CA) was run simultaneously on each gel and contained 5 µg each of phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme. Protein bands were stained overnight with 0.2% Coomassie blue and destained in 10% acetic acid, 25% methanol with continuous stirring. The stained protein bands were scanned in a Hoefer GS 300 Transmittance/Reflectance Scanning Densitometer. (Hoefer Scientific Instruments, San Francisco, CA). Apo A-I and apo B contents were calculated using a standard curve constructed with purified human apo A-I and apo B (Calbiochem-Behring, LaJolla, CA). Known quantities (6, 12, 18, 24 and 30 µg apo A-I and 5, 10, 15, 20 and 25 µg apo B) were applied to gels and run, stained and scanned as described above to construct the standard curve used in the quantitation. The same results for apo A-I and apo B were obtained by measuring areas under the peaks and calculating apoprotein concentration on the basis of total protein applied to the gel. The C and E apoprotein contents were determined using the peak area method because of their minimal contribution to total protein.

Statistical Analysis

A 2 x 2 x 3 factorial arrangement was set up to statistically analyze differences between the two diets, two sexes and three age

groups. Multivariate ANOVA was performed using the Statistical Package for the Social Sciences (SPSS) computer program. A least-squares fit was used to correct for missing data since cell sizes were unequal. Two separate age comparisons were analyzed: 1) the oldest group versus the two younger groups and 2) the adult group versus the youngest group. P-values less than 0.05 indicated significance.

RESULTS

Weight

Males were significantly heavier than females at all ages (Table 2). In both sexes, body weight was higher in adults as compared to younger animals; however, weight gain appeared to stabilize or even decline slightly once maturity was reached. No dietary effect was detected since similar body weights were observed in animals of all ages from both dietary treatments.

Hematological Data

Table 2 also shows the results of hematocrit, plasma and blood volume measurements. Both plasma and blood volumes, when expressed as ml/kg body weight, appeared to increase with increasing age. No significant dietary or sex effects were apparent. However, a three factor or second order interaction was associated with the hematocrit data. While the oldest females fed the test diet exhibited a drop in hematocrit, their counterparts fed the control diet experienced a rise. No significant differences were observed in males fed the control diet among the various ages. However, higher hematocrit levels were observed in the oldest males fed the experimental diet.

Table 2. Body Weight, Blood Volume, Plasma Volume and Hematocrit Levels of Three Age Groups of Macaca nemestrina Fed Either a Control or Test Diet.[1]

DIET	AGE	SEX[2]	Weight kg	Blood Volume ml/kg	Plasma Volume ml/kg	Hematocrit %
CONTROL	YOUNG	F	5.94 ± 0.60	72.9 ± 3.6	46.1 ± 1.8	37.3 ± 1.4
		M	11.46 ± 1.59	68.6 ± 6.7	41.8 ± 3.3	40.7 ± 0.4
	ADULT	F	6.72 ± 0.36	71.4 ± 5.6	43.5 ± 2.8	40.0 ± 1.7
		M	12.88 ± 1.40	80.2 ± 11.5	49.2 ± 5.8	40.9 ± 1.1
	OLD	F	6.45 ± 0.59	82.7 ± 12.0	43.6 ± 6.0	47.7 ± 2.5
		M	8.99 ± 0.74	94.9 ± 5.0	55.9 ± 2.5	40.2 ± 2.0
TEST	YOUNG	F	5.31 ± 0.40	78.4 ± 6.3	44.9 ± 3.2	43.5 ± 1.5
		M	11.65 ± 1.02	62.8 ± 6.0	36.9 ± 3.0	42.3 ± 0.3
	ADULT	F	7.45 ± 0.94	66.4 ± 12.4	40.0 ± 6.2	43.3 ± 3.3
		M	13.72 ± 0.56	76.0 ± 17.3	45.9 ± 8.6	40.0 ± 0.6
	OLD	F	6.00 ± 0.44	78.6 ± 7.2	48.5 ± 3.6	39.7 ± 1.1
		M	12.78 ± 1.74	96.0 ± 5.2	52.0 ± 2.6	46.6 ± 1.7

P-values for ANOVA Treatment Comparisons	df				
D=DIET	1				
S=SEX	1	0.001			
A_1=OLD vs YOUNG and ADULT	1		0.05	0.05	0.01
A_2=ADULT vs YOUNG	1	0.025			
Interactions	7				
DxS	1				
DxA_1	1				
DxA_2	1				
SxA_1	1				
SxA_2	1				
$DxSxA_1$	1				
$DxSxA_2$	1				<0.001

[1]Mean ± standard error of the mean; [2]M=Male, F=Female
df = degrees of freedom

Lipoproteins

Marked differences in plasma lipoproteins separated by agarose column chromatography were observed between animals fed the control and test diets. Two typical elution profiles representing control and test-fed animals are shown in Figure 1. In general, animals fed the test diet appeared to have higher lipoprotein levels as compared to control animals. This elevation was due primarily to increases in the LDL fraction. While HDL levels did not seem to decrease in the test animals, the relative concentration of HDL to LDL was reduced. No major differences were noted in VLDL levels between control and test animals.

Triglycerides

No significant dietary, sex or age effects were apparent for VLDL, LDL, HDL or total lipoprotein triglyceride levels (data not shown). The values obtained were approximately 50% lower than those reported by other researchers (Lacko and Hazzard 1979; McMahan et al. 1980). Although various first and second order interactions were noted, the patterns observed were inconsistent within diet, sex and age groups and therefore interpretation of the triglyceride data will not be discussed at length.

