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Triglyceride Synthesis in Normal

and HyperapoB Fibroblasts

Katherine M. Cianflone

A Thesis

in

The Department

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Chemistry / Biochemistry

Presented in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy at
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ABSTRACT

Triglyceride Synthesis in Normal and HyperapoB Fibroblasts

Katherine M. Cianflone, Ph.D.
Concordia University, 1989

The goal of this thesis was to examine the regulation of intracellular triglyceride synthesis from normals and HyperapoB patients using cultured skin fibroblasts as an experimental model. In a medium supplemented with lipoprotein-deficient serum, triglyceride synthesis and cholesterol esterification were reduced in the HyperapoB fibroblasts by 40% and 44% respectively as compared to the normal fibroblasts. These differences were due to differences in de novo synthesis, and not due to re-esterification or hydrolysis. However, unexpectedly, there was no difference in triglyceride synthesis between the cells from normals and HyperapoB when a serum-free supplemented medium was used.

The stimulatory factor in lipoprotein-deficient serum was then isolated from human plasma and characterized. The factor responsible is nondialyzable and trypsin sensitive, and its effects are both concentration and time dependent. The protein responsible has been purified to homogeneity and is a small (MW 14,000), basic (pI 9.0) protein. It has been named Acylation Stimulating Protein (ASP) because it markedly stimulates triglyceride synthesis and cholesterol esterification by 80% and 42% in normal human skin fibroblasts. Further ASP is specifically bound with a KD of

9.9×10^{-7} and is internalized and degraded by normal human skin fibroblasts.

In contrast, ASP has much less effect on triglyceride synthesis in HyperapoB fibroblasts with an average of only 25% triglyceride stimulation. This is consistent with its reduced maximal binding (51% of normal) and subsequent internalization into these cells. Although both cell groups demonstrate the same binding affinity for ASP (Scatchard analysis slope = $-.0725\ ug^{-1}$ normal and $-.080\ ug^{-1}$ HyperapoB) the maximal number of apparent binding sites is reduced in HyperapoB ($1.217\ mg^{-1}$ normal vs $0.621\ mg^{-1}$ HyperapoB). The ASP effect on cells from patients with Familial Hypercholesterolemia, however, is normal with regard to ASP stimulation of triglyceride synthesis (average 55% stimulation) and ASP binding to cells.

This research therefore has led to the description of a previously unrecognized protein in human plasma. This protein, ASP, appears to be the most potent stimulant of intracellular triglyceride synthesis yet described.

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1. Cianflone K, Kwiterovich P, Walsh M, Forse A, Rodriguez M, Sniderman A, Stimulation of fatty acid uptake and triglyceride synthesis in human cultured skin fibroblasts and adipocytes by a serum protein, Biochem Biophys Res Commun 144 94-100, 1987.

2. Cianflone K, Rodriguez M, Walsh M, Vu H, Sniderman A, The effect of a plasma protein fraction on lipid synthesis in cultured skin fibroblasts from normals and patienrs with HyperapoB, Clin Invest Med 11 99-107, 1988.

3. Cianflone K, Sniderman A, Walsh M, Vu H, Gagnon J, Rodriguez M, Purification and characterization of acylation stimulating protein, J Biol Chem 264 426-430,

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ABBREVIATIONS

apoB	apoprotein B
ASP	acylation stimulating protein
ATP	adenine triphosphate
BSA	bovine serum albumin
CE	cholesterol ester
Chol	cholesterol
CoA	Coenzyme A
DG	diglyceride
FABP	fatty acid binding protein
FFA	free fatty acid
FH	familial hypercholesterolemia
G3P	glycerol-3-phosphate
HDL	high density lipoprotein
HSA	human serum albumin
HyperapoB	Hyperapobetalipoproteinemia
IDL	intermediate density lipoprotein
IEF	isoelectric focusing
LDL	low density lipoprotein
LpL	lipoprotein lipase
ND	nor determined
NS	not significant
PAGE	polyacrylamide gel electrophoresis
PL	phospholipid

R	receptor
SD	standard deviation
SEM	standard error of the mean
SER	smooth endoplasmic reticulum
TG	triglyceride
VLDL	very low density lipoprotein

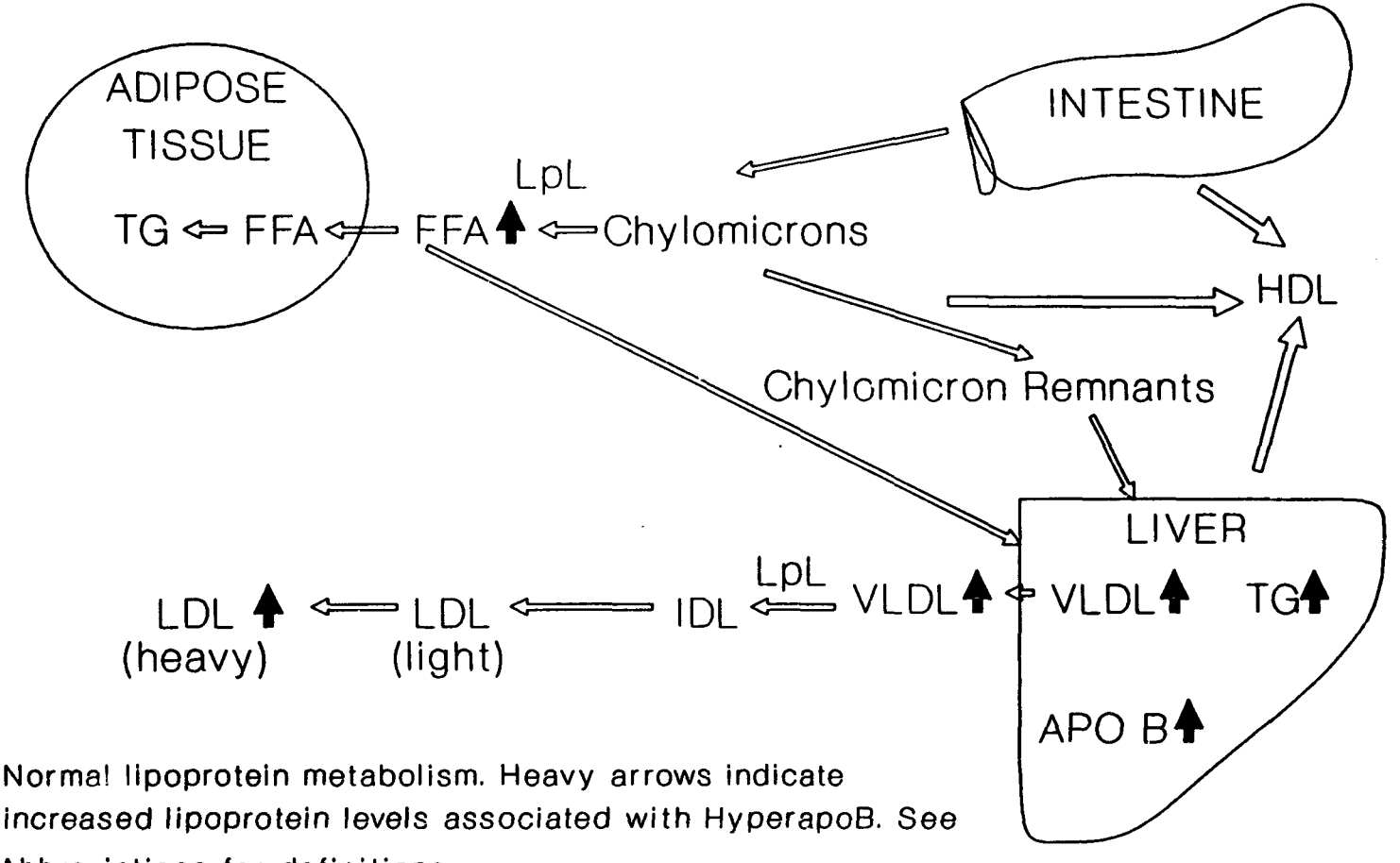
INTRODUCTION

1. LIPOPROTEIN METABOLISM

Cholesterol , esterified cholesterol and triglycerides are non-polar and, as such, they must be transported in blood in large complexes which also contain polar lipids and specific apoproteins, the latter with functions much beyond that of mere packaging. These complexes, which have been named lipoproteins (1: review), transport the non-polar lipids in the circulation in a water soluble form and are synthesized mainly by the liver and intestine and are catabolized by hepatic and extrahepatic tissues (Figure 1).

Chylomicrons are the largest of these lipoproteins. They are synthesized by the intestine in order to transport dietary triglyceride and cholesterol from the small intestine to most of the tissues in the body. The triglycerides in these particles are hydrolyzed within the plasma compartment by the action of lipoprotein lipase (LpL), an enzyme attached to the endothelial surfaces but synthesized by adipocytes and other cells. The fatty acids (FFA) liberated during hydrolysis are used as an energy source by many cells, such as the heart or skeletal muscle, or alternatively are taken up by adipocytes and stored within them as triglycerides.The lipoprotein particles thus generated are referred to as chylomicron remnants; they are relatively enriched in cholesterol and normally are rapidly cleared by the liver.

Figure 1 LIPOPROTEIN METABOLISM



Normal lipoprotein metabolism. Heavy arrows indicate increased lipoprotein levels associated with HyperapoB. See Abbreviations for definitions.

Very low density lipoproteins (VLDL) are also large triglyceride rich particles, synthesized in the liver, so as to transport both triglyceride and cholesterol from the liver to various tissues. Again, within the plasma compartment, VLDL triglycerides are hydrolyzed by lipoprotein lipase (and hepatic lipase) producing free fatty acids, and thereby generating a series of smaller, cholesterol-enriched lipoproteins, including intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). LDL represents the end product of VLDL catabolism, although not all VLDL reach this stage, with LDL being the major cholesterol-transporting lipoprotein in plasma. LDL are then cleared from the plasma both through hepatic and peripheral tissues via interaction with a specific receptor, the B,E receptor.

Lastly, high density lipoproteins (HDL) appear to arise from several sources, including the liver and the intestine. In addition, HDL or HDL precursors are generated in the plasma compartment as a result of lipolytic processing of dietary chylomicrons. It has been suggested that the HDL may be involved in reverse cholesterol transport, a pathway whereby HDL acquire cholesterol from peripheral tissues and then transport the cholesterol either directly or indirectly to the liver for excretion (1).

It should be emphasized that these lipoproteins are each heterogenous in size and composition, characteristics that are based on the rich traffic of lipids and apoproteins between the various classes of lipoproteins mediated by plasma enzymes, transfer proteins, and by the apoproteins themselves.

2. ATHEROSCLEROSIS AND LIPOPROTEINS

Epidemiological studies indicate that heart disease is the major cause of death in the North American adult population (2). Heart disease kills more people each year than all forms of cancer combined. Population studies have taught us that factors such as blood pressure, diet, weight, exercise and smoking habits are important in determining susceptibility to cardiovascular disease (3). However both epidemiologic data and clinical observations have shown that these variables alone do not explain susceptibility to cardiovascular disease among individuals. A large number of studies have now documented the strong association between lipoprotein concentrations in plasma and heart disease (4). Thus LDL concentrations correlate positively with coronary risk (5), whereas HDL correlate negatively with coronary risk (6).

Additionally, if the question is looked at from the other side, and the prevalence of dyslipoproteinemias among survivors of myocardial infarction or among individuals with

angiographically documented atherosclerotic lesions is ascertained, a sizable proportion are found to have abnormal lipoprotein levels (7,8). Lipid or lipoprotein levels exceeding the 95th percentile of the population distribution are classified as hyperlipidemic. This prevalence within the coronary artery disease population is not surprising since atherosclerotic lesions, which cause narrowing of the blood vessels, are characterized by depositions of lipoprotein-derived cholesterol in and around the cells of the arterial wall in addition to enhanced cellular proliferation and fibrosis (9). Thus epidemiologic evidence strongly supports a role for lipoproteins in atherosclerosis. In fact, recent clinical trials have demonstrated that altering plasma lipoprotein concentration (lowering LDL and raising HDL) using both diet and drugs lowers cardiovascular mortality (10) and reduces the rate of progression of disease (11).

3. ATHEROSCLEROSIS AND APOPROTEINS

The lipoproteins contain not only lipids but apoproteins which serve a variety of functions. These apoproteins provide not only structural support for the lipoprotein particle, but are necessary for their synthesis and secretion (1). Some also act as cofactors or activators of certain enzymes associated with lipid and lipoprotein metabolism and facilitate the exchange of lipids between the particles ultimately targeting the specific lipoprotein to tissues and

cells through interactions with cellular receptors. In 1971, Alaupovic (12) was the first to suggest that apoproteins should be considered when evaluating lipid disorders.

Since then a large number of studies have shown a strong association between elevated LDL apoB (the major apoprotein of LDL) and the risk of coronary heart disease, regardless of whether LDL cholesterol was elevated or normal (13). Patients with elevated plasma LDL apoB levels have been classified into two groups; those with elevated LDL cholesterol, and those with normal LDL cholesterol. The classic example of the first is Familial Hypercholesterolemia (FH), a disorder which is characterized by cholesterol-enriched LDL particles. This autosomal dominant disorder is due to any of a series of genetic defects in the LDL receptor all of which result in a greatly reduced plasma clearance rate of LDL. This pathway has been well characterized by Brown and Goldstein who received the Nobel Prize in 1985 for their contribution to the receptor hypothesis concept (14). The prevalence of this disorder within the general population is one in 500 for the heterozygous form, and one in one million for the homozygous form, and even within the coronary artery disease population the prevalence is low indeed. It should be noted that a clearance defect can be of two types: one present at a tissue site, such as a decrease in a receptor (as described above), but yet another may be in the lipoprotein particle itself that causes it to interact with

lesser affinity with the receptor. The recently described disorder, defective B100, is an example of the latter. (15,16).

4. HYPERAPOBETALIPOPROTEINEMIA

A far more prevalent phenotype within the coronary artery disease population, initially characterized by (i) an elevated LDL apoB (greater than the 95th percentile), (ii) a normal or near normal LDL cholesterol and (iii) a low LDL cholesterol/ apoB ratio was first recognized by Sniderman et al (17) and was called hyperapobeta-lipoproteinemia. This disorder is common in patients with premature coronary heart disease (18) and is often familial (19). Hyperapobetalipoproteinemia (HyperapoB) exhibits a number of similarities with another disorder, Familial Combined Hyperlipidemia. Familial Combined Hyperlipidemia was first described by Goldstein et al (20) and defined as both hypertriglyceridemia and hypercholesterolemia within the same family. More recently, this definition has been modified to include elevated plasma apoB (21) and it has been suggested that these two entities, Hyperapobeta-lipoproteinemia and Familial Combined Hyperlipidemia in fact usually represent two sides of the same entity (22).

The increased levels of apoB in plasma in HyperapoB appear to be due to hepatic overproduction of apoB containing lipoprotein particles (VLDL and LDL) (23,24) (Figure 1) as

opposed to increased levels resulting from decreased catabolism (14) as described previously in the case of FH. This evidence has been adduced from isotope kinetic studies that trace the input and exit of apoB containing lipoproteins within the plasma compartment (23,24). These studies are based on two characteristics of the apoB containing lipoprotein particles. First, there is only one apoB molecule per lipoprotein particle (25) and, second, unlike other apolipoproteins, the apoB molecule does not exchange between lipoproteins (26), but remains with its original lipoprotein particle until it is catabolized. As a result, quantification of apoB disappearance kinetics using ^{125}I (iodinated) apoB in VLDL or LDL accurately mirror the metabolism of these lipoprotein particles (23,24). In contrast to the apoB molecule, however, the lipid moieties of these lipoproteins do exchange with other lipoproteins (27). Specifically, in HyperapoB, the overproduction of hepatic particles results in an exaggeration of the normal neutral core-lipid exchange which occurs between circulating triglyceride-rich (i.e. VLDL) and cholesterol-rich (i.e. LDL) lipoproteins (28).

The increased net hepatic output of apoB lipoproteins may be the result of a primary or a secondary defect within the liver itself which results in overproduction of the lipoprotein components: apoB, triglyceride, cholesterol and phospholipid (Figure 1). However the causes of primary hepatic lipoprotein overproduction are, to date, unknown.

One possible cause of primary hepatic overproduction and secretion of apoB is a reduced capacity of the apoB molecule to bind to its lipid components (cholesterol, cholesterol ester, triglyceride and phospholipid) thereby reducing its effectiveness as a hepatic lipid exporter. Such avenues have been extensively explored using the techniques of molecular biology to examine the apoB gene. In any gene there is normally a certain amount of organism to organism variation in the allelic forms of the gene and therefore the gene product, for example, a protein such as apoB. A particular variation may be associated with an anomaly in protein structure and function. A number of groups have therefore examined the genetic (allelic) variation of the apoB gene itself in order to find associations between genetic variations in apoB DNA sequence and increased levels of plasma apoB. To date, the results are not persuasive, with some studies demonstrating an association between certain gene alleles and elevated levels of apoB (29), results which are at variance with other studies (30). Secondary causes of lipoprotein overproduction have been felt to include obesity with a high caloric intake, diabetes mellitus, and probably the nephrotic syndrome (31,32).

Alternatively, the increased secretion of apoB lipoproteins may be a hepatic response to an increased flux of lipids, either fatty acids or cholesterol,into the liver. In the case of fatty acids, this phenomenon might occur if

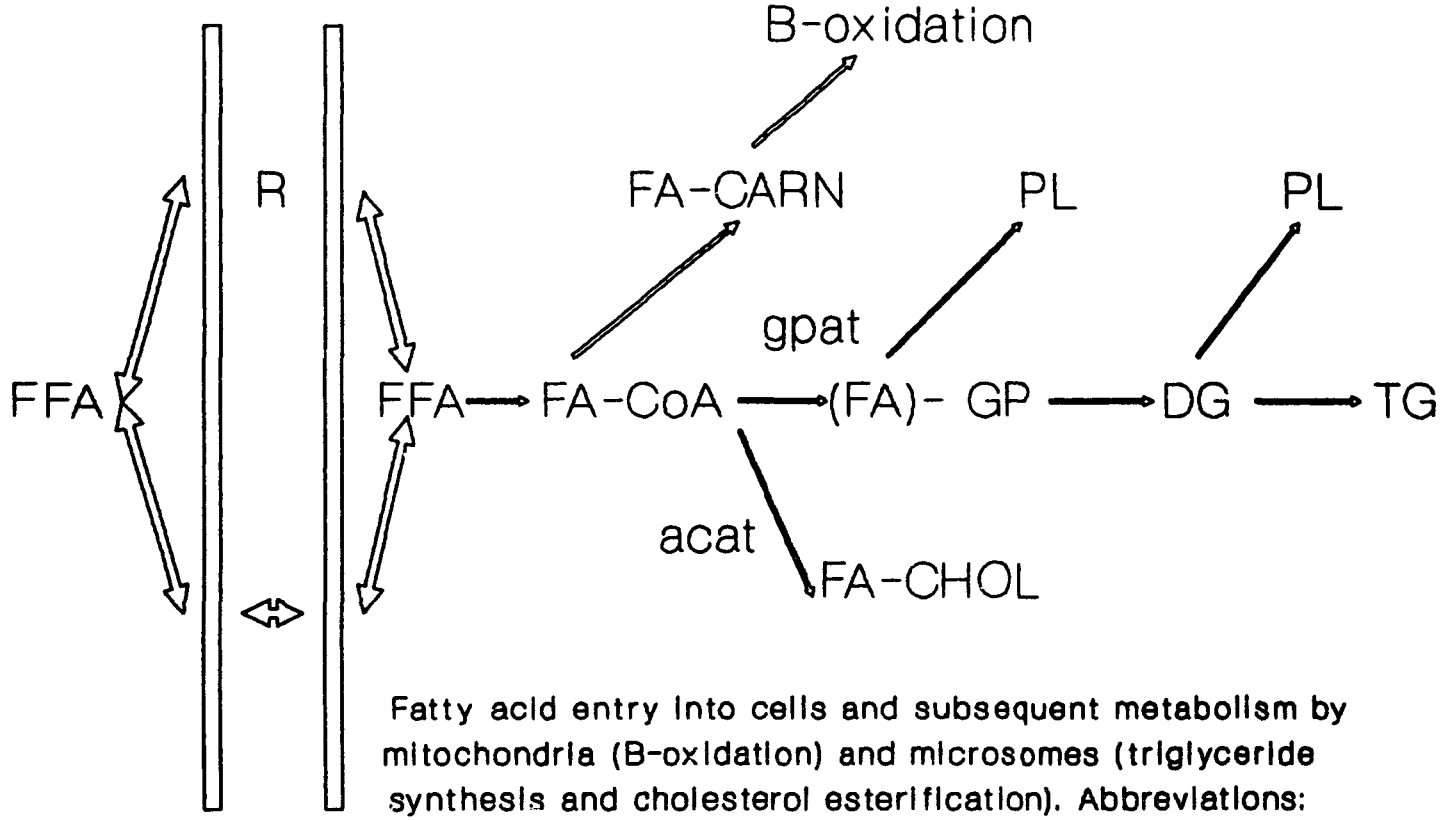
the rate of free fatty acid uptake into peripheral tissue such as adipose tissue were reduced. This hypothesis is supported by the reduced rate of in vitro fatty acid uptake and esterification which has been observed in adipose tissue obtained from patients with HyperapoB (33). Similarly, in vivo studies have also shown a delay in the clearance of exogenously derived triglycerides (chylomicrons) in patients with HyperapoB (34), a finding which is consistent with a peripheral tissue defect in the uptake and esterification of fatty acids. This reduced rate of peripheral tissue uptake and the resulting increased delivery to the liver of fatty acids might explain the increased secretion of VLDL and, subsequently, increased levels of LDL as the liver strives to maintain lipid homeostasis. As a result, the frequency of hypertriglyceridemia in patients with HyperapoB is not surprising (18). A similar line of reasoning has appeared in previous reports which have pointed to abnormal adipose tissue metabolism in patients with hypertriglyceridemia with either triglyceride synthesis diminished or triglyceride hydrolysis increased (35-37).

5. TRIGLYCERIDE SYNTHESIS IN ADIPOSE TISSUE

Extracellular fatty acids become available to the cell as a result of the hydrolysis of triglyceride rich particles (chylomicrons and VLDL) through the action of lipoprotein lipase. Lipoprotein lipase is synthesized by adipose tissue

and is attached to the endothelial cell surface where it is available to act on the lipoproteins in the circulation (38). Additionally, fatty acids bound to albumin are also present in the circulation and these also are available for uptake by the cell. Albumin acts as a transporter of fatty acids within the circulation, and almost all of the fatty acid present in the circulation is bound to albumin, to a maximum of 6 fatty acids bound per albumin molecule (39). Free fatty acids (FFA) can diffuse through the plasma membrane into the adipocyte by a non energy dependent process (Figure 2). Traditionally it has been thought that this was the only mode of entry of fatty acids into the cell (40,41); however, recently, a 40 kD plasma membrane protein (R) has been isolated from liver and cardiac tissue which binds fatty acids, consequently, it has been suggested that this protein may be involved in active fatty acid transport across the plasma membrane (42,43).

Figure 2. FATTY ACID METABOLISM



Fatty acid entry into cells and subsequent metabolism by mitochondria (B-oxidation) and microsomes (triglyceride synthesis and cholesterol esterification). Abbreviations: acat=acyl cholesterol acyl transferase, carn=carnitine, CoA=coenzyme A, gpat=glycerol phosphate acyl transferase GP=glycerol-3-phosphate, R=receptor, FA=fatty acid, CHOL= cholesterol,TG=triglyceride,PL=phospholipid, DG=diglyceride

Once within the cell, the fate of the fatty acid varies. Fatty acid can be transported to the mitochondria, to be oxidized so as to supply metabolic energy for the cell (44). Alternately, they can be directed to the microsome (smooth endoplasmic reticulum or SER) where they can be used for synthesis of both phospholipids (PL) and glycerolipids (triglyceride and diglyceride)(45). Phospholipids are synthesized for immediate use in the cell, principally as structural components of membranes. Within the adipocyte, the major portion of the fatty acids are esterified to form triglycerides (TG), which are then stored in the cells as lipid droplets (38). These fatty acids within the triglycerides can then be recovered when necessary through the action of triglyceride lipase, which releases the fatty acids according to the energy demands of the organism. It has been suggested that fatty acid binding protein (FABP), an intracellular protein present in a wide range of tissues, including liver and intestine, may play a role in the transport of fatty acid from the plasma membrane to the target site (mitochondria or microsome)(46). This protein belongs to a family of small molecular weight proteins which bind and transport hydrophobic ligands through the cytosolic milieu (47). Although these are principally thought to be intracellular proteins, preliminary evidence has suggested that some of these proteins may also be secreted (48,49).

At the endoplasmic reticulum, the fatty acids are first activated by the action of acyl CoA ligase to form acyl Coenzyme A (FA-CoA), and then sequentially esterified to a glycerol-3-phosphate (G3P) backbone in a series of tightly linked enzymatic reactions to form the end product, which, in the case of neutral lipid synthesis, is triglyceride (TG)(39),(Figure 2). Adipose tissue makes an important contribution to the overall metabolism of an organism through its ability to store fatty acids as triglycerides and then to make these metabolites available again when required (38). It is obviously important that the two opposing processes, fatty acid esterification and triglyceride hydrolysis, should be maintained under tight metabolic control (50).

A considerable body of knowledge exists concerning the direct activation of adipose tissue lipolysis by several hormones and the ability of insulin to antagonize these effects. On the other hand, current ideas about the control of triglyceride synthesis are concerned essentially with regulation of the supply of precursors to fat cells (50:review). With respect to hormonal regulation, one might predict that lipolytic hormones should inhibit esterification. In fact it is known that epinephrine, glucagon, adrenocorticotrophic hormone and thyroid stimulating hormone actually stimulate esterification as well as lipolysis (50), perhaps as a result of the release of free fatty acids. There is, however, considerable evidence that

insulin can increase the rate of triglyceride synthesis in adipose tissue, an effect that is not secondary to increased glucose uptake (50). The activity of lipoprotein lipase, the enzyme that initially determines fatty acid supply to the cell, appears to be subject to hormonal control, as are also several of the enzymes in the triglyceride synthetic pathway: acyl CoA ligase, glycerol phosphate acyltransferase (gpat), phosphatidate phosphatase and diacylglycerol acyltransferase (Figure 2). The rate-limiting step in this pathway has not been clearly identified largely because the enzymes and intermediates are membrane-bound, and the mechanism of hormonal effect has not been elucidated, although it has been postulated that covalent modification of the enzymes involved in triglyceride synthesis by phosphorylation-dephosphorylation may be involved (50).

Although adipose tissue is the tissue of interest with respect to triglyceride synthesis and storage within cells, methodological difficulties are encountered when working with these cells. For example freshly isolated adipocytes, which are normally used, may still be subject to previous influences within the organism from which they were obtained (51). Additionally, the adipocyte membrane functions are suspect due to the collagenase treatment used in the isolation procedure. Unfortunately, differentiated adipocytes are non-proliferating cells, and cannot therefore be

maintained in tissue culture for extended periods of time for repeated analysis.

6. TRIGLYCERIDE SYNTHESIS IN FIBROBLASTS

Human skin fibroblasts do not synthesize and store significant triglyceride in the absence of extracellular fatty acid (52). However, in the presence of an external fatty acid challenge, fibroblasts show a marked increase in lipid droplets and the accumulation of neutral lipid (52). Fibroblasts, therefore, have been examined as a potential model system for lipid synthesis. There is exchange of newly synthesized phospholipids with the preexisting phospholipid pool, however there is no net increase , whereas there is a net increase in triglyceride mass (52). A number of more recent studies have confirmed the classic earlier work which characterized cellular phospholipid as the "element constant" and cellular triglyceride as the "element variable". Triglyceride synthesis in fibroblasts appears to be under similar hormonal regulation as in adipose tissue, since it has been reported that insulin also increases triglyceride synthesis in fibroblasts (53), whereas epinephrine increases lipolysis (54).

Human skin fibroblasts have been widely used as a model of peripheral tissue to examine a variety of intracellular metabolic functions including DNA synthesis (55), protein synthesis, lipid synthesis (52) and extracellular

interactions involving hormonal influences and transport processes via plasma membrane receptors. The classical example of the latter is the developement of the LDL receptor hypothesis and its implications with respect to cellular lipid biosynthesis, lipoprotein metabolism and the atherosclerotic disease process (14). Other transport mechanisms with carrier functions, such as the transferrin receptor (56) and the asialoglycoprotein receptor have also been extensively examined using fibroblasts. The effect of hormones such as insulin (53) and epinephrine (54) has also been studied.

Classically, a peptide hormone action has been defined as the direct action of a protein on a cell mediated through an interaction principally on the plasma membrane itself. That is to say, it is not a requirement for the hormone to be endocytosed by the cell in order for there to be a consequent response. In contrast, a protein that functions as an extracellular transport protein to deliver a ligand to the cells must be endocytosed and processed in order to see a metabolic effect on the cell. Various experimental manipulations have been developed, therefore, in order to investigate the mechanism of action of proteins on fibroblasts. To differentiate a plasma membrane effect from an effect mediated subsequent to endocytosis, the process of internalization and processing can be blocked through the use of drugs such as monensin (57), chloroquine or by lowering

the ambient incubation temperature of the cells to 17^{0}C (58). These techniques have been used to investigate the effects of insulin (59), transferrin (56) and LDL (60), to name but a few. In conclusion, therefore, fibroblasts are a well-defined cell model which can be used to examine intracellular triglyceride synthesis, and protein-cell mediated interactions.

THESIS RATIONALE

The initial observations of reduced peripheral lipid synthesis in HyperapoB patients have been pursued and are the subject of the present work. Although adipose tissue is the principal site of triglyceride storage in the body, differentiated adipocytes are not proliferating cells and can, therefore, not be maintained in cell culture on a long term basis. Accordingly, human skin fibroblasts were chosen to study, since they are a well characterized biological system with a demonstrated consistency as a model for lipoprotein metabolism. Although in the absence of fatty acids in the cell culture medium they do not initiate intracellular triglyceride synthesis and storage, they are, however, known to synthesize and store triglycerides in response to extracellular fatty acids (52). These properties were confirmed in the introductory stages of the project where triglyceride synthesis in normal human skin fibroblasts was examined with respect to various parameters. These initial studies were performed in the presence of medium supplemented with human serum depleted of lipoproteins.

These studies demonstrated that human skin fibroblasts from patients with HyperapoB incorporated oleate into triglycerides to a lesser extent than the normal human skin fibroblasts. In contrast, when triglyceride synthesis was examined in a medium devoid of serum, but supplemented with

hormones, it was found that the discrepancy between the normal and the HyperapoB fibroblasts no longer existed.(61)

Examination of this paradox suggested that a factor present in serum was responsible for stimulation of triglyceride synthesis in the normal fibroblasts (62). Investigation of this lipogenic effect of serum on normal fibroblasts has led to the purification of a novel serum protein.

This protein has been named Acylation Stimulating Protein (ASP). ASP is a small (MW 14,000), basic (pI 9.0) plasma protein. (63). ASP stimulates triglyceride synthesis in normal human skin fibroblasts in a time and concentration dependent manner. In addition, the metabolic effect of ASP on cells appears to be mediated through specific receptor interaction and internalization of the protein. Preliminary evidence suggests that ASP does not appear to act as either a hormone or a fatty acid transport protein, yet its metabolic effect on triglyceride synthesis is greater than that reported for any other protein. This raises the possibility that ASP may be of physiologic importance in free fatty acid metabolism in humans. While the mechanism of ASP remains to be elucidated in further studies, the present work appears to be unique in that it is the first in which lipid synthesis has been stimulated to this extent by a purified human plasma protein acting on human cells.

The studies mentioned above have established the effect of ASP on normal human skin fibroblasts. The metabolism of ASP and its effect on synthesis of triglycerides has also been examined in human skin fibroblasts derived from patients with HyperapoB and other hyperlipidemias associated with coronary heart disease. Of primary relevance is the response, or rather lack of response, to ASP characteristic of the HyperapoB fibroblasts. As indicated by binding studies this, in turn, may be the result of a compromised receptor complement. In contrast, in another patient group examined, 6 patients with Familial Hypercholesterolemia (FH), both a normal metabolic response to ASP and an apparently normal complement of ASP receptors were evident in the cell studies. The normal response of the FH patient fibroblasts to ASP emphasizes the striking contrast provided by the HyperapoB cells. These findings may have implications, therefore, both for understanding the pathogenesis of HyperapoB as well as understanding the regulation of intracellular lipid synthetic reactions.

