LIPID METABOLISM OF PRIMARY CULTURES OF AORTIC SMOOTH MUSCLE CELLS

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By

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A Thesis

Submitted to the School of Graduate Studies

in partial fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

April 1976

MASTER OF SCIENCE (1976) McMASTER UNIVERSITY

(Biochemistry) Hamilton, Ontario

TITLE: Lipid Metabolism of Primary Cultures of Aortic Smooth Muscle Cells

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NUMBER OF PAGES: xi, 132

Abstract

Smooth muscle cells from pig aortic media were grown in tissue culture, in medium containing 10% calf serum. Lipid biosynthesis from radioactive substrates 1-^{14}C-acetate, U-^{14}C-D-glucose, 1-^{14}C-oleic acid and ^{32}P-phosphoric acid was measured. In addition, the influence of various sera, including pig serum, normolipemic human serum (NLHS), and hyperlipemic human serum (HLHS) on lipid biosynthesis from acetate and phosphoric acid was studied.

Compared to calf serum, all three test sera caused a stimulation of lipid synthesis in the lipid classes, phospholipid (PL), free fatty acids (FFA), triglycerides (TG) and cholesterol esters (CE), and an inhibition of cholesterol plus diglyceride (S + DG) synthesis. The extent of stimulation was least for pig serum and greatest for HLHS; the inhibition of S + DG was greatest for HLHS and least for pig serum. It was noted that the HLHS stimulation of CE synthesis was proportionately greater than the stimulation of the other lipid classes and that the HLHS inhibition of S + DG was significantly greater than that seen with the other test sera.

The morphology of cultured aortic smooth muscle cells grown in 10% calf serum and 10% HLHS was examined by means of scanning and transmission electronmicroscopy. It was observed that HLHS caused degenerative alterations in the morphology of the cultured smooth muscle cells, such as an abundance of lipid droplets and cellular debris. The implications of these results in relation to the development of atherosclerosis are discussed.

Acknowledgements

I wish to extend my appreciation to Dr. F.P. Bell for his superior advice and guidance and also for his encouragement and patience through the difficult periods I encountered. I would also like to thank Dr. C. Schwartz for his advice and interest concerning this research, and his help in preparing this manuscript; Drs. Gerrity and M. Richardson for their work on the ultrastructure and for providing the electron micrographs of the cultured cells; and to Professor M. Gent and Mrs. M. Johnson for assisting with statistical analyses.

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LIST OF ABBREVIATIONS

PL - phospholipids
S - sterols
DG - diglycerides
FFA - free fatty acids
TG - triglycerides
CE - cholesterol esters
LPC - lysophosphatidylcholine
Sph - Sphingomyelin
PC - phosphatidylcholine
PS - phosphatidylserine
PI - phosphatidylinositol
PE - phosphatidylethanolamine
NLHS - normolipemic human serum
HLHS - hyperlipemic human serum
Fil - filamentous projections
Su - subsurface globular elements
Cx - cytoplasmic extensions
SG - subsurface granular elements
H - hills
V - valleys
M - myofilaments
m - mitochondria
N - nucleus
n - nucleolus
Ly - lysosomes
L - lipid
G - Golgi apparatus
ECx - elongate cytoplasmic extensions
RER - rough endoplasmic reticulum
DB - dense bodies

Gp - globular projections

v - vesicle

SEM - scanning electron microscopy

TEM - transmission electron microscopy

MEM - minimum essential medium

TLC - thin layer chromatography

PBS - phosphate buffered saline

LDL - low density lipoprotein

VLDL - very low density lipoprotein

HDL - high density lipoprotein

HMG CoA reductase - 3-hydroxy-3-methylglutaryl Coenzyme A reductase

µg - microgram

DPM - disintegrations per minute

Introduction

I. Role of Smooth Muscle Cells in the Development of Atherosclerosis

Atherosclerosis is defined as a variable combination of changes of the intima of arteries consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes.[1]

The development of the early atherosclerotic lesion, although not clearly understood, appears to take place in several stages.[2] In the first stage, deposition of plasma lipid [3] can be observed within the intima. These lipid deposits are generally focal in nature; however, there are also areas of more diffuse fatty deposition which correspond to microscopic collections of extracellular lipid droplets which are scattered through an otherwise 'normal' intima.

The characterization of the 'normal' intima has some overlap with the atherosclerotic intima. In children, the intima of arteries is essentially limited to a single layer of endothelial cells overlying a thin subendothelial layer that contains variable amounts of glycosaminoglycans, and some collagen and elastin fibers. This subendothelial layer, which separates the endothelium from the internal elastic lamina, tends to thicken with age, such that by the third decade increased amounts of collagen and elastin are present as well as some smooth muscle cells. This diffuse intimal thickening [5] increases progressively with age, as does the accumulation of lipid in the form of fine extracellular perifibrous droplets. This accumulation of lipid is sometimes referred to as a fatty streak. It is, however, morpho-

logically different from the lesion containing numerous fat-filled-cells, or foamy macrophages which is also called a fatty streak.[4]

Another phase in the development of the atherosclerotic plaque is characterized by the presence of fatty streaks, which are composed of clusters of cells filled with lipid droplets. They may occur at any depth in the intima but most frequently are located in the luminal layers. It is believed that the lipid-containing cells are mostly smooth muscle cells[5] or modified smooth muscle cells.

Atherosclerotic plaques may evolve from the fatty streak, but some controversy on their interrelationships still exists. Extracellular lipid accumulates between and beneath the fat-filled cells. Gelatinous thickenings[6] are also formed. These thickenings consist of a rather loose structure with wide gaps between the smooth muscle cells and thick collagen bundles. It is believed that some fibrous plaques, a further stage in the development of the atherosclerotic plaque, may develop from gelatinous thickenings.[4] The importance of the gelatinous lesion as an intermediate in the pathogenesis of the atherosclerotic plaque has yet to be clarified.

Fibrous plaques are sharply raised, pearly lesions. Microscopically, there is a thick compact collagenous cap and some smooth muscle cells containing a few fat droplets.[7] There is also often a pool of amorphous atheroma lipid in the deeper layers.

