ABSTRACT

Terri D. Sells. RETARDATION OF U937 MACROPHAGE PROLIFERATION BY LOW DENSITY LIPOPROTEINS OF PATIENTS WITH TYPE II DIABETES. (Under the direction of Dr. Hisham A. Barakat) Department of Biology, August, 1990.

Low density lipoproteins (LDL), isolated from seven morbidly obese subjects with noninsulin-dependent diabetes mellitus (NIDDM) and seven lean nondiabetic women, were characterized chemically and physically. The chemical composition of LDL from NIDDM patients differed from that of the LDL of the lean controls in cholesteryl ester content (30.9 ± 1.53 for the NIDDM vs. 37.6 ± 2.33 for the controls, $P < 0.05$) and triglyceride content (10.6 ± 1.06 vs. 7.80 ± 0.807, $P < 0.05$). No differences in the protein, phospholipid or free cholesterol content of LDL of the two groups were observed. The LDL of both groups was subjected to polyacrylamide gradient (2-16%) gel electrophoresis (PAGGE), stained and scanned. The scans of the LDL of NIDDM patients showed multiple peaks, suggesting the existence of several LDL subpopulations, whereas scans of the LDL of the control group showed a single peak. These findings suggest that both the chemical and physical properties of LDL of patients with NIDDM are different than those of lean controls. The purpose of this work, therefore, was to assess the influence of such changes in the physical and chemical properties on their metabolism. To that end, the growth rate of the macrophage-like cell line, U937, was monitored following incubations with LDL from each group of subjects. The U937 macrophage requires exogenous sources of cholesterol, such as that from LDL, for growth and proliferation. Thus, U937 cells could be used as an experimental tool to evaluate the

biological integrity of LDL.

The increase in the number of U937 cells over 72 hours in response to incubations in the presence of 2, 3 and 10 ug of LDL protein was monitored. At 2 ug/ml of LDL-protein, total cell number in the presence of LDL of NIDDM was 1.7×10^5 cells/ml compared to 2.1×10^5 cells/ml ($P < 0.01$); at 3 ug/ml of LDL protein, 1.9×10^5 cells/ml for diabetics vs. 2.3×10^5 cells/ml for normal controls ($P < 0.01$) and at 10 ug/ml of LDL protein, 2.5×10^5 vs. 3.0×10^5 cells/ml for diabetic vs. control LDL respectively. This retardation of cell proliferation by LDL of diabetic patients may be the result of aberrations in binding, internalization or degradation of LDL. Thus, it is concluded that the changes in the physical, chemical and biological properties of LDL of patients with NIDDM may contribute to the increased risk and incidence of coronary heart disease associated with NIDDM.

RETARDATION OF U937 MACROPHAGE PROLIFERATION BY LOW DENSITY LIPOPROTEINS OF PATIENTS WITH TYPE II DIABETES

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Terri D. Sells

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RETARDATION OF U937 MACROPHAGE

PROLIFERATION BY LOW DENSITY LIPOPROTEINS

OF PATIENTS WITH TYPE II DIABETES

by

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INTRODUCTION

Atherosclerosis is a disease process characterized by the accumulation of cholesterol in the arterial wall, which subsequently leads to the formation of plaques that inhibit blood flow. Although atherosclerosis and the other clinical sequelae which accompany it are major complications of diabetes mellitus, the precise mechanisms underlying early atherogenesis in diabetic patients are not fully known.

It is well established that non-insulin dependent diabetes mellitus (NIDDM) is associated with a higher risk and incidence of coronary heart disease (CHD). Epidemiological studies such as the Framingham cohorts have noted a higher incidence of CHD among diabetics as compared to non-diabetics.[1] Furthermore, it has been reported that when diabetics are first diagnosed, they have already an increased tendency to develop CHD. Among the risk factors associated with this increased tendency are obesity, high triglyceride levels and low high density lipoprotein (HDL) concentrations.[2] Interestingly, the Framingham studies found that diabetes, especially in women, perniciously influences the preponderance of CHD about three times more than that among non-diabetic women.[1-3]

Many pathological sequelae are associated with NIDDM. As noted by Epstein, the precursor stages for this particular malady are characterized by hyperinsulinemia which results from tissue resistance to insulin. Thus, the tissues of patients with NIDDM may be exposed to increased circulating insulin levels for extensive time periods.[4,5] It has been suggested that hyperinsulinemia may lead to early atherosclerosis. The results from two population studies performed in

Finland showed that subjects suffering from CHD have high insulin levels following a glucose challenge.[4] In accordance with this finding, Stout states that high levels of circulating insulin may promote the development of atherosclerosis by the proliferation of smooth muscle cells, an inhibition of lipolysis and the synthesis of lipids.[6] Diabetics undergoing insulin treatment have additionally been found to develop atherosclerosis much more readily.[3]

As a result of insulin resistance, NIDDM patients also suffer from hyperglycemia. The relative impetus of this is based on epidemiological studies done by those such as Ostrander and coworkers in Tecumseh, Michigan. In this particular investigation, 5,140 individuals (16 years and older) were examined. Those individuals diagnosed with one or more manifestations of CHD were also found to have a significantly greater blood glucose level than persons in the normal category. Therefore, hyperglycemia, which is a characteristic feature of NIDDM, may contribute to CHD.[7]

The development of the atherosclerotic plaque begins with the accumulation of cholesterol in the arterial wall. The cholesterol in the plaque is derived from the cholesterol enriched low density lipoproteins (LDL) that circulate in the bloodstream. Epidemiological data have revealed that individuals with high levels of LDL are especially predisposed to atherosclerosis.[8,9]

Low density lipoproteins are spherical particles composed of an outer layer of phospholipid and unesterified cholesterol covering an inner core of cholesteryl esters. Embedded within the outer layer is an apoprotein called B-100 that is recognized and bound by specialized

glycoprotein receptors. These receptors project from the surface of cells and in doing so they perform two important functions: 1) Providing cholesterol for the cells by binding and internalizing LDL; and 2) removing LDL from the bloodstream.[9]

A deficient number of LDL receptors causes high levels of LDL to remain and accumulate in the bloodstream. The importance of the LDL receptor in atherosclerosis is especially seen in patients with familial hypercholesterolemia (FH). Sufferers of FH possess either one or two defective genes for a dominant trait in which the cells express few or no LDL receptors. As a result of this mutation, FH individuals have a circulating LDL level of more than six times higher than normal and they usually experience heart attacks from anywhere between the age of two to twenty.[9]

In addition to the aberrations in the LDL receptor's role in the development of atherosclerosis, the LDL particle itself has an equally menacing role. According to Sniderman, et al., some individuals suffering from CHD have LDL that are enriched in protein (apoprotein B-100) and triglycerides, but low in cholesteryl ester content.[10] As a result, these LDL particles are smaller and more dense than normal LDL. These denser particles may arise by a series of events in which the lipid transfer protein-mediated exchange of LDL-cholesteryl ester for very low density lipoproteins(VLDL)-triglyceride takes place. Furthermore, a decrease in the LDL size results from lipolysis of the LDL triglyceride molecules.[11,12] In vitro studies by Kinoshita, et al., have shown that by incubating LDL with VLDL in the presence of partially purified human plasma lipid transfer protein, a decrease of cholesteryl

esters and a three to fivefold increase of triglycerides are caused. Subsequently, the LDL diameters decrease and selective conformational alterations of several regions of the apoprotein B-100 occur.[13]

In the course of producing denser LDL particles, the molecular weight of the LDL also becomes altered. Crouse and coworkers have shown a close correlation between LDL molecular weight and coronary heart disease. Evidently, the lower the LDL molecular weight, the higher the atherosclerotic risk. This study also reported evidence for an inverse relationship between hypertriglyceridemia and LDL molecular weight.[14]

The metabolism of VLDL to LDL may produce a heterogeneous population of LDL particles with different sizes. Krauss and Burke, as well as Musliner and coworkers demonstrated by size analysis employing polyacrylamide gradient gel electrophoresis that there exists two subfractions, LDL_1 (IDL) and a smaller, more dense LDL fraction, in normal subjects. Some patients suffering from CHD appear to have a great amount of these smaller LDL particles, possibly rendering them more atherogenic than normal sized LDL.[15,16] Barakat, St. Clair and others have demonstrated that hypercholesterolemic serum and LDL isolated from hypercholesterolemic monkeys enhance cholesterol accumulation in cells in culture. Also, these LDL have been shown to be larger and contain an increased number of cholesteryl ester molecules per LDL particle.[17-25]

Much of what has been stated concerning LDL's role in atherosclerosis also applies to the LDL's relationship with diabetes. The prevalence of hypertriglyceridemia, hyperinsulinemia, and obesity are important factors that enhance diabetics' "sensitivity" for CHD as

compared to non-diabetics.[26]

Since LDL has been implicated as the atherogenic agent in the development of CHD, its metabolism has been thoroughly investigated. The precursor of LDL is the triglyceride enriched VLDL. Interestingly, the most common lipoprotein aberration in NIDDM is elevated VLDL levels as reflected by increased total triglyceride or VLDL triglyceride concentrations.[26] Furthermore, lipogenesis, particularly in the liver, is stimulated by the presence of insulin.[6] Therefore, in hyperinsulinemic states, VLDL levels are greatly increased. Even the triglycerides and the apoprotein B-100 moieties are over produced. After VLDL is secreted into the plasma by the liver, it is catabolized to remnants by lipoprotein lipase which then can be converted to LDL.[27] With an increased flux of VLDL into the plasma, as is seen in NIDDM, its catabolism may result in high levels of LDL. The composition of these LDL has been shown to be altered, which may be a consequence of changes in the catabolic pathway.[28] The precise biological pathway for the production of abnormal LDL has not been completely elucidated. However, Kinoshita and coworkers' experimental findings (discussed previously) may provide a possible mechanism. The reasoning behind this statement is that VLDL was an important substrate in the <u>in vitro</u> production of triglyceride-rich LDL and diabetics have modified LDL and high concentrations of VLDL.

NIDDM patients also have been reported to have five percent of the lysine residues of their LDL glucosylated. Steinbrecher and Witztum showed that the extent of this glucosylation that can occur in diabetics may inhibit the binding of the LDL particles to the LDL receptors,

thereby slowing its catabolism.[29]

Barakat and coworkers have investigated in detail the structural and compositional differences of diabetic LDL. In their studies, they found that the molecular weights of LDL of NIDDM patients were significantly lower ($2.78 \pm 0.07 \times 10^6$) from that of lean controls ($2.96 \pm 0.06 \times 10^6$). Another statistically significant finding produced from their studies showed that the diabetic's LDL were depleted of cholesteryl ester molecules per LDL particle (1442 ± 73) as compared to control LDL which were much more enriched (1672 ± 67). Consequently, the diabetic LDL are very dense particles. These abnormalities would suggest a higher atherogenic potential.[30] Although these differences are statistically significant, the biological significance of such alterations has not been demonstrated. Therefore, by joining Dr. Barakat's research efforts, he and I decided the direction of my thesis research should be to follow what others have done with respect to establishing biological significance.

Brown and Goldstein used fibroblasts and macrophages in studying the metabolism of LDL and discovered that fibroblasts bind, internalize and degrade LDL, but do not metabolize chemically modified LDL. When normal LDL was incubated with the macrophages, completely opposite results were observed. The macrophages did not metabolize the normal LDL normally, however, they readily metabolized chemically modified LDL.[31] This procedure is one way of determining the normality of various LDLs in a biological response setting.

Although the methods used by Brown and Goldstein establish the interaction between LDL and receptors or, in other words, LDL

metabolism, the procedures used to define the binding, internalization and degradation of LDL are usually cumbersome, difficult to perform and require large amounts of LDL, as well as include the labeling of LDL with a biological marker or isotope. Furthermore, to determine the biological response of the LDL, they evaluated enzyme activities such as ACAT (acyl-CoA:cholesterol acyltransferase) and determined the amount or degree of stimulation of ACAT or the inhibition of HMG-CoA reductase (beta-hydroxy beta-methyl glutaryl-CoA reductase). The ACAT enzyme catalyzes the esterification of excess cholesterol that has been internalized by the cell, whereas HMG-CoA reductase is the rate limiting enzyme in the de novo synthesis of cholesterol in certain cells.[31] These enzyme activities are very precise and extremely informative, but again they are time consuming and difficult to perform.

More recently, Frostegard and colleagues showed that normal LDL can induce the proliferation of a macrophage cell line, U937, after being serum starved for 24 hours. The U937 macrophages are actually a monoblastoid-premonocyte cell line that was derived from a histiocytic lymphoma. According to Frostegard and coworkers, the U937 cell line has no endogenous means of producing cholesterol, therefore the cells require cholesterol for proliferation and die when placed in a serum free media for longer than 48 hours. Their study showed that cell proliferation is stimulated by the cholesterol obtained from LDL. However, they reported that only LDL from normal individuals induced growth. LDL from persons with defective apoprotein B-100 and other abnormal lipoprotein phenotypes did not stimulate cell growth to the extent that the normal LDL did. Their findings suggest that LDL-induced

growth of U937 macrophages can be used as a novel method in determining biological response differences of LDL from various individuals.[32-34]

Since the focus of this thesis research is evaluating the biological significance of the alterations in structure and composition of diabetic LDL, we employed the latter procedure using U937 macrophages for this purpose.