MATERIALS AND METHODS

1. SUBJECTS STUDIED

Plasma samples (12 ml) were obtained from 12 hour fasted subjects. These samples were collected into tubes containing EDTA (1 mg/ml anticoagulant) and then were spun at 2000 rpm for 15 minutes to obtain plasma. In all, 10 normal subjects and 19 patients were examined. Plasma triglycerides and plasma cholesterol were measured with commercially available colorimetric enzymatic kits (Boehringer Mannheim,Germany). HDL cholesterol was measured in the supernatant following precipitation of apoB containing lipoproteins with heparin and magnesium sulphate according to Lipid Research Clinic methods (64). LDL cholesterol was calculated by the method of Friedewald (65). LDL apoB was measured by radial immunodiffusion (66). All normal subjects were in good health and all had plasma lipid and lipoprotein values within the normal range as defined by the Lipid Research Clinics study (67), and an LDL apoB < 120 mg/dl. All HyperapoB patients had an LDL apoB greater than 120 mg/dl with an LDL cholesterol to apoB ratio of less than 1.5:1 (68). Familial Hypercholesterolemia patients were defined as patients with an elevated total plasma cholesterol, elevated LDL cholesterol, LDL apoB, and an LDL cholesterol to LDL apoB ratio of greater than 1.8:1 (68) or, diagnosed based on the absence of functional LDL receptors, in the case of cells

obtained from the American Tissue Culture Collection (ATCC) cell bank.

2. SKIN FIBROBLASTS

2.1 CULTURE OF SKIN FIBROBLASTS

All subjects had forearm skin biopsies from which the fibroblasts were cultured according to standard techniques (69). All tissue culture media and solutions were obtained from Flow Laboratories. The cells demonstrated the characteristic elongated and flattened out appearance seen in fibroblasts. Cells were maintained in Eagle's Minimum Essential Medium supplemented with 10% (v/v) fetal calf serum and 100 IU of penicillin- streptomycin at 37° in a 5% CO_2 humid incubator. The cells were routinely subcultured at confluency in a 1:1 or 1:2 split ratio. Cells were detached from the wall of the flask by incubating with 0.25% trypsin in phosphate buffered saline (PBS) for 10 mins at 37°. Cell lines were used for experiments between passages 5 to 17. Prior to experiments, cells were dissociated in 0.25% trypsin (as above) and plated into 60 mm tissue culture treated dishes at a concentration of 1×10^5 cells per dish in 2.0 ml of medium (as described above). The experiments were initiated 6 days later.

2.2 LIPID SYNTHESIS: PREEXPERIMENTAL CONDITIONS

The general format for the experiments was the same in all cases : (1) On day 6 (following plating out of the cells on day 1 as described above), the medium was changed to 1.0-2.0 ml of a preincubation medium for 24 hours. This preincubation medium contained no radioactive oleate or other tracer.

(2) On day 7, radioactive tracer, usually ^{14}C oleate, was added to the cells and the incubation continued at 37°C for the indicated time periods. Following the incubation, the cells were harvested and analysed. All points for each cell line were always done in duplicate or triplicate (where indicated).

Preincubation medium consisted of either Eagle's Minimum Essential Medium supplemented with 2.5 mg protein/ml of lipoprotein- deficient serum (70) or a serum-free hormone supplemented medium (71). Lipoprotein-deficient serum is the plasma fraction with a density greater than 1.21 gm/ml, the lipoproteins having been removed by ultracentrifugation at 100,000g for 40 hours (72). This fraction was recentrifuged at 100,000g for 24 hours, dialyzed against isotonic saline (.15M NaCl), and defibrinated by incubation at 24°C with 20 IU/ml of thrombin followed by centrifugation (2000 rpm for 15 min) to remove the clot, with the final lipoprotein deficient serum concentration adjusted to 50 mg/ml protein. All plasma used to generate lipoprotein-deficient serum had normal levels of lipids and lipoproteins as defined above.

Free fatty acid concentration in the lipoprotein deficient serum was measured by enzymatic colorimetric method (Wako Chemicals, Japan) and the amount present was taken into account for calculation of specific activity in all the experiments.

The serum-free hormone supplemented medium consisted of a 1:1 mixture of Dulbecco's Minimum Essential Medium and Ham's F12 medium supplemented with 1% nonessential amino acids, 2.5 ug/ml insulin, 8 ug/ml biotin, 8 ug/ml calcium pantothenate, 5 ug/ml transferrin, 5 pg/ml triiodo-thyronine, and 17 ng/ml hydrocortisone as described by Amarosa, (71) 100 IU/ml penicillin-streptomycin and, where indicated, 1.25 mg/ml human serum albumin.

LDL was added to the preincubation medium for measurement of cholesterol esterification. LDL was isolated on a discontinuous gradient between densities 1.019 and 1.063 g/ml by preparative ultracentrifugation (72) using NaCl-KBr solution with 1mM EDTA, then recentrifuged and dialyzed against isotonic saline (.15M NaCl).

2.3 LIPID SYNTHESIS IN FIBROBLASTS

For the experimental incubation, on day 7, (1-^{14}C) oleate (52.6 mCi/mmol, New England Nuclear) in ethanol was evaporated to dryness under a stream of nitrogen and resuspended in a solution of 10 mM sodium oleate complexed to albumin (73) (with a molar ratio of oleate to albumin of

6:1) to yield an average specific activity of 4.1 dpm/pmol. Aliquots were added to the cells at the indicated concentrations of oleate. In some cases, D-(6-^{3}H(N)) glucose (33.2 Ci/mmol, New England Nuclear) or (Me-^{3}H) thymidine (20.0 Ci/mmol, New England Nuclear) were also used as tracers in conjunction with radiolabelled oleate. Radioactive glucose was added to medium which in all cases contained 5.1 mM glucose to yield a final specific activity of 0.8 dpm/pmol. For determination of endogenous lipid synthesis in the absence of exogenous fatty acid (oleate), ^{14}C acetate was included in the media at a concentration of 1 mM, specific activity 1.21 dpm/pmole (74). Oleate incorporation into lipids was measured following incubation in a 5% CO_2 humid incubator at 37°C for the times indicated.

2.4 ANALYSIS OF CELLULAR LIPID SYNTHESIS

Following the incubation, the cells were then washed three times with cold 0.02 M phosphate buffered saline (PBS), harvested by scraping with a rubber policeman in 2 ml of PBS, and aliquots (usually 0.5 ml of 2.0 ml total) removed for protein determination and counting in a scintillation counter. Cell protein was dissolved in 0.1 N NaOH and the protein content was determined by a modified Lowry assay for membrane protein (75) using bovine serum albumin as a standard. The range in protein content per dish was 30-70 ug cellular protein.

The remaining sample (1.5 ml) was extracted with a 5-fold volume of chloroform: methanol: 1N HCl (20:10:0.25) (76). After 30 min, the sample was centrifuged (2000 rpm) for 10 min and the two phases separated. The organic phase was evaporated under a stream of nitrogen and redissolved in a small volume (100 uL) of chloroform: methanol (2:1). The lipids (cholesterol ester, triglyceride, fatty acid, diglyceride, cholesterol and polar lipids) were separated by thin layer chromatography (77) on Silica Gel G plates (which were prewashed in chloroform: methanol 2:1), in hexane: ether: acetic acid (75:25:1), along with lipid standards, and visualized by exposure to iodine vapour. The spots were scraped into vials containing scintillation fluid and counted in a liquid scintillation counter (Beckman Instruments). Counts were automatically corrected for quenching and channel overlap in dual label experiments with an automatic quench curve program. Results were expressed as average (+standard error of the mean (SEM)) nmoles of oleate incorporated per mg of cell protein.

Hydrolysis of cell lipids was measured following preincubation of cells for 24 hours in lipoprotein-deficient serum medium and then incubation with 100 uM 1-^{14}C oleate in lipoprotein-deficient serum medium for 24 hours. The medium was then removed and the cells subsequently incubated for 2 hours in serum-free hormone medium containing albumin as fatty acid acceptor, during which time the rate of lipid

hydrolysis was determined. The cells were harvested and analysed as described above.

3. PURIFICATION AND CHARACTERIZATION OF ASP

3.1 COLUMN CHROMATOGRAPHY

ASP was purified by a three-step chromatographic procedure using normolipidemic defibrinated plasma.

STEP 1: Serum was applied to an Affigel-blue column (Biorad, California) at 1 g of serum protein (30 ml) per 100 ml of gel. The gel was washed with 3 column volumes of buffer A (0.02 M phosphate buffer, pH 7.2) followed by elution of the bound protein with 3 M NaCl in buffer A. The column was regenerated with 6 M urea in buffer A and re-equilibrated with 5 volumes of buffer A. The eluted peak was concentrated by dialysis (using 10 kDa molecular weight cutoff dialysis tubing) against 50% polyethylene glycol and dialysed against buffer A to remove excess salt.

STEP 2: The concentrated protein from the first step was rechromatographed on a Sephadex G-75 (Pharmacia, Sweden) column. Following application, fractions were eluted with buffer A and the second peak, which emerged at V_e/V_o= 3.4, was concentrated and dialyzed as above. This fraction emerged outside of the effective molecular weight range of the column as calibrated with albumin, beta-lactoglobulin, trypsinogen and cytochrome C (1 mg/ml of each protein).

STEP 3: The concentrated fraction 2 (above), was dialyzed against buffer B (0.025 M ethanolamine, 6 M urea, pH 9.7) and then applied to a PBE 96 chromatofocusing column (Pharmacia, Sweden). The column was eluted with Polybuffer 96 (Pharmacia, Sweden) diluted 1:10 in 6 M urea, pH 5.8, until a pH of 7 was reached. The first peak that emerged, at an average pH of 8.9, contained 67% of the total activity recovered. The active fraction was concentrated, dialyzed against saturated ammonium sulphate to remove excess polybuffer, and then dialysed extensively for 24 hours against 4 changes of 1 L of buffer A. Since the polybuffer is difficult to remove completely, an alternate method was also used. Following gel filtration (step 2), the protein fraction was dialyzed against 0.02 M phosphate, pH 7.5 (buffer C) and applied to an ion exchange CM Sepharose column (Pharmacia, Sweden). After washing with 3 column volumes of buffer C, the bound protein was eluted using 1 M NaCl in buffer C. The protein fraction was concentrated and dialyzed against buffer A. No differences in biological activity or physical properties were observed when the protein was isolated using either chromatofocusing or ion exchange chromatography. However, since the polybuffer might interfere with the amino acid analyses, for this purpose only samples purified by ion exchange chromatography were used. Aliquots of samples were frozen (-70°C) to minimize the aggregation which occurs when samples are maintained at 4°C.

3.2 ELECTROPHORESIS

Samples for electrophoresis were dialyzed against water and subsequently lyophilized. Lyophilized samples were redissolved in appropriate amounts of sample buffer (final protein concentration 1-2 mg/ml) and heated at 90°C for 5 mins. For molecular weight determinations, samples were electrophoresed in (1) SDS, 6 M urea 12.5% PAGE gels under nonreducing conditions (78) or (2) SDS 15% polyacrylamide gels (PAGE) under reducing conditions (79) simultaneously with molecular weight standards from 92 to 14 kDa (Bio-Rad, California). For isoelectric point determinations, samples were electrophoresed in 8 M urea, 7.5% PAGE isoelectric focusing gels (80) with a pH gradient of 3 to 10 for 18 hours in parallel with appropriate standards of pI range 4.6 to 9.6 (Bio-Rad, California). All gels were stained with Coomassie blue R250. Rf values and staining intensity were determined using a Transidyne RFT scanning densitometer (Transidyne Corporation, Michigan).

3.3 AMINO ACID ANALYSES

Aliquots of ASP purified by ion exchange chromatography and homogeneous by SDS PAGE for the 14,000 dalton protein were assayed for amino acid analysis by Dr Jean Gagnon of the Biotechnology Research Institute, Montreal, Canada. Samples were hydrolyzed at 150°C for 1,2,3 and 4 hours and

analyzed for amino acid composition on a Beckman 7300 Amino Acid Analyzer (Beckman Instruments, California) using citrate buffer. For quantitation of methionine and cysteine, samples were first subjected to performic acid oxidation followed by hydrolysis for 1 hour. Both serine and threonine values were correced to zero time. Valine, isoleucine, and leucine were corrected for maximum yield. Optimum amino acid residue number was calculated by least deviation from integer. The protein concentration of samples for amino acid analyses was measured by both the modified Lowry assay (75), using bovine serum albumin (BSA) as a standard, and the Bradford assay (81), using both IgG and BSA as a standard. It was found that either the Lowry assay or the Bradford assay using BSA as standard yielded comparable results to the protein concentration determined by amino acid analysis. Accordingly, the Bradford assay using BSA as standard was chosen as the standard assay and was used consistently for all protein determinations of ASP and experiments involving ASP.

4. LIPID SYNTHESIS AND METABOLISM OF ASP

4.1 EXPERIMENTAL CONDITIONS

For experiments, cells were plated out at 1 x 10^5 cells per 60 mm dish as described above. On day 6, cells were changed to a serum-free hormone supplemented medium without albumin. On day 7, (1-^{14}C) sodium oleate (average specific activity = 10 dpm/pmol) dissolved in isotonic saline was

added to the medium at the specified concentrations. The concentration of free oleate added to the incubation medium did not exceed 25 uM, a value which is below the critical micellar concentration for oleate (40). In addition, serum fractions or purified ASP were added simultaneously at the indicated concentrations. In cells where no ASP was added, an equal amount of buffer was added to serve as a control. The cells were incubated for specified times at 37°C in a humid incubator and then harvested and analysed as described above. Incubations at 4°C (82) were performed by placing the cells on a layer of ice and maintaining the cells in a cold chamber for the duration of the experiment. Experiments performed at 17°C (58) were performed by placing the cells on a platform in a temperature controlled centrifugal chamber. The temperature was also monitored with an independent thermometer. CO_2 was piped into the chamber to maintain an appropriate CO_2 content. Mild trypsinization of the cells without detachment from the plate was performed by incubating the cells in trypsin solution (as described above) for 5 minutes. Human serum albumin (HSA) was obtained from Sigma Chemicals, St. Louis. Fatty acid binding protein (FABP) was kindly provided by Dr. Mary Dempsey.

4.2 RADIOLABELLING OF ASP

For experiments on the metabolism of ASP, ASP was radioiodinated with ^{125}iodine by the chloramine-T method of

Elder (83). Briefly, 50-100 ug of ASP in 50 ul of 0.02 M phosphate buffer (buffer A) was mixed with 1 mCi of $Na^{125}I$ (in 10 ul) and 10 ul of 1 mg/ml chloramine-T in buffer A and vortexed at room temperature for 30 sec. Immediately, 10 ul of 10 mg/ml dithiothreitol and 150 ul of dialysis buffer were added to stop the reaction. The sample was then dialyzed extensively against dialysis buffer (0.02 M phosphate, pH 7.4, .9% NaCl, 1 mM EDTA, 5 mM dithiothreitol and 1 mM Na azide) for 24 hours against 4 changes of 1 L of buffer to remove excess ^{125}I. The average specific activity obtained was 79 cpm/ng protein. For binding of ASP to human skin fibroblasts, cells preincubated in hormone medium were precooled on ice (4°C) for 15 min. ^{125}I ASP was added to the cells at the desired concentrations (average specific activity 12.4 cpm/ng) and incubated at 4°C for 2 hours. The medium was then removed from the cells which were washed and collected by scraping. The protein was then dissolved in 0.1 N NaOH for 1 hour. An aliquot of the cell suspension was taken for counting in a gamma counter, and for protein determination. Non-specific binding was measured by adding a 20 fold excess of unlabelled ASP prior to the addition of labelled ASP (82). Specific binding was calculated as the difference between total binding and non-specific binding. Results are expressed as ng ASP bound/ mg cell protein.

4.3 INTERNALIZATION AND DEGRADATION OF ASP

Cells were preincubated in serum-free hormone media for 24 hours, followed by incubation at 37°C for 4-24 hours in the presence of (1-^{14}C) oleate and ^{125}I ASP at the indicated concentrations. Following the incubation, the medium was removed, the cells washed and harvested by scraping. Aliquots of the cell suspension were removed for ^{125}I counting in a gamma counter and for protein determinations, and the remaining sample was extracted in chloroform: methanol: HCl as described above for lipid analyses.

ASP degradation products in the medium were measured following addition of 1 mg/ml bovine serum albumin (final concentration) as a carrier protein and precipitation of remaining ^{125}I ASP with 60% trichloroacetic acid (final concentration 10%) (84). Following centrifugation (2000 rpm 15 min), free iodine was subsequently removed from the supernatant by a second precipitation with 5 % AgNO3 (final concentration 1.7%). The supernatant (after centrifugation as above) contains the degradation products, and an aliquot was counted in a gamma counter in a volume of 0.5 ml.

4.4 CELL HOMOGENATE EXPERIMENTS

Experiments measuring lipid synthesis in cell homogenates were performed essentially as described by Brown and Goldstein (60). In summary, cells were trypsinized (as above), collected and homogenized in a 200 mM Tris HCl buffer, pH 7.4. Aliquots of cell homogenate were incubated

in a final volume of 200 uL with 2.5 mM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM Coenzyme A, 0.4 mM glycerol-3-phosphate and 20 uM ^{14}C oleate. After a 30 minute incubation, the reaction is stopped by extraction with isopropanol: heptane (1:1). The neutral lipids are extracted and analysed by TLC as described above.

5. STATISTICAL ANALYSES

All results are expressed per mg cell protein. Averages, standard deviation and S.E.M. (standard error of the mean) were calculated using standard methods. For comparison purposes, mean t tests were used to compare different cell lines from the control and patient groups, and paired t tests were used to compare results within cell lines.

In the figures, except as noted in the figure legends, all smooth lines were drawn for ease of visualization and the data were not fit to any specific equation. Where indicated in the figure legends only, some lines were drawn based on linear regression analysis and the equations for these lines are given in the figure legends in the form y=ax+b where a= slope and b= y axis intercept with the appropriate correlation coefficients (r) given. Scatchard analysis for specific binding of proteins to the fibroblasts was performed according to the method of Scatchard (85). Significance levels (p values) of the regression analyses were calculated by Pearson's correlation.

RESULTS

1. LIPID SYNTHESIS IN SERUM SUPPLEMENTED VS HORMONE SUPPLEMENTED MEDIUM

1.1 LIPID SYNTHESIS IN NORMAL FIBROBLASTS

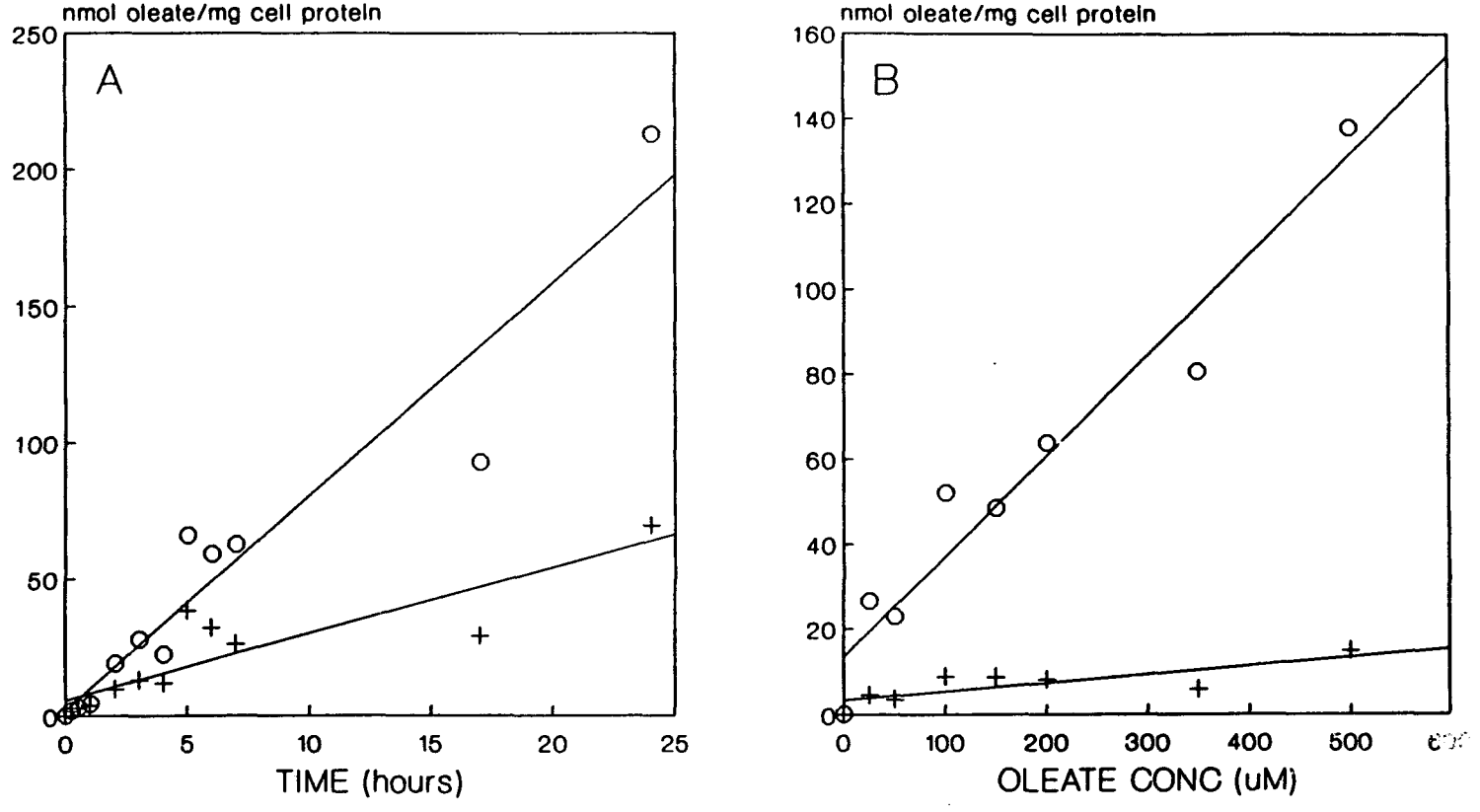
Human skin fibroblast cell lines were established in the laboratory. All normal subjects were adults in good health with normal lipid and lipoprotein profiles. The individual plasma lipid and lipoprotein values are shown in Table I. These normal skin fibroblasts were then examined for their ability to consistently synthesize triglycerides and other lipids to a measurable extent for use as a cell model. Cells were prepared for the experiments as described in Materials and Methods using the medium supplemented with lipoprotein-deficient human serum (referred to as SERUM medium) for both preincubation and incubation. Since various lipoproteins are known to affect endogenous lipid synthesis, particularly in the case of triglyceride and cholesterol synthesis, it is important to remove all of the lipoproteins from the human serum before use in order to examine intrinsic intracellular lipid synthesis. The cells incubated in the presence of 100 uM oleate complexed to albumin (molar ratio 6:1) synthesized triglyceride and phospholipid at a linear rate over 24 hours as shown in Figure 3A. Different fatty acid concentrations were also examined for their effect on the amount of lipid synthesized over a 24 hour incubation period. As shown in

Table I:PLASMA LIPIDS AND LIPOPROTEINS IN NORMAL SUBJECTS

CELL #	AGE	TC	TG	LDL C	HDL C	LDL B	C/B
		-----plasma concentration mg/dl----					
6000	66	156	80	100	40	100	1.00
7000	45	179	123	102	52	90	1.14
9000	44	180	122	118	38	99	1.19
5500	49	156	96	93	44	87	1.07
6500	27	170	43	106	55	54	1.96
average	46	168	93	104	46	86	1.27
S.E.M.	±6	±5	±15	±4	±3	±8	±.18

TC= plasma cholesterol, TG= plasma triglyceride
C= cholesterol, C/B= LDL cholesterol/ apoB

Figure 3. LIPID SYNTHESIS IN SERUM MEDIUM



Triglyceride (o) and phospholipid (+) synthesis in serum medium with oleate bound to albumin. Panel A: 100 uM oleate for 0 to 24 hours. Panel B: 24 hours from 25 to 500 uM oleate in a normal fibroblast cell line.

Figure 3B, triglyceride synthesis increased linearly up to the maximum concentration assayed of 500 uM oleate (complexed to albumin at a 6:1 molar ratio in all cases. Phospholipid synthesis however, remained fairly constant at all concentrations examined.

For the ensuing studies, therefore,a concentration of 100 uM oleate was chosen since this concentration produced a readily quantifiable amount of triglyceride well within the linear portion of the concentration curve.

1.2 NORMAL VS HYPERAPOB IN SERUM MEDIUM

The plasma profiles for the HyperapoB patients examined are shown in Table II. One patient with HyperapoB had plasma triglyceride levels greater than the 95th percentile, one had plasma cholesterol and LDL cholesterol greater than the 95th percentile, wnile two of seven patients had HDL cholesterol values below the 5th percentile. However, average age, total plasma cholesterol, LDL cholesterol, and HDL cholesterol did not differ statistically between the normal and HyperapoB groups, although a trend towards higher plasma levels was evident in the HyperapoB patients (Table II). Plasma triglyceride levels were slightly higher in the HyperapoB patients than in the controls ($p<0.01$). and there was a marked difference in their plasma LDL apoB levels, with the average plasma LDL apoB in the HyperapoB patients being almost double that

Table II: PLASMA LIPIDS AND LIPOPROTEIN IN HYPERAPOB SUBJECTS

CELL #	AGE	TC	TG	LDL C	HDL C	LDL B	C/B
		----plasma concentration mg/dl----					
3000	39	177	244	94	34	140	0.67
4000	40	249	329*	132	51	155	0.85
5000	43	223	172	125	64	156	0.81
8000	41	350*	132	289*	35	213	1.35
2500	56	215	231	143	26*	167	0.86
3500	50	137	173	87	15*	142	0.62
4500	37	195	148	119	46	142	0.84
average	44	221	204	141	39	159	0.86
S.E.M.	±3	±25	±26	±26	±6	±10	±.09
p value	NS	NS	0.01	NS	NS	0.0005	

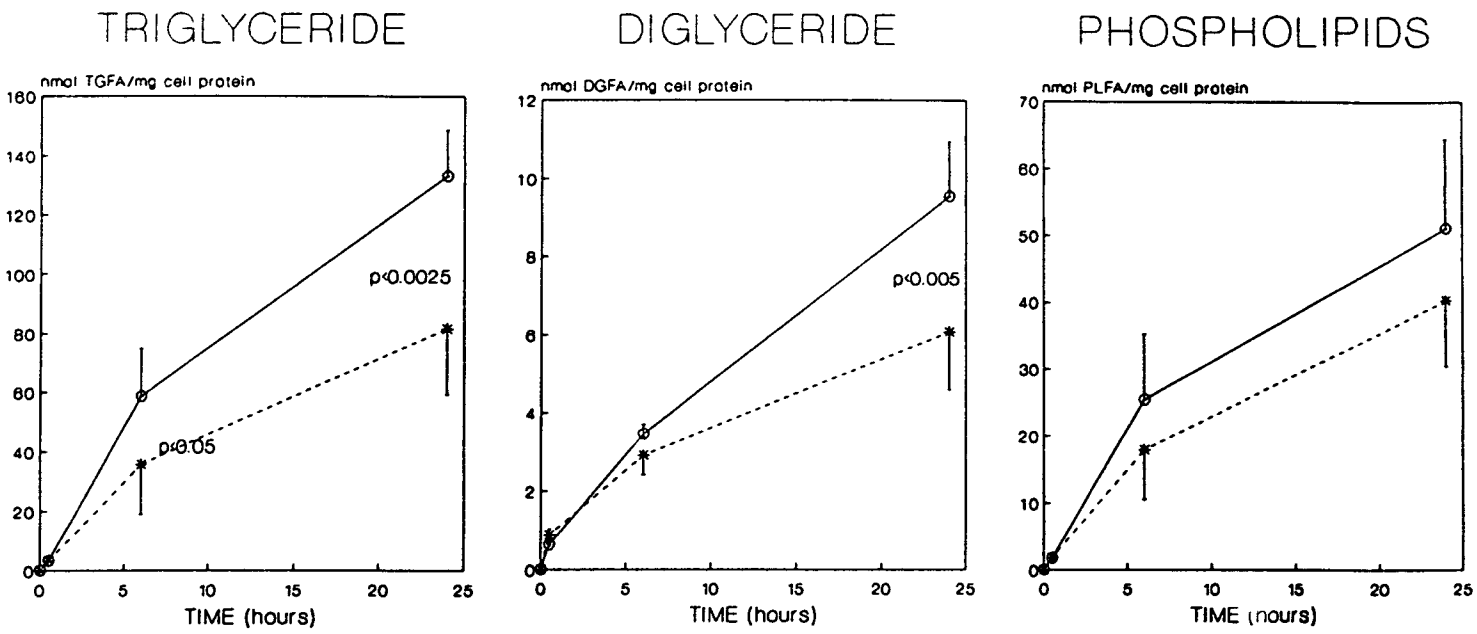
* › 95th percentile

TC= plasma cholesterol, TG= plasma triglyceride
C= cholesterol, C/B= LDL cholesterol/ LDL apoB

of the normal group (159±10 vs 86±8 mg/dL p<.0005). (1-^{14}C) Oleate incorporation into triglyceride, diglyceride, and phospholipid was measured in the normal and HyperapoB cells at three different time intervals. These three lipids constitute more than 95% of the oleate incorporated into the cells. The results are shown in Figure 4. At 30 minutes there is no difference in lipid synthesis between normal and HyperapoB cells; however at this point very little lipid has been synthesized. At both 6 hours and 24 hours there is a substantial difference in the amount of diglyceride and triglyceride synthesized in the HyperapoB cells as compared to the normal cells. At 24 hours in the HyperapoB cells, diglyceride levels are 64% ($p<0.005$) and triglyceride levels are 60% ($p<0.0025$) of the normal. Although there is a trend towards less phospholipid synthesis in the HyperapoB cells, it does not reach statistical significance.