Cholesterol

Significant dietary, age and sex effects were observed in the distributions of cholesterol among the lipoprotein fractions (Table 3). Overall, the test diet induced a two-fold increase in total plasma

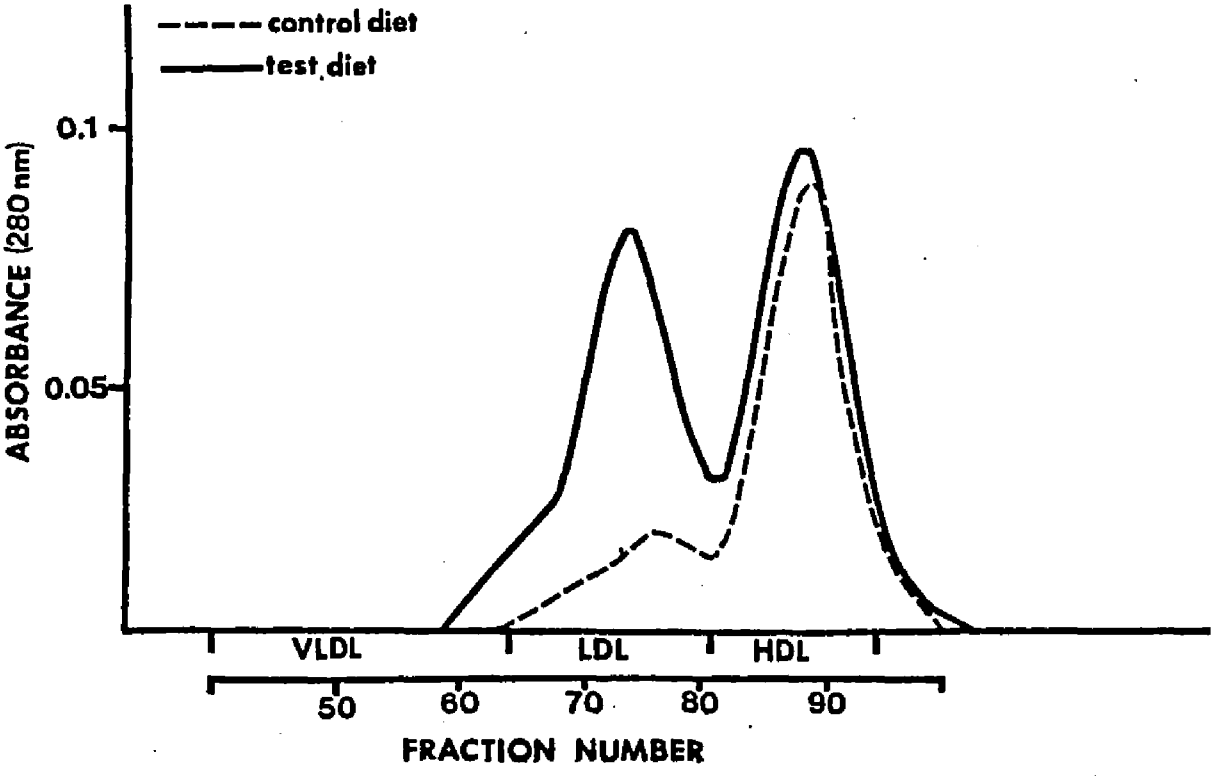

Figure 1. Elution Profiles of Monkey Plasma Lipoproteins Separated by Agarose Gel Chromatography on a Sepharose CL-4B Column (2.5 x 90 cm). Lipoproteins were separated from 10 ml plasma (density adjusted to 1.22 with solid KBr) by ultracentrifugation, applied to the column and eluted with 0.15 M NaCl, 0.01% EDTA, 0.02% NaAzide, pH 7.4, and run at a rate of 21 ml/hr. Each fraction contained 4.2 ml.

Table 3. Influence of Nutrition, Aging and Sex on Cholesterol Distributions in *M. nemestrina*.[1]

DIET	AGE	SEX[2]	CHOLESTEROL, mg/100 ml VLDL	LDL	HDL	TOTAL
CONTROL	YOUNG	F	0.09 ± 0.04	27.7 ± 8.5	46.8 ± 8.9	74.6 ± 11.8
		M	0.02 ± 0.01	16.7 ± 1.5	36.0 ± 3.1	52.7 ± 3.9
	ADULT	F	0.57 ± 0.33	33.5 ± 6.5	27.4 ± 4.9	61.4 ± 5.3
		M	0.49 ± 0.29	21.1 ± 3.9	38.4 ± 6.2	59.9 ± 4.9
	OLD	F	0.24 ± 0.13	31.8 ± 8.5	36.0 ± 5.6	68.1 ± 12.2
		M	0.09 ± 0.01	19.4 ± 1.5	30.8 ± 5.6	50.3 ± 5.7
TEST	YOUNG	F	1.24 ± 0.63	133.2 ± 67.2	56.9 ± 11.5	191.3 ± 59.1
		M	9.81 ± 8.83	144.8 ± 42.8	31.9 ± 4.6	186.5 ± 45.3
	ADULT	F	9.41 ± 7.65	117.8 ± 57.0	50.7 ± 15.7	177.9 ± 57.4
		M	19.81 ± 19.59	114.1 ± 48.0	47.5 ± 4.1	181.5 ± 65.0
	OLD	F	0.41 ± 0.15	60.8 ± 10.0	48.3 ± 4.2	109.5 ± 10.4
		M	0.27 ± 0.11	34.9 ± 4.9	46.9 ± 5.3	82.1 ± 6.4

P-values for ANOVA Treatment Comparisons	df				
D=DIET	1	(0.08)	<0.001	0.01	<0.001
S=SEX	1				
A_1=OLD vs YOUNG and ADULT	1		0.025		0.025
A_2=ADULT vs YOUNG	1				
Interactions	7				
DxS	1				
DxA_1	1		0.025		0.025
DxA_2	1				
SxA_1	1				
SxA_2	1			0.05	
$DxSxA_1$	1				
$DxSxA_2$	1				

[1] Mean ± standard error of the mean; [2] M=Male, F=Female
df = degrees of freedom

cholesterol levels. Analysis of the VLDL, LDL and HDL fractions revealed the elevation to be due primarily to a five-fold increase in LDL cholesterol. While only 40% of total plasma cholesterol resided in LDL in control animals, this value rose to 65% in test animals. Slight but significant increases in HDL cholesterol were also noted in the animals fed the test diet. However, the magnitude of increase was far less than that observed for LDL cholesterol. Differences between age groups were also present in the test animals (Figure 2). Although LDL cholesterol increased significantly in all three age groups fed the test diet, the magnitude of increase in the oldest group was significantly less than that observed in the two younger groups. This effect was not apparent in animals consuming the control diet. No male-female variability was observed in LDL cholesterol levels. However, an interesting sex-age effect was seen in HDL cholesterol (Figure 3). In the youngest group, concentrations were significantly higher in females than in males. This trend was not observed in the adult group, however, with males showing equal or higher HDL cholesterol levels than those in their female counterparts. Although VLDL cholesterol appeared to be higher in animals fed the test diet (Table 3), this trend was found to be statistically significant only at the $p < 0.08$ level.