MATERIALS AND METHODS

The subjects for this study consisted of fourteen females, seven of which served as lean normal controls and the remaining seven were morbidly obese (>100% over ideal body weight) individuals with non-insulin dependent diabetes mellitus (NIDDM). The diabetic subjects were part of the East Carolina University Diabetes Program Project. The normal controls were chosen based on several criteria including weight, body mass index, and lipid profile. None of the subjects were taking any medication that are known to influence lipid and carbohydrate metabolism. Determination of diabetes in the experimental group was based on their insulin responsiveness when subjected to an oral glucose tolerance test.

Approximately 50 ml of blood were collected into tubes from the subjects by venipuncture following a twelve hour fast. Each tube contained 0.75 ml of an anticoagulant cocktail which consisted of 5,5'Dithiobis-(2-Nitrobenzoic acid) (0.4 mg/ml), an inhibitor of Lecithin:Cholesterol Acyltransferase activity; aprotinin (10 Kallikrein inhibitory units/ml), a protease inhibitor, EDTA (0.1 mg/ml), a preservative, and NaN_3 (0.1 mg/ml), a preventative of bacterial contamination. The blood was subsequently centrifuged for 30 minutes at 1500 x g (2600 rpms) in an IEC Centra-7R centrifuge at 4^{o} C, and the plasma was removed from the red blood cells with a pasteur pipette and placed in a 25 ml graduated cylinder. For each 25 ml of plasma collected 20 ul of a protease inhibitor, Phenylmethylsulfonyl Fluoride (0.1 g PMSF in 1.0 ml Dimethylsulfoxide), was added to preserve the

plasma until further use.[30]

The quantitation of the plasma triglyceride concentrations was performed with a kit obtained from Sigma Diagnostics. This procedure is a slight modification of that described by Bucolo and David and involves four reactions. The initial reaction hydrolyzes the triglycerides by microbial lipase to glycerol and free fatty acid. Glycerol is subsequently phosphorylated by adenosine triphosphate (ATP), forming glycerol 1-phosphate (G-1-P) by the action of glycerol kinase (GK). Oxidative treatment of G-1-P with glycerol-1-phosphate dehydrogenase in the presence of nicotinamide adenosine dinucleotide (NAD) gives dihydroxyacetone phosphate (DAP and NADH). The NADH in this reaction reduces an equivalent amount of iodonitrotetrazolium violet, a reaction catalyzed by diaphorase. The product of this reaction is formazen, an intensely colored dye. The reactions are summarized below:

1. Triglycerides ----------> Glycerol + Fatty Acids
2. Glycerol + ATP ----------> G-1-P + ADP
3. G-1-P + NAD ----------> DAP + NADH
4. NADH + INT ---(diaphorase)---> NAD + Formazen

Thus, absorbance of formazen at 500 nm is directly proportional to the original concentration of glycerol, and hence, triglyceride content.[35,36]

Plasma total cholesterol content was determined using a kit obtained from Boehringer-Mannheim. In this assay, all cholesteryl esters present in plasma are hydrolyzed quantitatively into free cholesterol and free fatty acids by cholesterol esterase. The next reaction is oxidation of free cholesterol to cholest-4-en-3-one,

catalyzed by cholesterol oxidase in the presence of oxygen. Hydrogen peroxide formed in this reaction then reacts in the presence of peroxidase with phenol and 4-aminophenazone to form an o-quinone imine dye. The reactions are summarized below:

1. Cholesterol esters $\xrightarrow[\text{cholesterol oxidase}]{\text{cholesterase}}$ Cholesterol + Fatty Acids

2. Cholesterol + O_2 ----------> Cholest-4-en-3-one + H_2O_2

3. H_2O_2 + Phenol + 4-Aminophenazone -----> O-quinone Imine Dye + $2H_2O$

The intensity of the color formed is proportional to the cholesterol concentration and measured photometrically at 500 nm.[37]

In order to quantify HDL cholesterol it was necessary to perform a precipitation procedure described by Warnick, et al. In this procedure, 0.10 ml of a solution containing per liter, 10.0 g dextran sulfate and 0.5 M magnesium chloride was added to one ml of plasma and centrifuged at 1500 x g for 30 minutes. The dextran sulfate-magnesium chloride moiety binds to apo-B containing lipoproteins (i.e. VLDL and LDL) which precipitate out of solution upon centrifugation. The resulting supernatant contains the HDL subfraction.[38] An aliquot of the supernatant was subsequently used in the above procedure to measure HDL cholesterol.[37]

LDL cholesterol concentration was calculated by using the Friedewald equation:[39]

$$C_{LDL} = C_{Plasma} - C_{HDL} - TG/5$$

ISOLATION OF LDL

Isolation of LDL was achieved by following two methods. Havel, et al.'s method of sequential centrifugation[41] was used to isolate the LDL from the plasma of the normal lean controls, whereas the method of Rudel, et al.[42] employing a combination of ultracentrifugation and chromatography was used to isolate the diabetics' LDL. The reason the latter procedure was employed to isolate the diabetic LDL is because the chromatographic method, but not the sequential centrifugation, isolates other lipoproteins that were needed in different studies being undertaken in the laboratory.

Sequential centrifugation involves adjustment of the density of 20.0 ml of plasma (d = 1.006 g/ml) to a density of 1.019 by the addition of solid potassium bromide (KBr) according to the Radding-Steinberg equation.[40] The plasma was then poured into 13.2 ml polyallomer tubes and overlaid with a NaCl solution (150 mM NaCl, .24 mM EDTA, d = 1.006 g/ml). The tubes were centrifuged at 40000 rpms for 18 hours at 15° C in a Beckman SW 41 Ti rotor. The top 1.5 ml fraction containing VLDL and intermediate density lipoproteins (IDL) was removed by slicing the tubes with a Beckman tube slicer. The lower fraction which contains the LDL and HDL was collected and its density adjusted to 1.063 g/ml by adding KBr. It was then poured into tubes, overlaid with a solution of the same density (1.063 g/ml) and centrifuged at 40000 rpms for 40 hours at 15° C in a SW 41 Ti rotor. Following centrifugation, the tubes were sliced once more and the top portion which contains the LDL was recovered.[41]

The LDL of diabetic patients were isolated from plasma by a

combination of centrifugation and column chromatography as previously described by Rudel, et al.[42] Ten ml of plasma were adjusted to a density 1.225 g/ml by the addition of solid KBr, placed in 13.2 ml polyallomer tubes and overlaid with 3 ml of a solution of KBr with a density of 1.125 g/ml containing 0.1% EDTA. The tubes were centrifuged at 40000 rpms for 40 hours at 15° C in a Beckman SW 41 Ti rotor. The top 1.5 ml fraction containing all the floated lipoproteins was recovered by tube slicing. To the lipoprotein concentrate were added trace amounts of ^{125}I-LDL of known molecular weight (3.2×10^6 d) obtained from African Green monkey specifically bred for cardiovascular studies and was a gift from Dr. L. Rudel of the Bowman Gray School of Medicine, Winston Salem, NC. This fraction was then applied to a 4% agarose (Bio Gel A-15M, 200-400 mesh) column (1.5 x 90 cms) which had been equilibrated with a solution containing 0.15 M NaCl, 0.01% EDTA, 0.01% NaN_3, pH = 7.4. The lipoproteins were eluted from the column with the same buffer, with continuous monitoring of absorbance at 280 nm and a flow rate of approximately 8 ml/hr. The collected fractions were assayed for radioactivity, and the fractions corresponding to the peak of ^{125}I-LDL internal standard were pooled.

The LDL fractions collected from each procedure were dialyzed against 30 liters of buffer containing 150 mM NaCl and 0.24 mM EDTA, pH = 7.4, for 48-72 hours with five changes of buffer, six liters each. The dialyzed LDL fractions were placed in a pro-di-con apparatus which contained the same buffer that was used for dialysis and concentrated to protein concentrations of 0.5-1.0 mg/ml as determined by the Lowry, et al., method. Bovine serum albumin standards in concentrations of 2.5, 5,

10, 15, 20, 30, 40, 50, and 60 mg/ml were used in determining protein concentrations of the LDL.[4]

CHEMICAL AND PHYSICAL CHARACTERIZATION OF LDL

The chemical compositions of LDL included determination of triglycerides, total cholesterol, protein (previously described), phospholipids, free cholesterol, and cholesteryl esters. Physical characterization included the determination of LDL diameters.

LDL lipids were extracted by the method of Bligh and Dyer.[44] Equal volumes of methanol and chloroform and a volume of deionized water (a ratio of 2:2:1.8, CH_3Cl_3:MeOH:H_2O) were added to a known aliquot of each sample. The samples were thoroughly vortexed and centrifuged. After centrifugation, the top methanol:water layer was aspirated off and the lower chloroform layers were removed using a pasteur pipette and placed in 16 x 125 mm tubes. The samples were then evaporated to dryness under nitrogen. The residues were resuspended in five ml of chloroform:methanol (2:1) and heated at 40° C for 10 minutes.

By following the method of Fiske and SubbaRow, an aliquot was taken from the five ml of lipid extract to determine phospholipid phosphorous content of LDL.[45] This aliquot was placed in 16 x 125 mm acid washed tubes and evaporated to dryness. One ml of 60% perchloric acid was added to each dried extract and then the samples were heated for several hours on a hot plate until the samples had been digested, which was indicated by the clearness of the solutions. The working standards came from a stock solution (0.002% KH_2PO_4) which contained 250 ug of phosphorous per ml. The concentrations of the standards were 2.5, 5,

7.5, and 10 ug/ml and one ml of each was taken for the assay. Once the samples had cooled, four ml of deionized water were added and three ml of deionized water were added to the standards to bring them up to equal volumes. One ml of a 2.5% ammonium molybdate reagent and 0.5 ml of a 0.2% aminonapthosulfonic acid reagent were then added to both the samples and standards. The tubes were heated on an 80^{o} C heating block for exactly 20 minutes and then cooled to room temperature. Once cool, the samples and the standards were transferred to cuvettes and the absorbances measured at 700 nm. A standard curve was established from the absorbances of the working standards and the samples' absorbances were compared to this curve. The sample concentrations determined from the curve were multiplied by 25 to give an accurate value for the LDL phospholipid phosphorous content.

Free cholesterol and cholesteryl esters were measured by the orthophthaldehyde method described by Rudel and Morris.[46] In this procedure, a known amount of LDL-lipids previously extracted in 5 ml chloroform:methanol (2:1) was placed in conical tubes and dried down under nitrogen. The evaporated extract was resuspended in 100-200 ul of chloroform:methanol (2:1). The samples were spotted on thin layer chromatography plates and developed in a solution containing hexane, ethyl ether and acetic acid (146:50:4). Once the plates had been removed from the solvent tank and allowed to dry, they were placed in an iodine vapor tank. The free cholesterol and cholesteryl ester bands were stained by the iodine vapor and identified by comparing them to free cholesterol and cholesteryl ester standards. After the iodine had sublimed, the lipid bands were scraped from the plates and placed in 16

x 125 screw cap tubes. Ten ml of chloroform-methanol (2:1) were added to the scrapings and vortexed thoroughly in order to extract the free cholesterol and cholesteryl esters. The tubes were left to stand overnight. The next day the solvent extracts were removed from the silica gel, placed in 20 x 150 screw cap tubes and evaporated to dryness. The working standards came from a Boehringer-Mannheim cholesterol standard kit containing concentrations of 50, 100, 150, 200, 300, and 400 mg/dl. From each standard stock solution, 150 ul was saponified to yield final concentrations of 7.5, 15, 22.5, 30, 45, and 60 ug. The lipid residues and standards were saponified by adding 0.3 ml of 33% KOH and 3 ml of 95% ethanol and by placing them on a heating block at 60^{o} C for 20 minutes. Following saponification, 10 ml of hexane were forcefully added to each sample as well as 3 ml of deionized water. This mixture was stoppered and vortexed thoroughly. Two layers appeared in the tubes, a top hexane layer and a bottom ethanol-water layer. Four ml of the cholesterol-containing hexane layer from the lipid samples and one ml from the standards were recovered in duplicate and dried down under nitrogen on a 60^{o} C heating block for 10-15 minutes. Two ml of orthophthaldehyde reagent (50 mg orthophthaldehyde in 100 ml acetic acid) were added to each tube mixing thoroughly afterwards. Ten minutes later, one ml of sulfuric acid was slowly added to the tubes which were promptly vortexed resulting in a temperature increase. Once the tubes had cooled, the samples and standards' absorbances were quantitatively read at 550 nm.