These differences in synthesis determined solely on the basis of (1-^{14}C) oleate incorporation into triglyceride might have been attributable to different rates of re-esterification of diglyceride following partial hydrolysis of a pre-existing triglyceride pool, rather than to differences in de novo synthesis. To examine these possibilities both normal and HyperapoB fibroblasts were incubated simultaneously with (1-^{14}C) oleate and D-(6-^{3}H(N)) glucose. Following a 24 hour incubation in the

Figure 4. LIPID SYNTHESIS: NORMAL VS HYPERAPOB



Triglyceride, diglyceride and phospholipid synthesis was measured as described in Materials and Methods in serum medium with 100 uM oleate complexed to albumin Results are shown for 5 normal (o) and 7 HyperapoB (*) subjects with half error bars.

presence of 100 uM oleate complexed to albumin, cells were analysed for lipid content, and the molar amounts of (1-^{14}C) oleate and (^{3}H) derived from glucose contained in each lipid fraction were measured. The theoretical ratio of (^{14}C):(^{3}H) of 6:1 for triglyceride is calculated as follows. The three incorporated fatty acids have an equal chance of being radioactive. The 6 carbon glucose, labelled in the first carbon position only, will be split into a radioactive and a non-radioactive glycerol-3-phosphate pair for use as the triglyceride backbone. Significant re-esterification of a pre-existing pool would have resulted in a ratio greater than the theoretical one. As seen in Table III, these ratios for triglyceride in normal and HyperapoB cells are 4.5 ± 0.4 and 5.2 ± 0.3, respectively, both very close therefore to the theoretical ratio. Note though that significantly less (^{14}C) oleate is incorporated into triglyceride in the HyperapoB cells as compared to normal cells ($p<0.05$), a finding which is confirmed when glucose derived (^{3}H) incorporation into triglyceride is examined ($p<0.01$). Similarly, in the case of phospholipids, although the theoretically expected ratio cannot be calculated because the phospholipids represent a variety of molecular species, the ratios are the same in both the normal and the HyperapoB cells. Thus the difference in triglyceride synthesis between normal and HyperapoB cells appears to be due to de novo synthesis, and not solely due to re-

Table III. Oleate and Glucose Incorporation into Intracellular Triglyceride and Phospholipid

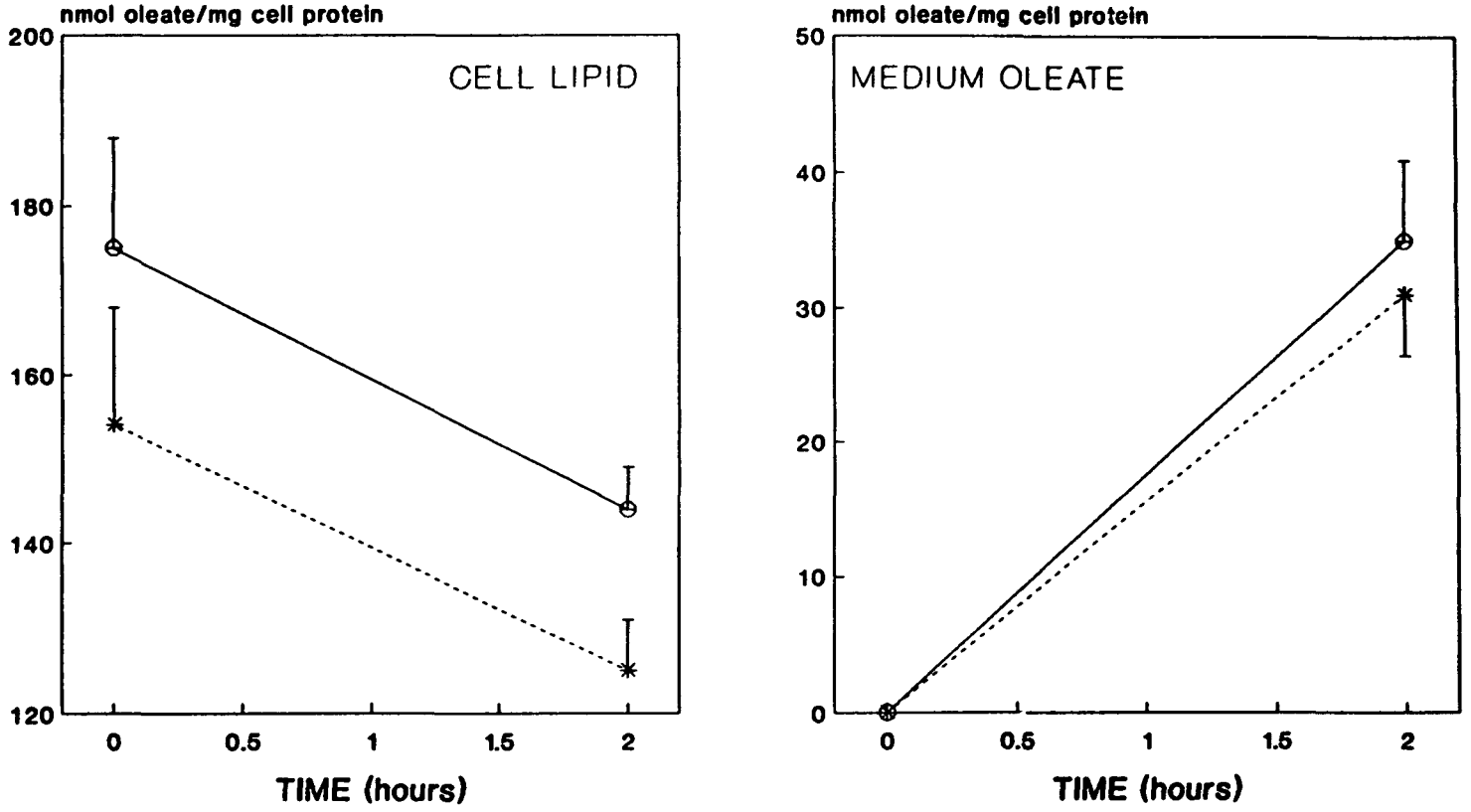
	TRIGLYCERIDE			PHOSPHOLIPID		
	^{14}C mol/mg cell	^{3}H protein	ratio	^{14}C nmol/mg cell	^{3}H protein	ratio
Normal (n=5)	171 ±16	41 ±6	4.5 ±0.4	56 ±9	43 ±11	1.6 ±0.2
HyperapoB (n=7)	139 ±6	27 ±1	5.2 ±0.3	44 ±3	27 ±2	1.7 ±0.01
p value	0.05	0.01	NS	NS	NS	NS

^{14}C-Oleate and ^{3}H-glucose incorporation into triglyceride and phospholipid in 5 normal and 7 HyperapoB cell lines. Cells were incubated for 24 hours in the presence of 100 uM oleate complexed to albumin in serum medium as described in Materials and Methods. Results are expressed as average nmole (oleate or glucose)/mg cell protein ± sem

esterification.

The differences between normal and HyperapoB cells in serum medium suggest that there is an effect on triglyceride synthesis or triglyceride hydrolysis to which normal and HyperapoB cells respond differently. Net oleate incorporation into triglyceride is a function of the rates of both triglyceride synthesis and hydrolysis. Lipid hydrolysis was examined in the following experiment: cells were preincubated in serum medium with 100 uM (1-^{14}C) oleate complexed to albumin in order to establish a pool of substrate available to lipase and thus allow the lipase to act maximally. Following this, cells were incubated for 2 hours in serum-free medium with albumin but no oleate present. The medium was analysed for (^{14}C) oleate released from the cells, and the cells extracted to measure the amount of (1-^{14}C) oleate remaining within them. The results are shown in Figure 5 . Note that there is a greater amount of lipid-associated (1-14C) oleate in the normal cells than in the HyperapoB cells, both before and after incubation. Thin layer chromatography (not shown) of the total cell lipids indicates that there is more (^{14}C) triglyceride in the normals compared with the HyperapoB cells (90 $\pm$ 5.3 vs 76 $\pm$ 4.2 nmol/mg cell protein,$p<0.025$) after the 2 hour hydrolysis period whereas there is no difference in (14C) phospholipids (54 $\pm$ 5 vs 50 $\pm$ 8 nmol/mg cell protein, p NS). The amount of (^{14}C) oleate released, however, is the

Figure 5. LIPID HYDROLYSIS



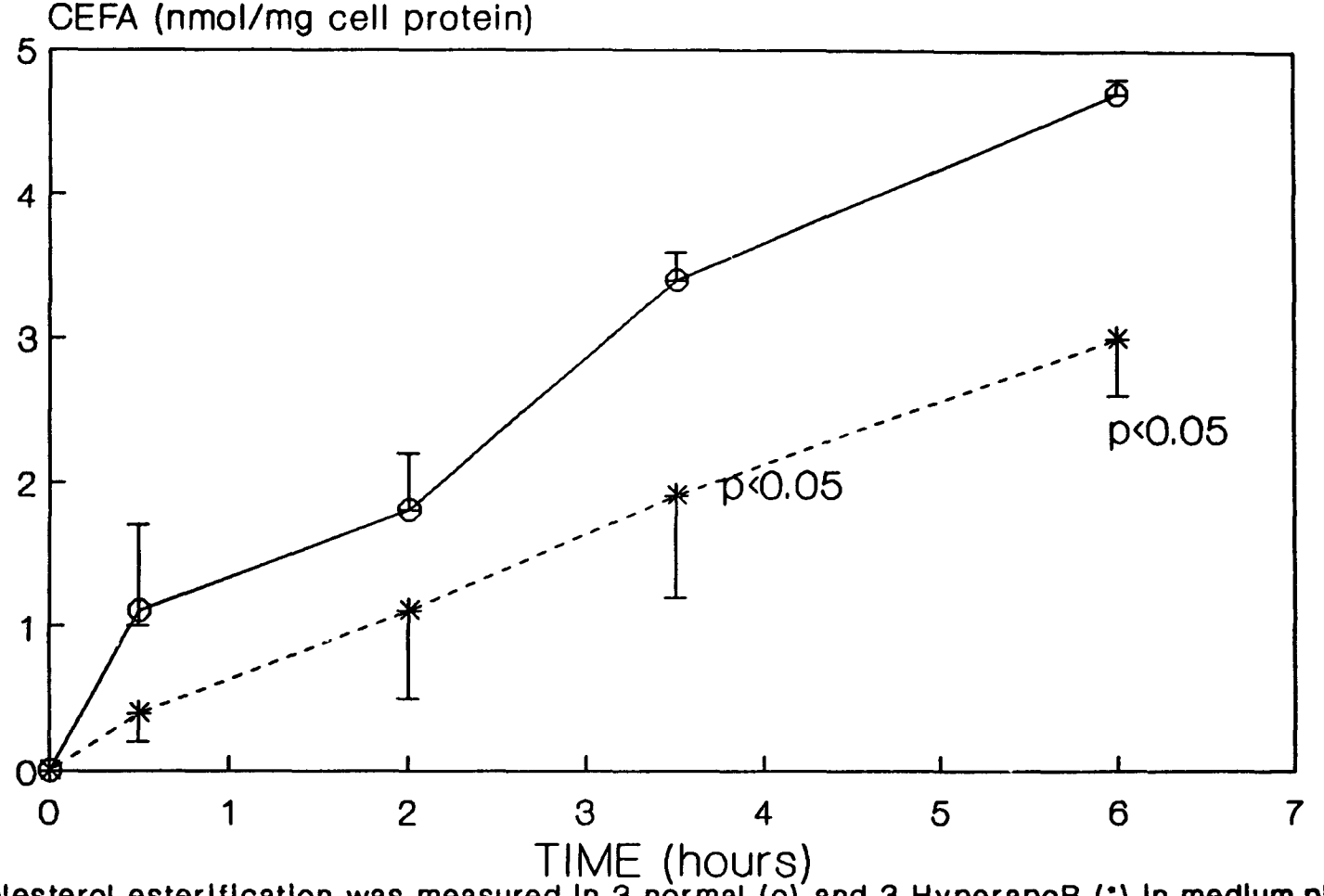
Lipid synthesis was measured following incubation in 100 uM oleate for 24 hours.Parallel Cells were then washed and incubated for 2 hours in serum-free medium. Cell lipid and oleate released were measured in 5 normal (o) and 7 HyperapoB (*) cell lines. Results are given as average + half error bars.

same in both normal and HyperapoB cells. Therefore, hydrolysis of lipids occurred at the same rate in both normal and HyperapoB cells.

Fatty acids are not only esterified to glycerol-3-phosphate to form storage triglycerides, but can also be linked to cholesterol to form cholesterol ester, which can be stored intracellularly (Figure 2). Therefore this reaction, cholesterol esterification, was also examined. In the absence of LDL in the medium, cholesterol ester synthesis in fibroblasts is minimal (71). Following preincubation in the presence of LDL, which results in cholesterol influx into the cells, cholesterol ester synthesis does increase substantially although (1-^{14}C) oleate incorporation into triglycerides and phospholipids still predominates. Cholesterol ester synthesis in normal fibroblasts was found to be linear up to 10 hours and was linear up to (^{14}C) oleate and LDL concentrations of 150 uM and 150 ug/ml, respectively (data not shown) . Accordingly, cells were preincubated in serum medium with 100 ug/ml LDL followed by incubation with 100 uM (^{14}C) oleate for the indicated time periods. As shown in Figure 6, cholesterol esterification was significantly reduced in the HyperapoB cells by 44% and 36% at 3 and 6 hours respectively ($p<0.05$).

These results have demonstrated that the HyperapoB cells are different from the normal cells with regard both

Figure 6. CHOLESTEROL ESTERIFICATION



Cholesterol esterification was measured in 3 normal (o) and 3 HyperapoB (*) in medium plus LDL (100 ug/ml) and 100 uM oleate for 0 to 6 hours as described in Materials and Methods

to the synthesis and storage of two acylation products: cholesterol ester and triglyceride.

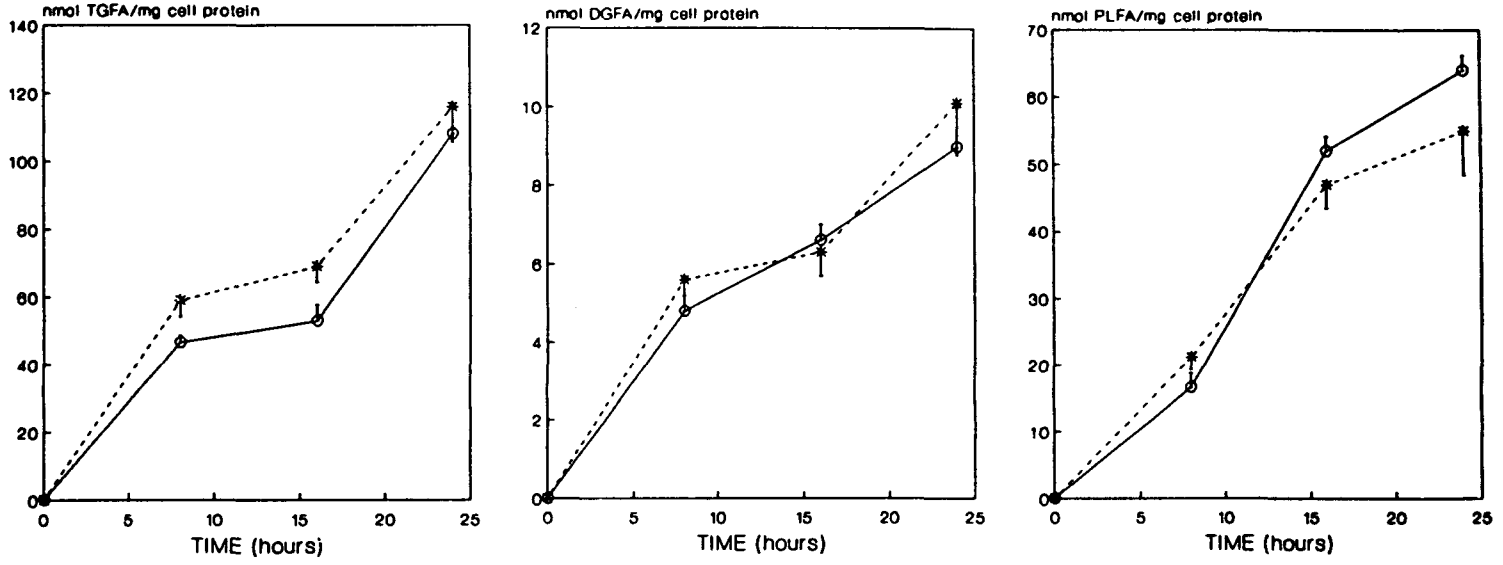
1.3 LIPID SYNTHESIS IN HORMONE MEDIUM

Lipid synthesis was examined in a serum-free hormone supplemented medium (referred to as HORMONE medium). This medium was devoid of serum but was supplemented with various vitamins and hormones including insulin, transferrin, biotin, Ca pantothenate and triiodothyronine as described in Materials and Methods. The amounts of hormones present were not necessarily at physiological concentration, but those shown by Amarosa et al (71) to maximize endogenous lipid synthesis. Assays indicated that optimal experimental conditions were similar to those chosen for lipid synthesis in serum medium (figure 3) as further discussed in section 1.5 (figures 10,11). Lipid and triglyceride synthesis increased up to 24 hours and 150 uM (^{14}C) oleate in normal cells (data not shown).

Lipid synthesis was examined in both normal and HyperapoB cells using the hormone medium in both preincubation and incubation stages. As shown in Figure 7, in contrast to the findings using serum medium (Figure 4), lipid synthesis was the same in both the normal and HyperapoB fibroblasts. This was the case for triglyceride, diglyceride, and phospholipid synthesis (Figure 7) at all the time points examined. This lack of difference between

Figure 7. HORMONE MEDIUM: NORMAL VS HYPERAPOB

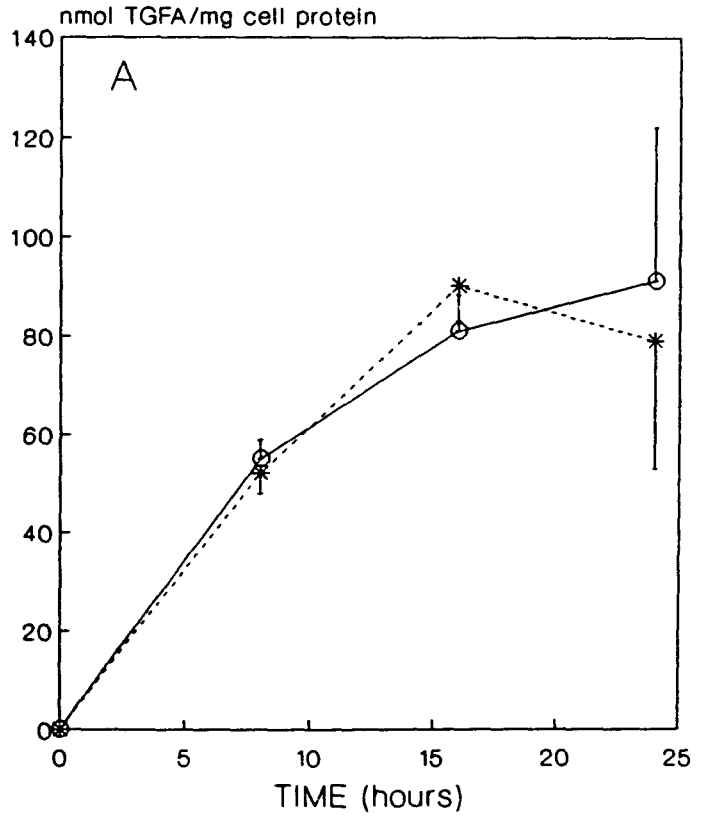
TRIGLYCERIDE DIGLYCERIDE PHOSPHOLIPIDS



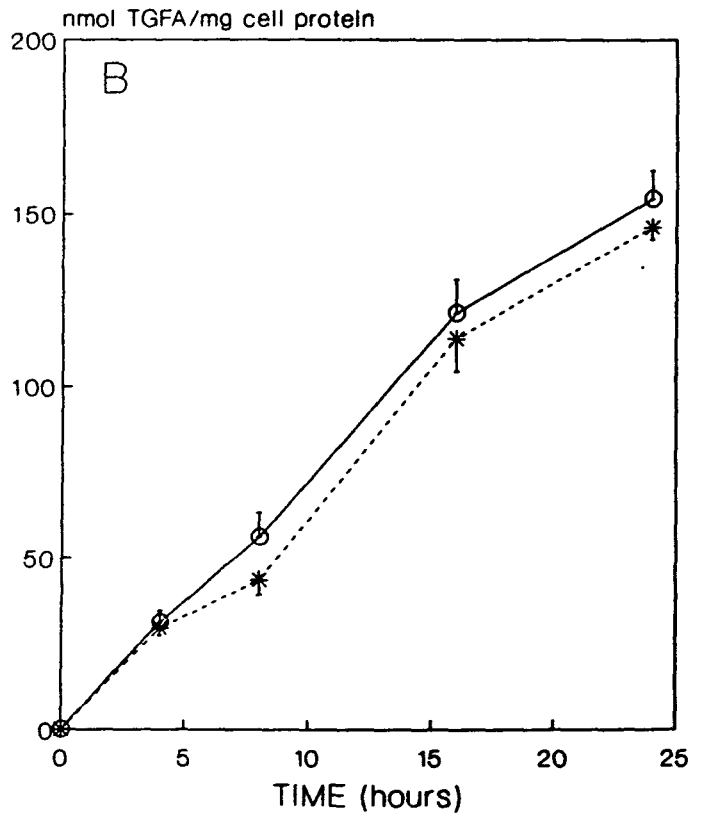
Triglyceride, diglyceride and phospholipid synthesis were measured in 5 normals (o) and 7 HyperapoB (*) in hormone medium with 100 uM oleate from 0 to 24 hours as described in Materials and Methods

Figure 8. TRIGLYCERIDE SYNTHESIS IN HORMONE MEDIUM

50 uM OLEATE



150 uM OLEATE



Triglyceride synthesis was measured in 5 normal (o) and 7 HyperapoB (*) in hormone medium with 50 uM (A) or 150 uM (B) oleate from 0 to 24 hours as described in Materials and Methods. Results are shown as average + half error bars.

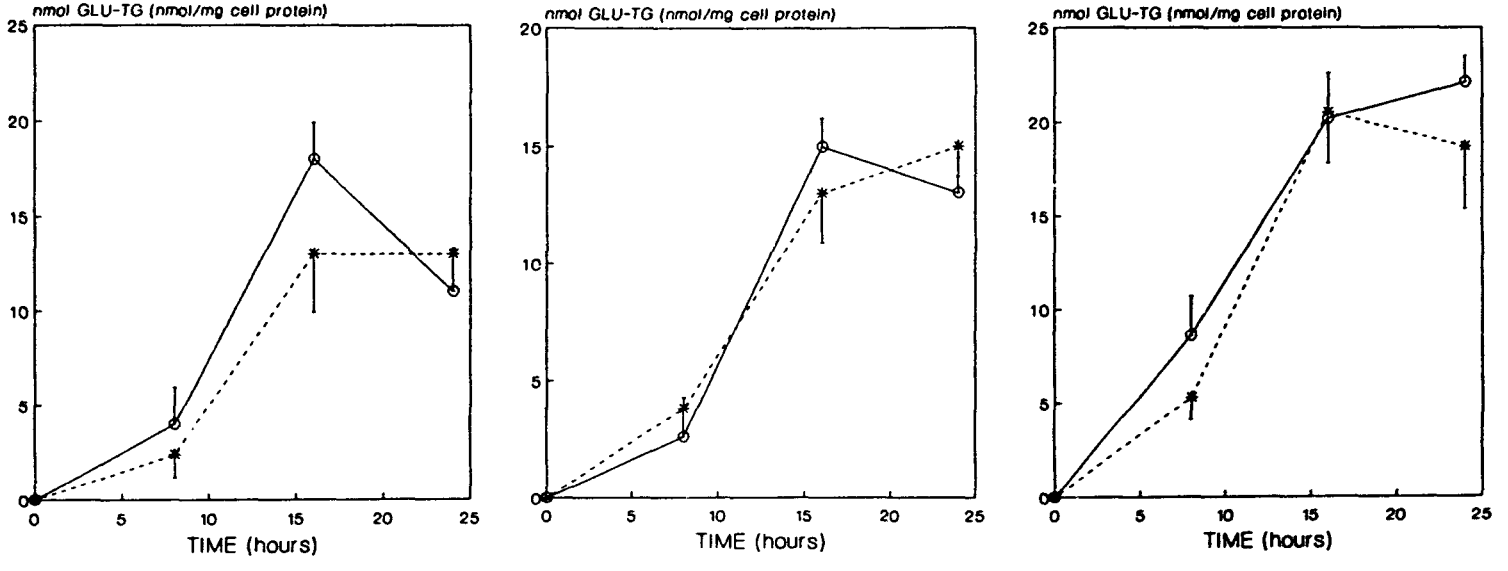
the normal and HyperapoB cells in hormone medium was confirmed using two other concentrations of (^{14}C) oleate, 50 uM and 150 uM, both within the linear range of lipid synthesis and these results for triglyceride are shown in Figure 8A and 8B. Similarly there was no difference in phospholipid synthesis. Determination of (^{3}H) glucose incorporation into triglycerides at these same oleate concentrations (50 uM, 100 uM and 150 uM) also demonstrated no difference between normal and HyperapoB cells (Figure 9). Additionally, the (^{14}C)oleate : (^{3}H) glucose ratio ranged from 6.3 $\pm$.7 in the normals and 6.8 $\pm$ .4 in the HyperapoB, close to the theoretical ratio of 6:1 as discussed above, reinforcing the conclusion that, in fact, in hormone medium there is no difference in the rate of triglyceride synthesis between the normal and HyperapoB fibroblasts.

1.4 SERUM MEDIUM VS HORMONE MEDIUM

Lipid syntheses in serum medium vs hormone medium at 24 hours and 100 uM oleate are directly compared in Table IV. Phospholipid synthesis in both cell groups was the same in either medium. However, in normal cells triglyceride synthesis is greater in serum medium than in hormone medium ($p<0.05$) whereas, by contrast, in HyperapoB cells, triglyceride synthesis is less in serum medium than in hormone medium ($p<0.025$).

Figure 9. GLUCOSE INCORPORATION INTO TRIGLYCERIDE

50 uM OLEATE 100 uM OLEATE 150 uM OLEATE



Glucose incorporation into triglyceride was measured as described in Materials and Methods in hormone medium containing 5.5 mM glucose and 50, 100 or 150 uM oleate complexed to albumin in 5 normal (o) and 7 HyperapoB (*) subjects from 0 to 24 hours. Results are shown as average + half error bars.

TABLE IV. LIPID SYNTHESIS: SERUM VS HORMONE MEDIUM

	TRIGLYCERIDE		DIGLYCERIDE		PHOSPHOLIPID	
	SERUM	HORM	SERUM	HORM	SERUM	HORM
	nmol/mg cell protein					
Normal (n=5)	133 ±8	108 ±9	10 ±1	9 ±1	51 ±7	65 ±2
p	0.05		NS		NS	
HyperapoB (n=7)	82 ±9	116 ±11	6 ±1	10 ±1	40 ±4	55 ±7
p	0.025		0.0125		NS	

Lipid synthesis was measured as described in Materials and Methods in serum or hormone medium supplemented with 100 uM oleate complexed to albumin for a 24 hour incubation time. Results are the average of 5 normal and 7 HyperapoB and are expressed as nmole oleate/mg cell protein ± s.e.m.
Serum= serum medium, Horm= hormone medium.

The search for other differences between normal and HyperapoB cells and between serum and hormone medium was pursued. The possibility that the difference in triglyceride synthesis might be due to different levels of intracellular glucose, the precursor to glycerol-3-phosphate, was explored. In experiments using labelled ^{3}H glucose to determine incorporation into triglyceride (as described above), the total amount of labelled cellular glucose in the cell suspension was also measured. Levels of cellular (^{3}H) glucose and (^{3}H) glucose derived products were the same in both normal and HyperapoB cells either with serum medium or hormone medium (910$\pm$ 71 Normal vs. 900 $\pm$28 HyperapoB nmol/mg cell protein in serum medium; 802 $\pm$115 Normal vs. 949 $\pm$46 HyperapoB nmol/mg cell protein in hormone medium).

Basal levels of endogenous fatty acid and lipid synthesis , that is, lipid synthesis in the absence of any added fatty acid in the medium, were also the same in normal cells and HyperapoB cells using either serum or hormone media. This is measured as (^{14}C) acetate incorporation into the various cell lipids as shown in Table V. Similarly, no difference in ($Me^{3}H$) thymidine uptake was detected. This is taken as an indication of DNA synthesis. Finally total amounts of cellular protein per tissue culture dish at the time of harvest were the same in both media. These last two points preclude a general cell

TABLE V. ACETATE INCORPORATION INTO LIPIDS

		TRIGLYCERIDE	CHOLESTEROL	PHOSPHOLIPID
		----------nmol/mg cell protein----------		
SERUM	Normal	4.2±1.2	5.7±1.5	11.6±3.1
	HyperapoB	3.4±0.5	4.4±1.0	10.0±1.6
	p	NS	NS	NS
HORM	Normal	3.6±0.8	1.9±0.5	19.9±8.5
	HyperapoB	3.1±0.7	1.8±0.4	18.3±4.3
	p	NS	NS	NS

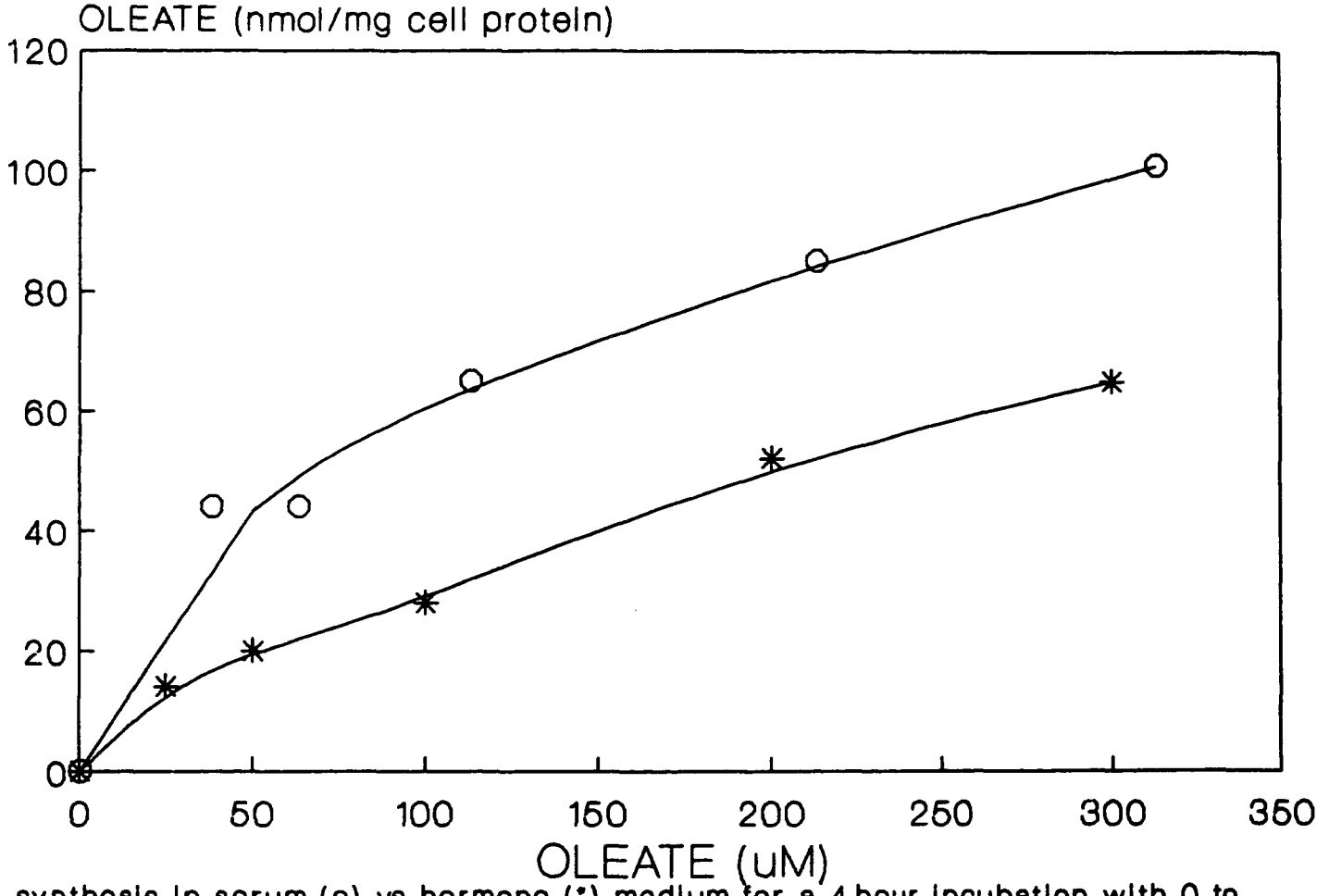
Acetate incorporation into lipids was measured as described in Materials and Methods in serum and hormone medium supplemented with 1 mM radioactive acetate incubated with 5 normal and 7 HyperapoB cell lines for 24 hours. Results are

proliferative effect as explanation for the increased lipid synthesis. Therefore, although a significant difference in triglyceride synthesis between normal and HyperapoB cells was evident in the presence of serum medium, that difference no longer existed in the presence of hormone supplemented medium. That difference was accordingly ascribed to a proposed factor present in the serum to which the normal cells were responding.