Atherosclerotic plaques can be complicated by ulceration, calcification, hemorrhage or thrombosis. Frequently the plaques rupture

and the resultant mural thrombosis, composed of platelets and fibrin, with variable quantities of blood cells, may lead to emboli or occlusions.[8]

The smooth muscle cell is of central importance in the development of atherosclerosis. The ultrastructural characteristics of the smooth muscle cell have been summarized by Getz, Vesselinovich and Wissler.[14] 1) It is an elongated cell with a centrally located nucleus often with indented contours; 2) Its cytoplasm contains prominent longitudinally arranged myofilaments; 3) Cytoplasmic organelles are scarce and concentrated particularly in the perinuclear region; 4) The plasma membrane contains a great number of micropinocytotic vesicles; 5) The cell has a basement membrane and a close association of the cell membrane with collagen and elastin fibres. Studies have shown that the majority of the cells in atherosclerotic lesions are modified smooth muscle cells, which appear to originate from the media of the artery.[8,9] These cells contain myofilaments, basement membranes, pinocytotic vesicles, and can be stained by fluorescent antibodies to smooth muscle actomyosin or myosin.[10-12] These cells have been called intimal smooth muscle cells, modified smooth muscle cells, multifunctional mesenchymal medial cells and medial smooth muscle cells.

Some investigators (Ross and Glomset)[13] propose that the key event in the development of atherosclerotic lesions is the proliferation of the smooth muscle cell. They believe that proliferation of smooth muscle cells precedes or accompanies both the deposition of lipid and accumulation of extracellular connective tissue matrix (the secretory

products of smooth muscle cells), since the lipid deposits occur either within the smooth muscle cells or outside them in association with the connective tissue matrix. These researchers also believe that the intact arterial endothelium acts as a barrier to plasma substances such as low density lipoproteins (LDL) and high density lipoproteins (HDL), which may promote proliferation of smooth muscle cells. Injury to the endothelium allows access of these substances to the vascular smooth muscle cells.

The arterial wall is able both to synthesize lipids and to accumulate lipids from the bloodstream. It is known that the concentration of lipids within the arterial wall increases with age;[4] in particular, cholesterol and cholesterol esters accumulate to the greatest extent. Many investigators believe that the major proportion of the accumulated cholesterol in the aorta is derived from the circulating blood.[15,16] Two theories for the transport of cholesterol across the endothelium have been proposed. Lipoprotein could pass through the interendothelial junctions or, alternatively, lipoproteins could be transported in plasmalemmal vesicles through the endothelial membranes.[15] The rate of removal of cholesterol and cholesterol esters also determines their concentration in the arterial wall. The catabolism of cholesterol and cholesterol esters[16] in the arterial wall is slow; in fact, cholesterol may not be catabolized at all, or perhaps only to cholestanol.[17] Also, the rate of removal of cholesterol esters appears to depend on the fatty acid esterified to cholesterol, i.e. the more saturated the fatty acid, the slower the removal of the cholesterol ester.[18]

It is evident that many factors are involved in the development of atherosclerosis, including among them the plasma lipoproteins, the metabolism and proliferation of the smooth muscle cell, the permeability of the arterial wall, injury to the vascular endothelium, and possible genetic factors. The purpose of this study is to examine one aspect of these factors, namely the lipid metabolism of cultured aortic smooth muscle cells and the influence of hyperlipemic serum on their metabolism.

II. Tissue Culture Techniques

Techniques have been developed for the study of smooth muscle cells in culture. The media of blood vessels which do not have vasa vasorum are composed only of smooth muscle cells and intracellular connective tissue fibers and glycosaminoglycans. The vasa vasorum of the aorta do not extend beyond 29 lamellar units into the inner one-third of the media; therefore, by stripping off the intima and adventitia and also the media containing the vasa vasorum, the remaining media is an excellent source of smooth muscle cells for preparing pure cultures.[19]

The procedure for culturing smooth muscle cells involves the use of tissue explants. The smooth muscle cells grow out from the explant. The morphology of the explant and the peripheral growth have been examined by Jarmolych (et al)[20] at various culture times. These researchers observed that as early as 24 hours after culture, changes in the shape, size and arrangement of the cells of the explant were noted. These modified cells were no longer oriented parallel to the elastic lamellae. At 48 hours, these changes were even more pronounced, and cellular degeneration was also observed. Three types

of cells were noted: primitive cells, fibroblast-like cells, and modified smooth muscle cells. The primitive cell had an irregular contour and frequent pseudopod-like processes. The nucleus showed a prominent nucleolus and a fine distribution of chromatin. The cytoplasm contained little or no endoplasmic reticulum, but large numbers of polysomes. The mitochondria were small and present in moderate number. The golgi apparatus was prominent. The fibroblast-like cell had few cell processes. The nucleus was oval with a regular contour and a fine distribution of chromatin. The cytoplasm contained various amounts of dilated endoplasmic reticulum. The mitochondria and golgi apparatus were similar to that seen in the primitive cell. The modified smooth muscle cell is an elongated cell with few cytoplasmic processes. The nucleus is elongated with marginal masses of chromatin and small nucleoli. There is a complete or partial basement membrane and a varying number of myofilaments and pinocytotic vesicles. They differ from mature smooth muscle cells of the media in that they contain fewer myofilaments and prominent dilated granular endoplasmic reticulum. Occasional cells, having the characteristics of fibroblast-like cells, were also found to have a few myofilaments and dense bodies, suggesting the possible transformation of a fibroblast-like cell to a modified smooth muscle cell. Alternatively, cells other than overtly contractile cells may also contain contractile protein.

At four days in culture, newly formed cells were seen on the periphery of the explant. Some of these cells were fusiform while others were rounded and morphologically similar to the modified cells

within the explant. In the explant itself, crowding of groups of modified cells was observed and cellular degeneration more pronounced than previously.

At 14 days, the changes in the cells of the explant were even more pronounced. However, most of the cells of the new growth were modified smooth muscle cells, containing a dilated endoplasmic reticulum. An increase in the number of lysosome-like structures and fat droplets was noted in these cultured cells. Degeneration was even more pronounced than in early cultures.

At 21 days, almost all of the cells were modified smooth muscle cells. The number of myofilaments per cell was increased, and cell degeneration was more prominent.

As the cultures grew, cells in some areas overlapped and began forming layers of cells, in some areas 10-13 cells thick. This growth pattern, termed "hills and valleys",[21] appears to be typical of arterial smooth muscle cell cultures.

III Lipid Biosynthesis in Cultured Cells

Lipid biosynthesis in cultured cells can be examined using various isotopically-labelled lipid precursors such as 1-^{14}C-acetate, U-^{14}C-D-glucose, 1-^{14}C-oleic acid, and ^{32}P-phosphoric acid. Since the various lipid classes have widely differing chemical structures and properties, each precursor can provide unique information about lipid biosynthesis by the cultured cells.

In these studies, five basic lipid classes were studied: (1) phospholipids, (2) sterols, (3) sterol esters, (4) free fatty acids and

(5) triglycerides. In addition, individual phospholipid classes were studied.

a) Phospholipid Metabolism

Phospholipid metabolism is of prime importance to cultured cells, primarily because of the role of phospholipids as structural components of membranes, and consequently their role in cell division since phospholipid synthesis would be a necessary step in membrane synthesis. There is only indirect evidence that cultured cells can incorporate phospholipids from the medium,[22] which perhaps indicates that biosynthesis is a more important source of phospholipid for the cell than exogenous phospholipid obtained from the medium.