The diameters of the LDL particles were determined by using 2-16% polyacrylamide gradient gel electrophoresis under non-denaturing

conditions. The gels and high molecular weight standards were obtained from Pharmacia Laboratories. The running buffer was 0.09M Trizma Base, 0.08M Boric acid, and 3mM EDTA at pH 8.35. The gels were placed in an electrophoresis chamber and allowed to equilibrate to 10-15^{o} C. After equilibration, 125 volts of electricity were applied to the gels for 30 minutes. The LDL samples were prepared by taking 25 ul (5 ug of protein) of the LDL and adding to them in microcentrifuge tubes 14 ul thyroglobulin, 1 ul bromophenol blue stain, 5 ul sucrose and 4 ul albumin. Once combs had been placed in the gels, 5-10 ul of each sample were loaded on the gels. High molecular weight standards with known diameters, thyroglobulin (diameter = 17 nm), ferritin (diameter = 12.2 nm), monkey LDL (diameter = 23.0 nm), and Lp(a) (diameter = 28.9 nm) were also added on a separate lane. The diameters of monkey LDL and Lp(a) (obtained from Dr. L. Rudel) were determined by electron microscopy. Fifty percent glycerol was added to any empty wells. Prior to electrophoresis, 70 volts of electricity were applied to the loaded gels for 20 minutes. The gels were then run vertically for 18-24 hours at 125 volts. After electrophoresis, the gels were fixed by placing them in a solution of 10% sulfosalicylic acid for at least one hour. Subsequently, the gels were stained with a Coomassie blue solution (3.5% perchloric acid and 0.04% Coomassie G-250) for 30 minutes to one hour. The destaining procedure required that the gels be placed in a 10% solution of acetic acid with several changes until the background of the gels were clear.[47]

The LDL diameters were measured after scanning the gels on a laser densitometer. Migration distances (R_f) of the LDL samples were

determined by comparing them to the migration distances of the high molecular weight standards, Lp(a) and monkey LDL.[47]

CELL CULTURE

The macrophage cell line, U937, was the gift of Dr. Kirk Ways, East Carolina University School of Medicine. They were grown in RPMI 1640 media supplemented with 10% fetal calf serum (Sigma Diagnostics) and cultured in a 37° C incubator chamber with a 5% CO_2 atmosphere. Fresh media was added twice a week and the cell density was maintained between 2-8 x 10^5 cells/ml.[32] The purpose of cell culture was to proliferate cells to be used in the assays.

ASSAY PROCEDURES

Cells grown in serum supplemented media, were washed in phosphate buffered saline (PBS) and starved for 24 hours in serum deficient RPMI 1640 media. After starvation, 1.5 x 10^5 cells/ml of media were plated in 35 mm plates in triplicate. It was first necessary to determine optimal conditions for growth by incubating cells with LDL according to protein and cholesterol concentrations. In these preliminary assays, triplicate plates of cells were incubated with varying concentrations of LDL protein. The LDL amounts added to the cells were 5, 10, 25, and 50 ug/ml of media for 48 hours. The same experiment was repeated by incubating the cells (in triplicate) in the presence of 10, 20, 40, 60, and 120 ug/ml LDL cholesterol. Cell numbers were determined after the 48 hour incubation period by counting, using a hemacytometer, and growth curves were constructed for each treatment. Maximal growth was obtained with 10 ug LDL protein, and 20 ug cholesterol. In subsequent

experiments, cells were incubated with submaximal and maximal concentrations of LDL protein (1, 2, 3, 5 and 10 ug/ml) for 72 hours. These concentrations allowed for visualization of differences in the growth curves of cells incubated with normal LDL as compared to the growth curves of cells incubated with diabetic LDL. After the incubation period, the cells in each plate were counted using a hemacytometer. Four counts were obtained for each of the triplicate incubations and an average of every treatment was calculated from these values. .LS1

STATISTICAL ANALYSIS

Statistical analysis of the data generated from the assays was performed by using the SAS statistical package. Although a T-test would have been normally employed to show significant differences between the two groups, the data from each group was subjected to the most stringent statistical test, analysis of variance (ANOVA).

RESULTS

The physical characteristics of the two groups of subjects in this study are shown in Table I. The lean controls had an average age of 25.9 ± 1.37 (mean ± SEM), whereas the average age of the NIDDM patients was 40.1 ± 4.28 ($P < 0.05$). As would be expected, body weight of the patients with NIDDM was significantly higher than that of the lean controls (128.4 ± 15.3 vs. 56.4 ± 2.09, $P < 0.05$). No differences in the height of the subjects in each group were observed. However, body mass index [BMI = weight/height^2 (kg/m^2)] was significantly higher in the patients with NIDDM compared to the controls. Body fat content, determined by hydrostatic weighing, accounted for 51% of the body weight of the diabetic patients. Percent body fat content of the lean group was not determined.

Table II shows the plasma lipid concentrations of each individual in the two groups, and the average values for these determinations are summarized in Table III. No statistically significant differences in plasma cholesterol levels were observed between the two groups (157.3 ± 9.39 vs. 161.1 ± 4.28, NIDDM vs. lean controls, respectively). High density lipoprotein cholesterol (HDL-CHOL) levels of the NIDDM patients were significantly lower than those of the lean controls (37.9 ± 3.86 vs. 56.9 ± 4.42). Triglyceride levels were elevated in the diabetic patients compared to the lean controls (119.9 ± 10.7 vs. 79.9 ± 9.79, $P < 0.05$). LDL-cholesterol levels were not different in the two groups. Both total cholesterol/HDL-CHOL and LDL-CHOL/HDL-CHOL ratios were higher in the diabetic group versus the control group. These ratios are more

reflective of CHD risk than the values for each lipoprotein alone. The accepted normal value of total cholesterol/HDL-CHOL for women is 3.5, above that value, CHD risk is suggested.

Table IV shows the average percent weight of the components of LDL from the diabetic and normal groups. The percent composition of protein (26.5 ± 1.20 vs. 25.8 ± 1.40), free cholesterol (8.74 ± .388 vs. 7.67 ± 1.59) and phospholipids (23.1 ± .944 vs. 21.3 ± 1.52) were not significantly different between the two groups. However, statistical differences were observed between the two groups for percent composition of cholesteryl esters and triglycerides. The percent content of cholesteryl esters was higher for the normal group (37.6 ± 2.23 vs. 30.9 ± 1.53, $P < 0.05$), whereas the percent content of triglycerides (10.6 ± 1.06 vs. 7.80 ± .807, $P < 0.05$) was higher for the diabetic group. It should be noted, however, that in comparing the percent lipid compositions to the normal range values reported in the literature the percent protein and cholesteryl ester values for both the diabetic and control groups fall out of the range of reported values. However, the other percent lipid categories are comparable to the cited values. The reason for the above discrepancies remains unclear. Nonetheless, there were chemical differences in the LDL composition between the two groups when the same methods were used in determining the composition of LDL. As for the LDL diameters, no significant differences were observed between the two groups. Even though the LDL diameters did not vary much, the scans of the gels revealed that the diabetics' LDL samples contained several subclasses of smaller sized LDL, whereas the scans of the normal subjects' LDL revealed only one subclass (Figure 1).

CELL CULTURE STUDIES

PRELIMINARY ASSAYS: Preliminary assays were performed in order to determine several things: 1) To repeat what had been done by Frostegard, et al., thereby proving that this method is a sound and viable test of differences in cell proliferation when incubated with LDL from normal and diabetic individuals; 2) to establish optimal culture conditions for maximum cell growth; and 3) to determine final experimental conditions which would consequently show differences in the ability of the two groups' LDL to stimulate maximum cell proliferation.

The preliminary studies involved incubating the U937 macrophages which had been serum starved for 24 hours with varying amounts of LDL according to protein and cholesterol concentrations from two normal individuals for 48 hours. By incubating the cells according to LDL protein and cholesterol, maximal and submaximal concentrations of LDL protein or cholesterol needed for cell growth were established. The growth curves generated by these experiments were comparable to those reported by Frostegard and coworkers. Figure 2 shows the average increase in cell numbers during incubations with varying amounts of LDL cholesterol for 48 hours. The concentrations added to the cells were 10, 20, 60 and 120 ug/ml of LDL cholesterol which corresponded to cholesterol concentrations of 0.025, 0.050, 0.150 and 0.300 mmol/l. By adding these amounts of LDL cholesterol, the cells were sufficiently loaded with cholesterol for maximal cell proliferation. Ten ug/ml of LDL cholesterol induced an average total growth of 2.0×10^5 cells/ml. When 20 ug/ml of LDL cholesterol was incubated with the cells, an increase of 2.5×10^5 cells/ml was observed. Cell proliferation of 2.6

x 10^5 cells/ml occurred when 60 and 120 ug/ml of LDL cholesterol were added to the cultures. The average percent increase in cell numbers over starting numbers when incubated with different LDL cholesterol concentrations is represented in Figure 3. Average percent increases of 33%, 67%, 73% and 73%, respectively, correspond to the LDL cholesterol concentrations of 10, 20, 60 and 120 ug/ml.

The average increase in cell numbers when incubated with 5, 10, 25 and 50 ug/ml concentrations of LDL protein (which correspond respectively to 0.0125, 0.025, 0.0625, and 0.125 mmol/l) for 48 hours is shown in Figure 4. This graph shows that when cells were incubated with 5, 10 and 25 ug/ml LDL protein, similar total increases of 2.0 x 10^5 cells/ml were observed, whereas 50 ug/ml of LDL protein induced a cell increase of 2.6 x 10^5 cells/ml. Figure 5 shows an average percent increase of cells above starting cell numbers when incubated with LDL protein concentrations. The average percent values which corresponded to the LDL protein concentrations of 5, 10, 25 and 50 ug/ml were 37%, 37%, 37%, and 53%, respectively.

Based on these findings, another preliminary assay was performed using the same two LDL samples. Unlike the first assay, LDL concentrations according to protein values of 1, 2, 3, 5 and 10 ug/ml were incubated with the cells. Since these concentrations were very minute, an incubation period of 72 hours was employed to ensure maximal cell growth. After the incubation period, the cells were counted and an average growth curve constructed (Figure 6). From this curve, the LDL protein concentrations of 2, 3 and 10 ug/ml were decided upon to use in the succeeding experimental assays because 2 and 3 ug/ml achieves

submaximal stimulation and 10 ug/ml can cause maximal stimulation of cell growth.

EXPERIMENTAL ASSAYS: In the following assays, it was important to incubate the cells with equal amounts of LDL protein from the diabetic and normal subjects because of the differences detected in the LDL with respect to cholesteryl ester content. Figure 7 shows the increases in cell numbers for each normal LDL sample incubated with the cells at concentrations of 2, 3 and 10 ug/ml of LDL protein. Similarly, Figure 8 shows the increases in cell numbers for each diabetic LDL sample added to the cells at the same concentrations as above. Figure 9 shows average cell increases for each group. As shown in Table V, there are significant differences ($P < 0.01$) between the diabetics' LDL and normal controls' LDL in their ability to stimulate cell growth at each concentration of LDL protein added. With the addition of 2 ug/ml of LDL protein, a total cell increase of $2.1 \pm 0.090 \times 10^5$ for the lean controls versus $1.7 \pm 0.117 \times 10^5$ for the NIDDM patients was observed. The lean controls' LDL on the average stimulated a cell increase of $2.3 \pm 0.085 \times 10^5$ cells/ml as compared to the diabetics' average cell increase of $1.9 \pm 0.0106 \times 10^5$ cells/ml when incubated with 3 ug/ml of LDL protein. The average cell increase for the lean control group when 10 ug/ml of LDL protein had been added was $3 \pm 0.048 \times 10^5$ cells/ml, a virtual doubling of the original cell number. The diabetic group's LDL at this concentration, however, did not stimulate cell proliferation to the same extent ($2.5 \pm 0.123 \times 10^5$ cells/ml). Figure 10 shows the percent increase of cell numbers above starting cell numbers. Clearly, the normal group's LDL on the average caused a greater percent increase

when compared to the diabetics' values. In fact, at the maximal concentration, the cells only proliferated up to 67% of that of the controls when incubated with the diabetic LDL. Similarly, in the submaximal concentrations, i.e. 2 and 3 ug/ml of LDL-protein, cell growth only had a 13 and 27 percent respective increase, whereas cell growth when incubated with normal LDL at these same concentrations was as high as 40 and 53 percent. In conclusion, the results show that under maximal or submaximal concentrations of LDL protein from diabetics, cell growth was retarded in comparison to the cell growth induced by normal LDL.

TABLE I. Characteristics of the Subjects in the Two Groups.

	NIDDM	LEAN CONTROLS
AGE (yrs)	40.1 ± 4.28	25.9 ± 1.37*
WEIGHT (kg)	128.4 ± 15.3	56.4 ± 2.09*
HEIGHT (m)	1.64 ± 2.76	1.66 ± 1.84
BMI (kg/m^2)	47.9 ± 5.05	20.5 ± .748*
% BODY FAT	51.0 ± .980	ND

*P < 0.05: ANOVA. Values are mean ± SEM.

TABLE II. Concentrations of Lipids in the Plasma of each Individual in the Two Groups.

SUBJECTS	CHOL	HDL-CHOL	TRIG	LDL-C	CHOL/HDL-C	LDL-C/HDL-C
NORMAL (mg/dl)						
TS	149	57	132	66	2.61	1.16
CB	150	61	52	79	2.46	1.30
JF	168	52	76	101	3.23	1.94
JB	168	64	61	92	2.63	1.44
GM	152	48	88	86	3.17	1.79
MW	179	40	80	123	4.48	3.08
DK	162	76	70	72	2.13	0.947
DIABETIC						
RS	119	56	127	38	2.13	0.679
DS	163	37	90	108	4.41	2.92
BP	179	40	127	114	4.48	2.85
MT	187	32	166	122	5.84	3.81
LM	171	38	87	116	4.50	3.05
JK	149	22	139	99	6.77	4.50
RS	133	40	103	72	3.33	1.80

Table III. Concentrations of Lipids in the Plasma of the Two Groups.