1.5 SERUM EFFECT ON TRIGLYCERIDE SYNTHESIS IN NORMAL FIBROBLASTS

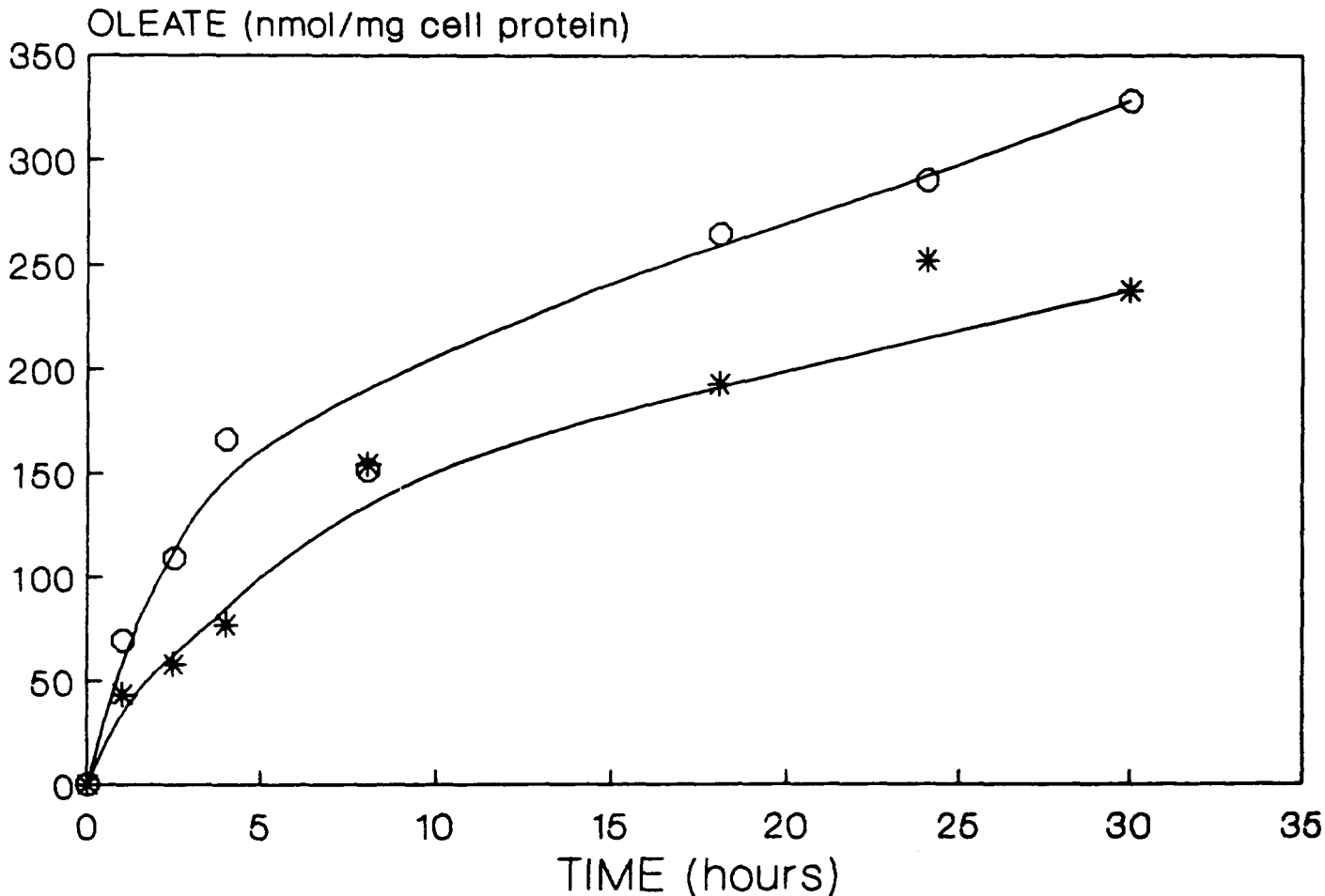
This effect was, therefore, further characterized. Serum medium and hormone medium were compared under exactly the same experimental conditions using normal fibroblasts. In Figure 10, the effect of oleate concentration on fibroblast lipid synthesis is shown over a 4 hour time period. Oleate (bound to albumin) was increased systematically from 25 uM to 300 uM. Serum concentration in the medium was maintained at 2.5 mg protein/ml medium. In the hormone medium the concentration of albumin was 1.25 mg/ml, approximately the amount present in medium supplemented with 10% (v/v) serum. Free fatty acid uptake and incorporation into lipids increased as the concentration of oleate in the medium increased. However, at each point, oleate incorporation into cell lipid was significantly higher in the serum medium as compared to the

Figure 10. LIPID SYNTHESIS: OLEATE CONCENTRATION



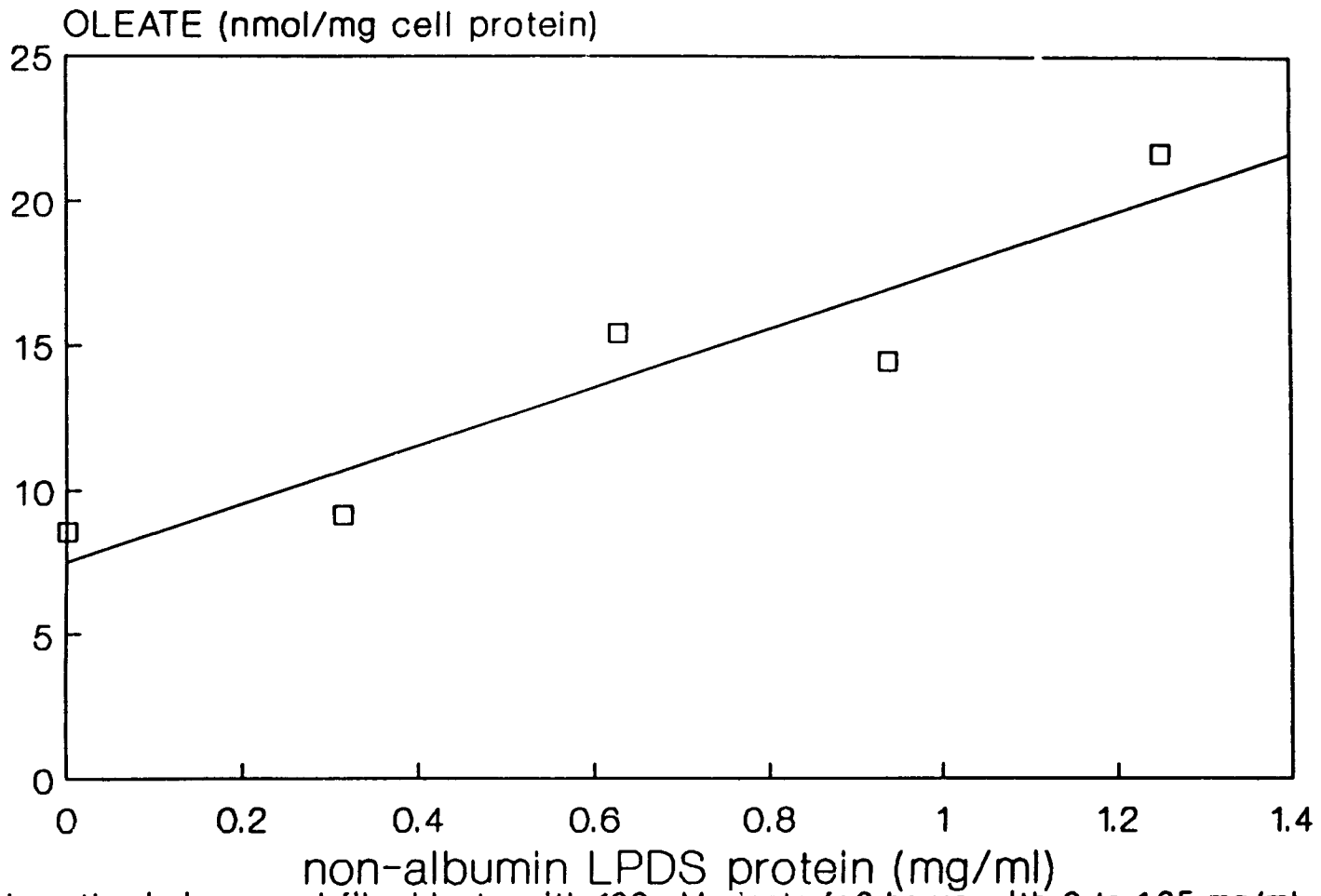
Lipid synthesis in serum (o) vs hormone (*) medium for a 4 hour incubation with 0 to 300 uM oleate, as described in Materials and Methods.

Figure 11. LIPID SYNTHESIS VS TIME



Lipid synthesis in serum (o) vs hormone (*) medium at 100 uM oleate for 0 to 30 hours, as described in Materials and Methods.

Figure 12. LIPID SYNTHESIS: EFFECT OF LPDS PROTEIN

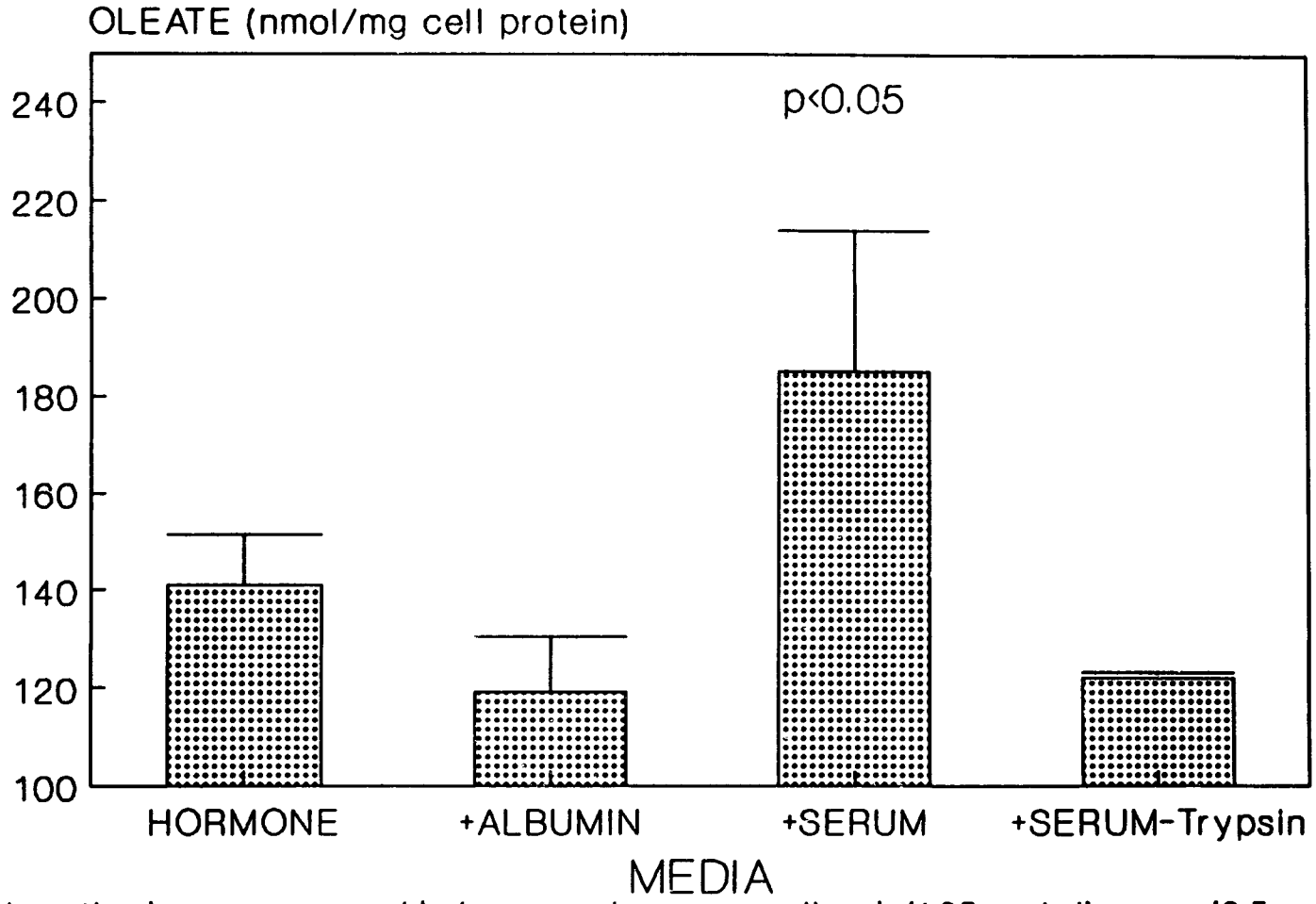


Lipid synthesis in normal fibroblasts with 100 uM oleate for2 hours with 0 to 1.25 mg/ml non-albumin LPDS protein, as described in Materials and Methods

hormone medium. Similarly, at all incubation times examined up to 30 hours, the difference between serum and hormone medium was maintained (Figure 11).

Next, the effect of medium composition itself on oleate incorporation into total lipids was examined. The data are shown in Figure 12. Albumin concentration in the medium was maintained constant at 1.25 mg/ml while the non-albumin protein in the serum was increased successively from 0 to 1.25 mg/ml. Care was taken to maintain the albumin concentration constant in order not to produce effects due solely to a changing free fatty acid to albumin ratio (92). Note that oleate incorporation into total lipid was a linear function of the amount of non-albumin serum protein, increasing from 7.5 to 21.6 nmol oleate incorporated/ mg cell protein over this range. On the other hand, serum pretreated with trypsin did not stimulate oleate incorporation into lipids whereas dialyzed serum did as shown in figure 13.

Figure 13. LIPID SYNTHESIS: EFFECT OF MEDIUM



Lipid synthesis was measured in hormone, hormone + albumin(1.25 mg/ml),serum (2.5 mg/ml) or trypsinized-serum medium with 100 uM oleate for 24 hours as in Materials and Methods

1.6 DISCUSSION

These studies have shown that fibroblasts can be used as a model to study triglyceride synthesis and storage and that these cells are consistent and reproducible in their response to various media. As a rule, fibroblasts do not store phospholipids; phospholipids are synthesized for immediate use only, however, the cells will respond to an excess free fatty acid influx by esterifying the fatty acids to produce storage triglyceride. The major fatty acid in adipose tissue triglyceride is oleate, which represents more than 50% of total triglyceride fatty acids (86). Oleic acid, when incubated with fibroblasts in cell culture, promotes triglyceride synthesis and deposition but even at high concentrations and for extended periods of time remains non-toxic to the cells, in contrast to other fatty acids (87). It should be noted that the concentrations of fatty acid used were well within the physiological range of fatty acid concentrations found in plasma . Both medium supplemented with serum, and serum-free hormone medium were examined. The use of serum-free medium has gained popularity in tissue culture because it allows greater consistency in batch-to batch preparation, and of greater relevance, it allows examination of the effect of serum factors on lipid synthesis. Unfortunately, it is not well tolerated by many cells. The serum-free medium used here does not represent a physiologic medium, since the concentration of supplements,

particularly insulin at 2.5 ug/ml is far in excess of serum concentrations of 2.5 ng/ml. It was chosen, however, since it had been developed for maximal lipid synthesis in human skin fibroblasts (71). The data reported above indicate that serum can stimulate lipid, and in particular, triglyceride synthesis in normal human skin fibroblasts. The factor is trypsin-sensitive, non-dialyzable and operates over a wide range of fatty acid concentrations and incubation times. The effect is suggestive of a protein action and demonstrates a concentration dependent response. Additionally, the stimulation is specific for lipid synthesis, since hydrolysis is not affected, and a general cell proliferative response has also been excluded. The lipogenic effect of serum has been previously reported. It was shown that pre-adipocyte differentiation was dependent on the presence of serum in the media, and was species sensitive (89). This effect could not be exactly reproduced by inclusion of insulin or other hormones in the media. This "lipogenic factor", however, has not been characterized or isolated.

However, the responses that characterize the normal skin fibroblasts are either absent or markedly attenuated in similar cells derived from patients with HyperapoB. This difference is present consistently regardless of the marker of intracellular triglyceride synthesis used, either the fatty acid moiety (^{14}C oleate) or the glycerolipid backbone moiety (^{3}H glucose). This is even more striking because lipid

synthesis in a hormone medium is identical in both cell groups, as are the other cellular parameters that were examined. In the absence of LDL in the medium, cholesterol esterification in minimal (71). In the presence of LDL, cholesterol influx into the cells increases. Cholesterol esterification in the HyperapoB cells under these conditions was found to be markedly reduced as compared to the normal fibroblasts. The difference between normal and HyperapoB cells is restricted principally to the acylation reactions which produce triglyceride and cholesterol ester, both being lipids which are stored intracellularly (Figure 2).

These findings may have important implications for the understanding of intracellular fatty acid metabolism as well as further delineation of the pathogenesis and genetic basis of HyperapoB. Additionally, the data are consistent with previous work which showed a reduction in the rate of fatty acid uptake and esterification in adipose tissue from HyperapoB patients (33) and Hypertriglyceridemic patients (35-37). These findings are also consistent with the hypothesis that, in vivo, peripheral tissues in such patients do not incorporate free fatty acids at a normal rate, thus creating the potential, particularly postprandially, for an increased free fatty acid flux to the liver, resulting, in turn, in increased VLDL production. These results, therefore, support the hypothesis set out in the introduction.

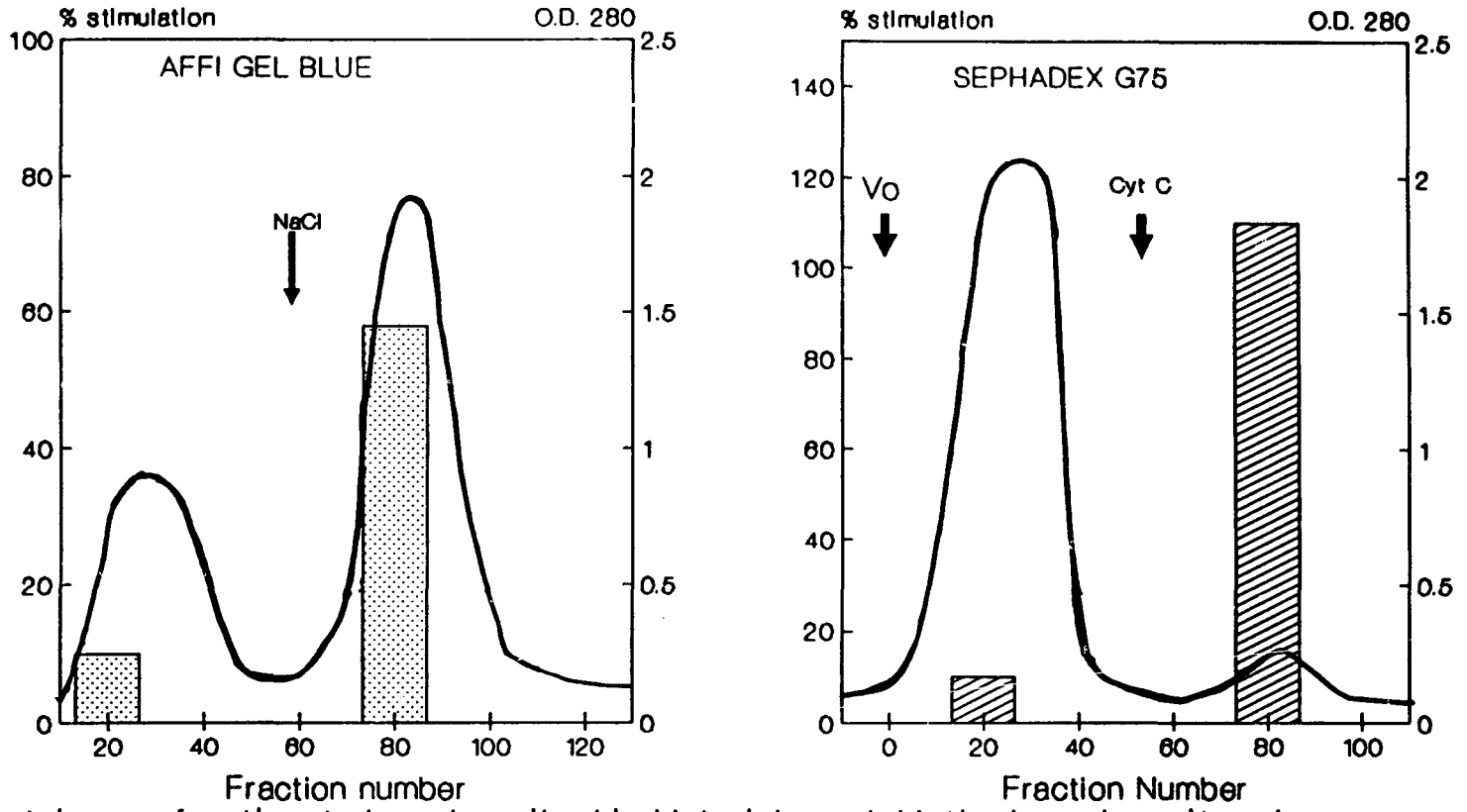
Clearly, however, before the proposed protein's mode of action could be elucidated, or before further testing of these hypotheses with respect to HyperapoB, complete purification of the active factor had to be achieved.

2. PURIFICATION AND CHARACTERIZATION OF ASP (ACYLATION STIMULATING PROTEIN)

2.1 PURIFICATION OF ASP

Purification of ASP (acylation stimulating protein) was achieved by a three-step chromatographic procedure. At each step, the capacity of each column fraction to stimulate incorporation of radiolabelled oleic acid into cell lipids was assayed using cultured normal human skin fibroblasts as described in Materials and Methods. Following defibrination, normolipidemic human serum was applied to a dye-binding Affi-gel Blue column. This resulted in two fractions, one fraction which eluted directly from the column (unbound fraction) and a second fraction which remained bound to the column and was eluted from the column by addition of 3 M NaCl to the starting buffer (Figure 14A). Both fractions were concentrated, dialyzed and the protein content determined. Each fraction constituted about 50% of the recovered protein as determined by protein assay. The effect of the fractions (1.25 mg protein/ml cell medium) on (^{14}C) oleate incorporation into total lipids in normal fibroblasts is indicated by the bars in Figure 14A. Virtually all of the acylation stimulating activity was confined to the second peak. This fraction was then applied to a Sephadex G-75 column (Figure14B) and resolved into two peaks. The first, and by far the largest, eluted close to the void volume at

Figure 14. ISOLATION OF PARTIALLY PURIFIED PROTEIN



Protein was fractionated as described in Materials and Methods and monitored at O.D. 280 (solid line). Fractions were pooled and assayed for lipid stimulation on normal fibroblasts.in hormone medium with 10 uM oleate for 20 hours (bars).

a molecular weight corresponding to that of albumin. That this peak was indeed primarily albumin was confirmed by both SDS 15% polyacrylamide gel electrophoresis and by double immunodiffusion (95) against anti-albumin antibody (Boehringer-Mannheim). Acylation stimulating activity, however, was confined to the smaller second peak which eluted after the total column volume at Ve/Vo=3.4, pointing to interaction of this fraction with the gel. This fraction constituted < 1 % of total applied protein and this step produced an 80-fold increase in specific activity as compared to the starting material, lipoprotein deficient serum (Table VI); as well, little activity was lost.

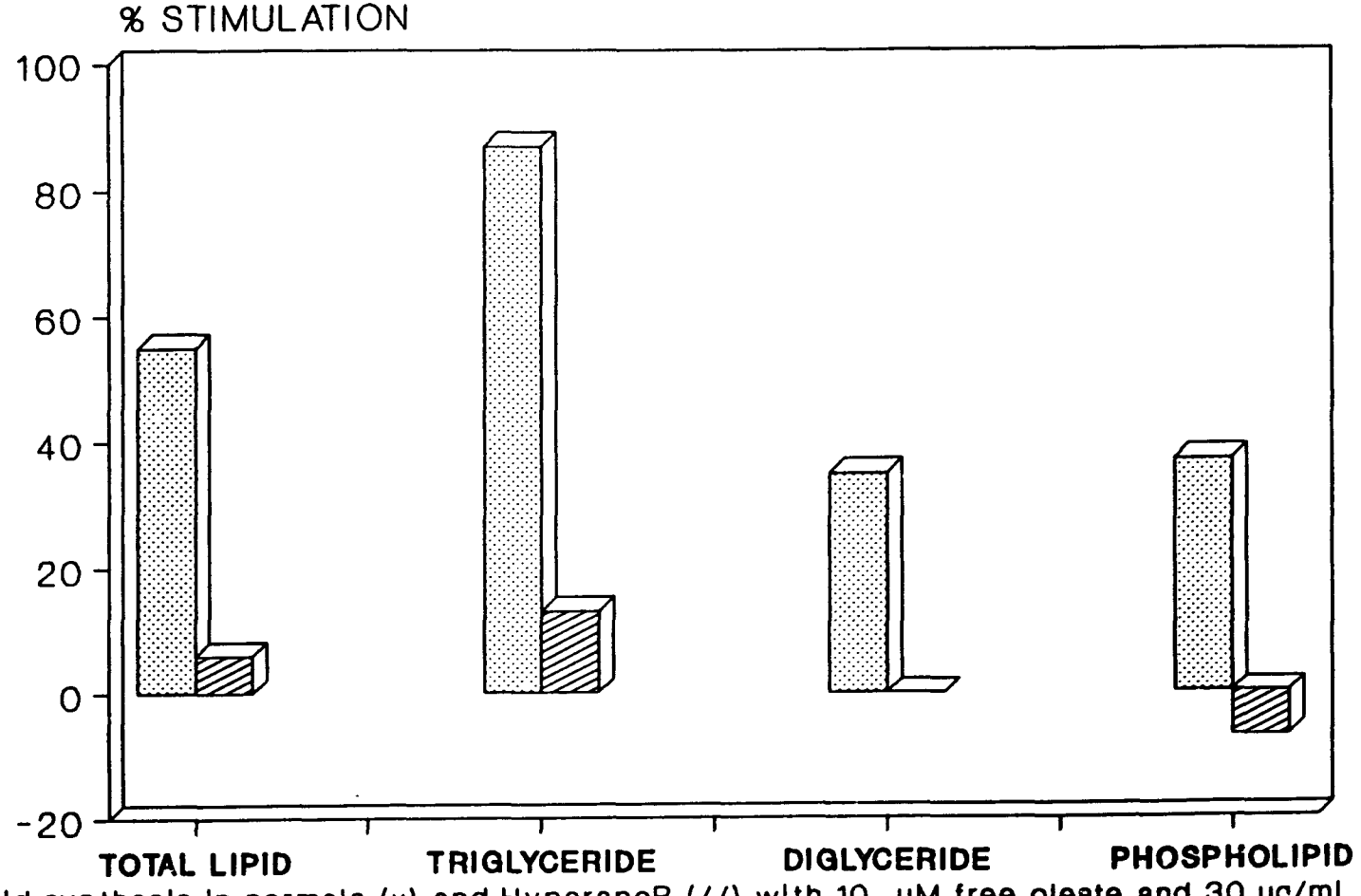
During purification, the object was not only to purify the factor to which the normal cells were responding, but also to monitor the response (or lack of response) of the HyperapoB cells to the serum fraction. These results are shown in Figure 15. With incubation times of 4 hours and 24 hours (not shown), there is consistent stimulation of triglyceride ($p<0.005$, and $p<0.01$) synthesis in the normal cells. Also, at 4 hours, there is significantly higher diglyceride ($p<0.005$) and phospholipid synthesis ($p<0.0125$) in the presence of the serum plasma fraction (30 ug/ml). By contrast, lipid synthesis does not increase in the HyperapoB cells in the presence of the protein fraction, as reflected by triglyceride, diglyceride, and phospholipid synthesis, but in some cases actually shows a downward

TABLE VI: PURIFICATION OF ASP

FRACTION	PROTEIN (mg)	SPECIFIC ACTIVITY (units/mg)	TOTAL ACTIVITY (units)	PURIFICATION (FOLD)	YIELD (%)
PLASMA	1645	28±7	46060	-	-
AFFIGEL	910	46±6	41860	1.6	91%
SEPHADEX G75	10	3680±76	34960	131.0	76%
CHROMATO-FOCUSING	0.5	12200±610	6340	436.0	14%

ASP was isolated as described under Materials and Methods. Fractions from each step were assayed for activity on normal fibroblasts with a 20 hour incubation in hormone medium with 10 uM oleate. The amounts of protein used were: plasma 2.5 mg/ml; Affi-gel fraction 1.25 mg/ml; Sephadex G-75 fraction 20 ug/ml and chromatofocusing fraction 10 ug/ml. One unit of ASP activity equals a 1% stimulation of oleate incorporation into lipids.

Figure 15. effect of partially purified fraction

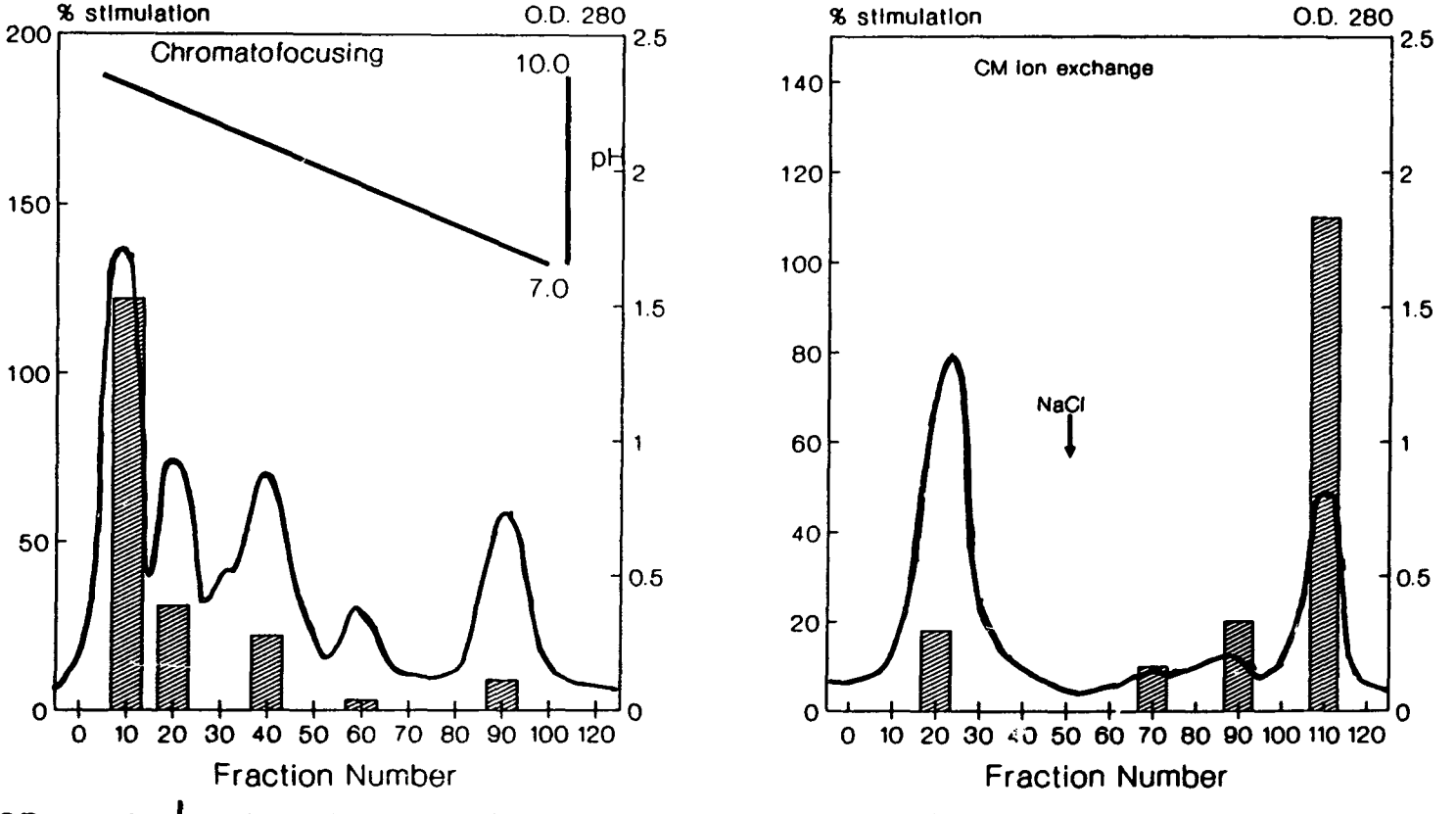


Lipid synthesis in normals (::) and HyperapoB (//) with 10 uM free oleate and 30 ug/ml partially purified fraction (peak II,Sephadex column,fig 14) for 4 hours, as in methods.

trend. In fact, this was also seen when serum medium was compared to hormone medium in the HyperapoB cells (Table IV). When normal cells are directly compared to HyperapoB cells in the absence of serum plasma protein, there is no difference in baseline lipid synthesis. However, when lipid synthesis between normal cells and HyperapoB cells is compared in the presence of the serum protein fraction, there is significantly more triglyceride ($p<0.05$) and phospholipid ($p<0.025$) in the normal cells.