Mammalian cells possess three main pathways for phospholipid synthesis;[23] 1) de novo synthesis from phosphatidic acid; 2) esterification of fatty acids onto lysophosphatides; 3) the condensation of two lysophosphatides. The de novo pathway predominates in cultured cells, as demonstrated in studies of phospholipid synthesis.[24] These pathways are shown in Figure 1.

^{32}P-phosphoric acid is an ideal phospholipid precursor. It can be incorporated into ATP and CTP, and hence incorporated into such intermediate products as dihydroxyactone phosphate and CDP-diacylglyceride. The radioactively-labelled intermediates are then further metabolized to form the individual phospholipids, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidylcholine (PC), depending on the requirements of the cell.

^{14}C-acetate is also an excellent phospholipid precursor, since

the fatty acids providing the acyl chains of the phospholipids are synthesized from acetate. In this respect, the free fatty acid, oleic acid, is also a lipid precursor because it can be esterified to the lysophosphatides. U-^{14}C-D-glucose is another phospholipid precursor since it can provide (via glycolysis) the α-glycerol phosphate required for synthesis of the glycerol phosphatides such as PC, PS, PE and PI.

Interconversion of phospholipid subclasses also occurs in cultured cells. It is thought that sphingomyelin is synthesized via transfer of phosphorylcholine from phosphatidylcholine to sphingosine.[25] Other pathways of interconversion of various phospholipids are shown in Figure 2.

The turnover is another important aspect of phospholipid metabolism. In this respect, the phospholipids have been divided into two subclasses[25]; (1) the "unstable" class consists mainly of phosphatidylcholine; (2) the "stable" class consists of most of the sphingomyelin. The unstable class has a very high turnover rate, estimated to be as short as 7½ minutes for some phospholipids.[26]

Phospholipid metabolism varies with the cell cycle and there is stimulation of phospholipid turnover following cell division.[27] Phospholipid metabolism is also associated with cell population and contact inhibition.[28] Studies on 3T3 cells showed that there was no change in total ^{32}P-incorporation into phospholipids after confluency. However, upon stimulation of cell division there was a rapid increase in the turnover of all phospholipids within the first two hours.

Phospholipid synthesis can be interpreted as a sign of active

metabolism, an increased rate of synthesis indicating logarithmic growth, and a constant rate of synthesis indicating stationary growth phase. An increased rate of phospholipid synthesis could also indicate stimulated cell growth, with or without accompanying cell division.

b) Fatty Acid and Triglyceride Metabolism

Fatty acids can be taken up by cultured cells from the serum in the culture medium,[29] and the medium can provide almost the total cellular requirement for fatty acids in most cases. Fatty acids can also be synthesized by the cell if required. Several labelled lipid precursors, such as U-^{14}C-D-glucose and 1-^{14}C-acetate can be incorporated into fatty acids by cultured cells. Acetate provides the 2-carbon acetyl units from which the fatty acids are synthesized. Glucose, more indirectly, also supplies acetyl-CoA for fatty acid synthesis. The rate of fatty acid synthesis appears to be under control of the enzyme acetylCoA-carboxylase,[30] which in turn is regulated by levels of fatty acylCoA in the cell as a result of fatty acid uptake.

There are basically two types of fatty acid synthesis in mammalian cells;[31] "de novo", in which the entire fatty acid is produced from 2-carbon units, and chain elongation, in which 2-carbon units are attached to pre-existing fatty acids. In general, chain elongation is the method used to produce the longer (C18-C24 carbon atoms) fatty acids, whereas the shorter fatty acids (C14-C16) are synthesized de novo.

Within the cell, fatty acids, whether derived from synthesis or uptake from the culture medium, can be esterified to glycerol phosphate to form mono-, di-, and triglycerides, or be incorporated into phospho-

lipids or cholesterol esters. The fatty acids can also be degraded by β-oxidation to provide energy for the cell.

Tissue culture cells are also capable of taking up monoglycerides, and even triglycerides from the culture medium. In the case of triglycerides, the majority of the triglycerides is taken up intact, without prior hydrolysis.[32]

Cellular triglyceride stores are used as a source of fatty acids for both energy production and phospholipid synthesis.

c) Sterol Metabolism

Under normal growth conditions, most of the sterol required by cultured cells for growth and maintenance of cellular integrity (e.g. membrane structure) is obtained from the serum lipoproteins in the culture medium.[33] The sterol in the serum of all mammalian species is mainly cholesterol, present in both the free and esterified forms. Sera from different species vary in the total sterol content as well as in the lipid and protein composition of the individual lipoprotein classes; there can be considerable variation in lipoprotein composition within a single species. Lipoprotein composition can also be modified by the nutritional state of the animal.

Many studies have shown that the various tissue culture lines and strains are able to incorporate labelled precursors such as acetate and mevalonate into cholesterol.[33] Serum seems to have an inhibitory effect on cellular sterol synthesis,[34] and it is now generally believed that the serum cholesterol, as low-density lipoprotein (LDL), regulates sterol synthesis by a feedback mechanism at the level of HMGCoA

reductase, the rate-limiting step in sterol synthesis.[35,36] HMGCoA reductase (3-OH-3-methylglutaryl CoA reductase) is the enzyme converting HMGCoA to mevalonate, an obligatory precursor of sterols. Evidence indicates that cholesterol inhibits the synthesis of HMGCoA reductase.

A series of events have been postulated to explain the mechanism by which LDL cholesterol regulates this enzyme.[37] According to the theory advanced by Goldstein et al, plasma LDL binds to a specific cell surface receptor.[38,39] This binding regulates the sterol content of cells by modulating the rates of uptake, esterification and synthesis of cholesterol. After the initial binding of LDL to the receptor, the LDL-cholesterol is transferred into the cell and accumulates in both the free and esterified forms.[40] The rate of esterification with long chain fatty acids is enhanced,[41] whereas the activity of HMGCoA reductase, the rate-limiting enzyme in the biosynthesis of cholesterol, is suppressed,[38,42,43] as a result of the binding of LDL to the receptor. Degradation of the apo-LDL protein[39] takes place after binding of LDL to the receptor as a result of incorporation of bound LDL into endocytotic vesicles which then fuse with lysosomes. In cultured cells, ^{14}C-acetate is a good precursor for the study of cholesterol biosynthesis,[44,45] since carbon atoms of the cholesterol molecule are derived entirely from acetate. ^{14}C-glucose also serves as a sterol precursor[44,45] in cultured cells since it can be metabolized to ^{14}C-acetyl CoA, via the glycolysis pathway to ^{14}C-pyruvate (in cytoplasm) and then by conversion of pyruvate to ^{14}C-acetyl CoA, a reaction which takes place in the mitochondria.