	NIDDM	LEAN CONTROLS
	mg/dl	
CHOLESTEROL	157.3 ± 9.39	161.1 ± 4.28
HDL-CHOL	37.9 ± 3.86	56.9 ± 4.42*
TRIGLYCERIDES	119.9 ± 10.7	79.9 ± 9.79*
LDL-CHOL	95.6 ± 11.4	88.4 ± 7.29
CHOL/HDL-C	4.49 ± .576	2.96 ± .293*
LDL-C/HDL-C	2.80 ± .475	1.67 ± .269*

* $P < 0.05$: ANOVA. Values are mean ± SEM.

TABLE IV. Percent Weight of the Components of LDL from the Two Groups.

VALUES	NIDDM	LEAN CONTROLS	LIT.
PROTEIN	26.5 ± 1.20	25.8 ± 1.40	18-22
FREE CHOLESTEROL	8.74 ± 0.388	7.67 ± 1.59	6-8
CHOLESTERYL ESTERS	30.9 ± 1.53	37.6 ± 2.23*	45-50
TRIGLYCERIDES	10.6 ± 1.06	7.80 ± 0.807*	4-8
PHOSPHOLIPIDS	23.1 ± 0.944	21.3 ± 1.52	18-24
LDL DIAMETERS (nm)	25.2 ± 0.188	25.3 ± .213	23-26

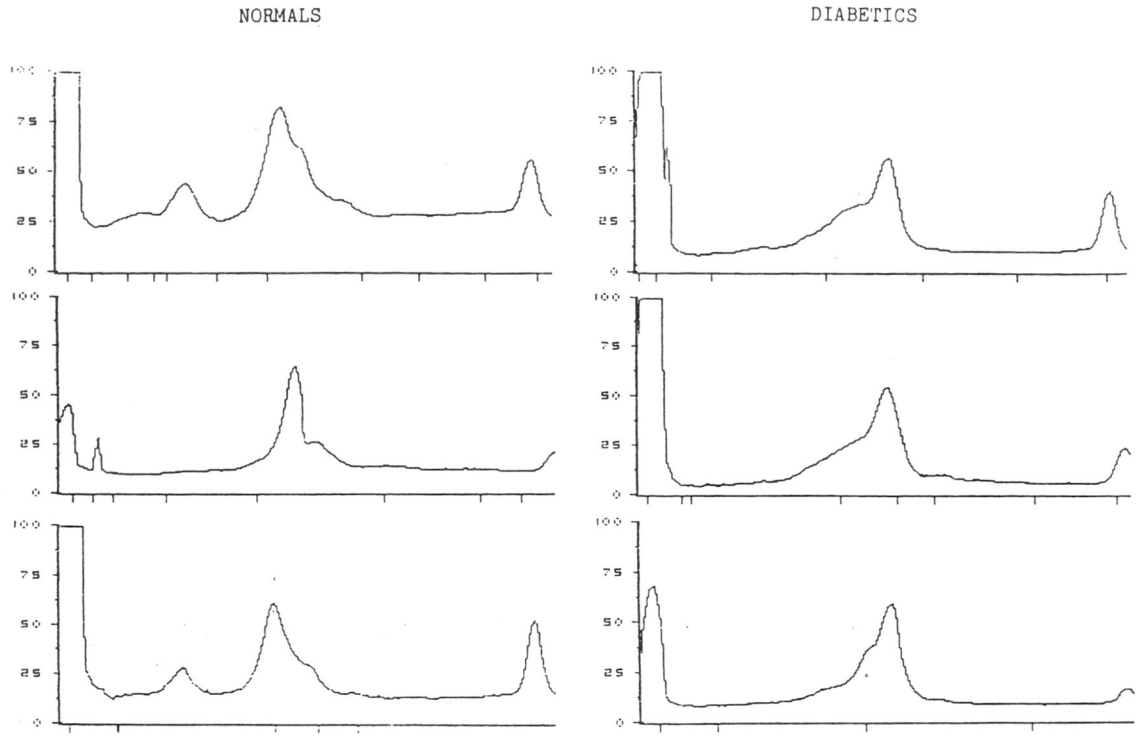
* $P < 0.05$: ANOVA. Values are mean ± SEM.

TABLE V. Average Cell Counts at the Various Concentrations for the Two Groups.

PROTEIN CONCENTRATIONS (ug/ml)	NIDDM	LEAN CONTROLS
TWO	$1.7 \pm 0.117 \times 10^5$	$2.1 \pm 0.090 \times 10^{5*}$
THREE	$1.9 \pm 0.106 \times 10^5$	$2.3 \pm 0.085 \times 10^{5*}$
TEN	$2.5 \pm 0.123 \times 10^5$	$3.0 \pm 0.048 \times 10^{5*}$

* $P < 0.01$: ANOVA. Values are mean ± SEM.

Figure 1. Densitometric scans based on polyacrylamide gradient gel electrophoresis of low density lipoproteins (LDL) from three individuals in each group, diabetic and normal lean. The lower illustrations taken from Austin and coworkers' study[50] shows the densitometric scans of LDL with different subclass patterns. Pattern A is representative of normal LDL, pattern B is indicative of persons with CHD.



Densitometric scans based on gradient gel electrophoresis of low-density lipoproteins (LDL). Top, Controls and cases with LDL subclass pattern A, defined as having major peak of relatively large LDL particles and secondary peak of smaller LDL particles. Bottom, Controls and cases with LDL subclass pattern B, characterized by major peak of small LDL particles and skewing of curve toward larger particle diameters.

(Austin, et. al., 1988)

Figure 2. The effect of LDL cholesterol concentrations on U937 cell growth. Serum starved U937 cells (1.5×10^5) were seeded out in 1 ml of RPMI 1640 media and allowed to grow for 48 hours in the indicated concentrations of LDL according to cholesterol from two normal lean controls. The cells were then removed from the plates by pipetting and final cell number was determined by using a hemacytometer. Each value represents the mean of triplicate cultures.

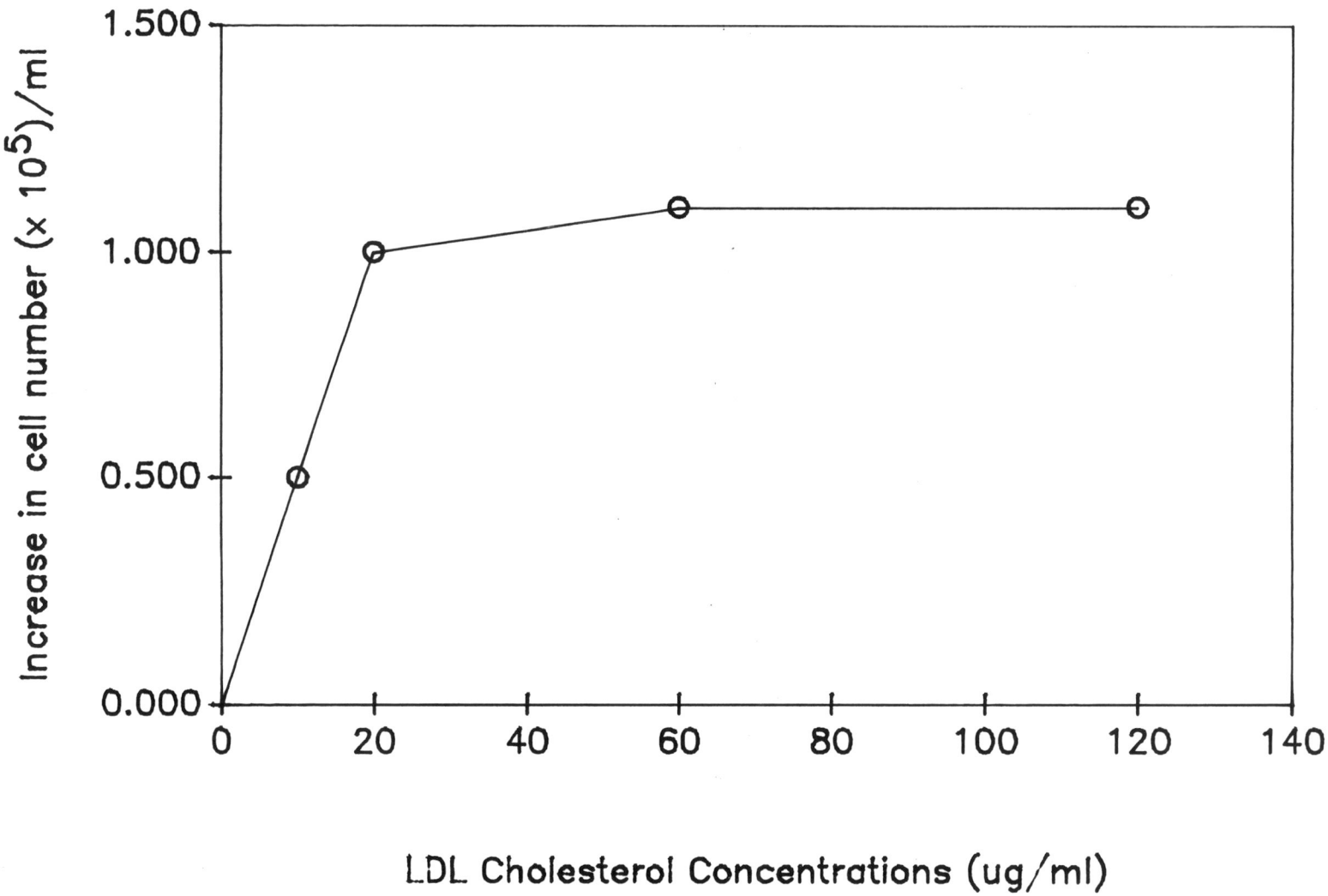

1.500
1.000
0.500
0.000
Increase in cell number (x 10^5)/ml
0
20
40
60
80
100
120
140
LDL Cholesterol Concentrations (ug/ml)

Figure 3. The effect of LDL cholesterol concentrations on U937 cell growth. The values are expressed as percent of the mean increase in cell number over starting cell numbers.

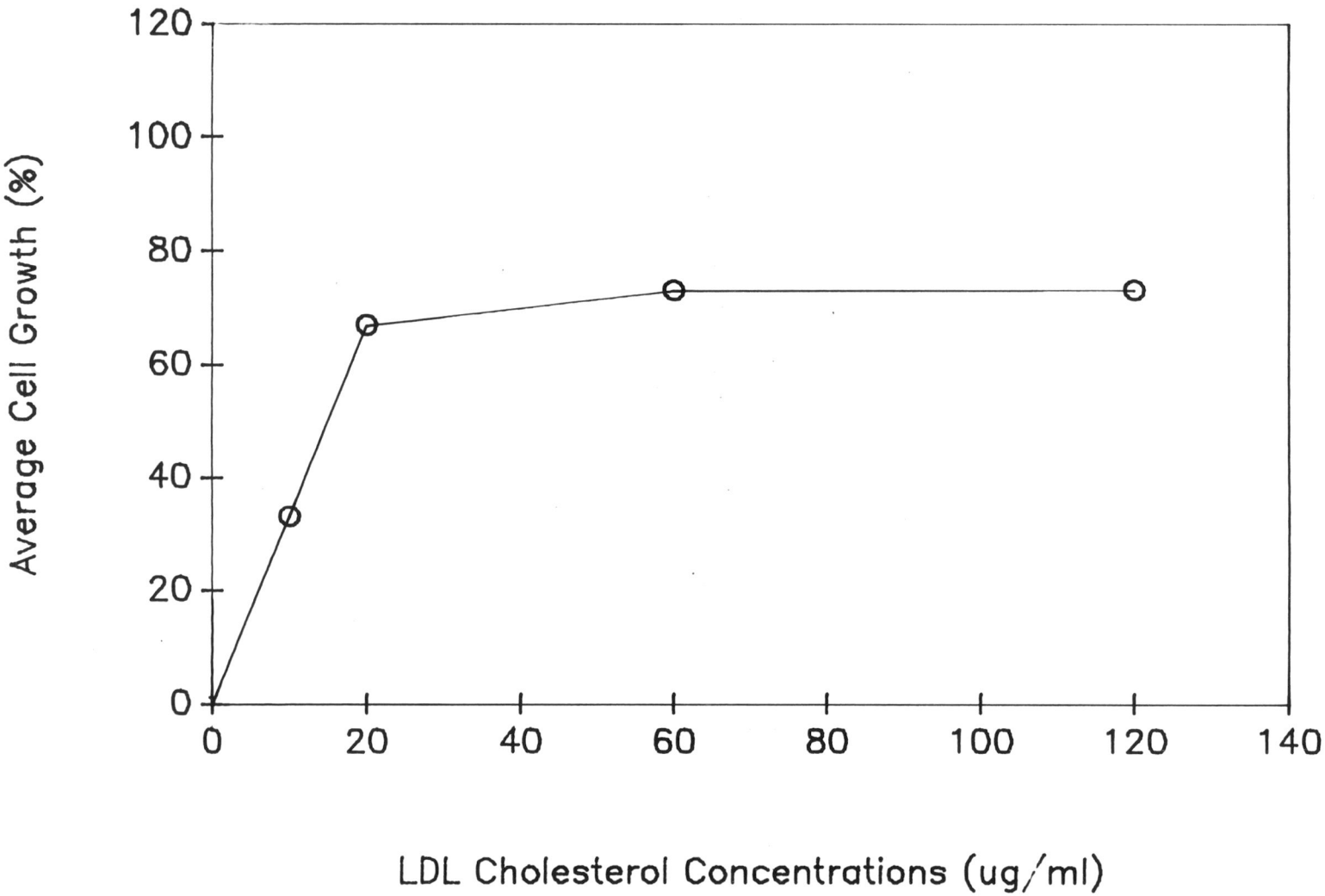

Average Cell Growth (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
LDL Cholesterol Concentrations (ug/ml)

Figure 4. The effect of LDL protein concentrations on U937 cell growth. The experimental conditions were as described in the legend to Figure 2.