A number of different chromatography media were assayed in order to separate the protein fraction into its various components. Purification to homogeneity was achieved by chromatofocusing in 6 M urea with a pH gradient of 9.7 to 7.0. This yielded the profile shown in Figure 16A. Acylation stimulating activity was predominantly associated (67%) with the first peak which emerged at a pH of 8.9 $\pm$0.1. A considerable amount of activity was lost during this step (Table VI) possibly due to irreversible inactivation of the protein following exposure to urea. In the absence of the urea, the fraction emerged from the chromatofocusing column as a single peak, which however contained multiple bands by SDS gel electrophoresis. Subsequently, this procedure was modified and purification to homogeneity was achieved by ion exchange chromatography on CM Sepharose at pH 7.5 in 0.02M phosphate buffer without urea, as shown in Figure 16B. At this pH, ASP binds to the

Figure 16. ISOLATION OF ASP



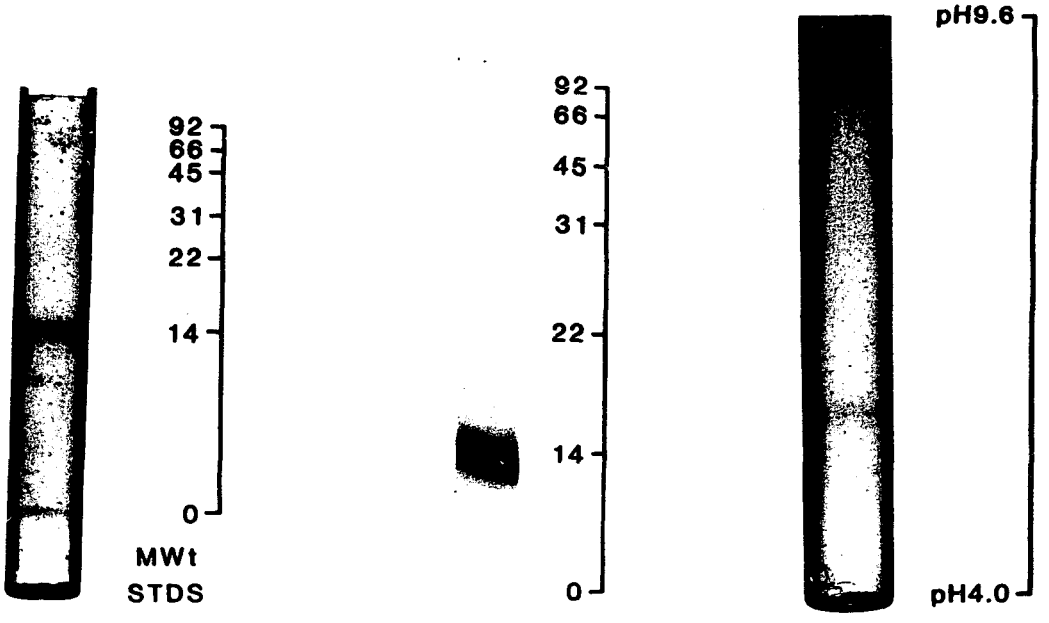
ASP was isolated as described in Materials and Methods. Protein was separated on chromatofocusing (left) or CM ion exchange (right) and monitored at O.D. 280 (solid line). Fractions were pooled at assayed for lipid stimulation on normal cells incubated for 20 hours in hormone medium with 10 uM oleate.

column and is subsequently eluted with 1M NaCl in 0.02M phosphate. Activity is confined to this bound protein.

2.2 PHYSICAL CHARACTERIZATION OF ASP

When ASP isolated by either chromatofocusing or ion exchange chromatography was analyzed by SDS 15% polyacrylamide gel electrophoresis with Coomassie Blue R250 staining, a band with a molecular mass of 14,000 ±400 daltons, n=26 (Figure 17) was demonstrated. Usually only a single band was evident but occasional preparations contained minor contaminants of 52 and 27 kDa molecular mass. If so, a second gel filtration using Sephadex G-75 was employed and complete purification achieved. Biologic activity was restricted to the fraction which contained the 14,000 molecular weight protein, which has been named Acylation Stimulating Protein (ASP). Of note, a single band was evident with both urea/non-reducing SDS conditions as well as reducing SDS gel electrophoresis. However, a comparison of reducing vs non-reducing conditions (with no urea) reveals that ASP migrates slightly more rapidly under reducing conditions. Further, as shown in Figure 17, isoelectric focusing of ASP using a pH 3-10 gradient also revealed only a single band. The pI of this band based either on gel pH or by calibration against an isoelectric focusing standard was 9.0 ±0.3 n=5, a value consistent, therefore, with the observed pH at which the protein was isolated by chromatofocusing.

Figure 17. ASP
molecular weight and pI



ASP was electrophoresed as described in Materials and Methods for molecular weight determinations: Left: 17 ug ASP isolated by chromatofocusing and Center: 20 ug ASP isolated by CM ion exchange; and for isoelectric point determination: Right: 25 ug ASP isolated by CM ion exchange.

The yield of ASP, the increase in purification, and the retention of biologic activity during the purification of ASP are summarized in Table VI. Starting with 1.6 g of plasma protein (50 ml), 0.5 mg of ASP was obtained, a purification of 436 fold, based on specific activity. Total yield of activity of the final fraction was 14%.

The physical characteristics of ASP are summarized in Table VII. ASP has a molecular weight of 14,000 $\pm$400 and a pI of 9.0 $\pm$0.3. The molecular weight determination from amino acid analysis integer residue number optimization is 14,500, a value in close agreement with the average SDS polyacrylamide gel electrophoresis value. The relative number of polar residues of 0.49 is close to the characteristic value for a large number of soluble proteins, that is, above 0.47 (91). The average hydrophobicity, H0=886 calories per residue, calculated as described by Bigelow (92), falls within the range for globular proteins. The amino acid compositional analysis reveals a high content of cysteine residues which may result in intramolecular association via disulfide bridges. Some of these cysteine residues in the oxidized form may be essential for ASP activity, since when ASP purification is performed in buffers containing 2-mercaptoethanol, activity is lost. The apparent lower molecular weight demonstrated by SDS PAGE under reducing conditions also suggests that

Table VII.
PHYSICAL CHARACTERISTICS OF ASP

Molecular Weight: PAGE (daltons) (n=26 determinations)	14,000±400
pI (n=5 determinations)	9.0±.3
Polarity Index	0.489
Hydrophobicity HO (cal/residue)	0.886

Amino Acid Composition:
(purified by ion exchange chromatography)

	mole %	number of residues
ASP/ASN	10.3	14
THR	4.9	7
SER	6.6	9
GLU/GLN	14.1	19
GLY	11.7	16
ALA	8.2	11
CYS	5.4	7
VAL	4.8	6
MET	1.5	2
ILE	2.7	4
LEU	6.2	8
TYR	2.7	4
PHE	3.2	4
HIS	1.4	2
LYS	5.2	7
TRP	n.d.	n.d.
ARG	5.8	8
PRO	5.2	7

number of residues	135
molecular weight	14514

ND not determined

intramolecular cysteine bridges are an important component of the structure.

2.3 BIOACTIVITY OF ASP

The primary effects of ASP on lipid synthesis in normal fibroblasts are shown in Table VIII. Following incubation for 20 hours in medium containing 10 uM (^{14}C) oleate and 10 ug/ml ASP, fibroblast lipids were extracted and resolved by thin layer chromatography. Oleate incorporation into triglyceride, diglyceride and phospholipid constituted at least 95% of total fatty acid incorporated into cell lipids. In the absence of ASP, triglyceride is the major lipid synthesized. In the presence of ASP this trend is even more pronounced with the amount of oleic acid entering triglyceride synthesis increasing significantly ($p<0.0025$). From the data in Table VIII it is evident that ASP also significantly increases diglyceride synthesis ($p<0.05$). In normal fibroblasts, while phospholipid synthesis was increased on average, the difference was not statistically significant.

The effect of ASP on oleate incorporation into triglyceride is linear with incubation periods ranging from 1 to 24 hours. Interestingly, when linear regression of the data with and without ASP is performed the intersection

Table VIII. ASP EFFECT ON LIPID SYNTHESIS % STIMULATION AND LIPID DISTRIBUTION

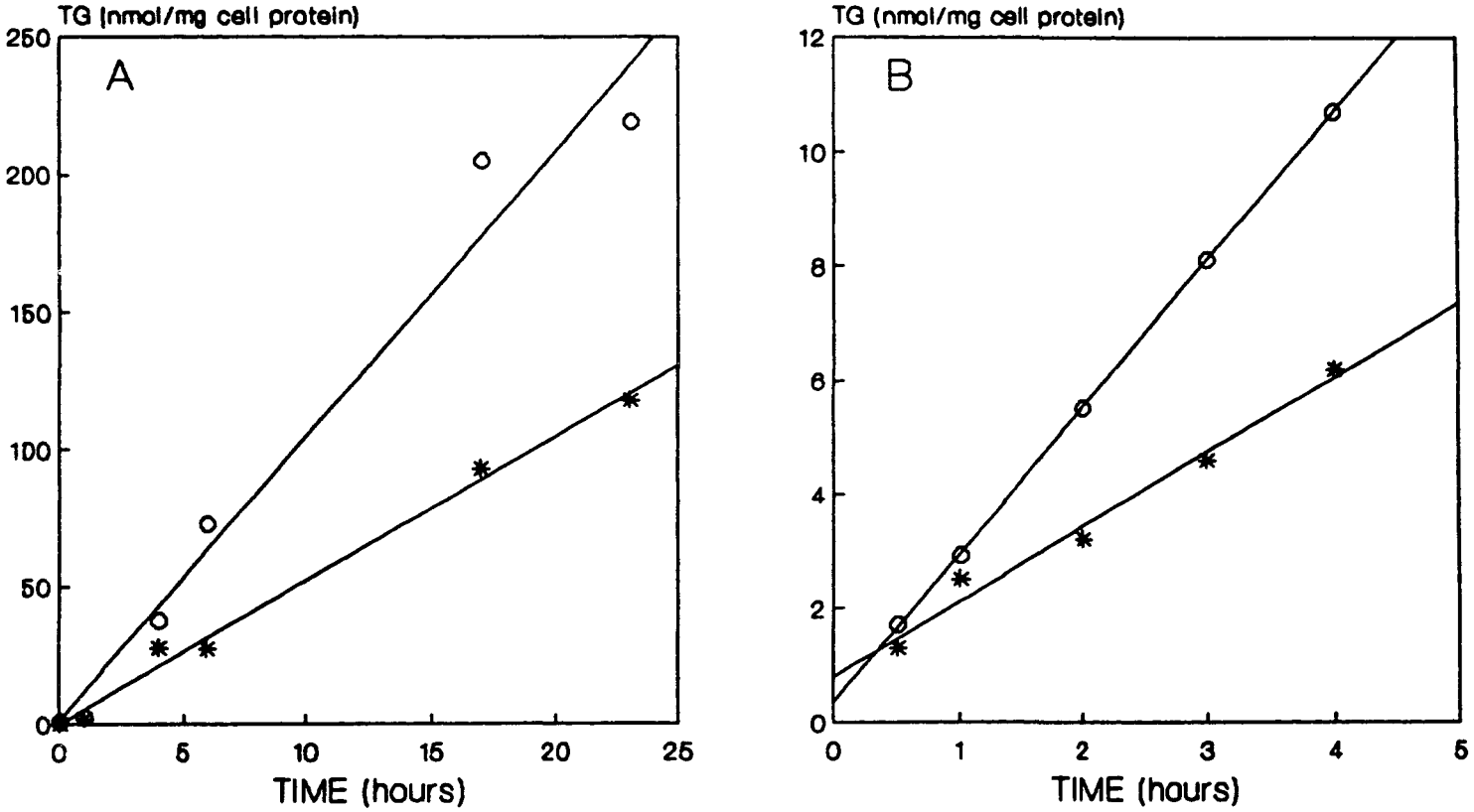
	TRIGLYCERIDE	DIGLYCERIDE	PHOSPHOLIPID
	nmol/mg cell protein		
(-) ASP	19.0±3.7 *	1.7±0.3	16.5±3.5
	(50%)	(5%)	(43%)
(+) ASP	34.8±6.2	2.4±1.3	23.3±5.5
	(56%)	(4%)	(37%)
% stimulation	110±36	41±29	39±9
p	<0.0025	<0.05	NS

Normal fibroblasts (n=7) were incubated for 20 hours with 10 ug/ ml ASP and 10 uM oleate as described in Materials and Methods.

* Percent distribution of TG, DG, and PL within total lipids.

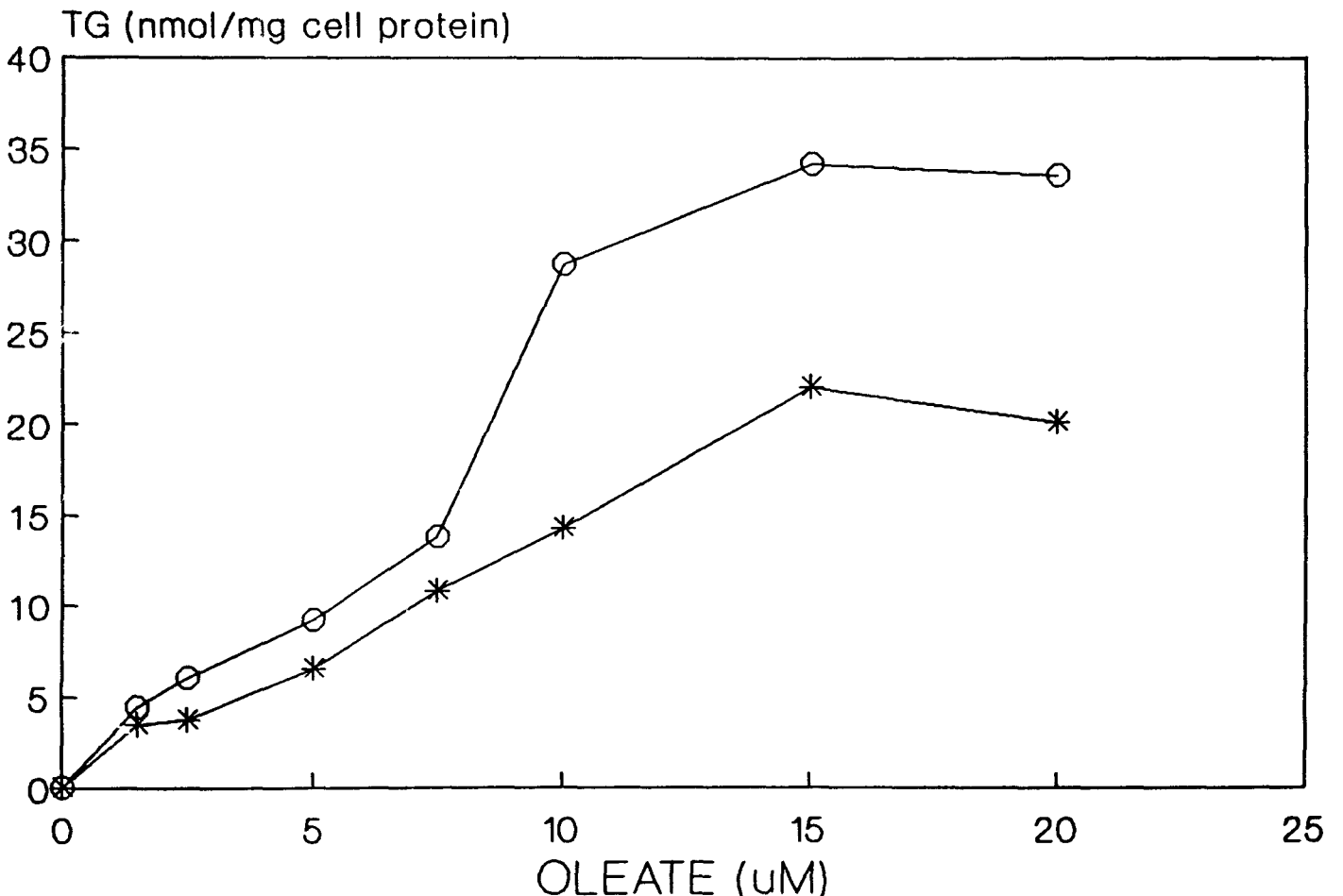
Percent stimulation is the increase in activity above baseline calculated as an average of 8 experiments.

Figure 18. ASP EFFECT ON TRIGLYCERIDE SYNTHESIS TIME CURVE



ASP effect on triglyceride synthesis in normal fibroblasts incubated in hormone medium with 10 uM oleate and 15 ug/ml ASP (o) or without ASP (*) for 0 to 24 hours (A) or 0 to 4 hours (B) (o) y=2.6x+.46 r=.99 (*) y=1.23x+.95 r=.97

Figure 19. ASP: EFFECT OF OLEATE CONCENTRATION



Triglyceride synthesis in hormone medium plus 10 uM free oleate without (*) or with (o) 10 ug/ml ASP for 6 hours in normal cells as described in Materials and Methods

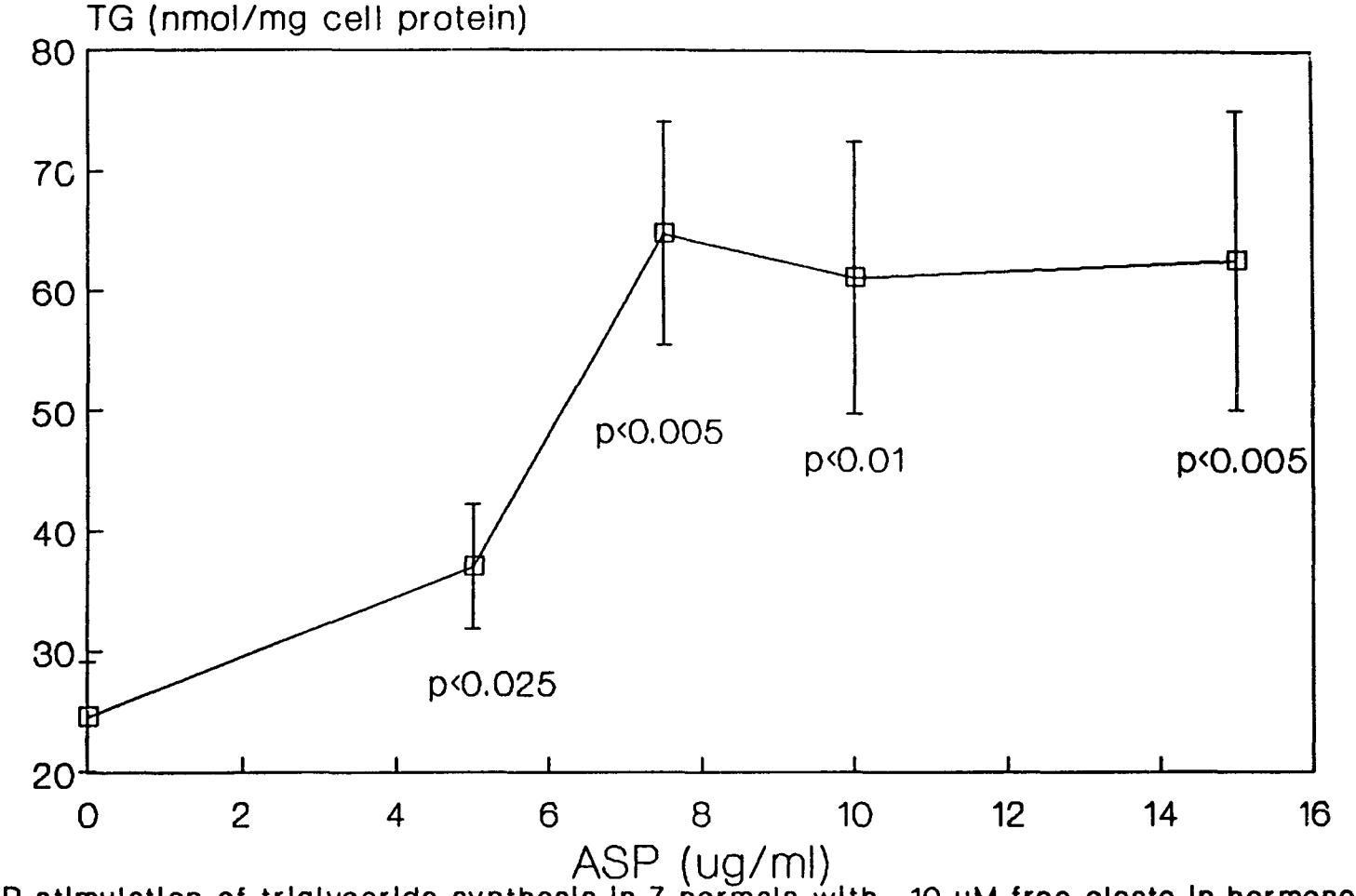
occurs at 30 mins, suggesting a lag time before the effect of ASP becomes apparent. This is true in experiments examining the time relation of triglyceride synthesis over both long (Figure 18A) and short time periods (Figure 18B).

As shown in Figure 19 the ASP effect is also present over a range in fatty acid concentrations (2-20 uM) with the percentage stimulation remaining fairly constant over the range (54%). The upper limit of fatty acid concentration used was 20 uM, below the critical micellar concentration limit of 25 uM (40).

The effect of varying the concentration of ASP on lipid synthesis in normal fibroblasts is shown in Figure 20. With increasing amounts of ASP, triglyceride synthesis increased, reaching a maximum of 165 $\pm$30% stimulation at a concentration of 7.5 ug/ml (.54 uM) with a 20 hour incubation. At this concentration, the range of stimulation seen in 7 normal fibroblasts examined varied from 71% to 228%. This plateau level may reflect either saturation of triglyceride synthetic enzymes or saturation of membrane receptor-mediated ASP action.

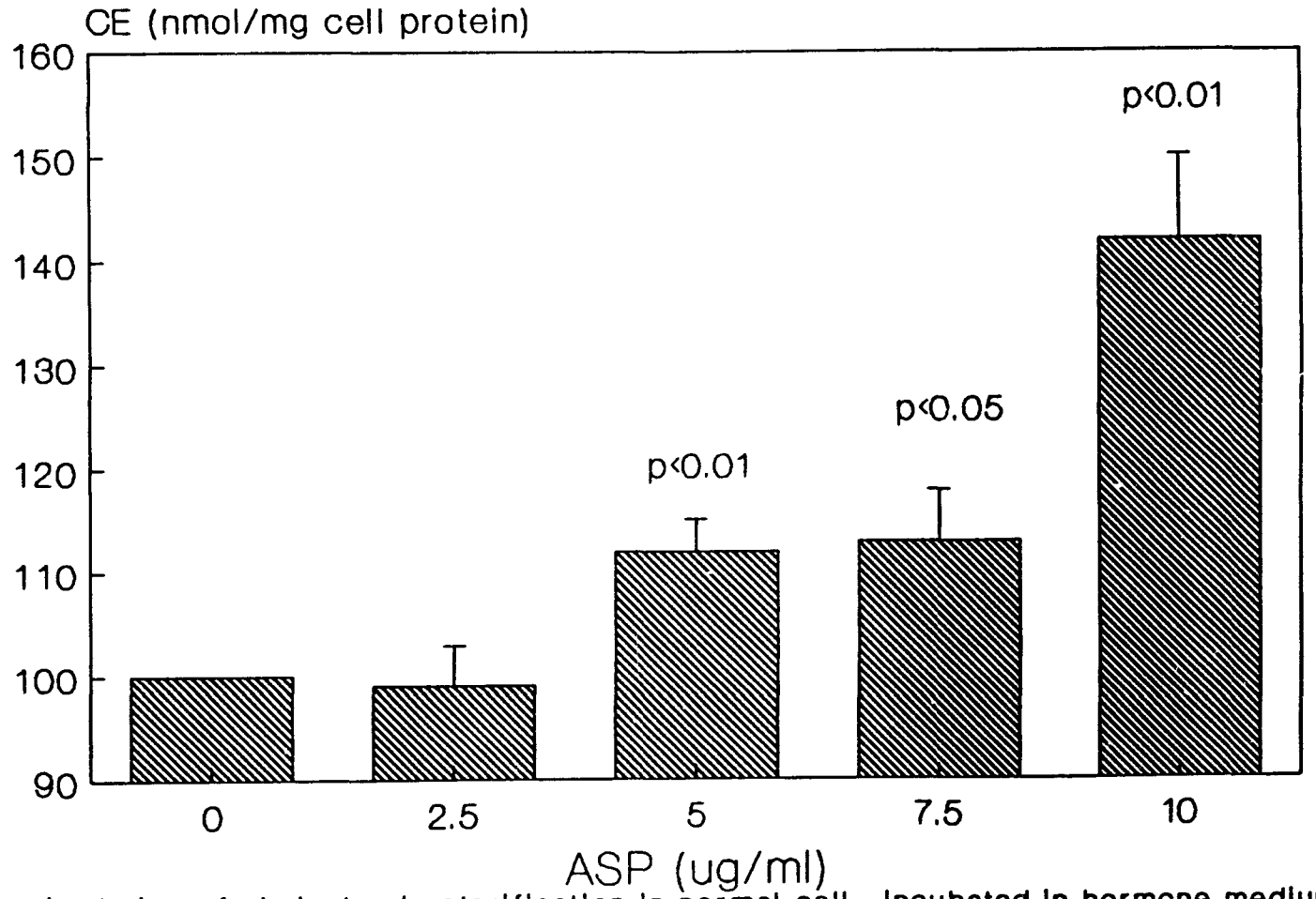
As was noted before, in the absence of low density lipoprotein (LDL), relatively little cholesterol ester is formed in fibroblasts. The effect of ASP on cholesterol esterification was therefore examined in fibroblasts in the presence of 50 ug/ml LDL added to the preincubation medium.

Figure 20. ASP: EFFECT OF CONCENTRATION



ASP stimulation of triglyceride synthesis in 7 normals with 10 uM free oleate in hormone medium for 20 hours as described in Materials and Methods (average ± s.e.m.)

Figure 21. ASP EFFECT ON CHOLESTEROL ESTERIFICATION

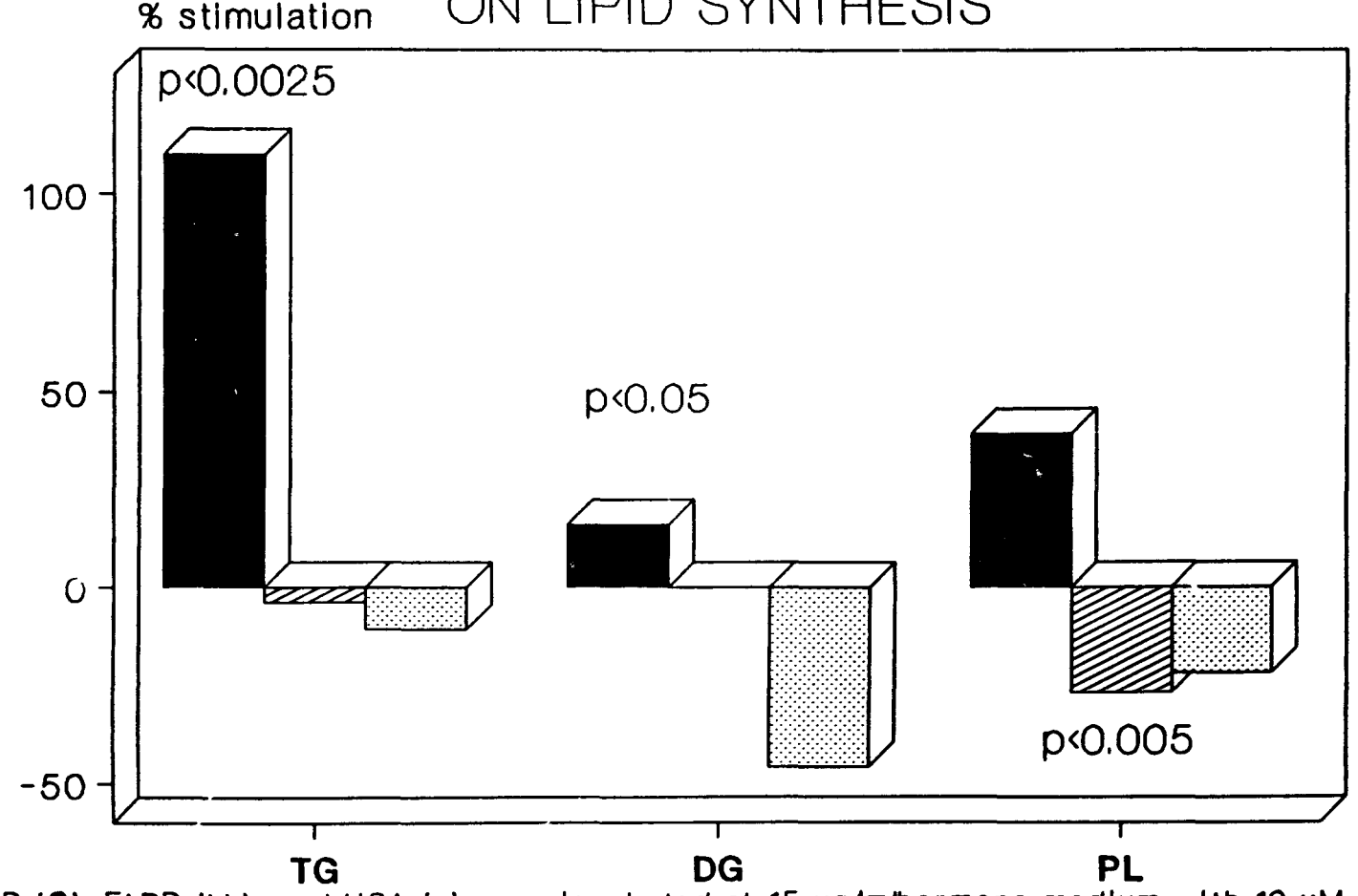


ASP stimulation of cholesterol esterification in normal cell Incubated in hormone medium with 10 uM free oleate and LDL (50 ug/ml) for 20 hours. Results are average ±sem

Baseline activity was 6.3 ±1.7 nmol cholesterol ester /mg cell protein with LDL as compared to 0.2 ±.2 nmol cholesterol ester/mg cell protein without LDL. In Figure 21 it can be seen that increasing amounts of ASP stimulate significantly the acylation of cholesterol, resulting in a 42% increase in cholesterol ester in the presence of 10 ug/ml of ASP. Thus ASP not only stimulates acylation of glycerol and/or glycerolipids to form triglyceride and diglyceride, but also the acylation of cholesterol to form cholesterol ester.

Various fatty acid binding proteins either of plasma origin (albumin) or intracellular origin (fatty acid binding protein-FABP)(47) have been implicated in fatty acid transport and lipid synthesis. The effects of albumin (HSA) and FABP on oleate incorporation into triglyceride were compared simultaneously with those of ASP. As seen in Figure 22, neither human serum albumin (HSA) nor FABP show any stimulatory effect on fatty acid incorporation into lipids in fibroblasts. Although the results shown here are at a concentration of 15 ug/ml, the effect of these proteins was investigated at concentrations varying from 5 to 30 ug/ml, and no effect of either albumin or FABP was found at any concentration (results not shown). This is in direct contrast to ASP which, when present in comparable amounts to the albumin and FABP, produces a considerable increase in lipid synthesis. Moreover, comparison of the

Figure 22. EFFECT OF FATTY ACID BINDING PROTEINS ON LIPID SYNTHESIS



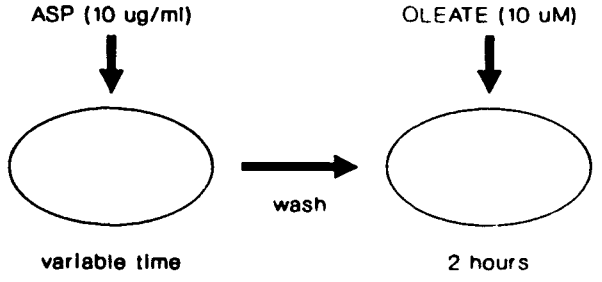
ASP (●), FABP (\\), and HSA (::) were incubated at 15 ug/ml hormone medium with 10 uM free oleate for 20 hours in normal cells as described in Materials and Methods

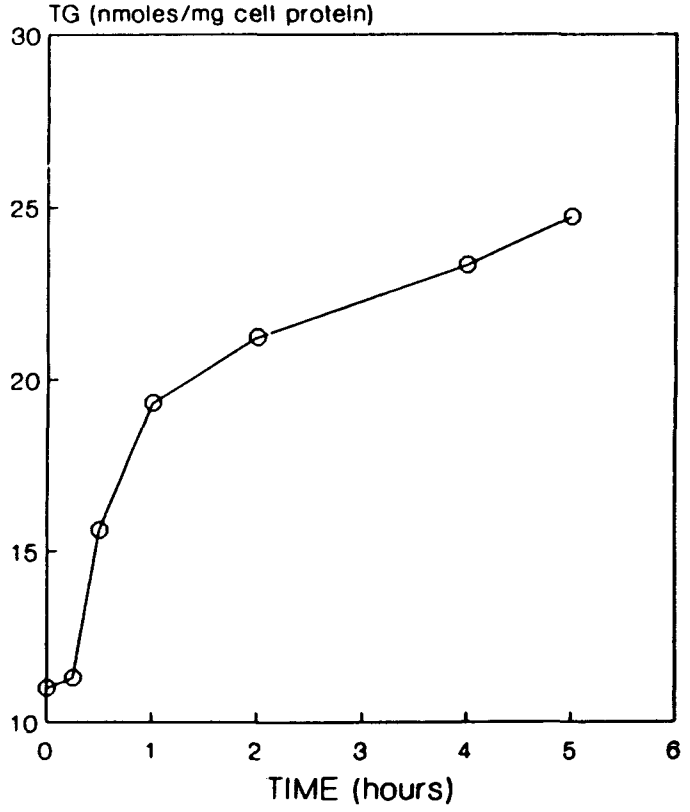
physical properties of ASP to those of various cytosolic and plasma proteins and apoproteins shows little similarity.