Not all of the cholesterol entering the cell or synthesized by

the cell remains as unesterified cholesterol; some becomes esterified to fatty acids to form cholesterol esters.[33] Cholesterol esterification by smooth muscle cells has important implications in the atherogenic processes. In the development of atherosclerotic lesions, both cholesterol and cholesterol esters accumulate within the artery, although cholesterol esters accumulate at the greater rate. Together, cholesterol and cholesterol esters form the greatest percentage (up to 80%)[46] of lipid in the atherosclerotic plaque.

Investigations have shown that under normal growth conditions esterification of exogenous cholesterol occurs only to a limited extent. However, studies have indicated that sterol esterification is greatly stimulated by the addition of hyperlipemic serum to the growth medium.[47,48]

The mechanism(s) of this stimulation of cholesterol ester synthesis is uncertain, but a number of theories have been presented.

Results from experiments conducted by a research group, led by R.W. Wissler,[48,49] indicate that the type of serum, i.e. hyperlipemic versus normolipemic, has a more important influence on the cholesterol ester content of cultured smooth muscle cells than does the cholesterol content of the medium. This group found that cholesterol esterification was greatly stimulated by even very small amounts of hyperlipemic serum (2%), whereas normal serum, even in high concentrations (40%), in which the cholesterol concentration was two times that of the 2% hyperlipemic serum, caused only small changes in cholesterol content of the cells. Of the various lipoprotein fractions of hyperlipemic serum, LDL showed the most stimulatory effect, and the researchers hypothesize that the LDL of

hyperlipemic serum is abnormal in some way. They do not propose a mechanism by which the abnormal LDL stimulates cholesterol esterification.

As mentioned earlier, the theory proposed by Goldstein et al.,[37-43] attempts to explain the stimulation of cholesterol esterification. Low density lipoprotein, bound to a specific high affinity cell surface receptor, stimulates the rate of esterification of cholesterol with long chain fatty acids. This enhancement of the enzyme system for cholesterol esterification parallels the accumulation of cholesterol esters within the cell, resulting from more free cholesterol being transferred inside the cell from receptor bound LDL in medium containing hyperlipemic serum than medium containing normolipemic serum.

Another research group, led by P.J. Bailey[47] suggests that hyperlipemic serum stimulates fatty acid synthesis in cultured cells. This results in an increased rate of esterification of the free cholesterol which has been derived from the medium.

In view of the correlation between hyperlipoproteinemia and arterial smooth muscle cell proliferation in the development of atherosclerosis, the following studies were undertaken to examine the influence of various sera and hyperlipoproteinemia on lipid metabolism of arterial smooth muscle cells in culture.

Figure I

Biosynthesis of Phospholipids

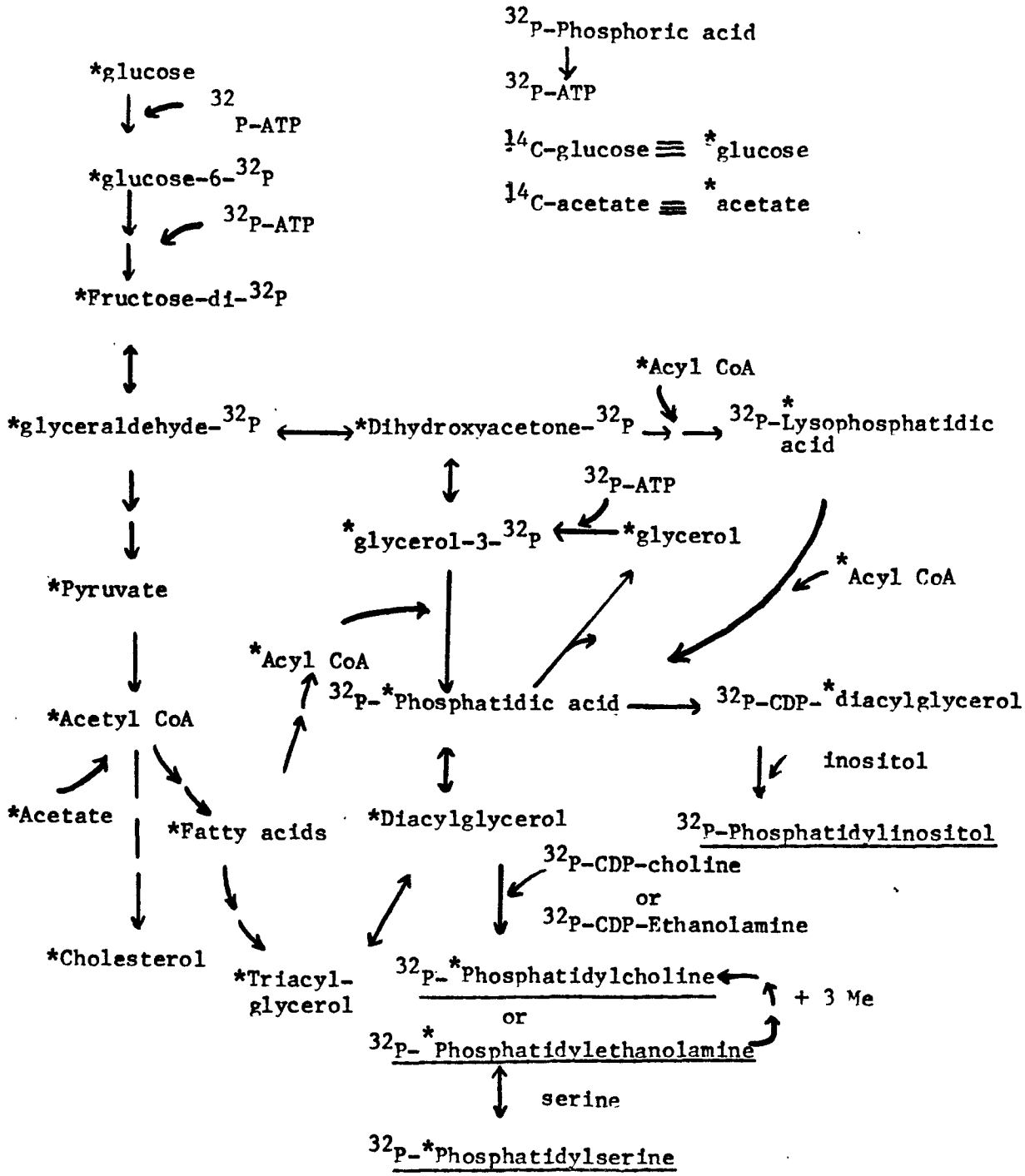
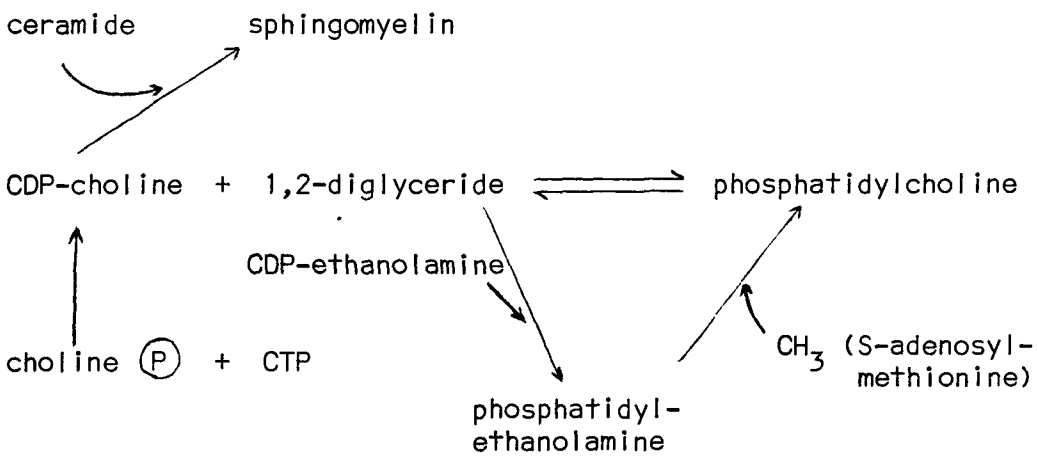