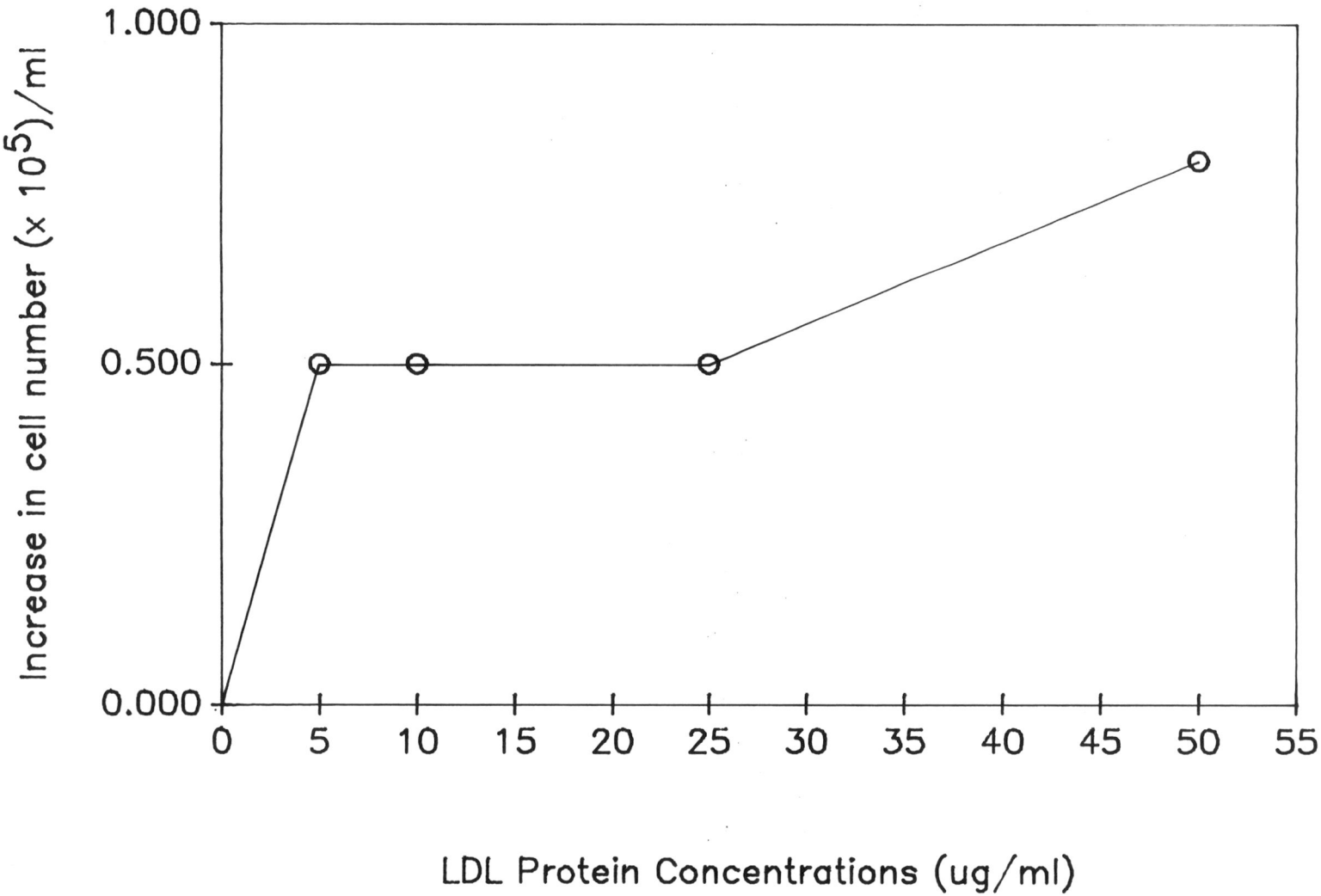

1.000
0.500
0.000
Increase in cell number (x 10^5)/ml
0
5
10
15
20
25
30
35
40
45
50
55
LDL Protein Concentrations (ug/ml)

Figure 5. The effect of LDL protein concentrations on U937 cell growth. The values are expressed as percent of the mean increase in cell number over starting cell numbers.

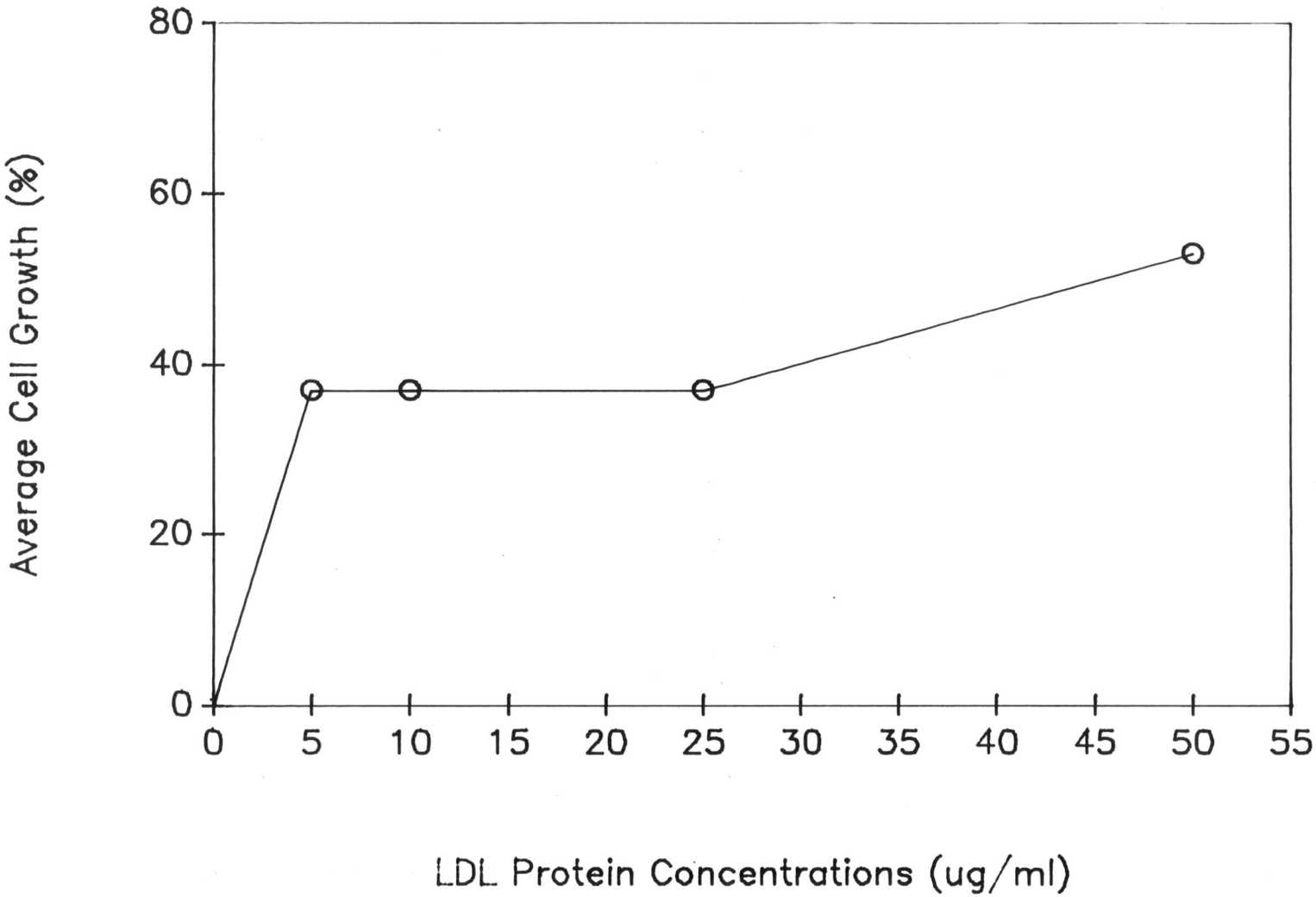

Average Cell Growth (%)
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
55
LDL Protein Concentrations (ug/ml)

Figure 6. The effect of LDL protein concentrations on U937 cell growth. Serum starved U937 cells (1.5×10^5) were seeded out in 1 ml of RPMI 1640 media and allowed to grow for 72 hours in the indicated concentrations of LDL according to protein from two normal lean controls. Subsequent experimental conditions were as described in the legend to Figure 2.

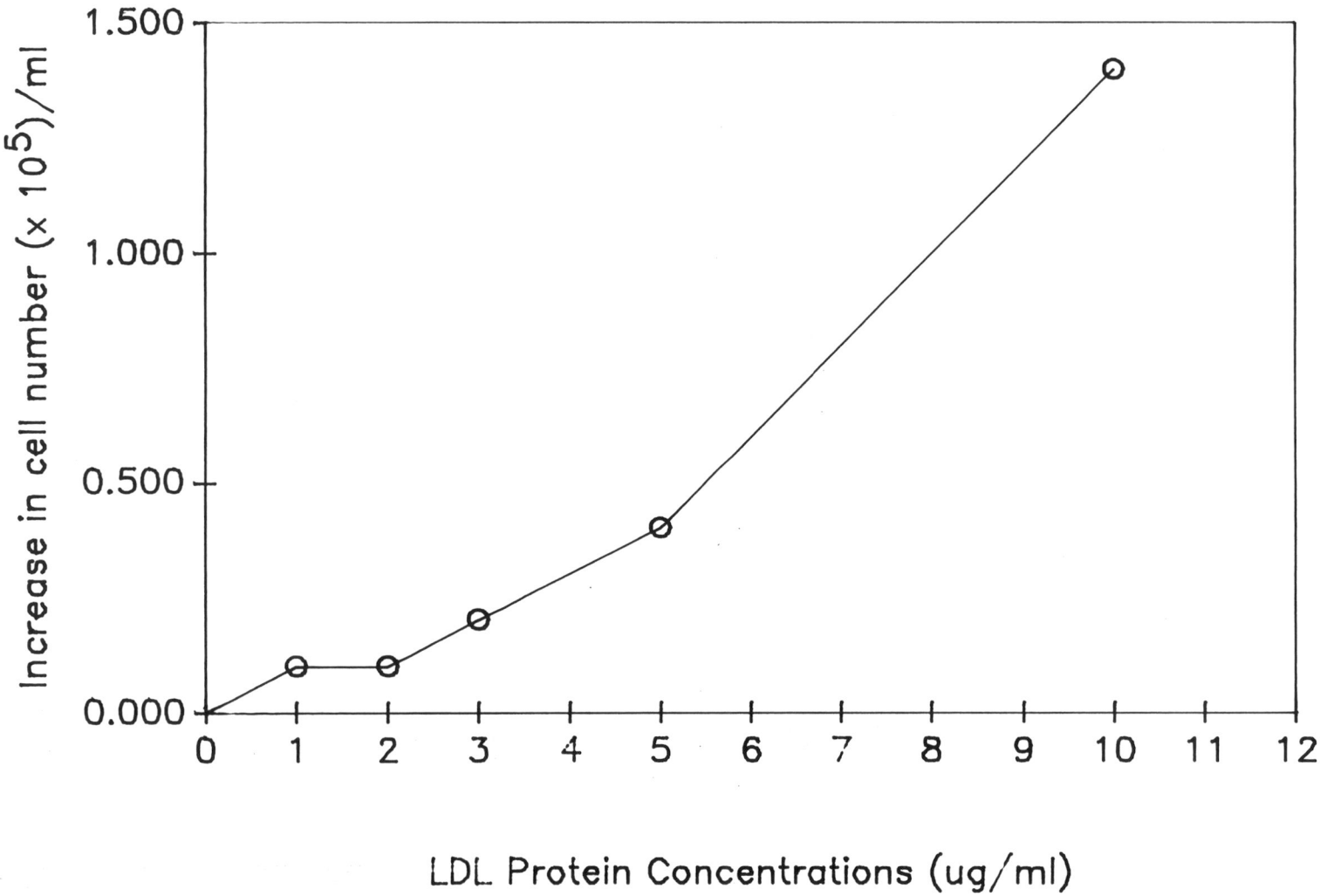

1.500
1.000
0.500
0.000
Increase in cell number (x 10^5)/ml
0
1
2
3
4
5
6
7
8
9
10
11
12
LDL Protein Concentrations (ug/ml)

Figure 7. The effect of LDL protein concentrations from seven normal lean controls on U937 cell growth. The concentrations of 2, 3 and 10 ug/ml of LDL protein were incubated with serum starved cells for 72 hours. Additional experimental conditions were as described in the legend to Figure 2.