Protein

Patterns similar to those seen in cholesterol distributions were observed for protein concentrations among the various lipoprotein

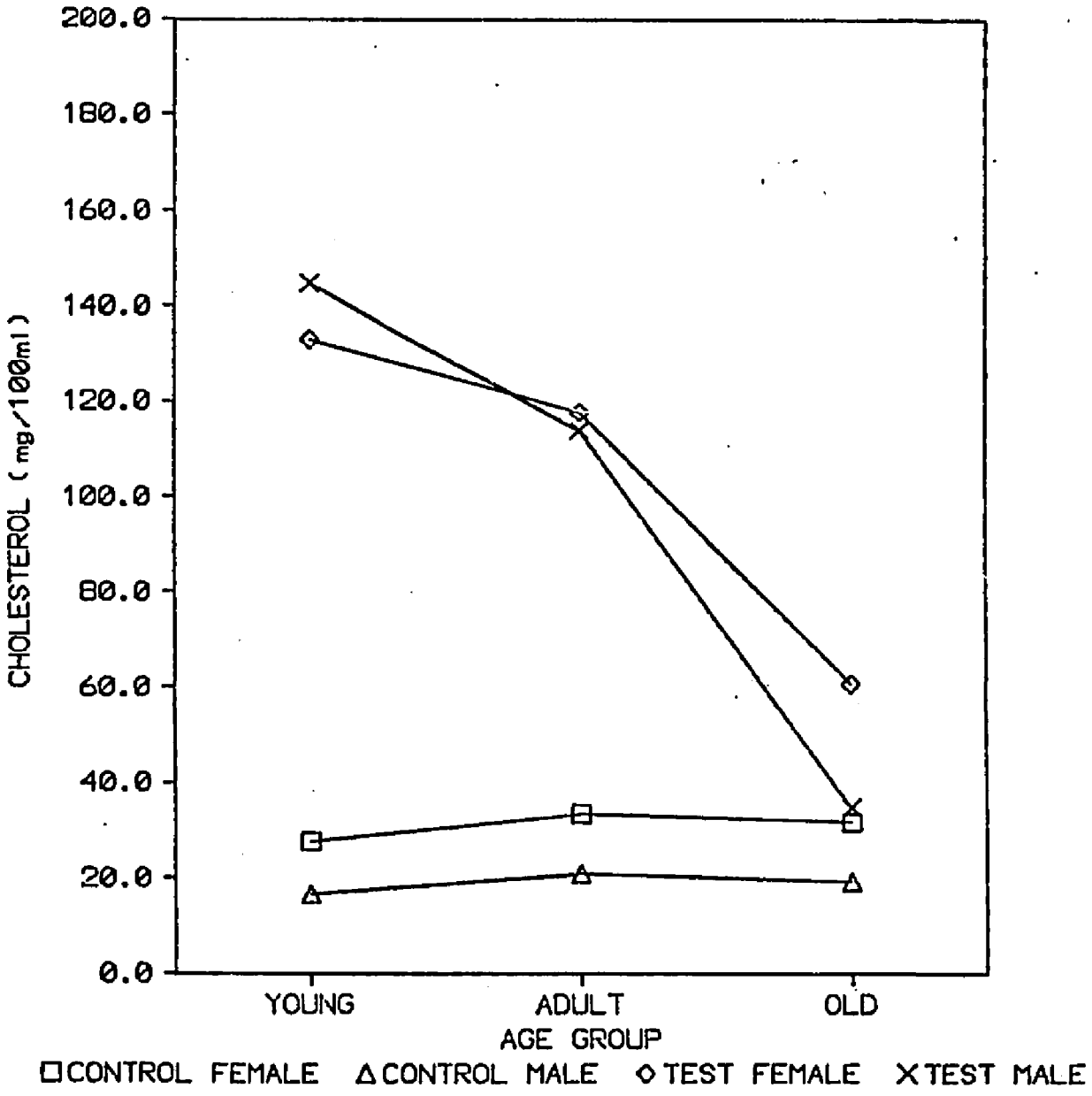

Figure 2. LDL Cholesterol Concentrations in Three Age Groups of Male and Female Macaca nemestrina Fed Control and Experimental Diets.