Figure 2

Interconversion of Phospholipids



from: Howard, B.V., W.J. Howard. (1974)
Lipid Metabolism in Cultured Cells.
Adv. in Lipid Res. 12:65

Methods

1. Preparation of Medium

The basal medium, Minimum Essential Medium (MEM), with Hank's salts (GIBCO # F-16) was prepared according to the manufacturer's instructions.

One package of powdered medium (10.8 gm) was dissolved in sterile distilled water (~ 800 ml). To this was added 0.35 gm sodium bicarbonate ($NaHCO_3$ - Certified A.C.S. Fisher Scientific); 10 ml BME Vitamins (GIBCO # 104, 100X); 10 ml penicillin/streptomycin (GIBCO # 514, 10,000 units penicillin, 10,000 mcg streptomycin/ml); 10 ml Kanamycin (10,000 mcg/ml (100X), GIBCO # 516). The pH was adjusted to approximately 7 with 1N - NaOH. The volume was then made up to 1000 ml with sterile distilled water, and subsequently sterilized by vacuum filtration through a microfilter (0.20 μ-pore size, millipore GSWP04700). For routine use, calf serum (GIBCO # 617), previously filtered through a 0.20 μfilter (Nalgene # 120), was added to the medium to give a final concentration of 10% serum.

Immediately prior to use in preparing cultures or changing medium, the medium was warmed to 37°C and Mycostatin (1 ml/100 ml of medium, GIBCO # 532, 10,000 units/ml in PBS) was added.

2. Culturing of Aortic Smooth Muscle Cells

Aortas from young male Yorkshire pigs, weighing approximately 30 kg, sacrificed by an overdose of sodium pentobarbital, were used as

the tissue source. The entire aorta from the arch to the trifurcation was excised and placed in sterile culture medium. All subsequent handling of the aortic tissue was carried out aseptically in sterile medium under germicidal ultraviolet lamps. Peri-adventitial debris was removed from the aorta. The aorta was then cut into sections of approximately 1 cm^2 and the inner one-third of the media obtained by carefully stripping off the adventitial-outer medial layer and an intimal-medial layer. Complete removal of the adventitia is essential to avoid the introduction of other cell levels derived from the adventitia. Inner media is used to avoid contamination by endothelial cells derived from the vasa vasorum of the artery media. Sections of inner media were then placed in fresh sterile medium.

Tissue explants were prepared for culture by punching discs of tissue from the aortic medial segments by means of a 2 mm diameter skin biopsy punch (Keyes Skin Punch, Ingram and Bell, Ltd, Toronto, Ont). The explants, in lots of 5 to 10, were then placed on sterile filter paper to absorb the excess medium and then quickly transferred with a sterile glass rod to 75 cm^2 culture flasks (Falcon Plastic Culture Flasks, Cat # 3024), and attached to the bottom of the flask by slight pressure from the glass rod. Thirty-five explants were placed in each flask. Culture medium (8 ml) was then carefully pipetted over the explants, being careful not to dislodge them, and the flask placed on end until transfer to the incubation.

Cultures were incubated at 37°C in a 5% CO_2/air atmosphere to maintain the pH at 7.4. After approximately four days the smooth muscle

cells grew out radially from the explants; by four weeks, the cultures measured approximately 5 mm in diameter.

3. Addition of Radioactive Substrates

All explants were removed from the culture flask prior to addition of the labelled materials, leaving only the outgrowth of smooth muscle cells to participate in the experiment. The medium was then decanted and the cells washed with 8 ml warmed (37°C) sterile medium, and the wash discarded. Fresh medium (8 ml) was then pipetted into the flasks, omitting the mycostatin, and the desired radioactive substrate added by means of a microsyringe (Pressure-Lok liquid syringe, Series C-160, Precision Sampling Corp, Baton Rouge, Louisiana, U.S.A.) and well-mixed with the medium. The cultures were then incubated for 5 hours at 37°C in a 5% CO_2/air atmosphere.

4. Harvesting of Cells

After five hours of incubation, the labelled medium was decanted from the culture flasks and the cells washed twice with 10 ml sterile isotonic saline to remove residual traces of isotopic precursor. Trypsin EDTA solution (8 ml) (GIBCO # 540, 10X, diluted with sterile saline) was then pipetted into the flasks and the cultures were incubated at 37°C for 20 min or until the cells detached from the bottom of the flask. The cells were then transferred to clean 15 ml conical centrifuge tubes; the flasks were washed twice with 2 ml of sterile isotonic saline and the washes transferred to the centrifuge tubes. A rubber policeman was used to dislodge any residual adhering cells from the flask. The cells were collected in a refrigerated centrifuge (International Equipment Co.,

Model PR-J) at 1000 Xg for 15 min at 4°C. The supernatant was discarded and the cells subsequently stored at -10°C to await subsequent lipid extraction and analyses.