2.4 ASP CELL INTERACTIONS

These experiments have all examined the effect of ASP on triglyceride synthesis with the simultaneous incubation of ASP and oleate with the fibroblasts. This raises the question as to whether ASP is acting via independent interaction with the cells, or solely by increasing free fatty acid transport across the plasma membrane. If there is no specific ASP-cell interaction whatsoever, then a preincubation in the presence of ASP alone, without oleate, prior to incubation with oleate alone would not be expected to affect lipid synthesis. The hypothesis was that ASP would enter the cell during the preincubation period and that as a consequence, oleate incorporation into triglyceride would still be increased even though only free fatty acid and not ASP was present in the medium for the second phase. The outline and results of such an experiment are shown in Figure 23. Cells were incubated with 10 ug/ml of ASP (for maximal activity) for various times, followed by a 2 hour incubation with 10 uM oleate in the absence of ASP. It was felt that a 2 hour time interval provided sufficient time for the effect to be measured before all of the ASP could be metabolized. Note that there

Figure 23. ASP PREINCUBATION: EFFECT ON TG SYNTHESIS





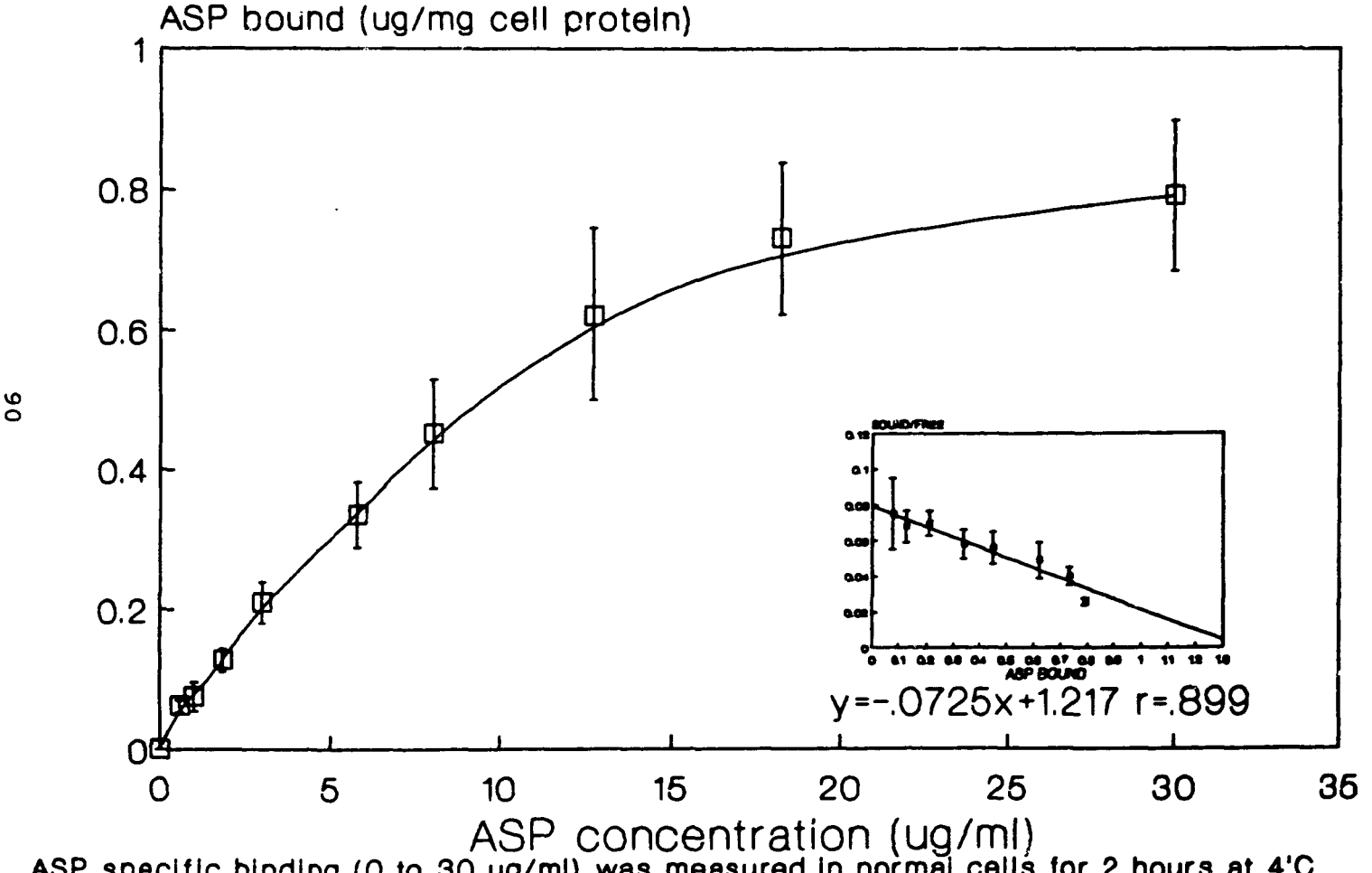
Normal cells were preincubated with ASP (10 ug/ml) in hormone medium for 0 to 5 hours followed by incubation with 10 uM free oleate for 2 hours. as described in Materials and Methods

is a substantial increase in triglyceride synthesis mediated by the ASP, and that this effect is maximal after 2 hours incubation (190 %). Additionally, there is a 30 min lag time before the effect of ASP becomes apparent, consistent with the previous time curves with ASP (Figure 18A and B).

The interaction of ASP with normal fibroblasts was further examined using radio-iodinated ASP and both total binding and non-specific binding were measured as described in Materials and Methods. At 4°C saturable specific binding of ^{125}I ASP is obtained as shown in Figure 24. Specific binding constituted 90% of total binding. Scatchard analysis of the binding data produces a linear isotherm consistent with a single class of binding sites (Figure 24 inset) with a K_D OF 9.9×10^{-7} M.

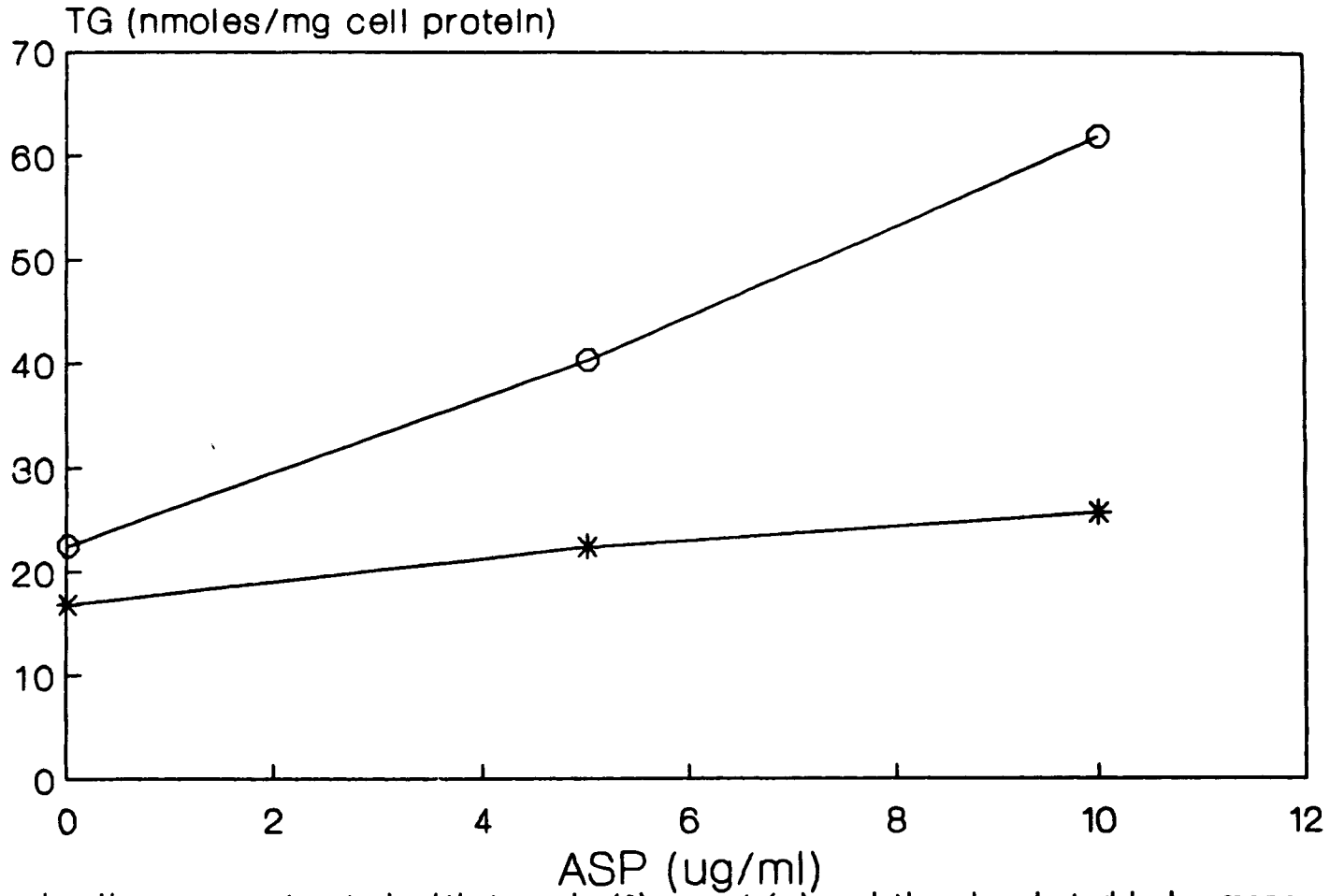
These binding studies indicate a specific receptor interaction. This was further examined by pretreating the cells in order to hydrolyze the cell surface proteins that might be involved in a receptor interaction with ASP (93). Following a mild trypsinization, the cells were incubated for 4 hours with 10 uM oleate and various concentrations of ASP. The results in the absence of trypsin treatment are shown in the circles and the results with trypsin treatment in the stars in Figure 25. In the absence of ASP (zero point) there is little difference in the amount of triglyceride synthesized between trypsin treated and

Figure 24. ASP SPECIFIC BINDING TO NORMAL FIBROBLASTS



ASP specific binding (0 to 30 ug/ml) was measured in normal cells for 2 hours at 4'C as described in Materials and Methods Inset: Scatchard linear regression analysis

Figure 25. EFFECT OF TRYPSIN TREATMENT ON ASP STIMULATION



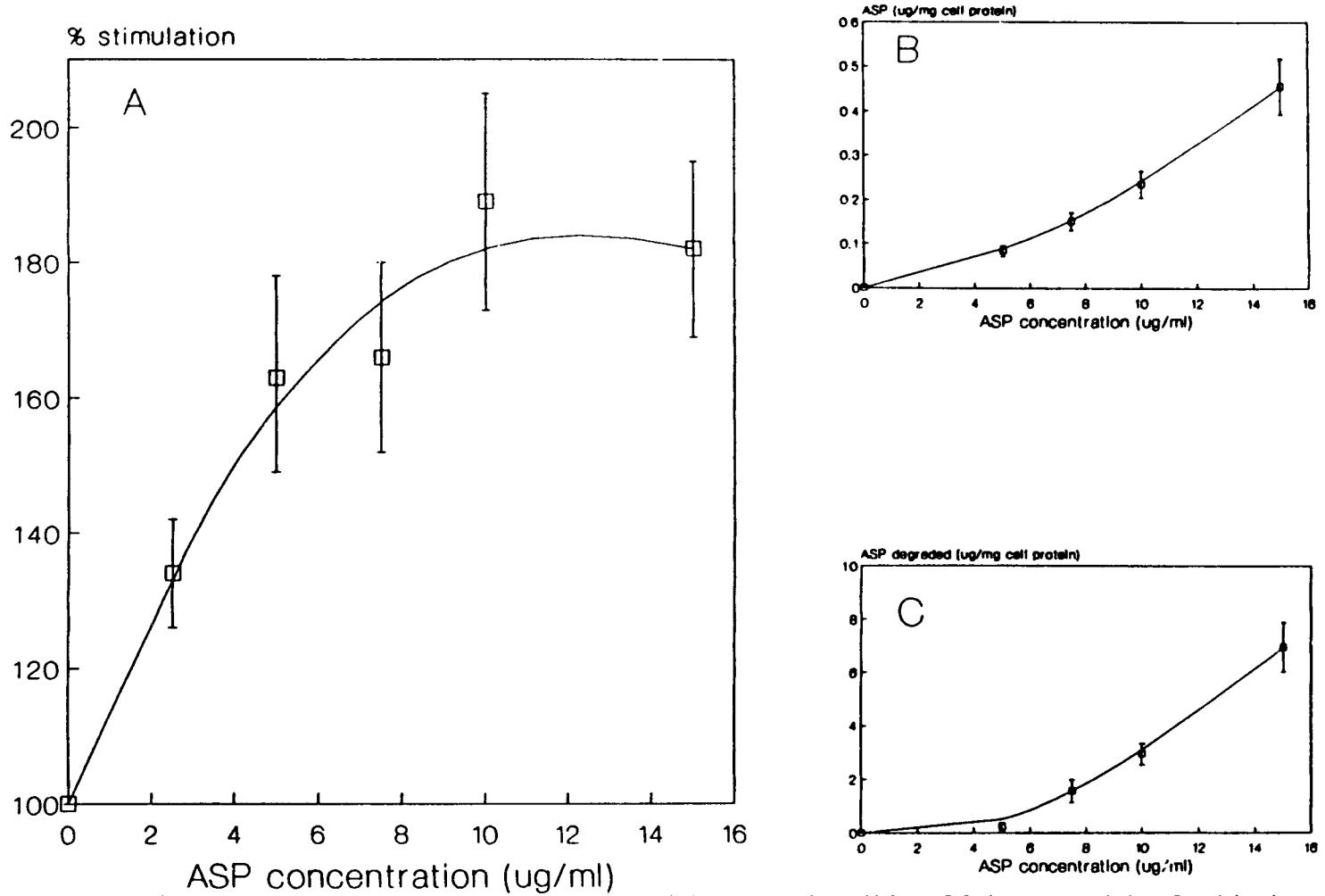
Normal cells were pretreated with trypsin (*) or not (o) and then incubated in hormone medium with ASP (0 to 10 ug/ml) and 10 uM free oleate for 4 hours (Materials and Methods)

untreated cells (all points were assayed in triplicate). Additionally, cells were microscopically examined to verify cell attachment to the dish and the cells from one dish were assayed for viability by Trypan Blue exclusion. In the untreated cells, 10 ug/ml ASP produces a 2.5-fold increase in radiolabelled triglyceride from 22.5 to 61.9 nmol/mg cell protein. In the treated cells, however, there is only a 22 % increase in triglyceride synthesis from 17 to 25.7 nmol/mg cell protein. This represents a 78% reduction in the ASP activity. These results indicate that ASP does interact with the plasma membrane, probably through interaction with a cell surface protein, and that this interaction is necessary to achieve an ASP effect.

2.5 ASP INTERNALIZATION AND DEGRADATION

An ASP-cell interaction suggests that the ASP may well also be internalized and degraded by the cells. To observe this, radiolabelled ASP was incubated with normal fibroblasts under the same conditions which produced the lipid synthetic stimulation. As shown in Figure 26B and C, ASP is both internalized and degraded by the cells. Additionally, there is concurrent stimulation of triglyceride synthesis as shown in Figure 26A. Although the levels of ASP internalized and degraded have not reached saturation levels, in spite of this, triglyceride synthesis appears to be saturated. This may be related more to the

Figure 26. ASP STIMULATION, INTERNALIZATION AND DEGRADATION



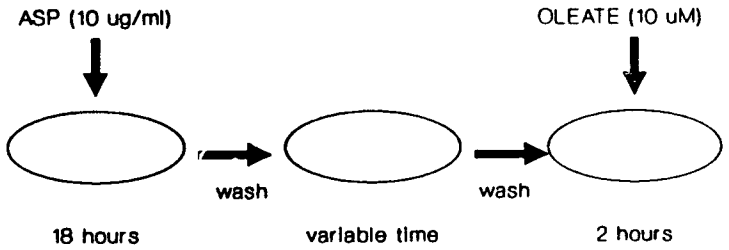
Iodinated ASP (0 to 14 ug/ml) was incubated with normal cell for 20 hours with 10 uM oleate
Results are average ± sem of 7 cells (see Materials and Methods

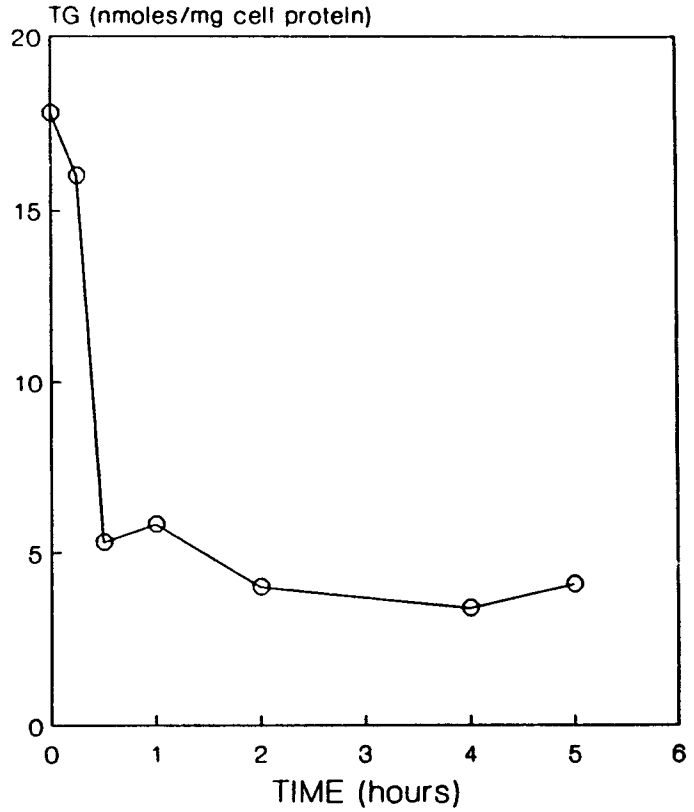
limited amount of triglyceride synthetic enzymes available in the fibroblast than to ASP metabolism per se. Note that, at the maximum level of ASP degradation, this represents only 2 % of the total medium ASP. However, of the total amount of ASP metabolized by the cell (internalized and degraded), 95 % has been degraded and the metabolites exported to the medium, suggesting reasonably rapid turnover of ASP within the cell.

This concept was pursued in the following experiment. Cells were preincubated with ASP for 18 hours. The medium was changed to one containing neither ASP or oleate and the cells were maintained in this medium, as shown in Figure 27, from 0-5 hours. Next , triglyceride synthesis was measured during a 2 hour incubation with ^{14}C oleate alone. There is a substantial and rapid drop in the amount of triglyceride present, implying as would be expected from the radioiodinated ASP data, rapid degradation of ASP by the cell.

The ASP effect therefore appears to be mediated through interaction with the plasma membrane, and ASP is in turn hydrolyzed within the cells. This satisfies the requirements both for a hormone mediated action and for a transport process. However, this does not answer the question as to whether internalization of ASP is necessary to provoke the metabolic response. To examine this issue, fibroblasts were treated in various ways to prevent

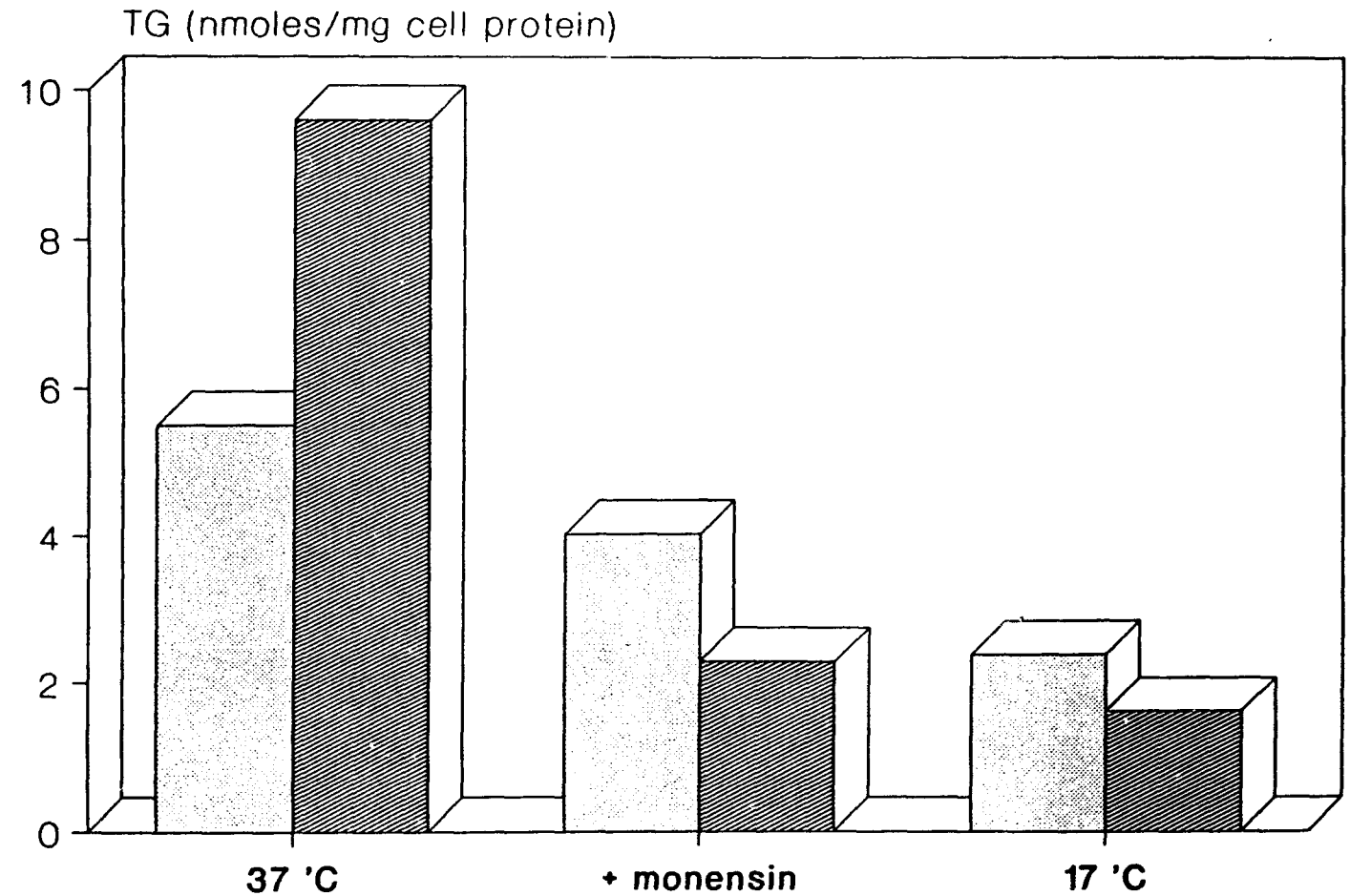
Figure 27. ASP POSTINCUBATION: EFFECT ON TG SYNTHESIS





Normal cells were incubated with ASP (10 ug/ml), followed byincubation with 10 uM oleate in hormone medium for 2 hours. Inbetween there was a wash time of 0 to 5 hours

Figure 28. ASP: EFFECT OF BLOCKING ENDOCYTOSIS



Normal cells were incubated with (//) or without (::) ASP 10ug/ml) in 10 uM oleate in hormone medium for 2 hours at 37'C, 17'C or monensin (5 uM)(see Materials and Methods)

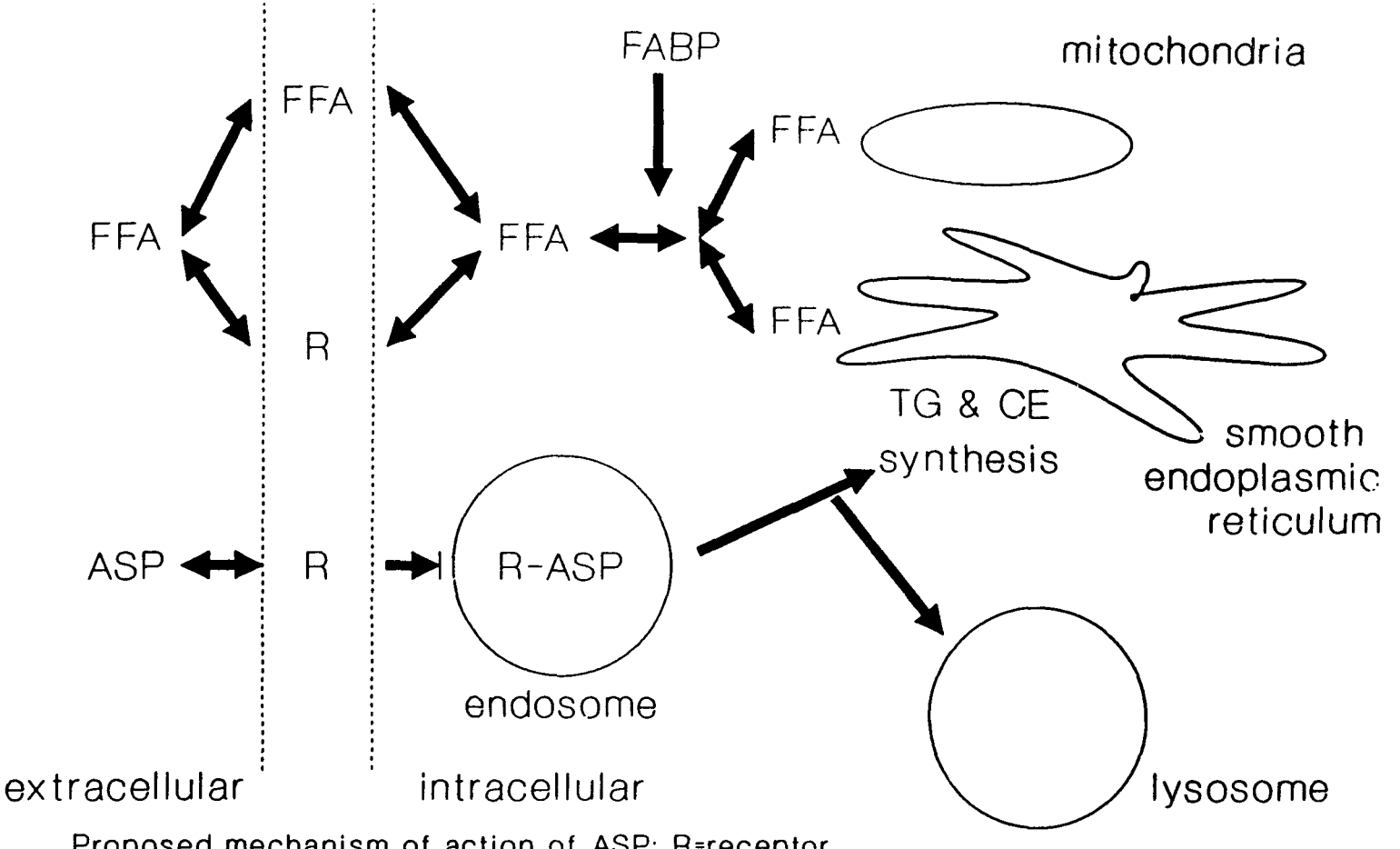
endocytosis. This was achieved by incubation in the presence of 5 uM monensin (57) or at 17°C (58), with or without ASP. As shown in Figure 28, with untreated cells ASP stimulates triglyceride synthesis by 180%. Incubation with monensin or incubation at a temperature of 17°C, both of which effectively block endocytosis, also completely block the ASP effect. These results imply that internalization of the protein is necessary for it to achieve its specific effect on the fibroblasts.

2.6 DISCUSSION

ASP is a small (14,000 Da), basic (pI 9.0) protein which has been purified 436 fold from human plasma to yield a homogeneous protein. The data presented above establish that ASP, a purified plasma protein, stimulates triglyceride synthesis and cholesterol esterification in cultured human skin fibroblasts. The effect is time-dependent and concentration dependent and is consistent with the results that were obtained using serum medium. In addition, the effect is demonstrated over a range of free fatty acid concentrations (2-20 uM). Although the experiments were performed with free oleate at low concentrations, previous experiments with serum medium showed that a similar effect was achieved when cells were incubated with higher concentrations of oleate complexed to albumin. The concentration of oleate used in the majority of the experiments (10 uM) is the calculated amount of free fatty acid present in a solution of 100 uM oleate complexed to albumin at a molar ratio of 6:1 (39).

The hypothesis is that specific binding to the plasma membrane appears essential to the metabolic mechanism of action of ASP (Figure 29). Trypsin pretreatment of the cells eliminates the ASP specific response indicating that ASP-cell interaction is necessary for the effect. In addition, ASP is internalized and degraded by fibroblasts. Hormones, for example, are well known to affect

Figure 29. MECHANISM OF ACTION OF ASP



Proposed mechanism of action of ASP: R=receptor
see list of abbreviations for definitions

intracellular lipid synthesis with catecholamines stimulating lipolysis (54), insulin promoting synthesis (53). That ASP stimulation exceeds that of insulin (53,63), where insulin has previously been thought to be the most potent known stimulator of triglyceride synthesis, has been demonstrated above. Certain cytosolic proteins have also been reported to increase microsomal triglyceride (94), cholesterol (46,47) and cholesterol ester (95) synthesis. However, none of these proteins examined appear to be physically similar to ASP, and, in this experimental system, one of these proteins, fatty acid binding protein (FABP), did not mimic the action of ASP. Therefore, altering intracellular lipid synthesis to this extent, consequent to the action of an extracellular protein, appears to be a novel observation.

What mechanism or mechanisms might be responsible, and why would the effect be more obvious for storage lipids such as cholesterol ester and triglyceride, but not as marked for those such as phospholipids which are not stored, but used immediately in the cell's ongoing metabolism? Since hormones, specifically insulin, have been shown to increase fatty acid esterificaton to form triglyceride, a stimulation which is not secondary to increased glucose transport, it is possible that ASP is operating in the same manner. Surprisingly, in spite of extensive research into the mechanism of action of insulin, the process by which it acts is still far from understood. Nonetheless, insulin appears to act on cells

primarily through interaction with the plasma membrane. In marked contrast with insulin, there is no ASP effect in the absence of internalization of ASP as shown in Figure 28. And indeed, the experiments designed to inhibit endocytosis indicate that the metabolic effect of ASP occurs after its interaction with the cell membrane, presumably after it actually enters the cell itself. These data therefore are not completely consistent with the hormonal model in which the ultimate intracellular effect occurs consequent to a secondary messenger cascade initiated solely by the initial ligand-plasma membrane receptor interaction. In addition, the effect of insulin is rapid, whereas the effect of ASP exhibits a 30 minute lag time before the effect is apparent, a phenomenon more characteristic of a process which involves secondary processing. This model is supported by the similar time lag before the drop in ASP effect (Figure 27) although this does not necessarily differentiate between hormone vs non-hormone mediated actions. Additionally, direct activation of the enzymes involved in triglyceride synthesis occurs by dephosphorylation (45) while acyl-cholesterol-acyl-transferase activity is increased by a phosphorylation reaction (96). Thus it is unlikely that a single stimulus acting in such a manner directly on enzymes would promote both reactions.

The data suggest that ASP enters the cell as a consequence of specific receptor binding (Figure 29). This

conclusion is based on the studies of binding, internalization and degradation using iodinated ASP. Clearly, endocytosis is also necessary in order to mediate the ASP action. With regard to triglyceride synthesis, the data make it clear that it is the synthesis of the total glycerolipid molecule that is affected by this activity, not merely re-esterification and/or glucose uptake into the cell. In general, enzyme reactions are product-inhibited, providing economical self-regulation. However, synthesis of cholesterol ester and triglyceride to be stored within cells cannot be so regulated, and control of these processes much more likely rests on substrate availability.