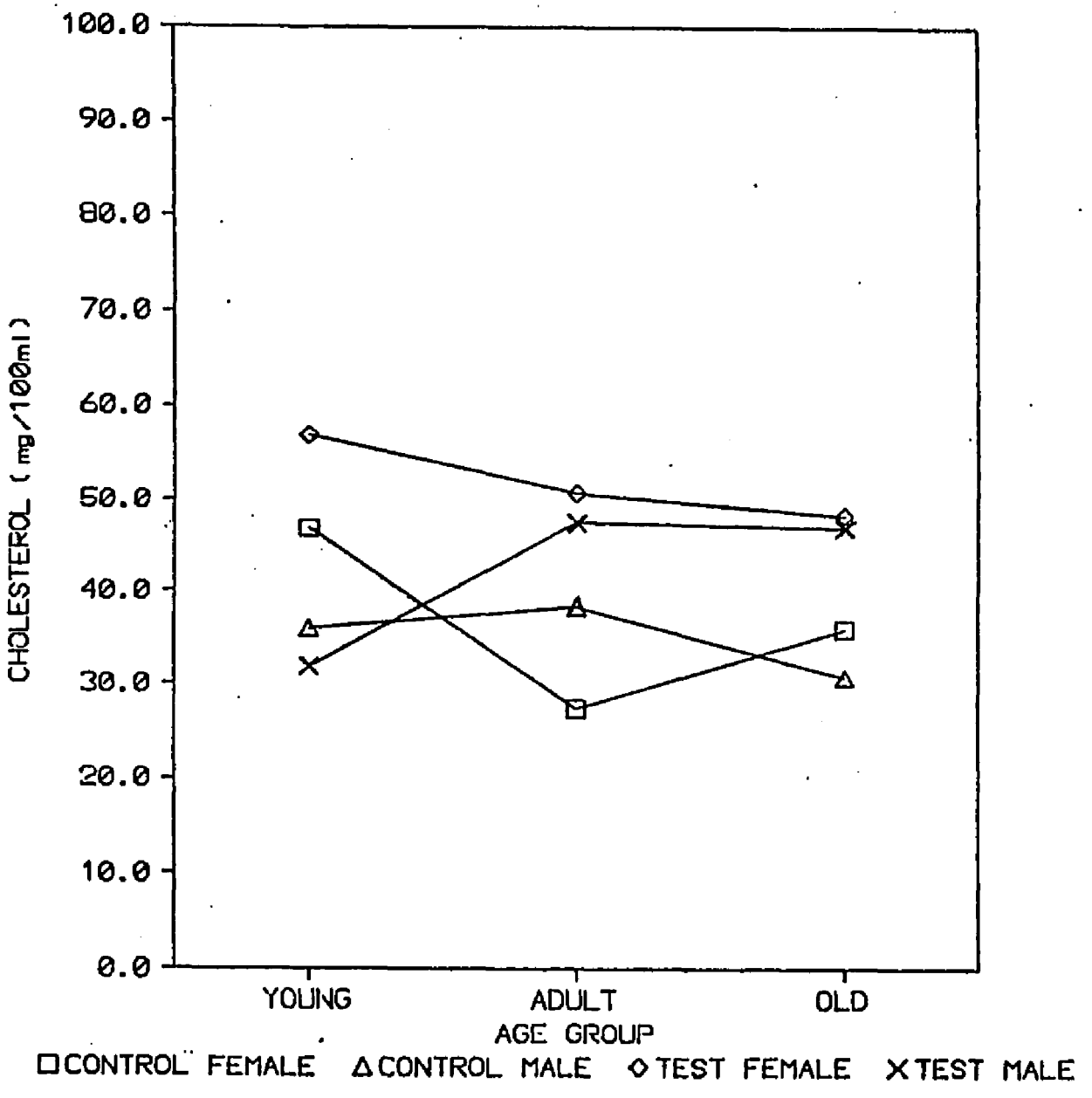

Figure 3. HDL Cholesterol Levels in Three Age Groups of Male and Female Macaca nemestrina Fed Control and Experimental Diets.

fractions (Table 4). Overall, total protein content of plasma lipoproteins was increased by the test diet in all animals. Analysis of VLDL, LDL and HDL protein levels revealed a two-fold increase in LDL protein and slight but significant elevations in VLDL and HDL protein in test animals. Again, a diet-age interaction was observed for LDL protein (Figure 4). While all animals fed the test diet experienced a rise in LDL protein, the magnitude of increase was significantly less in the older group as compared to the younger animals. No sex differences were noted for LDL protein concentrations. However, male-female differences were apparent for HDL protein and were similar to those reported for HDL cholesterol. In animals fed the test diet, HDL protein was lower in males of the youngest group as compared to females. This trend was not observed in the adult group.

Apolipoproteins

Separation of LDL and HDL apolipoproteins by SDS-PAGE and their subsequent quantitation by densitometric scanning revealed significant dietary and sex effects (Table 5). The major apoprotein of LDL, apo B-100, was significantly elevated by the test diet in all animals. A pattern emerged that was similar to the age effect seen in LDL cholesterol and protein concentrations. The magnitude of increase in apo B-100 levels appeared to be less in the oldest group as compared to the two younger groups; however, this trend was found to be statistically significant only at the $p < 0.09$ level. Apo A-I, the major apoprotein in HDL, followed the same patterns described for

Table 4. Influence of Nutrition, Aging and Sex on Protein Levels in *M. nemestrina*.[1]

DIET	AGE	SEX[2]	PROTEIN, mg/100 ml VLDL	LDL	HDL	TOTAL
CONTROL	YOUNG	F	0.38 ± 0.07	30.8 ± 4.4	130.6 ± 16.8	161.8 ± 17.0
		M	0.13 ± 0.08	22.1 ± 1.7	123.0 ± 16.3	145.2 ± 16.8
	ADULT	F	1.54 ± 0.71	39.2 ± 5.8	91.2 ± 10.6	131.8 ± 8.9
		M	0.78 ± 0.35	27.6 ± 4.3	118.9 ± 8.9	147.3 ± 6.2
	OLD	F	0.94 ± 0.43	40.3 ± 5.3	130.9 ± 15.3	172.1 ± 18.2
		M	0.41 ± 0.20	25.7 ± 1.5	98.9 ± 6.7	125.0 ± 5.3
TEST	YOUNG	F	2.16 ± 1.13	92.3 ± 34.4	155.5 ± 18.7	250.0 ± 25.2
		M	5.87 ± 4.04	78.5 ± 17.1	92.4 ± 11.0	176.8 ± 15.9
	ADULT	F	6.32 ± 4.70	74.9 ± 25.6	138.6 ± 35.3	219.8 ± 31.5
		M	7.85 ± 7.28	68.2 ± 18.3	142.1 ± 15.4	218.2 ± 22.0
	OLD	F	1.02 ± 0.24	56.8 ± 6.4	131.0 ± 4.5	188.8 ± 7.7
		M	0.70 ± 0.36	38.6 ± 5.8	149.9 ± 13.5	189.2 ± 16.2

P-values for ANOVA Treatment Comparisons	df				
D=DIET	1	0.05	<0.001	0.05	<0.001
S=SEX	1				
A_1=OLD vs YOUNG and ADULT	1				
A_2=ADULT vs YOUNG	1				
Interactions	7				
DxS	1				
DxA_1	1		0.05		
DxA_2	1				
SxA_1	1				
SxA_2	1			0.05	
$DxSxA_1$	1				
$DxSxA_2$	1			0.05	

[1] Mean ± standard error of the mean; [2] M=Male, F=Female
df = degrees of freedom