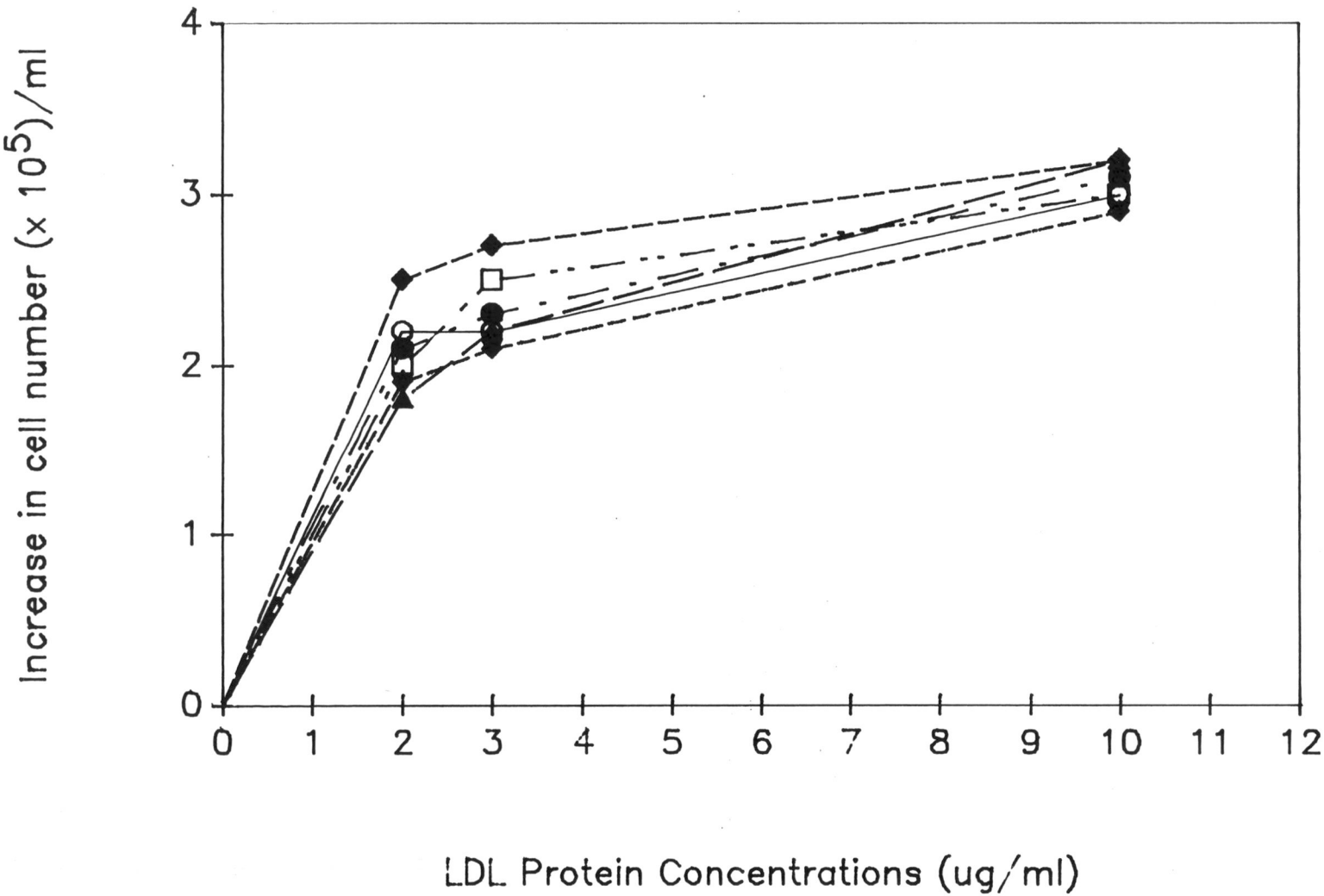

Increase in cell number (x 10^5)/ml
0
1
2
3
4
0
1
2
3
4
5
6
7
8
9
10
11
12
LDL Protein Concentrations (ug/ml)

Figure 8. The effect of LDL protein concentrations from seven patients with NIDDM on U937 cell growth. The concentrations of 2, 3 and 10 ug/ml of LDL protein were incubated with the serum starved cells for 72 hours. Additional experimental conditions were as described in the legend to Figure 2.

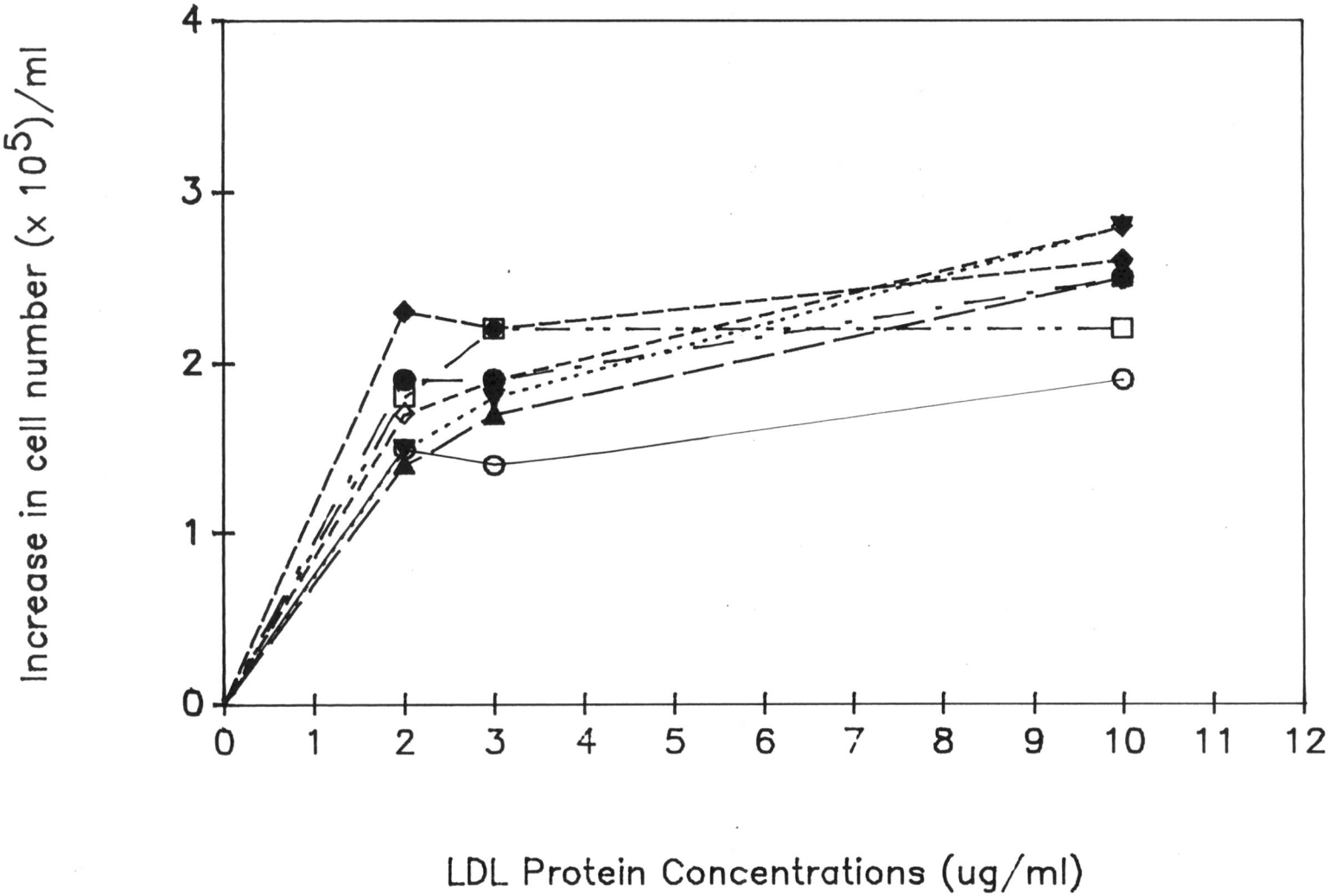

Increase in cell number (x 10^5)/ml
0
1
2
3
4
0
1
2
3
4
5
6
7
8
9
10
11
12
LDL Protein Concentrations (ug/ml)

Figure 9. The average effect of LDL protein concentrations from the NIDDM and normal lean groups on U937 cell growth.

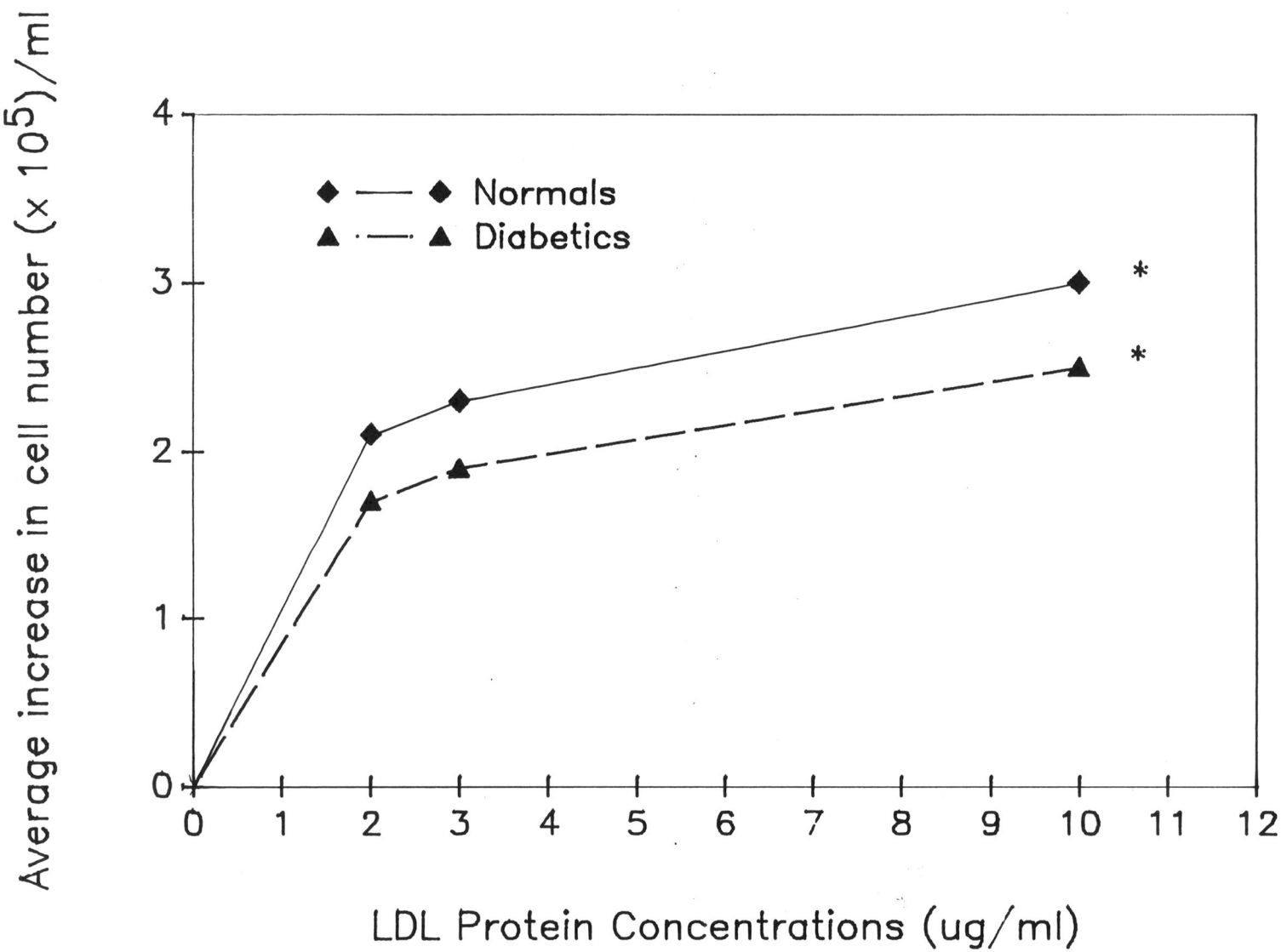
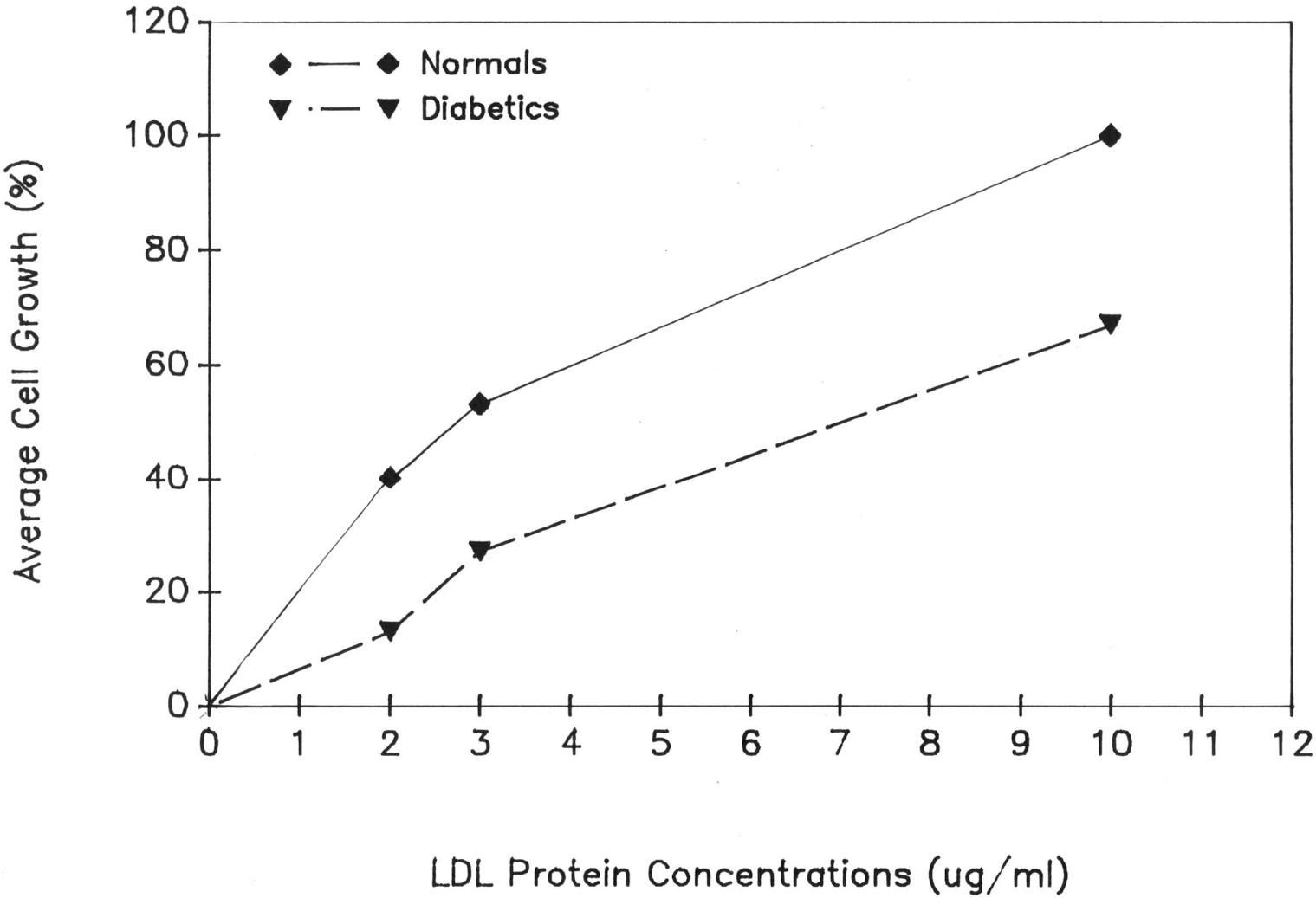