Theoretically, the protein might influence free fatty acid uptake into the cell and subsequent transport within the cell, or activity of the triglyceride synthetic enzyme complex located within the smooth endoplasmic reticulum. More is known of the last step than the first. Thus the processes by which free fatty acids enter cells remain surprisingly controversial, with most opinion favoring passive or membrane protein mediated diffusion (40-43), although others still suggest that binding of albumin to specific receptors occurs first, followed by inward transfer of free fatty acid. Similarly, how free fatty acid moves from the cell membrane through the cytoplasm to be targeted either to the mitochondria or smooth endoplasmic reticulum also remains unclear, although such a role has been suggested for certain

intracellular fatty acid binding proteins (46,47). However, there is no evidence that ASP is acting primarily by increasing free fatty acid entry into the cell. In the first place, its fatty acid binding properties are weak and non-specific (personal communication, M.A. Rodriguez) and in the second, as shown in both preincubation experiments (Figures 23 and 27), the metabolic effect of ASP is evident, even if no free fatty acid is present during preincubation. Lastly, other fatty acid binding proteins, both plasma and intracellular (albumin and fatty-acid-binding-protein), have no apparent effect on triglyceride synthesis.

Though the effect of ASP seems clear, its mechanism of action is not, and that is of considerable interest.What is apparently left is a protein that does not appear to work as a hormone, nor does it apparently work as a transport protein in the classical concept. The substrate availability at the critical intracellular site, the endoplasmic reticulum, may well be an important control of the synthetic rate of storage lipids. The hypothesis that will be tested in future studies is that the plasma protein, ASP, responsible for the activities identified here, has its impact principally on the presentation of substrates: specifically acyl-CoA, to the enzymes in the smooth endoplasmic reticulum. Although the precise mechanism of action of ASP remains to be elucidated, ASP may well be important in the regulation of intracellular triglyceride synthesis.

3. ASP EFFECT ON HYPERAPOB FIBROBLASTS

3.1 TRIGLYCERIDE SYNTHESIS IN HYPERAPOB

ASP has been shown to have a marked effect on triglyceride synthesis in normal cultured human skin fibroblasts. In addition, the data suggest that ASP is bound, internalized and degraded by the cells. Preliminary investigations with respect to its mechanism of action indicate that the protein produces its effect on the cells only after endocytosis. This stands in contrast to a classical hormone effect, further the data also do not support the model of a typical transport protein (Figure 30).

The initial experiments designed to compare lipid, and in particular, triglyceride synthesis in normal vs HyperapoB fibroblasts demonstrated that, using serum (Figure 4) or partially purified protein fraction (Figure 15), the HyperapoB cells did not respond to the stimulus in the supplemented media, whereas the normal cells did. Following purification of ASP, the HyperapoB cells were examined for their response to ASP.

The plasma lipid values for the normal and HyperapoB subjects are given in Table IX and X. The definition of HyperapoB is based on increased plasma LDL apoB accompanied by an LDL cholesterol to apoB ratio less than 1.5:1.0 (68) and is principally a functional definition. It can be seen that all of the patients have LDL C/B ratios less than 1.5

Figure 30. ASP MODEL

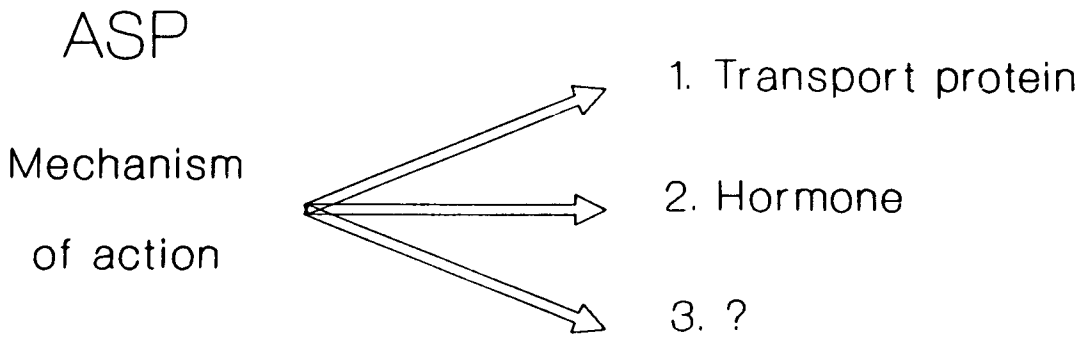


Table IX. PLASMA LIPIDS AND LIPOPROTEINS IN NORMAL SUBJECTS

CELL #	AGE	TC	TG	LDL C	HDL C	LDL B	C/B
		----plasma concentration md/dl-----					
2000	27	156	80	58	82	56	1.04
6000	66	156	80	100	40	100	1.00
9000	45	180	122	118	38	99	1.19
6500	27	170	43	106	55	54	1.96
1000	42	146	100	88	38	70	1.24
1300	41	128	63	42	73	35	1.21
2700	39	193	84	91	85	64	1.42
0000	39	156	82	89	51	65	1.36
average	41	161	82	87	58	68	1.30
S.E.M.	±4	±7	±8	±8	±7	±8	±.10

TC= plasma cholesterol, TG= plasma triglyceride
C= cholesterol, C/B= LDL cholesterol/ apoB

Table X. PLASMA LIPIDS AND LIPOPROTEINS IN HYPERAPOB SUBJECTS

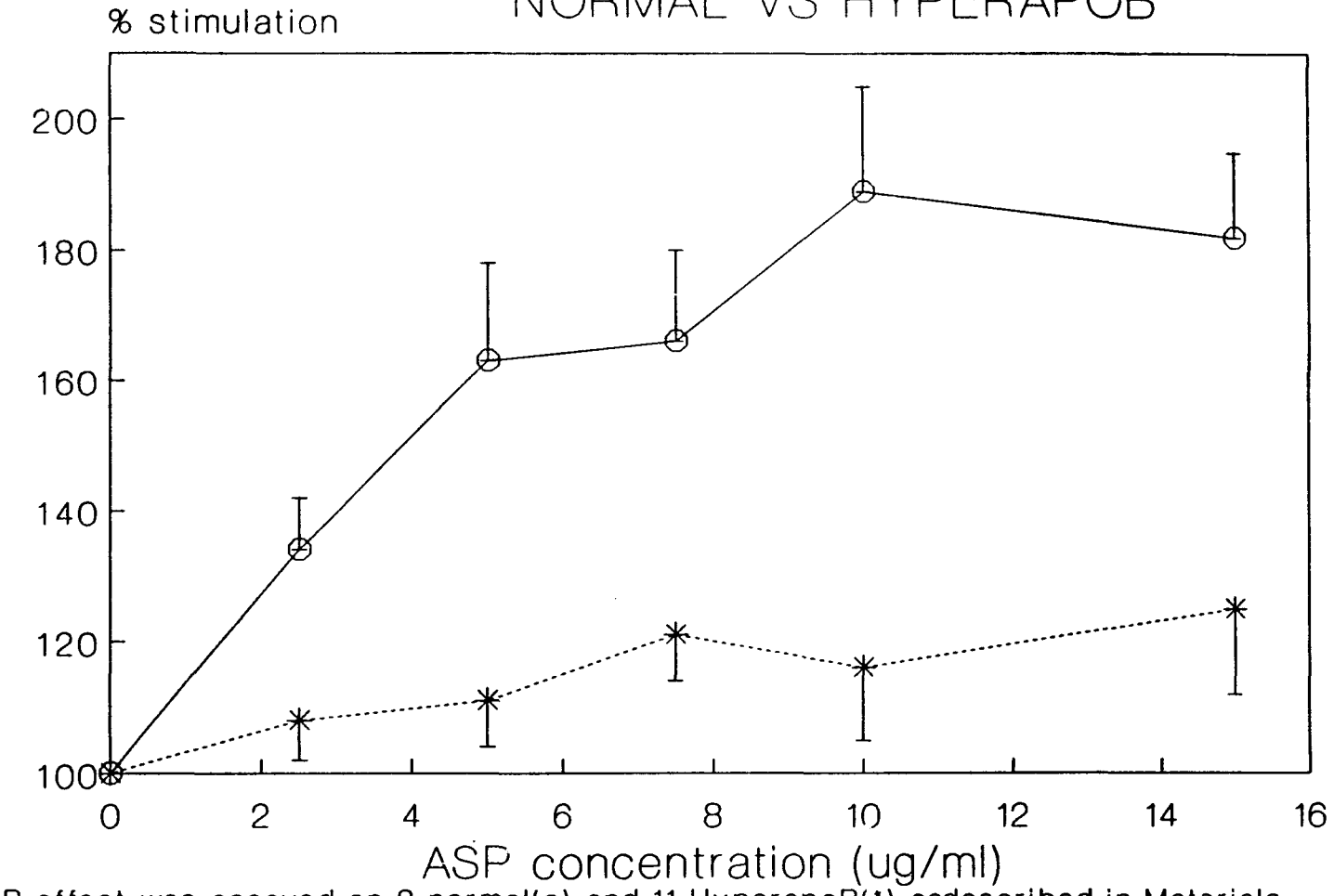
CELL #	AGE	TC	TG	LDL C	HDL C	LDL B	C/B
		----plasma concnetration md/dl-----					
3000	39	177	244	94	34	140	0.67
4000	40	249	329*	132	51	155	0.85
5000	43	223	172	125	64	156	0.81
8000	41	350*	132	289*	35	213	1.35
4500	37	195	148	119	46	142	0.84
9500	54	261	122	173	64	156	1.11
1100	54	236	199	160	36	113	1.42
1500	33	296*	170	207*	55	160	1.29
1400	59	368*	311*	262*	44	184	1.42
2400	62	361*	531*	216*	39	149	1.45
2600	59	299*	355*	190	38	146	1.30
average	46	272	236	178	47	157	1.12
S.E.M.	±3	±21	±38	±19	±3	±8	±.10
p value	NS	.0005	.0025	.0005	NS	.0005	NS

* >95th percentile

to 1, ranging from 0.67 to 1.45. Average plasma cholesterol, LDL cholesterol, plasma triglyceride, and LDL apoB were all significantly higher in the HyperapoB patients. HDL cholesterol, however, was not different.

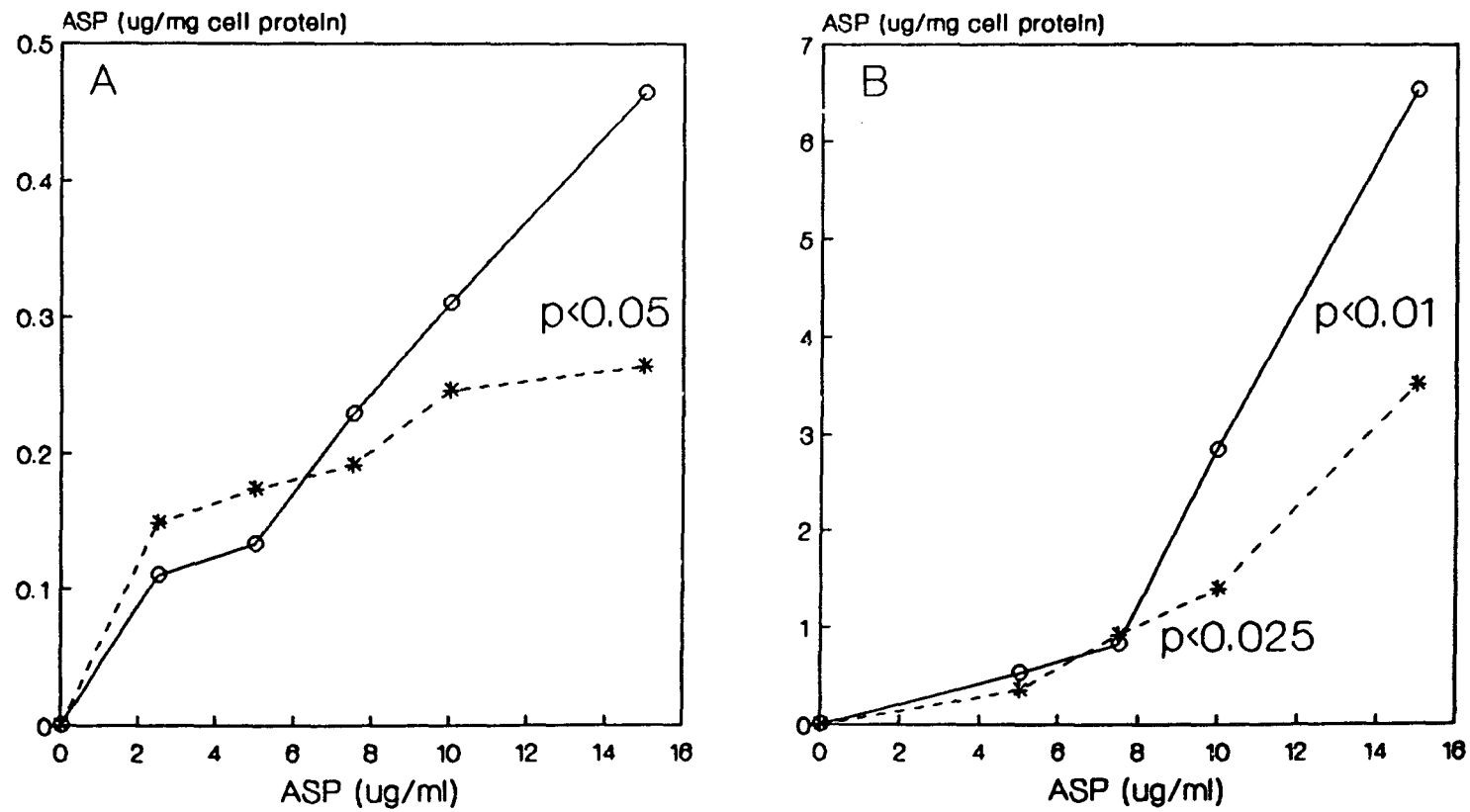
The ASP effect on triglyceride synthesis is shown in Figure 31. In the normal cells, with increasing concentrations of ASP, there is an increase in triglyceride synthesis, reaching a maximum of 180 % of baseline (range 131 to 270), that is a stimulation of 80%. In contrast, the HyperapoB cells demonstrate much less response to ASP, with an average maximum activity 125% (range 78 to 187%), that is, 25% stimulation. It should be noted that the absolute baseline values, that is, nmol of triglyceride synthesized per mg cell protein in the absence of ASP, are no different between normal and HyperapoB cells. The metabolism of iodinated ASP was followed concurrently with the effect on triglyceride synthesis. The amount of ASP that was associated with the cells at time of harvest was determined, as was the amount of ASP that had been degraded by the cells during the incubation period, which in all cases was 24 hours. The results indicate that much less ASP had been degraded by the HyperapoB cells (Figure 32B). Similarly, although not as pronounced, there is a trend towards less ASP associated with the HyperapoB cells as compared with the normal cells (Figure 32A), and this

Figure 31. ASP EFFECT ON TRIGLYCERIDE SYNTHESIS NORMAL VS HYPERAPOB



ASP effect was assayed on 8 normal(o) and 11 HyperapoB(*) asdescribed in Materials and Methods in hormone medium with 10 uM oleate for 20 hours

Figure 32. ASP INTERNALIZED AND DEGRADED
NORMAL VS HYPERAPOB



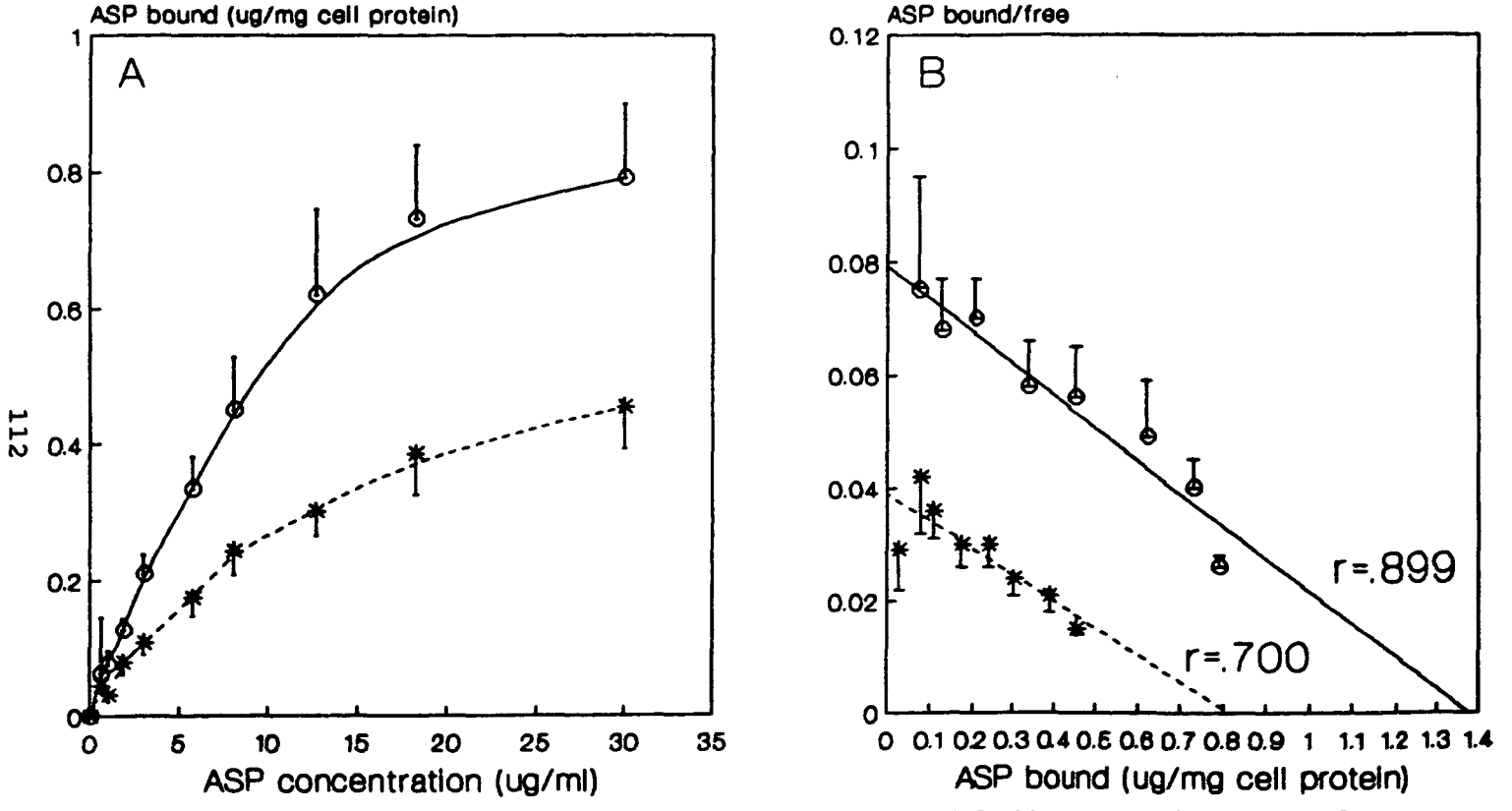
Internalization and degradation of Iodinated ASP was assayed In 8 normal (o) and 11 HyperapoB (*) over 20 hours In hormone medium with 10 uM oleate as described In Materials and Methods

represents ASP which may be bound to the cell surface as well as internalized in the cells.

3.2 ASP BINDING TO HYPERAPOB CELLS

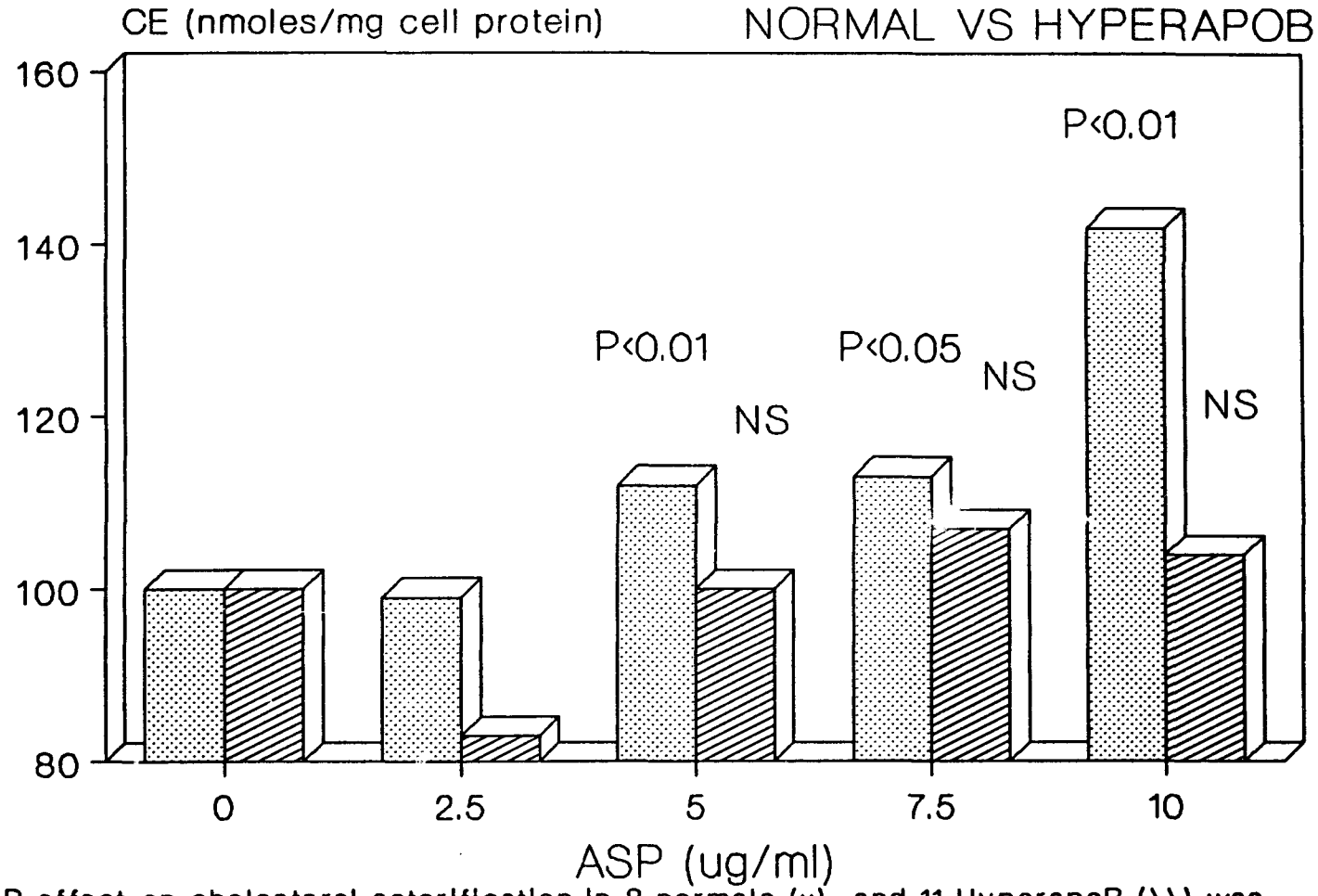
At 37°C, proteins that bind to cells may be internalized and degraded. However at 4°C, proteins will bind to cells, but will not be internalized and metabolized by the cells (82). The interaction of the protein with the cells can be either specific, or non- specific. Non-specific binding, or association of a protein to the cells is via interaction with the cell membrane surface. Specific binding is the interaction of a protein with a particular membrane protein on the cell surface. The membrane protein is present at a fixed concentration, and binding of ASP is, therefore, a saturable process. Binding of ASP to normal and HyperapoB fibroblasts has been examined. The results shown in Figure 33A indicate that ASP binds saturably and specifically to both normal and HyperapoB cells. However, although the binding of ASP to the HyperapoB fibroblasts is saturable, that saturation is attained at half the maximum level of the normal cells. A Scatchard analysis (Figure 33B) permits quantitation of both affinity constants and estimates of receptor number. Overall, the HyperapoB cells demonstrate the same binding affinity for ASP as the normal cells as indicated by the parallel slopes (-0.0725 ug^{-1} Normal, and -0.080 ug^{-1}

Figure 33. ASP SPECIFIC BINDING: NORMAL VS HYPERAPOB



ASP specific binding (A) was assayed in 8 normal (o) and 9 HyperapoB (•) at 4'C for 2 hours. Binding isotherms (B) were calculated by Scatchard linear regression where (o) y=-.0725x+1.217 and (•) y=-.080x+.621

Figure 34. ASP CHOLESTEROLESTERIFICATION:



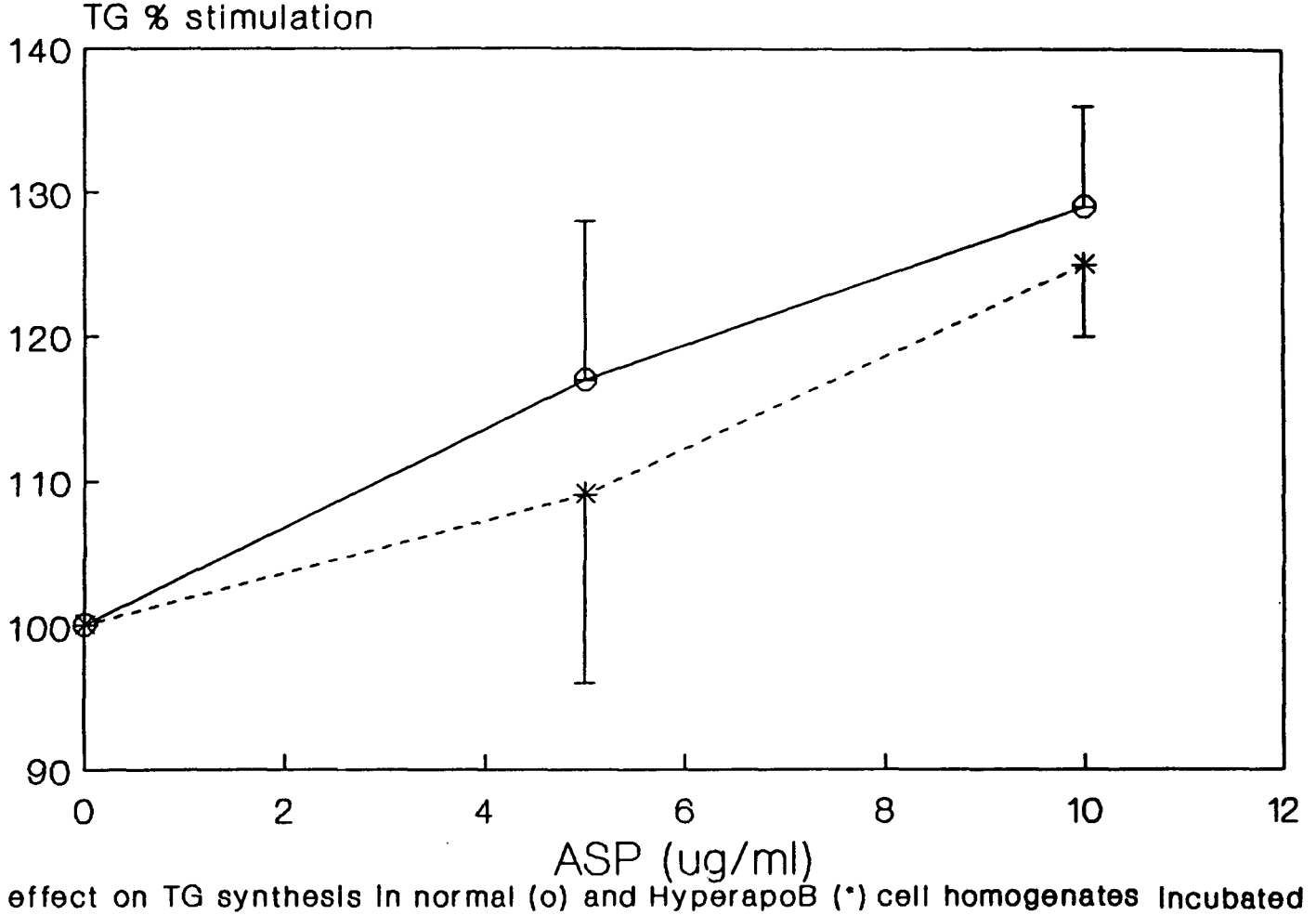
ASP effect on cholesterol esterification in 8 normals (::) and 11 HyperapoB (\\) was assayed in hormone medium with 10 uM oleate and 50 ug/ml LDLfor 20 hours (see Methods)

HyperapoB), however the x intercept on the graph, resulting from linear regression of the data, indicate that the number of binding sites is half that seen in the normal cells, the value for the normal cells being 1.217 mg^{-1} and for the HyperapoB cells 0.621 mg^{-1}. The individual values for the normals and HyperapoB cells are given in Table XI). It is interesting to note that although the binding of ASP to the HyperapoB cells appears to be half normal, the resulting triglyceride stimulation appears to be less than half that of the normal cells. One possible explanation for this is that the ASP in the medium, which is being taken up to a lesser extent by the HyperapoB cells, is acting as a fatty acid sink even though the fatty acid binding properties appear to be weak and non-specific. Therefore, reducing the effective fatty acid concentration available to the cells.

3.3 CHOLESTEROL ESTERIFICATION IN HYPERAPOB

Previously, it has been shown that ASP can also stimulate another acylation reaction, cholesterol esterification, when the cells are incubated under the appropriate conditions . This was also examined for the HyperapoB cells under the same conditions (Figure 21). In the absence of LDL cholesterol ester formed is .2 $\pm$.2 nmol/mg cell protein in the normals and 1.4 $\pm$.4 nmol/mg cell protein in the HyperapoB cells. In the presence of LDL

Figure 35. ASP STIMULATION IN CELL HOMOGENATES



ASP effect on TG synthesis in normal (o) and HyperapoB (*) cell homogenates incubated for 30 mins with 20 uM oleate, CoA, ATP, and G3P (as described in Materials and Methods)

but without ASP cholesterol ester formed is 6.3 ±1.7 nmoles CE/mg cell protein in the normal cells vs 7.3 ±.9 nmoles/mg cell protein in the HyperapoB cells. However, as was the case for triglyceride synthesis, in the presence of ASP, HyperapoB cells also esterified less cholesterol than the normal cells (Figure 34), consistent with the results found using lipoprotein deficient serum (Figure 6).

3.4 TRIGLYCERIDE SYNTHESIS IN CELL HOMOGENATES

If the HyperapoB cells are less responsive due to a decreased complement of receptors, and therefore an inability to internalize the ASP, then it is of interest to examine lipid synthesis in a cell free system, that is in a cell homogenate, where the ASP is available to the triglyceride synthetic enzymes. As shown in Figure 35, there was no apparent difference in triglyceride synthesis in the normal cells as compared to the HyperapoB cells. However, the amount of stimulation is relatively low in both the normal and HyperapoB homogenates, although this may simply be a function of the assay conditions (for example the short incubation time).

3.5 ASP EFFECT IN FH FIBROBLASTS

Therefore, the HyperapoB cells are consistently less responsive to ASP than the normal for both triglyceride synthesis and for cholesterol esterification, and this

Table XII. PLASMA LIPIDS AND LIPOPROTEINS IN FH SUBJECTS

CELL #	AGE	TC	TG	LDL C	HDL C	LDL B	C/B
		-----plasma concnetration mg/dl----					
4100	63	560	239	478	34	247	1.94
6100	18	349	185	267	45	112	2.38
1200	52	499	403	375	43	181	2.07
3813	25						
3609	13		cell lines from ATCC:				
3411	16		no functional receptors				
average	31	469	275	374	41	180	2.13
S.E.M.	±8.6	±63	±66	±61	±2	±39	±.13
p value	NS	.0005	.0005	.0005	NS	.0005	.0005

TC= plasma cholesterol, TG= plasma triglyceride
C= cholesterol, C/B= LDL cholesterol/ apoB

decrease in stimulation can be explained by a reduced amount of ASP bound and internalized by the cells. The question then is, is this a specific characteristic of HyperapoB cells, or, since all of these cell lines were derived from patients with coronary artery disease, is this lack of response to ASP a more general phenomenom unrelated to the dyslipoproteinemia HyperapoB. Accordingly, cells derived from patients with the homozygous or heterozygous form of Familial Hypercholesterolemia were examined for their response to ASP. Three patients had heterozygous FH as defined by total plasma cholesterol and LDL cholesterol above the 95th percentile and an LDL cholesterol-to-LDL apoB ratio greater than 1.8 to 1 (68) (Table XII). All of the plasma parameters are significantly increased as compared to the normals (Table XIII). Three other cell lines were obtained from the ATCC (American Tissue Culture Collection) and these cell lines were from adult patients with homozygous FH as determined by LDL receptor binding studies which indicated that their cells contained no functional LDL receptors. Therefore, these cells are derived from patients whose phenotypes demonstrate increased circulating levels of LDL apoB, but this increase is due to a decreased catabolism, as opposed to an increased production.