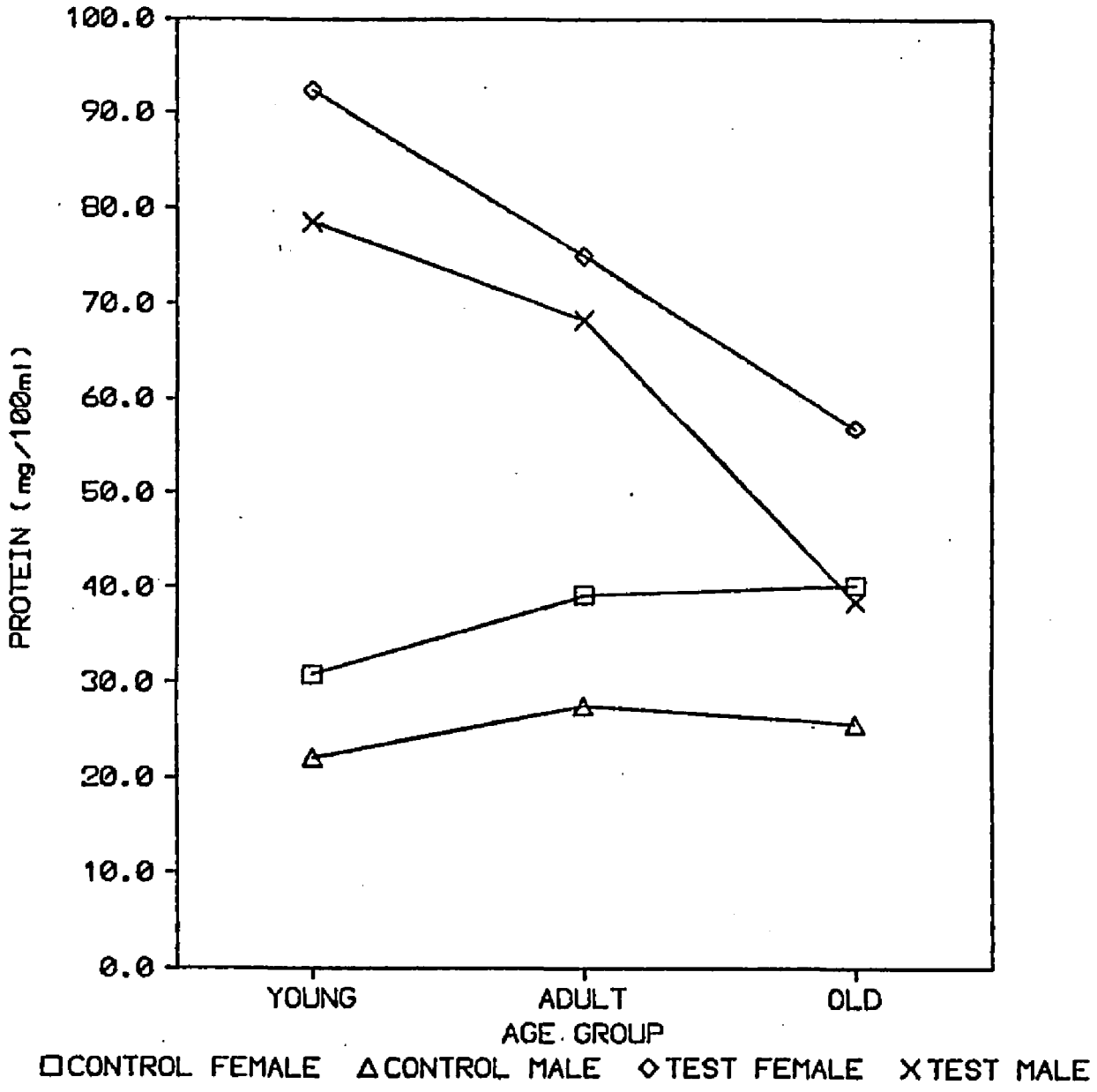

Figure 4. LDL Protein Levels in Three Age Groups of Male and Female Macaca nemestrina Fed Control and Test Diets.

Table 5. Influence of Nutrition, Aging and Sex on LDL apo B-100 and HDL apo A-I Levels in *M. nemestrina*.[1]

DIET	AGE	SEX[2]	LDL APO B-100 mg/100 ml	HDL APO A-I mg/100 ml
CONTROL	YOUNG	F	25.9 ± 3.8	84.1 ± 14.1
		M	16.6 ± 1.9	61.8 ± 7.8
	ADULT	F	20.2 ± 2.6	61.2 ± 9.9
		M	19.1 ± 3.2	76.0 ± 8.4
	OLD	F	30.2 ± 3.9	75.4 ± 15.6
		M	16.1 ± 1.6	64.7 ± 6.8
TEST	YOUNG	F	55.6 ± 25.8	94.1 ± 26.0
		M	61.5 ± 22.3	54.7 ± 12.7
	ADULT	F	65.6 ± 27.9	91.4 ± 38.3
		M	56.9 ± 16.0	99.7 ± 9.6
	OLD	F	43.1 ± 16.6	90.1 ± 5.4
		M	28.1 ± 6.5	103.5 ± 10.9

P-values for ANOVA Treatment Comparisons	df		
D=DIET	1	<0.001	0.025
S=SEX	1		
A_1=OLD vs. YOUNG and ADULT	1		
A_2=ADULT vs. YOUNG	1		
Interactions	7		
DxS	1		
DxA_1	1	(0.09)	
DxA_2	1		
SxA_1	1		
SxA_2	1		0.05
$DxSxA_1$	1		
$DxSxA_2$	1		

[1]Mean ± standard error of the mean; [2]M=Male, F=Female
df = degrees of freedom

HDL cholesterol and protein concentrations (Table 5). Significant increases in apo A-I levels were observed in the animals fed the test diet, with the magnitude of increase less than that observed in LDL apo B-100 concentrations. Again, male-female variability was apparent, in that significantly lower apo A-I was observed in the youngest males as compared to their female counterparts (Figure 5). This trend was not observed in the adult animals. No significant differences due to diet, sex or age were apparent in HDL apo C or LDL apo E concentrations. However, it should be noted that apo E was detected in only four animals in this study; all four animals were from the young or adult groups consuming the test diet. Male-female differences were not evident since these animals with apo E were of both sexes. In all cases the apo E that was seen appeared after SDS-PAGE of the LDL rather than HDL fraction. However, differences in apo E concentrations were found to be statistically insignificant since the number of observations was so limited.