Average increase in cell number (x 10^5)/ml
Normals
Diabetics
*
*
0
1
2
3
4
0
1
2
3
4
5
6
7
8
9
10
11
12
LDL Protein Concentrations (ug/ml)

Figure 10. The average effect of LDL protein concentrations from the NIDDM and normal lean groups on U937 cell growth. The values are expressed as percent of the mean increase in cell number over starting cell numbers.


Normals
Diabetics
Average Cell Growth (%)
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
LDL Protein Concentrations (ug/ml)

DISCUSSION

In this study, two groups of subjects were chosen for several reasons. The normal lean subjects obviously served as a control group and the morbidly obese subjects with NIDDM served as the experimental group. It could be argued that another control group, specifically a morbidly obese group without NIDDM, was needed to show that differences found with the diabetic plasma lipid levels and LDL were due to the diabetics' morbid obesity. Barakat and coworkers, however, have shown that the plasma lipid levels of morbidly obese individuals who are not diabetic are comparable to that of lean individuals. It is also important to note that the LDL molecular weight of the morbidly obese subjects is very close to that of the lean subjects ($2.96 \pm 0.06 \times 10^6$ vs. $2.87 \pm 0.05 \times 10^6$). In contrast, they observed significant differences between diabetics and normal lean individuals in their plasma lipid concentrations and in the physical and chemical properties of their LDL, particularly in molecular weight ($2.78 \pm 0.07 \times 10^6$ vs. $2.96 \pm 0.06 \times 10^6$, NIDDM vs. normal lean controls, respectively). Based on these differences, it was concluded that the diabetic condition, rather than obesity, was the attributing factor to the observed differences in plasma lipid levels and LDL characteristics.[30]

The lipid profiles of the diabetic patients who participated in this study were not unlike those reported in other investigations (Table III). The fact that the total plasma cholesterol and LDL-cholesterol was fairly normal agrees with previous findings that diabetic individuals, although at high risk for CHD, do not necessarily have

elevated cholesterol concentrations.[48] However, there is not a clear consensus on the nature of LDL in NIDDM. According to Kissebah, there are several reasons for the difficulty in estimating the prevalence of abnormal plasma LDL concentrations in NIDDM. They are as follows:

> "(1) Most studies either do not include lipoprotein analysis , or those that include quantitative lipoprotein analysis and a control population lack uniform 'normal' limits. (2) Most studies continue to report LDL as the density fraction 1.006-1.063, although this includes the intermediate density lipoprotein (IDL), which is composed largely of remnants of VLDL metabolism. (3) The type of patient in the diabetic population under study can be strongly influenced by the specific interest of the investigator, e.g. studies of patient populations followed in lipid clinics or by cardiologists are likely to contain more hyperlipoproteinemia subjects than those derived from diabetic clinics. (4) Common conditions that affect lipoprotein metabolism such as renal disease, use of diuretic reagents, or beta-adrenergic blocking drugs that are particularly prevalent in diabetic patients may influence a study's conclusions."[49]

In initiating this study, these reservations were carefully considered. First our patient population was comprised of women with morbid obesity and NIDDM, and thus was relatively homogeneous. Second, the LDL was isolated in a consistent and defined manner, such that LDL with a density range of 1.019-1.063 g/ml was only included. Third, all patients that were included in this study were not on any medications that influence carbohydrate or lipid metabolism, and none of the patients had any clinical complications such as renal disease. Therefore, these precautions should have minimized bias.

Much evidence from the literature implicates subpopulations of plasma lipoproteins with accelerated atherosclerosis and CHD. In particular, the LDL particle appears to influence perniciously this diseased state. This is predicated on the research done by such investigators as Musliner and coworkers, Krauss and Burke who have

revealed that patients with CHD have an abundance of smaller more dense LDL.[15-16] Sniderman, et. al., have also demonstrated that some CHD patients have a prevalence of protein and triglyceride enriched, cholesteryl ester depleted LDL which consequently make them smaller carrier molecules.[10] Austin and coworkers have shown through gradient gel electrophoresis that individuals at high risk for myocardial infarction have distinct LDL subclass patterns in which there is a preponderance of small dense LDL particles.[50] Changes in the lipid composition of the LDL consequently may cause conformational alterations which affect several regions of the apoprotein B-100 thereby decreasing the LDL's ability to bind to the cell receptors and decreasing its eventual catabolism.[13] More recently, Galeano, et. al., have reported that small LDL which were cholesteryl ester poor and triglyceride rich, showed decreased immunoreactivities with a monoclonal antibody that recognizes the apoprotein B-100 receptor binding site. Additionally, displacement studies of ^{125}I-LDL on cultured fibroblasts have shown that small LDL had a lower affinity for the LDL receptor.[51] Thus, alterations in the physical and chemical properties of LDL may negatively affect its metabolism rendering it more atherogenic.

Crouse and coworkers have shown a negative correlation between LDL size and plasma triglyceride levels to exist in hypertriglyceridemic individuals.[14] Barakat, et. al., have demonstrated comparable results in diabetic patients. They have additionally reported that high plasma insulin levels appear to have an independent inverse relationship with LDL molecular weight in patients with NIDDM.[30] Because these inverse relationships of hypertriglyceridemia and hyperinsulinemia to low LDL

molecular weight are independent suggests that there may be different mechanisms governing the alterations in LDL size.

These previous findings are closely related to the results of this study in that the hyperinsulinemic subjects with NIDDM had low HDL-cholesterol and high triglyceride levels. Although the patients with NIDDM were hypertriglyceridemic in comparison to the normal subjects, their triglyceride levels were appreciably lower than what is considered to be a dangerously high clinical value (<150 mg/dl). Why then would these diabetics be at high risk for CHD if by accepted standards they have fairly normal lipid levels? The answer to this possibly lies in the fact that these individuals are diabetic and consequently hyperinsulinemic which may have some bearing on the alterations of the LDL particle.

Even though the actual mechanism for production of small dense LDL remains unknown, <u>in vitro</u> studies by Kinoshita and coworkers may shed light on it. They showed that when LDL is incubated with VLDL and partially purified human lipid transfer protein, the LDL becomes triglyceride rich and cholesteryl ester poor.[13] Conceivably, this may be what is happening in patients with NIDDM. Their hyperinsulinemic state may also have a stimulatory effect on the lipid transfer protein causing an enhanced transferral of triglycerides to LDL from VLDL. Furthermore, since diabetics often have high levels of triglycerides, this may provide ample substrate for the lipid transfer protein to act upon. At any rate, the elucidation of this mechanism presents another avenue of research.

In order to determine whether the LDL particles from these diabetic

individuals were sufficiently altered in some way to render them more atherogenic, the chemical and physical compositions were measured. The results show that the LDL from the patients with NIDDM were on the average triglyceride enriched and cholesteryl ester depleted. The physical properties in terms of LDL size, however, revealed that the diabetic LDL were not significantly different from the normal LDL (Table IV). The ramifications of these results could only be further understood with a study of the biological interaction of the LDL with cells. Therefore, the goal of this study was to determine if there were differences in the biological activity of the diabetic LDL molecules in comparison to that of normal LDL by incubating them with the U937 macrophage cell line.

As mentioned earlier, the U937 cell line does not have the ability to synthesize cholesterol de novo, and thus requires cholesterol from exogenous sources for growth and proliferation. It has been shown that LDL can serve as a source for cell growth, that this cell line possesses an LDL-receptor, and that provision of the cells' need for cholesterol is mediated via the LDL-receptor pathway.[32] Because of these properties of the U937 cell line, it was reasoned that these cells could be used as an experimental tool in determinig whether the aberrations in the chemical properties of LDL would influence their metabolism. It was postulated that cell growth and proliferation will be diminished if LDL of diabetic patients are sufficiently altered to reduce binding, internalization or degradation of these LDL. Therefore, cell numbers were monitored in response to incubations with normal or diabetic LDL, under well defined culture conditions. Results indicated that there are

differences in the diabetic LDL's ability to stimulate cell proliferation to the same extent as the normal LDL. Furthermore, the comparatively higher standard error of the mean values associated with the cell counts from the assays using diabetic LDL are another indication that the diabetic LDL population is very heterogeneous which may affect its biological integrity (Table V).

In attempting to explain the differences in cell proliferation when incubated with normal and diabetic LDL, it is postulated that the diabetic LDL bound to the cell receptors as did the normal LDL, but the deliverance of cholesterol by the diabetic LDL was evidently different. In other words, upon binding to the macrophage LDL receptors, the diabetic LDL, because they were cholesteryl ester depleted, were unable to deliver to the cells the cholesterol that was needed by them to proliferate. Another possible explanation for the diabetic LDL's decreased ability to stimulate cell proliferation is that the shape of the LDL or apoprotein B-100 was sufficiently altered in that the LDL was not able to bind effectively to the cell receptors. Even when incubated with saturating concentrations (10 ug/ml of LDL protein) the cells only proliferated to 67% of the maximum level. Quite possibly, the U937 cell line chosen for this study may not have been the best model. But, the method described by Frostegard and coworkers in which this cell line was employed is well established and appears to be a valid <u>in vitro</u> test of detecting differences in the metabolism of LDL from normal and diabetic individuals. In order to evaluate further this cell model and the affinity of the diabetic LDL for the cell receptors, binding assays would need to be performed. Subsequent assays which would involve

incubating the diabetic LDL with fibroblasts to measure HMG-CoA reductase and ACAT activity would help to determine differences in internalization and degradation of the LDL. These procedures may help to elucidate further the biological interaction of LDL with cells, particularly diabetic LDL.

In conclusion, this study was able to demonstrate that LDL from patients with NIDDM are sufficiently altered which causes their biological interaction with cells to be abnormal. The significance of these *in vitro* findings to an *in vivo* situation in diabetics is that if the LDL are altered and consequently not recognized by the LDL receptors, they may remain in the circulation longer. If the circulating residence time of the LDL is increased, the LDL may be further modified or seep into the lining of arteries which may lead to the formation of atherosclerosis and CHD.