5. Lipid Extraction

Lipids were extracted with chloroform:methanol according to the procedure of Folch et al.[50] The cells were first disrupted by sonication in 3 ml $CHCl_3$:MeOH (2:1) for 3-5 min (Sonifer - Ultrasonics, Inc.) and then centrifuged at 1000 Xg at 4°C for 15 min to concentrate the cell residues. The supernatant was transferred to clean 15 ml conical centrifuge tubes and the pellet re-extracted with 1 ml $CHCl_3$:MeOH (2:1 v/v), and re-centrifuged as above. The resulting supernatant was removed and combined with the supernatant from the first extraction; the cell residue was saved for protein determination by the method of Lowry.[51] The $CHCl_3$:MeOH extracts were washed according to the method of Folch et al.[50]

Lipid standards were added to the washed lipid extracts to facilitate visualization and identification of the various lipid classes after fractionation by thin-layer chromatography. The lipid extracts were reduced to dryness under N_2 then redissolved in a measured volume of $CHCl_3$:MeOH (2:1) for application to TLC plates. One aliquot of the lipid extract was used to fractionate total lipid into five subclasses; another portion used to fractionate phospholipids into five subclasses.

6. Fractionation of Lipid Extracts by Thin-Layer Chromatography

a) Total Lipids

An aliquot of the lipid extract was applied to thin-layer plates

coated with Silica Gel-G (Brinkman, 250 μ thick, 20 cm x 20 cm) in a band approximately 2 cm long and 2-3 cm from the edge of the plate. The plates were developed in the solvent system hexane:diethyl ether:glacial acetic acid (146:50:4) for approximately 30 min. The plates were then air-dried and sprayed with a solution of Rhodamine 6G (0.05% in ethanol) to detect the positions of the individual lipid bands which are visible under ultraviolet light. Chromatography in this system fractionates lipids into five distinct classes, namely the phospholipids (PL), sterols + diglycerides (SADG), free fatty acids (FFA), triglycerides (TG) and sterol esters (CE); phospholipids remain at the origin of the plate.

The lipid zones were scraped into counting vials containing 15 ml scintillation counting fluid (0.3 gm dimethyl-POPOP, 5 gm PPO, in 1 l toluene) and assayed for radioactivity in a liquid scintillation spectrometer (Intertechnique, Model SL30). Quench corrections were made by the external standard method.

b) Phospholipids

Phospholipids were fractionated by the method of Skipski et al.[52] With this method, the phospholipids are usually separated into five subfractions; lysophosphatidylcholine (LPC); sphingomyelin (Sph); phosphatidylcholine (PC); phosphatidylserine (PS) and phosphatidylinositol (PI); phosphatidylethanolamine (PE); the neutral lipids travel with the solvent front.

Aliquots of the lipid extracts (containing the phospholipids) dissolved in $CHCl_3$:MeOH were applied to Silica Gel H-coated TLC plates (activated at 100°C for 30 min prior to use), in a band 3-4 cm long and

½ cm wide, at 2-3 cm from the edge of the plate. (These TLC plates were prepared in the laboratory: 80 gm Silica Gel H mixed with 195 ml 1 mM - Na_2CO_3 (aq); dried at room temp for 1-2 hours; then dried overnight at 100°C in an oven). After application of lipid the plates were dried in an oven at 100°C for 20 min and then allowed to cool in a dessicator. The plates were developed in the solvent system chloroform: methanol:glacial acetic acid: water (25:15:4:2) for approximately 2 hours, or until the solvent front was within 2-3 cm of the top of the plate. After developing, the plates were air-dried for one hour and the individual phospholipid bands were then detected with iodine vapour. The position of the bands were marked and the iodine allowed to sublime before the bands were scraped into counting vials for radioactive assay as described above. In addition to the five phospholipid bands, the origin and solvent front (the latter containing the neutral lipids and cardiolipins) were also taken for radioactive assay.

7. Protein Determination

The amount of protein per incubation flask was determined by the method of Lowry et al.[51] A total protein assay was made on the tissue residues remaining after lipid extraction. A standard curve was prepared using bovine serum albumin. Determinations were in the range of 0.075 mg to 0.30 mg total protein/flask.

TABLE I

Composition of MEM (Eagle) - with Hanks Salts

Component	Concentration (mg/l)
NaCl	8000.0
KCl	400.0
$Na_2HPO_4 \cdot 2H_2O$	60.0
KH_2PO_4	60.0
$MgSO_4 \cdot 7H_2O$	200.0
$CaCl_2$ (anhyd)	140.0
glucose	1000.0
$NaHCO_3$	350.0
phenol red	10.0
L-arginine	105.0
L-cystine	24.0
L-glutamine	292.0
L-histidine	31.0
L-isoleucine	52.5
L-leucine	52.4
L-lysine	58.0
L-methionine	15.0
L-phenylalanine	32.0
L-threonine	48.0
L-tryptophan	10.0
L-tyrosine	36.0
valine	46.0
choline (Cl)	1.0
folic acid	1.0
i-Inositol	2.0
nicotinamide	1.0
D-Ca pantothenate	1.0
Pyridoxal HCl	1.0
Riboflavin	0.1
Thiamine HCl	1.0

Results

I. Lipid Synthesis in Cultured Smooth Muscle Cells

(a) Culture Time

As a preliminary investigation of the aortic smooth muscle cells grown in culture, the incorporation of 1-^{14}C-acetate into the various lipid fractions , was examined after periods of 4,5 and 7 weeks in culture. In all cases the concentration of the label was 1.87 µCi/ml culture medium (specific activity 59 mCi/mM). A similar set of cultures, but grown in medium containing a BME Vitamin supplement, was also studied at similar culture times. In these experiments, the lipid fractions were separated by thin layer chromatography, as described, and the radioactivity in each lipid fraction assayed. The results are shown in Figure 3 and Tables 2 and 3.

In Table 2, the percentage distribution of the incorporated 1-^{14}C-acetate in the five lipid fractions is shown. There appears to be little variation in the metabolism of the cells of different ages since the distribution of the label did not change significantly with the age of the cultures. In all cases, the greatest percentage of the label was incorporated into phospholipids(~40%) and the least into cholesterol esters (~3%). Additional supplementary vitamins have not significantly altered the percentage distribution of the incorporated acetate.

However, the incorporation of 1-^{14}C-acetate, expressed as dpm ^{14}C/µg protein/5 hr incubation, did vary considerably with cultures

of differing ages. As shown in Figure 3, incorporation of 1-^{14}C-acetate into total lipids, was greatest at 4 weeks and decreased significantly in PL, S and TG by seven weeks. This decrease in acetate incorporation could reflect basic differences in the logarithmic versus stationary growth phase, the latter occurring after some five weeks in culture. In the logarithmic growth phase, the cells are dividing at a much greater rate than in the stationary growth phase, and, consequently might have a much greater requirement for lipids, especially those utilized in membrane synthesis such as phospholipids and sterols.

Supplementary vitamins also have a stimulatory effect on the incorporation of 1-^{14}C-acetate, as shown in Table 3, at 4 and 5 weeks; this effect is negligible at 7 weeks.