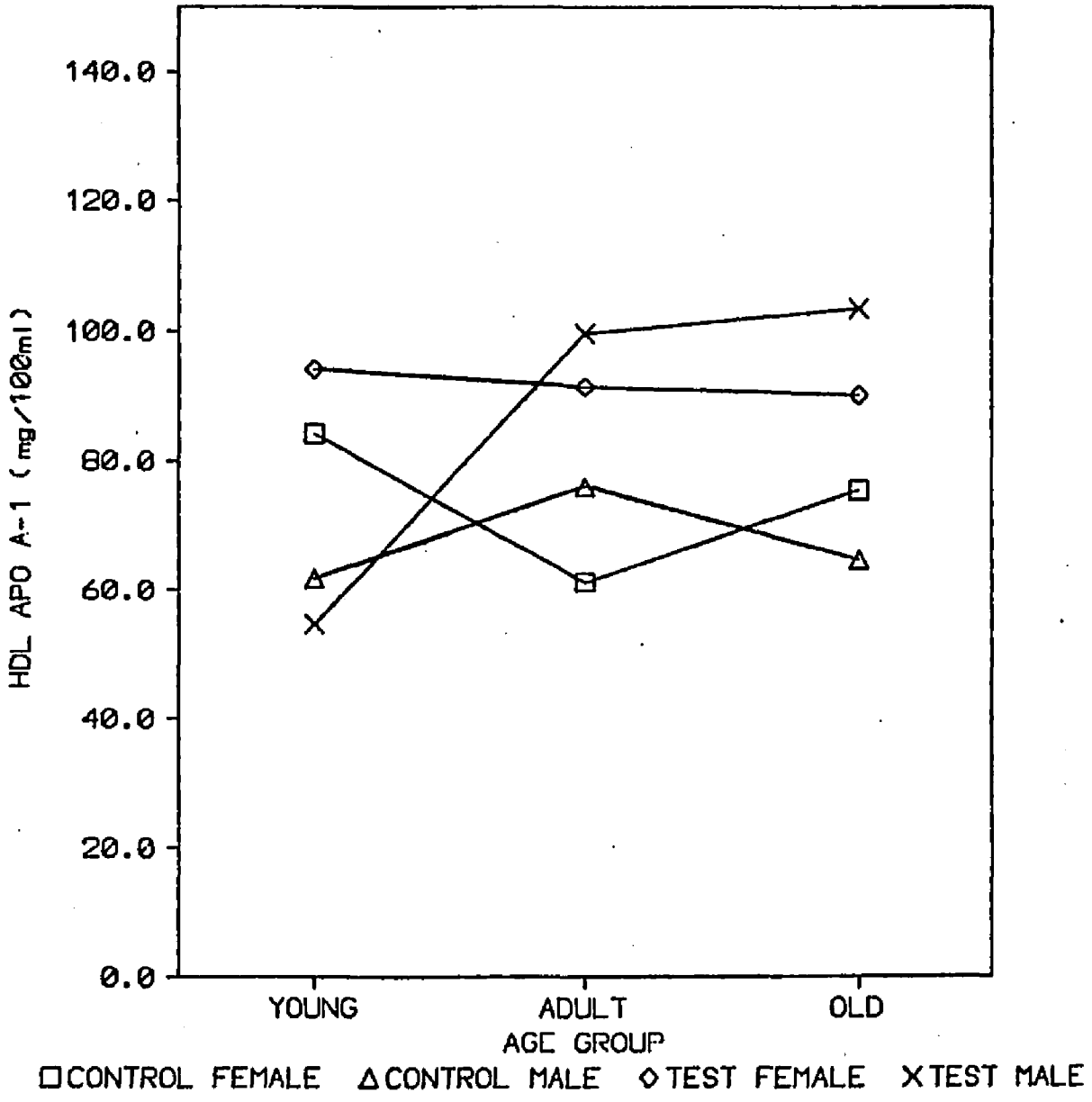

Figure 5. HDL apo A-I Levels in Three Age Groups of Male and Female Macaca nemestrina Fed Control and Experimental Diets.

DISCUSSION

Total plasma cholesterol levels for control animals were within the ranges reported by Blakley et al. (1973) for newly-imported Macaca nemestrina. The distribution of cholesterol within lipoprotein fractions was slightly lower than that observed by Lacko and Hazzard (1979) but consistent with the findings of McMahan et al. (1980). Although sex and age effects were seen in this study, diet appeared to play the most significant role in modifying plasma lipoproteins in M. nemestrina. The "typical American diet," fed to the test animals for three years, greatly elevated LDL protein, LDL cholesterol and total cholesterol levels ($p < 0.001$). Also, HDL cholesterol and protein were increased but at a lesser magnitude ($p < 0.01$ and 0.05 respectively). The elevations appear to be a direct result of diet composition rather than differences in food consumption since body weights, which would reflect variations in calorie intake, were not significantly different between control and test animals.

These results were not surprising since cholesterol feeding, in almost every type of laboratory animal model, commonly leads to hypercholesterolemia. While animals such as rats and dogs are clearly more resistant to hypercholesterolemia than nonhuman primates (Mahley 1978), a cholesterol-enriched diet will lead to elevated plasma cholesterol levels almost without exception (Goldstein and Brown 1982). However, in many of these animal studies the level of cholesterol

enrichment was on the order of 2 to 5% (Kovanen et al. 1981; Mahley et al. 1981) so that hypercholesterolemia could be induced in a relatively short period of time. In contrast, the typical Western diet contains approximately 0.1% cholesterol by weight (Connor and Connor 1972). The experimental diet used in this study was designed to more closely approximate actual human consumption. As a result, the elevations in LDL, HDL and total cholesterol were not as dramatic as those reported by Rudel and Lofland (1976), who fed M. nemestrina a 0.5% cholesterol diet for two years. However, McMahan et al. (1980) employed a diet similar to that used in this study and observed comparable increases in LDL, HDL and total cholesterol.

In spite of the more moderate elevations, it was apparent that the experimental diet induced a condition of hypercholesterolemia in the experimental animals. However, "hypercholesterolemia" in the test animals is referred to only in terms of comparison to the control animals. The test animals would not be considered hypercholesterolemic as compared to humans since their LDL and total cholesterol levels are well within ranges reported for "normal" Americans (Havel et al. 1980; Brown and Goldstein 1984). "Normal" does not necessarily imply optimal, however, especially when consideration is given to the positive correlation between plasma cholesterol levels and accelerated atherosclerosis (Kannel et al. 1971) and the high incidence of heart disease in Western nations (Connor and Connor 1972). Although the animals fed the experimental diet were not severely hypercholesterolemic, they exhibited lipoprotein profiles that in humans are associated with an increased risk of mortality from coronary heart disease.

Genetic factors also influence the way in which an organism will respond to dietary cholesterol. More specifically, the terms hypo- and hyper-responsiveness have been used to describe variations observed between members of the same species when challenged with dietary cholesterol. Typically hyper-responders develop severe hypercholesterolemia whereas the same dietary regimen will produce only a moderate rise in plasma cholesterol in hypo-responders. Heritability studies in mice and rabbits have shown this variability to be due in part to genetic factors (Weiburst 1973; Roberts and West 1974; Dunnington et al. 1977). This type of variation in response to dietary cholesterol has also been observed in many nonhuman primates, including several species of the Macaque family (Rudel and Lofland 1976). Stills and Clarkson (1979) attributed 65% of the variation in plasma cholesterol levels in squirrel monkeys to genetic factors.