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APPENDIX A

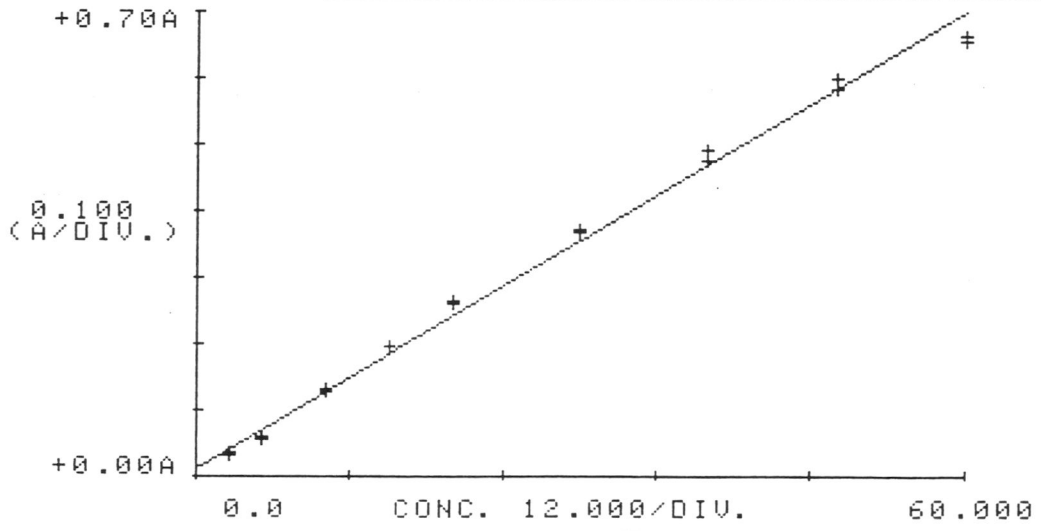
A Representative Standard Curve of Protein Determination by Lowry's Method.

CALIBRATION WORKING CURVE DISPLAY Y/N ?

STD. No.	CONC.	ABS.
1	0.0000	0.000
2	0.0000	0.000
3	2.5000	0.036
4	2.5000	0.035
5	5.0000	0.055
6	5.0000	0.055
7	10.000	0.132
8	10.000	0.130
9	15.000	0.197
10	15.000	0.197
11	20.000	0.262
12	20.000	0.263
13	30.000	0.367
14	30.000	0.372
15	40.000	0.477
16	40.000	0.493
17	50.000	0.586
18	50.000	0.601
19	60.000	0.665
20	60.000	0.657

10:38 8/23 '90 700.0NM -0.001A

WORKING CURVE C=K*ABS+B K= 87.681 B=-1.2256



10:39 8/23 '90 700.0NM 0.000A

APPENDIX B

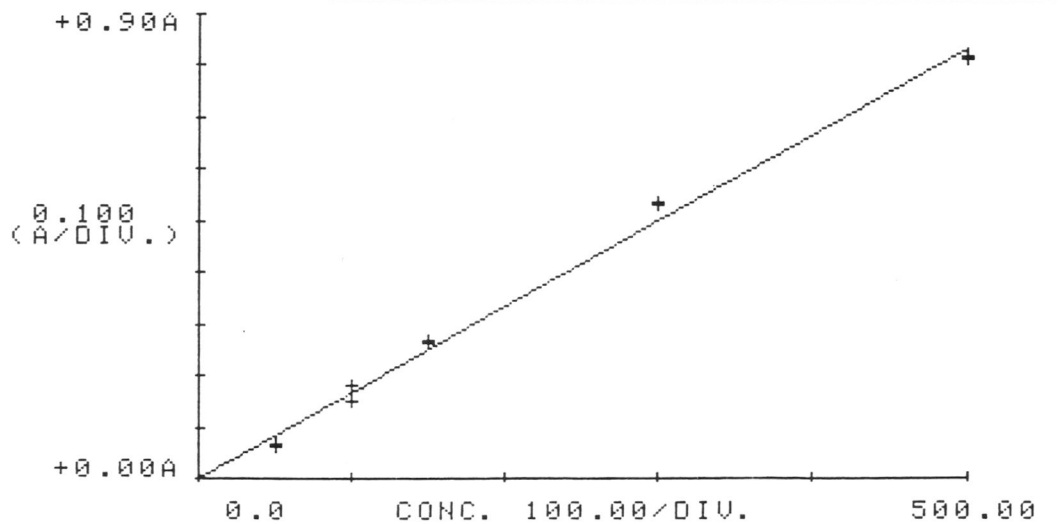
A Representative Standard Curve of Triglyceride Determination by Enzymatic Kit Method.

CALIBRATION WORKING CURVE DISPLAY Y/N ?

STD. No.	CONC.	ABS.
1	0.0000	0.000
2	0.0000	0.000
3	50.000	0.060
4	50.000	0.066
5	100.00	0.147
6	100.00	0.179
7	150.00	0.267
8	150.00	0.260
9	300.00	0.533
10	300.00	0.528
11	500.00	0.816
12	500.00	0.810

11:11 8/23 '90 500.0NM -0.000A

WORKING CURVE C=K*ABS+B K= 597.88 B= 0.7094



11:17 8/23 '90 500.0NM -0.000A

APPENDIX C

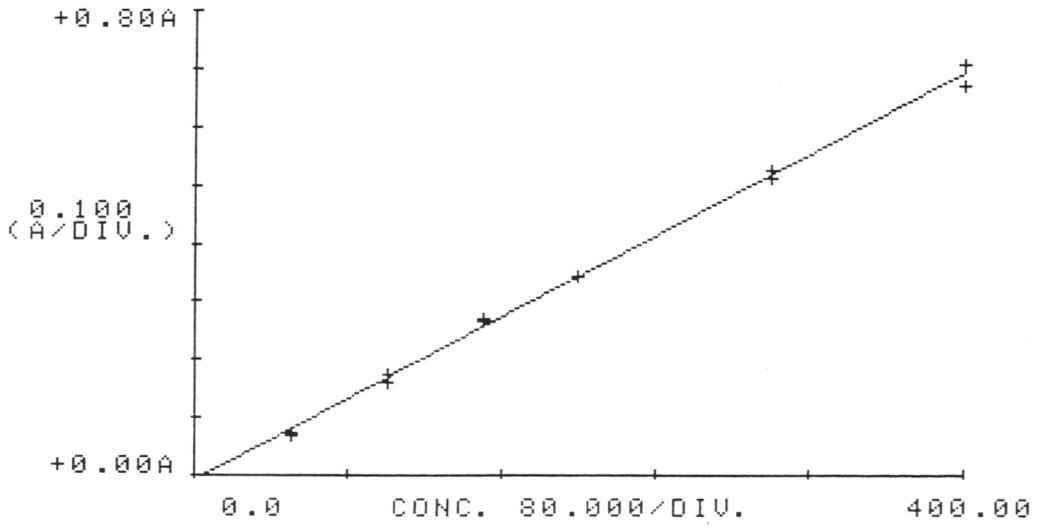
A Representative Standard Curve of Cholesterol Determination by Enzymatic Kit Method.

CALIBRATION WORKING CURVE DISPLAY Y/N ?

STD. No.	CONC.	ABS.
1	0.0000	0.000
2	0.0000	0.000
3	50.000	0.068
4	50.000	0.071
5	100.00	0.172
6	100.00	0.160
7	150.00	0.267
8	150.00	0.270
9	200.00	0.343
10	200.00	0.344
11	300.00	0.510
12	300.00	0.528
13	400.00	0.671
14	400.00	0.710

1:22 8/23 '90 500.0NM -0.000A

WORKING CURVE C=K*ABS+B K= 571.32 B= 3.5708



11:23 8/23 '90 500.0NM -0.001A

APPENDIX D

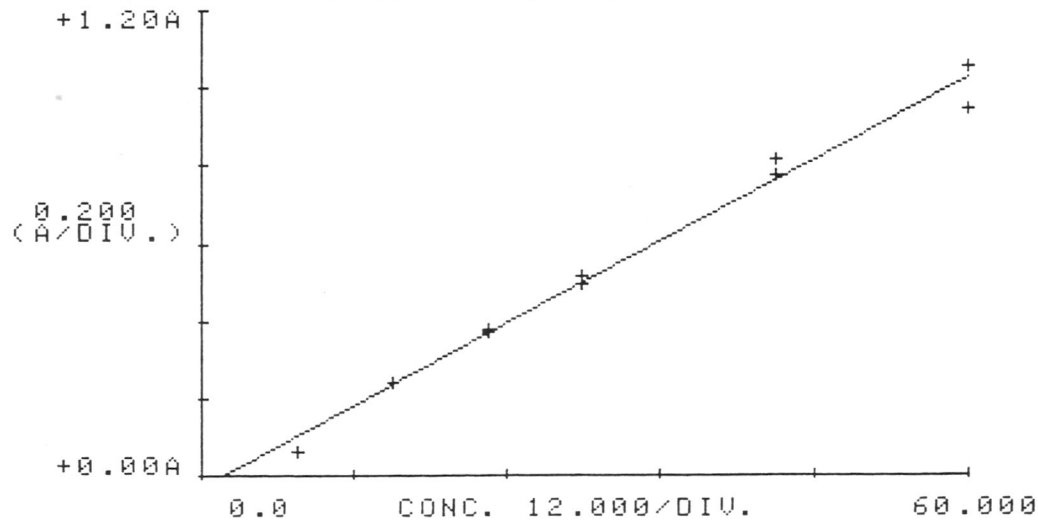
A Representative Standard Curve of Free and Esterified Cholesterol Determination by Orthophthaldehyde Method.

CALIBRATION WORKING CURVE DISPLAY Y/N ?

STD. No.	CONC.	ABS.
1	0.0000	0.000
2	0.0000	0.000
3	7.5000	0.062
4	7.5000	0.060
5	15.000	0.238
6	15.000	0.242
7	22.500	0.370
8	22.500	0.375
9	30.000	0.495
10	30.000	0.514
11	45.000	0.777
12	45.000	0.814
13	60.000	1.055
14	60.000	0.946

12:34 8/23 '90 550.0NM -0.000A

WORKING CURVE C=K*ABS+B K= 56.655 B= 1.6467



12:35 8/23 '90 550.0NM -0.000A

APPENDIX E

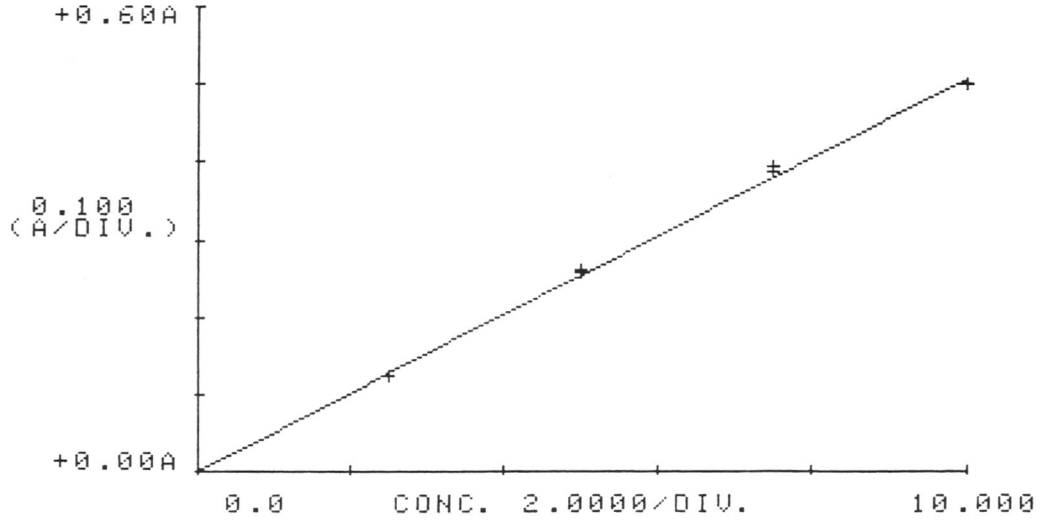
A Representative Standard Curve of Phospholipid Phosphorous Determination by the Method of Fiske and SubbaRow.

CALIBRATION WORKING CURVE DISPLAY Y/N ?

STD. No.	CONC.	ABS.
1	0.0000	0.000
2	0.0000	0.000
3	2.5000	0.1245
4	2.5000	0.1255
5	5.0000	0.2557
6	5.0000	0.2557
7	7.5000	0.3739
8	7.5000	0.3839
9	10.000	0.5002
10	10.000	0.499

12:48 8/23 '90 700.0NM 0.000A

WORKING CURVE C=K*ABS+B K= 19.704 B=-0.0214



12:51 8/23 '90 700.0NM -0.000A

APPENDIX F

A Representative Photograph of Polyacrylamide Gradient (2-16%) Gel Electrophoresis of LDL from Normal Lean Individuals.

ABBREVIATIONS LIST

NIDDM-Non-Insulin Dependent Diabetes Mellitus

CHD-Coronary Heart Disease

HDL-High Density Lipoproteins

LDL-Low Density Lipoproteins

FH-Familial Hypercholesterolemia

VLDL-Very Low Density Lipoproteins

ACAT-Acyl-CoA:Cholesterol acyltransferase

HMG-CoA Reductase-Beta-Hydroxy Beta-Methyl Glutaryl-CoA Reductase

ATP-Adenosine Triphosphate

G-1-P-Glycerol 1 Phosphate

NAD-Nicotinamide Adenosine Dinucleotide

DAP-Dihydroxyacetone Phosphate

IDL-Intermediate Density Lipoproteins