As a result of these experiments, four weeks was chosen as the culture time for all further biochemical experiments. In addition to optimum acetate incorporation, the size of the cultures was adequate and the 4 week culture time convenient. Supplementary BME vitamins were added to the culture medium for all further studies.

(b) 1-^{14}C-acetate, U-^{14}C-D-glucose, 1-^{14}C-oleic acid and ^{32}P-phosphoric acid incorporation.

The lipid biosynthesis from 4 radioactive lipid precursors, 1-^{14}C-acetate, 1-^{14}C-oleic acid, U-^{14}C-D-glucose, and ^{32}P-phosphoric acid, was studied in cultured smooth muscle cells grown for four weeks. As outlined in the Introduction, the different lipid classes are synthesized by various metabolic pathways, and each precursor can give different information about the metabolism of these cells. Acetate, which is not present in the culture medium, is a precursor for de novo

lipid synthesis in all classes. Glucose is also a de novo precursor, but mainly from the metabolite glycerol. Oleic acid, as a free fatty acid, supplies information about esterification by the cells, to phospholipids, triglycerides and cholesterol esters. ^{32}P-phosphoric acid is a precursor for de novo synthesis of the various phospholipids. These preliminary studies on the metabolism of aortic smooth muscle cells grown in culture are necessary because one must first characterize these cells, both morphologically (presented later in the chapter on ultrastructure) and metabolically, before more sophisticated metabolic studies can be performed. The results of these studies are shown in Tables 4 and 5.

1-^{14}C-acetate was shown to be a "good" general lipid precursor, in that it was incorporated into all the lipid classes and phospholipids. The largest proportion was incorporated into phospholipids (approximately 60%), with roughly 50% in phosphatidylcholine. About 20% of the total acetate incorporated was into sterols and diglycerides, the main sterol being cholesterol, and approximately 13% was incorporated into triglycerides. Only small amounts of labelled acetate were incorporated into free fatty acids and cholesterol esters (<2% respectively).

The absolute incorporation of U-^{14}C-D-glucose into lipids (expressed as DPM ^{14}C/µg protein/5 hr) was less than that of acetate, although the concentration of the radioactive label was roughly equivalent in both cases. However, dilution of the labelled glucose by "cold" glucose present in the culture medium (approximately 8 mg) is the most likely reason for this difference; as with acetate, the greatest per-

centage of labelled glucose was incorporated into phospholipids, accounting for 46% of the total; incorporation into triglycerides was essentially similar, at 44% of the total. The other three fractions incorporated only about 10%; sterols and diglycerides, 8%, free fatty acids, 1%, cholesterol esters, 1%.

1-^{14}C-oleic acid, as predicted, was also incorporated mostly into phospholipids (75%) and triglycerides (22%). The remainder (~2%) was incorporated into the sterols plus diglycerides and cholesterol esters.

The cultured aortic smooth muscle cells are very efficient in the utilization of free fatty acids in the medium. The concentration of the labelled oleic acid was only about 1/6 that of ^{14}C-acetate (0.31 μCi ^{14}C/ml medium for ^{14}C-oleic acid versus 1.87 μCi ^{14}C/ml medium for ^{14}C-acetate), yet the total incorporation of oleic acid into the lipid classes was only 38% less than the total 1-^{14}C-acetate incorporation into total lipids.

Incorporation of 1-^{14}C-acetate into the phospholipids (Table 4) showed that almost 50% of the label was incorporated into phosphatidylcholine, with almost equal and smaller amounts into phosphatidylserine + phosphatidylinositol and phosphatidylethanolamine (17-21%) and again, almost equal, but even smaller amounts into lysophosphatidylcholine and sphingomyelin (6%).

The distribution of ^{32}P-phosphoric acid into phospholipids was different from that of labelled acetate. Phosphatidylcholine accounted for the majority of the label (65%), phosphatidylserine + phosphatidyl-

inositol (24%), lysophosphatidylcholine and phosphatidylethanolamine (~5% each) and sphingomyelin (~2%).