In the present study, the variation in plasma cholesterol levels seen in animals fed the test diet might be due, at least in part, to this phenomenon of hypo- and hyper-responsiveness. The young and adult groups in particular exhibited a wider range of values for total and LDL cholesterol than did the older group. McMahan et al. (1980) observed a similar degree of variation in cholesterol levels in M. nemestrina fed a 0.1% cholesterol diet and attributed the differences to genetic individuality in response to the diet. Breeding studies performed on squirrel monkeys have indicated that the trait is heritable (Lofland et al. 1974). Similar studies would have to be performed on M. nemestrina in order to gain further information on possible genetic individuality in response to dietary cholesterol.

Fiber content in the diet can also influence plasma cholesterol levels. Both the control and experimental diets used in this study contained a relatively high level of dietary fiber, 6.3% by weight, which is necessary for optimal growth of nonhuman primates. In this respect the experimental diet does not resemble the low-fiber Western diet which typically contains only 0.5 to 3% fiber (Robertson 1972). Certain types of dietary fiber have been shown to indirectly lower plasma cholesterol levels by increasing fecal excretion of bile acids (Keys 1961). Theoretically, the liver compensates by utilizing more cholesterol for bile acid synthesis so that less cholesterol enters the plasma via circulating lipoproteins (Trowell 1972). Hence, the high level of dietary fiber may have provided additional protection to the monkeys against developing more severe hypercholesterolemia than was observed.

The elevated plasma cholesterol levels seen in M. nemestrina were due primarily to increases in the LDL fraction with slight but significant elevations in HDL cholesterol as well. Similar results have been reported for this species as well as for rhesus, green and cynomolgus monkeys (McMahan et al. 1980; Ershow et al. 1981; Klein and Rudel 1983). In all of these studies, however, moderate levels of cholesterol enrichment were used (0.1 to 0.2%) and total plasma cholesterol in the animals never exceeded 300 mg/dl. Higher levels of cholesterol enrichment (0.5 to 1.0%) appear to induce either a biphasic response or a decline in HDL cholesterol levels. Nelson et al. (1983) reported a 50% rise in HDL cholesterol in the Old World

monkey Cercocebus atys until total plasma cholesterol levels reached 450 mg/dl; at this point HDL cholesterol gradually fell to 46% of baseline concentrations. Kushwaha et al. (1982) reported a similar decline in HDL cholesterol in M. nemestrina once mean total plasma cholesterol levels exceeded 500 mg/dl. While the mechanism responsible for this drop in HDL has not yet been elucidated, the reduction seems to be correlated, at least in some species of nonhuman primates, with a high level of dietary cholesterol and a condition of severe hypercholesterolemia (Nelson et al. 1983).

Although changes in lipoprotein cholesterol distributions with cholesterol feeding have been well-documented, effects on lipoprotein protein composition have not been studied as extensively. In this study, LDL, HDL and total protein levels followed the patterns observed for cholesterol distributions. Animals fed the experimental diet exhibited highly significant elevations in LDL and total protein ($p < 0.001$) and moderate increases in both VLDL and HDL protein ($p < 0.05$). Significant elevations were also observed for LDL apo B-100 and HDL apo A-I contents ($p < 0.001$ and 0.05 respectively). Given that these apoproteins are the major surface proteins in LDL and HDL respectively, and that concomitant increases in cholesterol were also observed, it is reasonable to conclude that the experimental diet enhanced the number of these lipoprotein particles. Elevations in LDL and HDL protein were observed in cholesterol-fed rhesus monkeys (Ershow et al. 1981) although individual apoprotein analysis was not performed. Srinivasan et al. (1984) reported an increase in LDL apo B-100 content in cholesterol-fed cynomolgus monkeys which was accompanied by either no change

or a decline in HDL apo A-I levels. Although the latter conflicts with results presented in this study, different dietary regimens could be responsible for the discrepancy. Srinivasan et al. (1984) fed their animals a 0.5% cholesterol diet which resulted not only in decreased apo A-I levels, but also a reduction in HDL cholesterol. In contrast, the more moderate level of dietary cholesterol used in this study led to increased HDL cholesterol accompanied by elevated apo A-I levels. Apparently changes in apo A-I levels directly corresponded to changes in HDL cholesterol regardless of whether the diet enhances or depresses HDL concentrations. Further evidence for this correlation was shown by Kushwaha et al. (1982), who reported a decline in both HDL cholesterol and apo A-I content in cholesterol-fed M. nemestrina.

Although in this study diet appeared to play the most significant role in changing lipoprotein profiles in M. nemestrina, interesting age effects were also observed in animals consuming the experimental diet. While all animals exhibited increases in LDL and total cholesterol, the levels were elevated in the oldest group at a magnitude significantly less than that observed in the two younger groups ($p < 0.025$). Similar trends were apparent for LDL protein ($p < 0.05$) and LDL apo B-100 content ($p < 0.09$). This age effect was not reported by McMahan et al. (1980) in cholesterol-fed M. nemestrina since only animals ranging in age from 1 to 8 years old were included for their study. Therefore the age effect observed in this study would not have been apparent since it occurred in animals 18 years and older. Evidently a cholesterol-enriched diet is necessary to induce this trend since the older animals fed the control diet in this study did not

exhibit significantly lower LDL and total cholesterol levels than their younger counterparts. Further evidence for this implication is provided by Lacko and Hazzard (1979) who noted no significant differences in LDL and total cholesterol in 4, 10 and 20 year old female M. nemestrina fed a basal low-cholesterol diet.

The nature of the decline in LDL and total cholesterol concentrations in the older animals fed the experimental diet can be explained by several possible mechanisms. An increased rate of LDL clearance may be responsible but it is highly unlikely since LDL receptor expression has been shown to decrease with increasing age in swine, in dogs and in human diploid fibroblasts in culture (Hui et al. 1981; Mahley et al. 1981; Lee et al. 1982). Theoretically, LDL levels in the monkeys should have increased with increasing age due to this depression in receptor activity. Equally unlikely is the possibility that a selective process based on efficient cholesterol clearance may be responsible because animals in the old group were at least 18 years old prior to beginning the 3 year treatment period. Since it is doubtful that lower plasma LDL levels in the old monkeys may have resulted from more rapid clearance by the liver and peripheral tissues, the most logical explanation is that the cholesterol did not enter the plasma via the lipoprotein pathway. This failure could have resulted from either an inability of the liver to package the cholesterol into lipoproteins, or from intestinal malabsorption of the added dietary cholesterol.