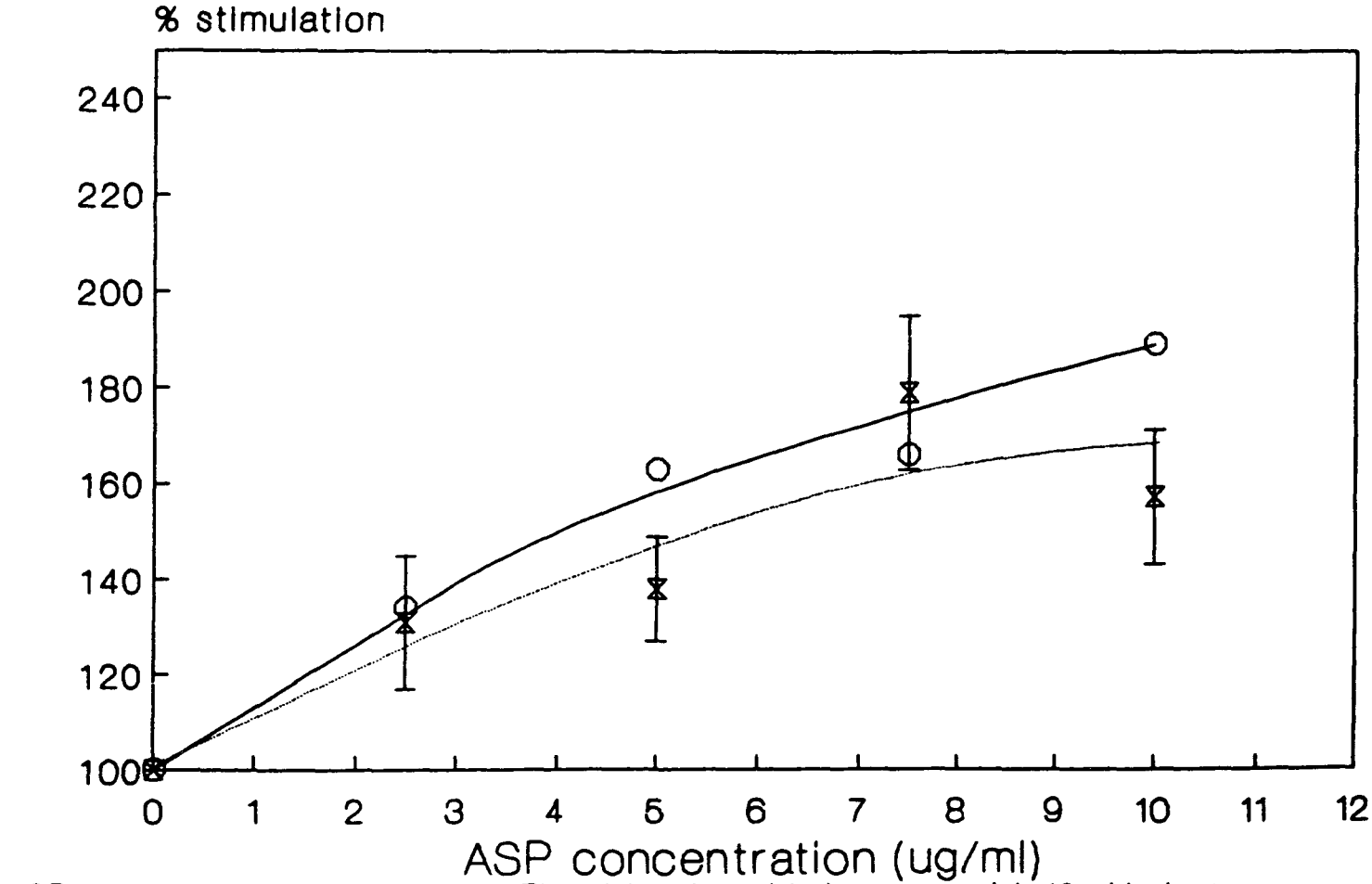
The response of the FH cells to ASP is shown in Figure 36. Triglyceride synthesis increases with increasing ASP

Table XIII. PLASMA LIPIDS AND LIPOPROTEINS IN ALL SUBJECTS

	AGE	TC	TG	LDL C	HDL C	LDL B	C/B
		----plasma concentration mg/dl----					
Normals	41	161	82	87	58	68	1.30
n=8	±4	±7	±8	±8	±7	±7	±.10
Hyperapo B	46	272	236	178	47	157	1.12
n=11	±3	±21	±38	±19	±3	±8	±.09
FH	31	469	275	374	41	180	2.13
n=6	±9	±63	±66	±61	±2	±39	±.13

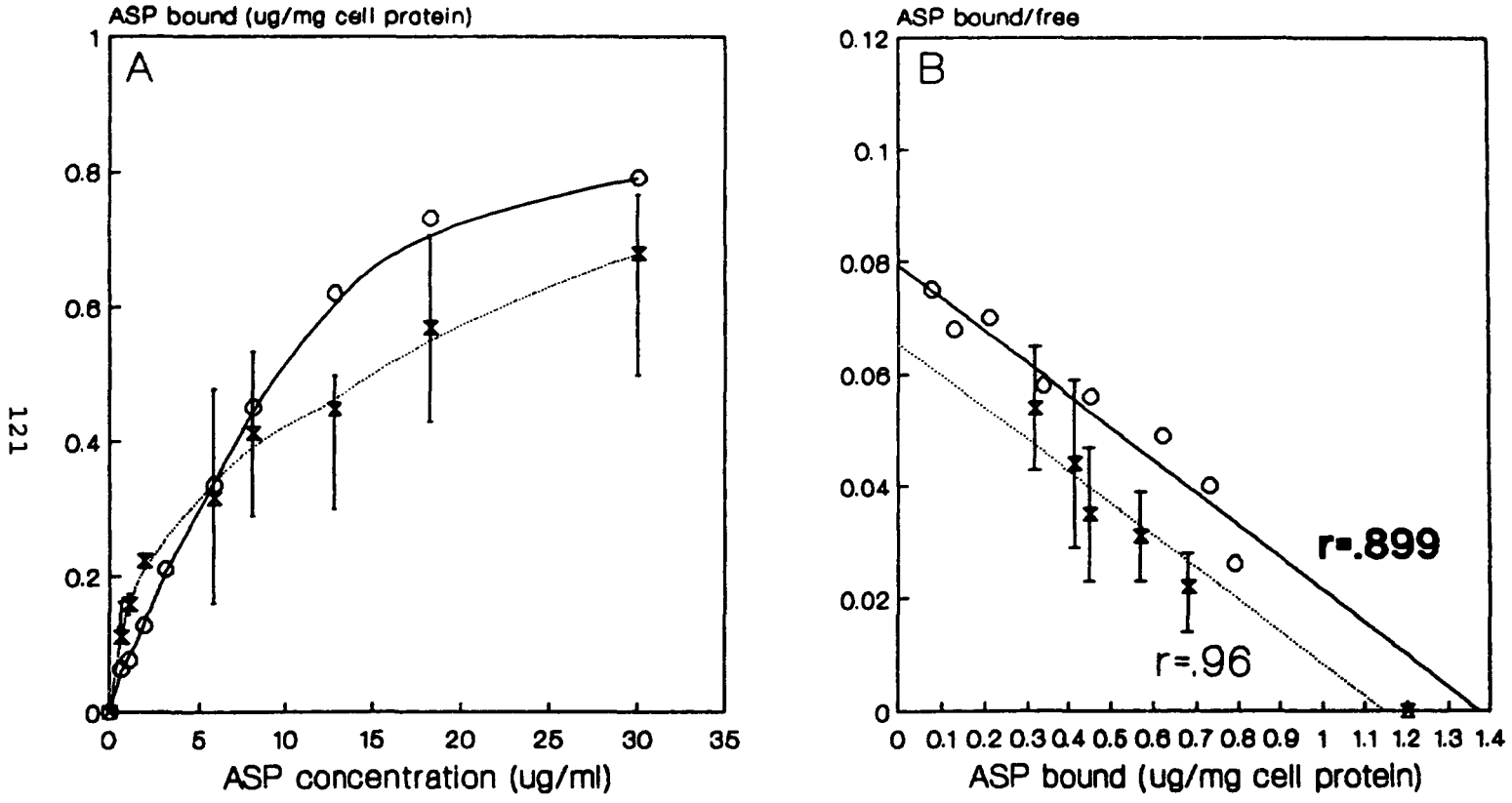
TC= plasma cholesterol, TG= plasma triglyceride
C= cholesterol, C/B= LDL cholesterol/ apoB

Figure 36. ASP TG STIMULATION: NORMAL VS FH



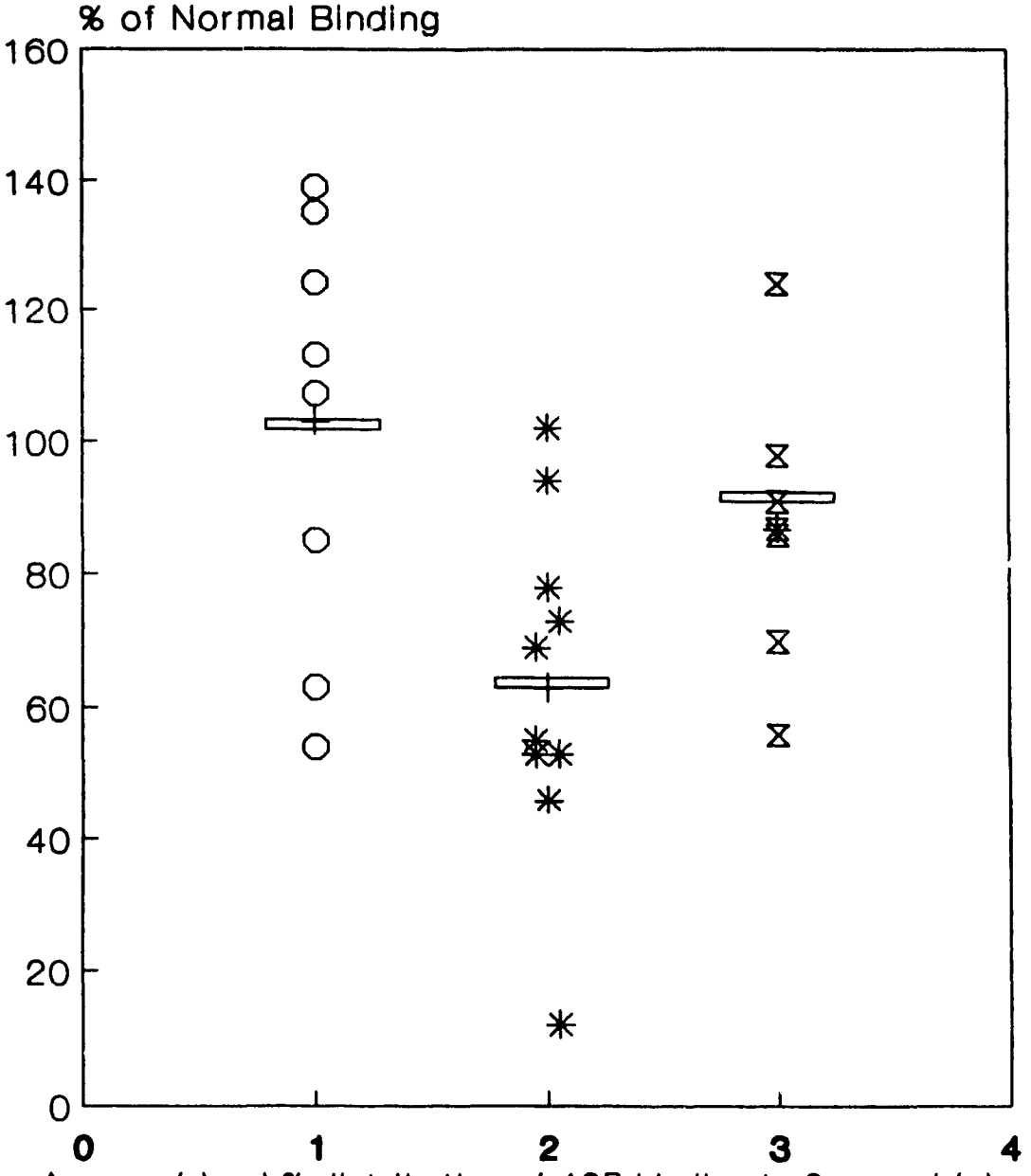
ASPeffect on 8 normal (o) and 6 FH (x) incubated in hormone with 10 uM oleate for 20 hours as described in Materials and Methods

Figure 37. ASP SPECIFIC BINDING: NORMAL VS FH



ASP specific binding (A) in 8 normal (o) and 6 FH (x) at 4'C for 2 hours
Binding isotherms (B) were calculated by Scatchard analysis as described in Methods where (o)y=-.**0725x+1.217 and (•)y=-.0768x+1.187**

Figure 38. ASP BINDING DISTRIBUTION IN ALL SUBJECTS



Average (=)and % distribution of ASP binding to 8 normal (o) 10 HyperapoB (*) and 6 FH (X) fibroblasts expressed as % of average normal binding (see Materials and Methods)

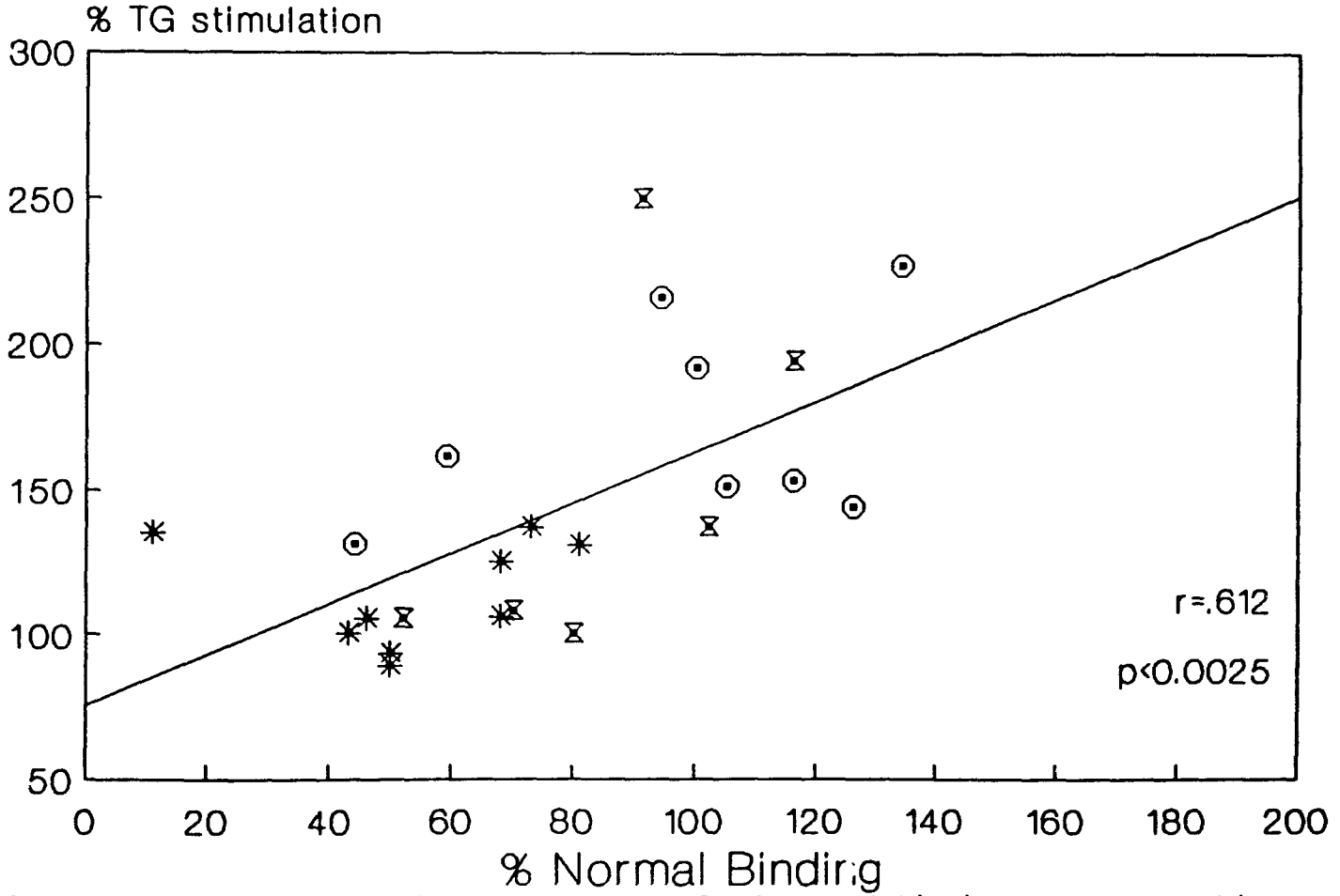
concentration to an average maximum of 155% stimulation (range 89 to 250%) and there is no apparent difference between the normal and the FH. Additionally, baseline synthesis is similar to that seen in the normal and HyperapoB cells . Specific binding of ASP and Scatchard analysis of the specific binding also show very similar results to the normal cells (Figure 37A and B). There is no indication that the FH heterozygotes, or the FH homozygotes demonstrate any difference in ASP binding or subsequent triglyceride response.

3.6 ASP BINDING DISTRIBUTION

All of the data to date on ASP effect on triglyceride synthesis and ASP binding in the normal, HyperapoB and FH cells have been shown as averages of groups. This however, provides very little insight into the range of the response to ASP, the consistency of response within a cell line, and additionally the association between binding and triglyceride synthesis, or conversely, reduced binding and lack of triglyceride synthesis. Ultimately, the goal is the classification of the individual cell lines based on ASP response, with respect to ASP binding and triglyceride synthesis.

The values for the ASP saturation binding and for the ASP triglyceride response are an average of at least 2-3 experiments for each cell line. The variation between

Figure 39. ASP BINDING VS ASP TG STIMULATION IN ALL SUBJECTS



Relationship between ASP binding expressed as % of normal binding and the resulting ASP effect on TG synthesis stimulation in 8 normal (O) 9 HyperapoB (*) and 6 FH (X)

experiments for both binding and triglyceride response in each of the cell groups is 26% and 14.5% for the normals, 16% and 17% for the HyperapoB and 16% and 13% for the FH group. Note that the deviation is relatively the same for each of the 3 different cell groups.

In Figure 38, the distribution of the saturable ASP binding levels is shown for each of the three cell groups. The level of saturable ASP binding is the plateau level of the specific binding curve as shown in Figures 33A and 37A. It is normally estimated by linearization by Scatchard analysis and the x axis intercept is calculated by linear regression as shown in Figures 33B and 37B. Each cell line was assessed individually (as shown in Table XI, and the average for all of the normal cell lines was determined. Using this as the 100% level, the saturable binding level for each of the normal, HyperapoB and FH cell lines was determined. The normal cell lines range from 60% to 140% of the normal average. The HyperapoB cell lines average 63% $\pm$ 7 of the normal cell lines, ranging from 11% to 80% of the normals ($p<0.0025$). Note that 8 of the 11 cell lines fall outside of the normal range of ASP binding (defined as average $\pm$ 1 standard deviation). In contrast, the FH cell lines fall primarily within the range of the normals, average FH binding 87% $\pm$8 (p N.S.). Of the 6 cell lines examined, 5 are within the normal range. A similar

Table XII. SCATCHARD LINEAR REGRESSION ANALYSIS

CELL #	SLOPE	INTERCEPT	CORRELATION
NORMAL			
2000	-.050	1.680	.49
6000	-.054	.780	.70
9000	-.087	1.157	.62
6500	-.125	1.207	.82
1000	-.058	1.153	.74
2700	-.053	1.454	.79
0000	-.050	1.760	.60
average	-.068	1.313	.68
s.e.m.	±.01	±.13	
HYPERAPOB			
3000	-.066	1.242	.66
4000	-.080	.760	.86
5000	-.062	.660	.80
4500	-.053	.740	.55
9500	-.098	.810	.90
1100	-.013	1.260	.47
1500	-.053	.720	.48
1400	-.046	.110	.65
2400	-.049	.610	.42
2600	-.266	.750	.90
average	-.080	.760	.67
s.e.m.	±.02	±.10	
p value	N.S.	«.0025	

Scatchard analysis of binding isotherms by least squares linear regression in normal and HyperapoB cell lines as described in materials and methods.

distribution of the ASP effect on triglyceride synthesis was obtained.

Lastly, the association of ASP binding with the subsequent triglyceride synthesis stimulation was examined for all of the cell lines. If there is a direct link between ASP binding and internalization into the cells and a subsequent stimulation of triglyceride synthesis, then a linear correlation would be anticipated. And indeed, as shown in Figure 39, there is a strong linear relationship between ASP binding and triglyceride stimulation. ($r=.612$ and $p<0.0025$).

3.7 DISCUSSION

Compared to the results obtained in normals, decreased responsiveness to ASP occurred in the skin fibroblasts cultured from patients with HyperapoB. The findings in terms of altered binding and altered cell metabolic response were concordant. That is to say, in HyperapoB, specific binding, internalization, and degradation were all reduced, and these changes correlated with decreased triglyceride synthesis by the HyperapoB fibroblasts. These findings support the hypothesis that the metabolic effect of ASP is dependent upon specific interaction with the cell.

These results may provide the opportunity to determine the genetics of HyperapoB itself. It has been established that HyperapoB is a familial disorder with roughly one child in three at risk being affected (19). This, of course, does not mean that HyperapoB is genetic in origin. The many points of similarity between HyperapoB and Familial Combined Hyperlipidemia have been noted. The latter disorder was originally characterized by multiple lipoprotein phenotypes within a family and thought to be autosomal dominant in character (20). Only recently has an elevated apoB been recognized as the common thread amongst those affected with this disorder (21). The genetics of disorders with variable expression plus variable age of expression is complex to say the least. If reduced ASP responsiveness is sustained as characteristic of at least a subset of patients with

HyperapoB, it will allow independent,and hopefully, less ambiguous examination of the genetics of HyperapoB. It will at the same time allow the issue to be settled as to whether if ever, and if so , how often, HyperapoB and Familial Combined Hyperlipidemia are one and the same disorder.

These results are important as well in that they sustain the hypothesis that peripheral fatty acid metabolism is abnormal in HyperapoB. They are consistent with the observations of a reduced rate of triglyceride synthesis in adipocytes from patients with HyperapoB as well as the observations of a reduced rate of clearance of chylomicron triglyceride in patients with HyperapoB. If the rate of fatty acid uptake in key peripheral tissues were, indeed, reduced in patients with HyperapoB then increased influx to the liver and subsequent increased hepatic apoB production by the liver would be possible. It should be noted that although such results are consistent with the thesis, they by no means establish it.

In summary, these data indicate that a decreased response to ASP is characteristic of the HyperapoB cells. In contrast, FH cells demonstrate no abnormal response to ASP.

The association between dyslipidemias and heart disease is now a well defined and well accepted concept (4-8). The search for the underlying causes of elevated levels of lipoproteins in the circulation has focused on the two

hypotheses of reduced peripheral tissue catabolism and increased hepatic production.

As a result of the studies of Brown and Goldstein (14) that demonstrated reduced LDL catabolism and reduced cholesterol esterification in FH patients, other groups have also examined LDL binding and/or cholesterol esterification in peripheral tissues in patients with other dyslipidemias or patients with coronary artery disease using human skin fibroblasts as a model. However, to date, the only other abnormality described has been the recently described defective apoB100, which is a defect in the lipoprotein particle, and not the cellular receptor (15,16).

No studies have examined lipid synthesis, and in particular triglyceride synthesis in human skin fibroblasts in patients with dyslipidemias or in patients with coronary heart disease.

SUMMARY

THESIS SUMMARY

The observation that normal lipoprotein deficient serum stimulates triglyceride synthesis in human skin fibroblasts more than serum free media was serendipitous. The decision to pursue it was not. A committed effort to identify the factor responsible led to the purification and initial characterization of a unique plasma protein responsible for the metabolic effects on these cells. The protein has not previously been recognized, and has been named Acylation Stimulating Protein. ASP is a small molecular weight (14,000 daltons) basic (pI 9.0) protein that stimulates triglyceride synthesis in normal human skin fibroblasts.

Relatively little is known about the regulation of triglyceride synthesis, such that to date, insulin has been the only substance known which can stimulate fatty acid esterification to increase triglyceride synthesis. ASP, however, stimulates triglyceride synthesis to a much greater extent than insulin under the experimental conditions used.

The effect of ASP was first recognized in experiments designed to examine lipid synthesis in normal fibroblasts under defined conditions. In the presence of lipoprotein deficient serum medium, triglyceride synthesis was greater than in the presence of a serum-free hormone supplemented medium. This effect was trypsin sensitive, and not affected by dialysis. Further, the effect was dependent on the

concentration of lipoprotein-deficient serum in the medium, and apparent over a wide range in fatty acid concentration and incubation times.

A partially purified protein fraction was achieved through column chromatography using Affi-gel partitioning, followed by molecular weight gel filtration. Although this fraction contained 76% of the activity, it was composed of multiple proteins. Initial attempts at further purification using various chromatographic media did not successfully separate the different protein components of this partially purified fraction. Ultimately, however, complete purification was achieved by chromatofocusing with a pH gradient. Subsequently, this final step was modified, and complete purification is now acheived through the use of a CM cellulose ion exchanger.

The experimental data demonstrate that ASP stimulates triglyceride synthesis in a concentration dependent manner over a range of fatty acid concentrations and incubation times. Under appropriate conditions, in the presence of LDL, cholesterol esterification is also stimulated. Moreover, ASP is bound by specific high affinity receptors following which it is internalized and then degraded by fibroblasts.

In contrast to the stimulation of triglyceride synthesis seen in the normal cells, cells derived from patients with HyperapoB are much less responsive to ASP. This lack of response is paralleled by a decrease in specific ASP binding.

However, cells derived from patients with Familial Hypercholesterolemia demonstrate normal binding and triglyceride stimulation comparable to the normal cells.

ASP response may not only serve as a cell marker for HyperapoB but may also be important in the pathophysiology of HyperapoB. Previous work has shown that HyperapoB is characterized by a decreased clearance of chylomicrons (34), and in vitro studies have suggested that this may be due to a reduced uptake of fatty acids by peripheral tissues (33). These results suggest, first, ASP may be important in vivo in the clearance of triglyceride from the circulation through stimulation of fatty acid uptake and esterification, and second, if the peripheral tissues of HyperapoB patients have a decreased response to ASP, then there may be an increased fatty acid flux to the liver. This in turn may result in the overproduction of hepatic apoB containing lipoproteins which is characteristic of HyperapoB (24).

Experiments have been undertaken to examine the mechanism of action of ASP. Preliminary results indicate that ASP must be internalized in order to produce its metabolic effect, since blocking endocytosis also blocks this effect. This, of course, stands in marked contrast to the effect of insulin on triglyceride synthesis which is mediated primarily through interaction with the plasma membrane. Moreover, this hypothesis is consistent with the time lag evidenced before

a metabolic effect is apparent. ASP does not, therefore, appear to be acting via a classical hormone mechanism.

Similarly, ASP does not appear to be acting either solely, or even principally, to transport fatty acids into the cell, since preincubation of the cells with ASP in the absence of fatty acids serves to prime the cells, such that in a subsequent incubation with fatty acids and no ASP, the level of triglyceride synthesis is increased. Thus ASP appears to be acting via a mechanism other than the two well characterized classical pathways. Obviously, though, the exact mechanism by which ASP acts must be explored in depth in further experiments.

ASP: ADDITIONAL INFORMATION

The present body of work deals solely with the effect of ASP on human skin fibroblasts. However the action of ASP has also been investigated in other systems, and this associated work is presented here in order to provide a more comprehensive overview of ASP and its possible mechanism of action. The action of ASP has also been investigated in primary human adipocytes (62,63). As in the fibroblasts, there is a marked effect on triglyceride synthesis, but to a much greater extent. Since ASP is bound, internalized and degraded, it was of interest to examine the effect of ASP directly at the site of triglyceride synthesis. Microsomal fractions were prepared from human adipose tissue and assayed

for the effect of ASP on fatty acid incorporation into triglycerides. Marked stimulation which was significantly greater than that seen in the fibroblast homogenates, was observed (unpublished data).

LIMITATIONS

There are certain limitations to the work which must be enunciated. Preliminary evidence, based on the physical characterization to date, indicates that the protein appears to be a unique protein. However, to confirm this, and also to further characterize the protein and its site(s) of synthesis, its amino acid sequence must be determined and compared to that of other proteins, not only to determine if this is a unique protein, but also to elucidate information about its structure and function based on comparison analysis with proteins of known amino acid sequence, structure and function.

Although preliminary experiments on the mechanism of action of ASP have been completed, a detailed experimental analysis of the mechanism of action of ASP is required.

Additionally, and more importantly, all of the evidence to date deals with the effect of ASP on in vitro cell systems, the cultured human skin fibroblast and the primary isolated adipocyte. It remains to be seen whether, in the organism as a whole, ASP will exert a physiological effect. Since ASP is a plasma protein, a first step will be to

quantitate its levels in plasma and to examine whether these levels can be modified under conditions that are relevant, such as dietary fat loads. To that end, a polyclonal antibody has been produced, and a preliminary assay has been developed. It should be noted that, based on the estimate of ASP in plasma using this antibody assay, the experiments to measure the ASP biological response were conducted under physiological ASP concentrations.

ASP: HYPOTHESIS

It would appear that ASP is acting in a unique manner to stimulate triglyceride synthesis and it is interesting to speculate on the possible mechanism of action (Figure 29). The present hypothesis is an attempt to integrate the function of ASP with other recent published experimental work on fatty acid uptake and esterification. This suggests that fatty acid enters the cell via either diffusion or a membrane receptor. Once within the cell these may be transported via fatty acid binding protein to either the mitochondria for oxidation or the microsome for esterification. At the microsome, the fatty acid must be activated by acyl CoA ligase, before partaking in the series of enzymatic reactions that produce triglyceride. ASP, in turn, is bound to the cell membrane via a specific receptor, and is endocytosed into the cell. One hypothesis is that ASP is acting directly at the site of triglyceride synthesis, the smooth endoplasmic

reticulum, to stimulate the enzymes involved, possibly by improving substrate presentation (specifically acyl CoA) or by some effect on the membrane enzymes themselves such as acyl CoA ligase, glycerol-phosphate-acyltransferase (gpat) or diacylglycerol-acyltransferase (dgat) and this is currently the working hypothesis for the mechanism of action of ASP.

The concept of intracellular targeting of ligands is not new, but very little has, in fact, been elucidated concerning this mode of action. This would also imply a specific plasma membrane receptor protein in order to effect this targeting. Thus, with respect to the mechanism of action of ASP, a great deal of experimental work remains to be done.

ASP may not only be relevant physiologically, but also pathologically. The lack of response of the HyperapoB cells demonstrates that there is a specificity to the action of ASP, and that it may play a part in HyperapoB. In contrast, cells derived from patients with FH responded normally to ASP. As outlined above, a reduced ASP receptor number in HyperapoB may help to explain the increased levels of LDL apoB. Since the postulated defect in some cases of HyperapoB is suggested to be based on a reduced response to ASP, it is interesting to imagine what might be the effect of an increased response to ASP, or to the effect that increased levels of plasma ASP might have on the organism. Obesity, as a potential example of the latter, immediately comes to mind.

In conclusion, ASP is a small, basic human protein that has been purified from human plasma. ASP stimulates triglyceride synthesis in both normal skin fibroblasts and FH fibroblasts, an effect that is mediated through specific binding and uptake into the cells. In contrast, HyperapoB cells show markedly reduced response to ASP both with respect to triglyceride synthesis stimulation and ASP specific binding.

ASP, therefore, may be relevant not only in the pathogenesis of HyperapoB, but may also be important physiologically in the regulation of triglyceride synthesis, and also, and at least of equal interest, ASP may yield insights into what must be the wide variety of ways proteins interact with cells.

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