Based on what has been observed in aging humans, the latter hypothesis is probably a more plausible explanation for the decline in LDL cholesterol in the oldest animals. LDL cholesterol levels in humans tend to increase with increasing age until age 70 whereupon they begin to decline; both cross-sectional and longitudinal studies have shown similar findings, suggesting that the phenomenon is not due to a process of selection (Rifkind et al. 1979; Hershcopf et al. 1982). The decline in LDL cholesterol beyond age 70 could be attributed to a reduction in the efficiency of fat absorption. Webster et al. (1977) compared lipid absorption between young (mean age of 26) and old (mean age of 82) human subjects by administering an oral fat tolerance test. A significant reduction in fat absorption in the elderly was observed as compared to their younger counterparts. However, when pancreatic enzymatic extracts were administered to the elderly subjects along with the fat load, differences in absorption between the two age groups were minimized and found to be statistically insignificant. Therefore, Webster et al. (1977) concluded that reduced pancreatic function could be partially responsible for decreased fat absorption in the elderly.

Although a fat tolerance test was not performed on the test animals in this study, it is possible that malabsorption may be responsible for the observed decline in LDL cholesterol. Pancreatic insufficiency could have contributed to the malabsorption, as could have atrophy of the intestinal mucosa which is often seen in aging monkeys (Lapin et al. 1979). The animals in this group were at least 18 years old at

the start of the project; some of the animals were as old as 23 years when samples were collected three years later. Relatively speaking these monkeys were very elderly since the maximum lifespan of M. nemestrina has been estimated conservatively at 25 years (Bowden and Jones 1979). However, no conclusions can be drawn since fat absorption was not measured in this study. The possibility still exists that the lower LDL cholesterol levels may have resulted from an inability of the liver to package and/or excrete lipoproteins into the plasma. This hypothesis seems less plausible than malabsorption, however, since metabolic and kinetic studies in humans have shown little variation in LDL production throughout the lifespan (Miller 1984).

In addition to diet and age effects, variation between sexes was also observed in this study. The differences were confined to animals in the young and adult groups and were apparent only in HDL cholesterol, protein and apo A-I contents. In both control and test animals, males in the young group exhibited significantly lower HDL cholesterol, protein and apo A-I concentrations ($p < 0.05$) than females of the same age. This trend was not apparent in the adult group where males had equal or slightly higher values than their female counterparts. These variations appear to be true sexual differences since they were apparent regardless of the dietary treatment imposed. No sexual variations were observed in the oldest animals. This sexual variation in lipoprotein profiles is comparable to that observed in humans. During adolescence, males experience a

decline in HDL cholesterol whereas levels for adolescent females remain relatively constant. After sexual maturation is achieved HDL levels in males typically begin to rise so that by the mid-twenties the values for males and females are fairly close (Rifkind et al. 1979). Sex hormones are thought to be responsible for the male-female variability in lipoprotein profiles. Estrogens tend to elevate plasma HDL while androgens have the opposite effect (Furman et al. 1958). Therefore the drop in HDL in adolescent males has been attributed to increased androgen secretion; once sexual maturation is completed, androgen secretion stabilizes at a lower rate and the HDL equilibrate at a higher level (Morrison et al. 1979).

Differences in circulating levels of sex hormones may have contributed to the variation in HDL observed in male and female monkeys in this study. In nonhuman primates maximum fertility is generally achieved by age 6 (Lapin et al. 1979); in the present study animals in this age range would be classified as young (3 to 4 years old at the start). Therefore, if circulating sex hormones were highest during this period of peak fertility, HDL levels in females would be elevated whereas those in males would be depressed due to the opposite effects of estrogens and androgens on HDL concentrations. In keeping with this hypothesis, it is possible that males and females in the adult group, past the period of maximum fertility, exhibited similar HDL levels due to a reduction in sex hormone secretion. No definite conclusions can be drawn, however, since this hypothesis is based solely on what is seen in humans. Although male-female variability in lipoprotein

profiles has been documented in several species of nonhuman primates (Rudel and Pitts 1978; McMahan et al. 1980), few studies have characterized changes in lipoprotein profiles during and after sexual maturation.

SUMMARY

A colony of 66 male and female pig-tailed macaques (Macaca nemestrina) were divided into three age groups representing young, middle-aged and elderly animals. The monkeys were fed either a control diet or a diet resembling that of a typical American for three years. Plasma lipid, protein and apolipoprotein concentrations were analyzed to determine the interacting influences of sex, aging and nutrition on lipoprotein metabolism.

The experimental diet greatly elevated LDL cholesterol, protein and apo B-100 concentrations, with slight but significant increases occurring in HDL cholesterol, protein and apo A-I contents as well. Age effects were also observed in animals fed the experimental diets. Although all animals fed the test diet experienced a rise in LDL cholesterol, protein and apo B-100 concentrations, the magnitude of increase in the oldest animals was significantly less than that observed in the two younger groups. Sexual variability in lipoprotein distribution was present in younger animals and appeared regardless of the dietary treatment imposed. Males in the young group had significantly less HDL cholesterol, protein and apo A-I than did their female counterparts. This differences was not apparent in the adult group, however, where males showed equal or slightly higher levels than females. No significant sexual variability was observed in the oldest group.

These results, when compared to data derived from both animal and human studies, suggest that M. nemestrina may be a suitable model for studying not only how sex, aging and nutrition interact to modify lipoprotein metabolism, but also how these factors can contribute to the development of atherosclerosis. Dietary cholesterol appears to modify lipoprotein distributions in M. nemestrina to resemble profiles seen in humans consuming a cholesterol-containing iet. Although the diet-induced response in the oldest animals was of a lesser magnitude than observed in younger animals, the difference more likely resulted from a reduction in fat absorption rather than from an increase in plasma lipoprotein metabolism. M. nemestrina may also be a suitable model for studying the so-called "female protection" against atherosclerosis since sexual differences comparable to those seen in humans were observed in the younger animals in this study.

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