Figure 3

Incorporation of 1-^{14}C-acetate into Lipids at Various Culture Times

a) No Vitamin Supplement

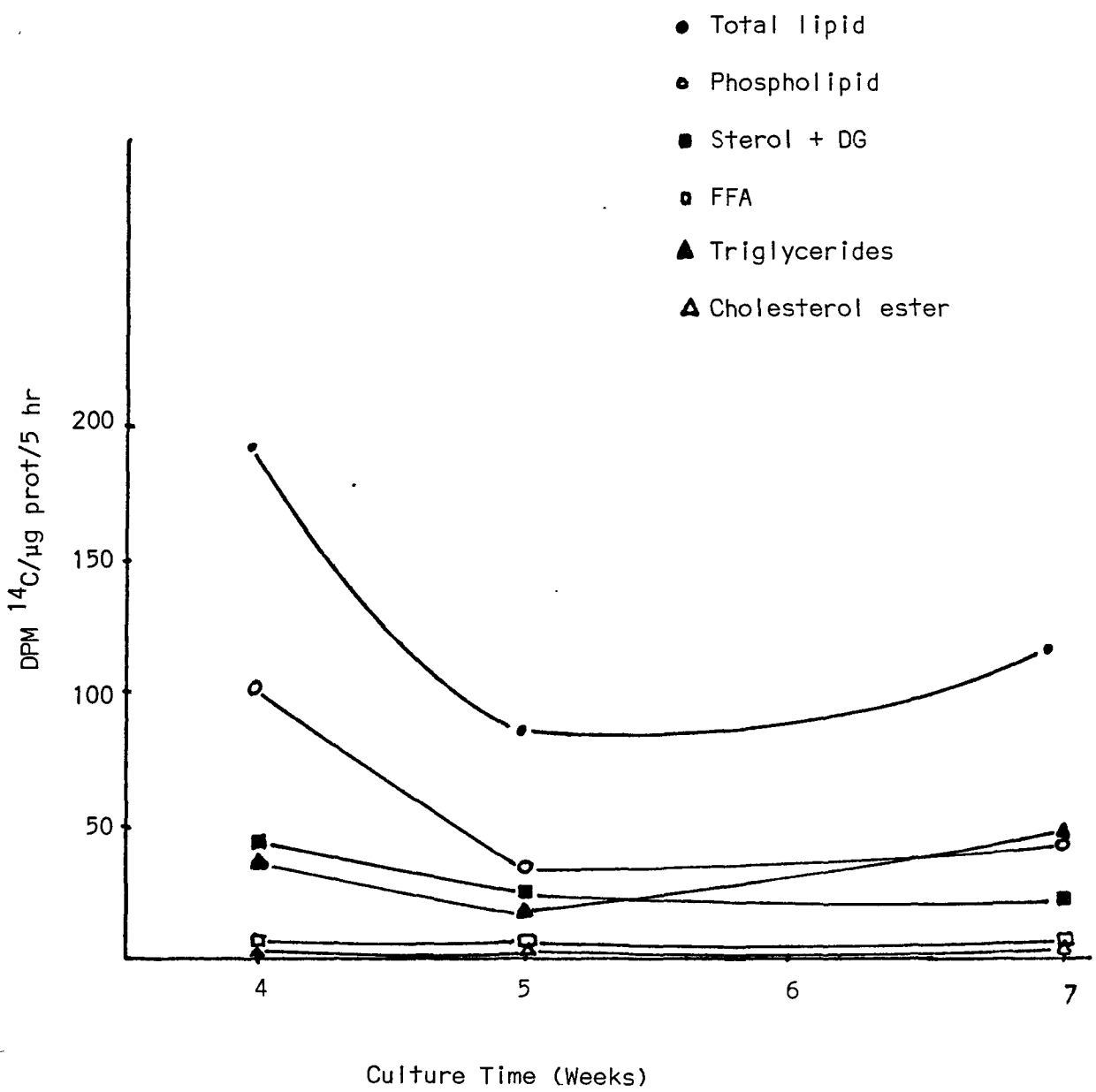


Figure 3

Incorporation of 1-^{14}C-acetate into Lipids at Various Culture Times

b) With Supplementary Vitamins

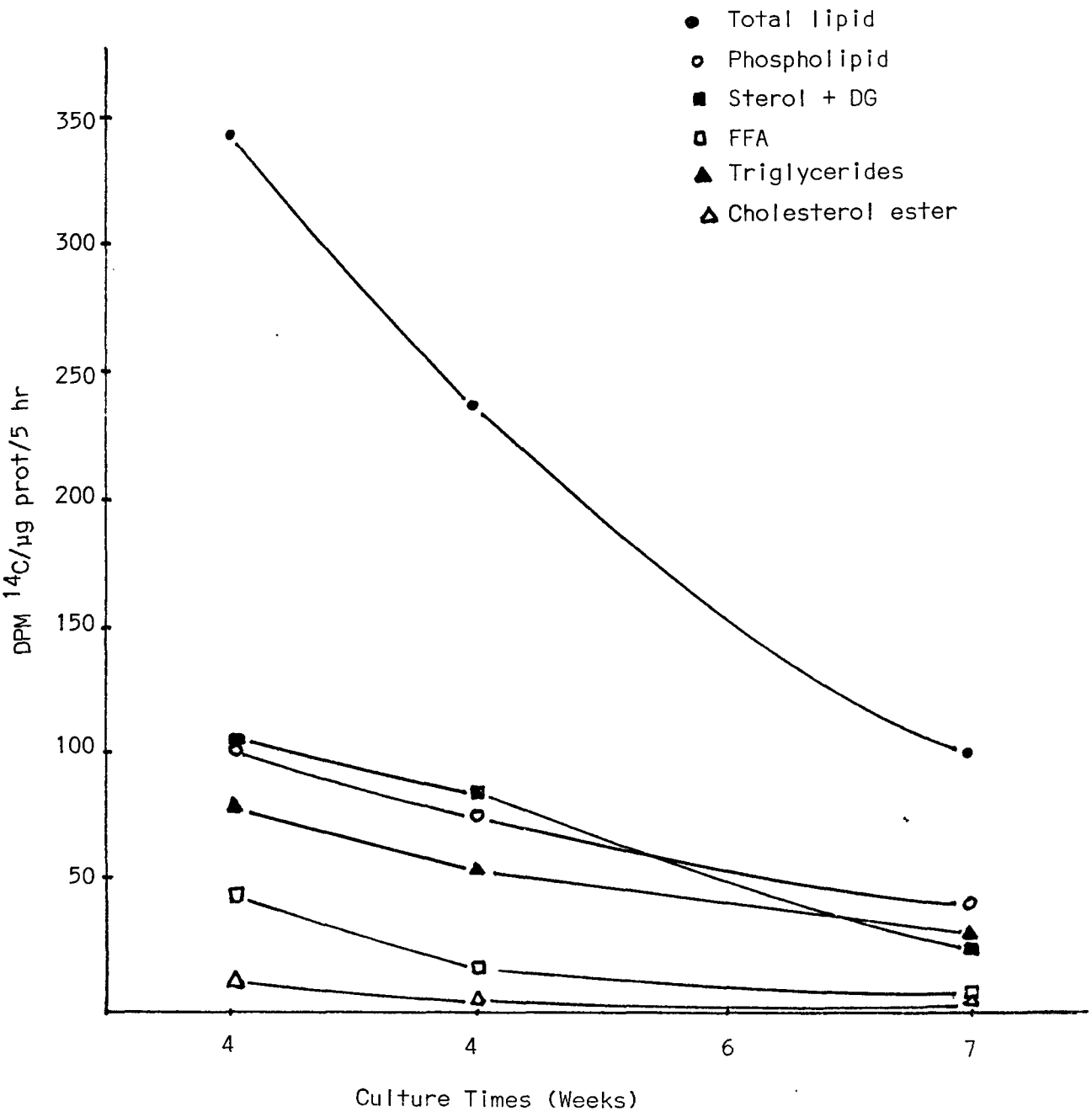


TABLE 2

PERCENTAGE DISTRIBUTION OF 1-^{14}C-ACETATE AMONG THE LIPID CLASSES IN PRIMARY CULTURES OF AORTIC SMOOTH MUSCLE CELLS

LIPID CLASS	Culture Time (No Supplementary Vitamins) (weeks)			Culture Time (with Supplementary Vitamins) (weeks)		
	4	5	7	4	5	7
Phospholipid	51.14 ± 1.73	35.77 ± 0.94	36.11 ± 3.59	29.02 ± 1.17	34.54 ± 2.36	40.93 ± 1.90
Sterol + Diglyceride	22.56 ± 1.28	31.87 ± 3.51	18.69 ± 1.70	32.47 ± 1.04	33.56 ± 4.58	21.69 ± 3.26
Free Fatty Acids	3.49 ± 0.12	5.33 ± 0.58	3.71 ± 0.57	11.91 ± 1.86	5.61 ± 1.34	6.16 ± 0.49
Triglycerides	21.40 ± 1.43	24.10 ± 2.39	38.06 ± 2.34	23.41 ± 1.82	24.01 ± 3.41	29.68 ± 1.48
Cholesterol Esters	1.39 ± 0.03	2.92 ± 0.25	3.12 ± 0.39	3.13 ± 0.14	2.24 ± 0.14	1.65 ± 0.14
Number of Observations	6	8	6	10	7	7

TABLE 3

RATIO OF INCORPORATION OF 1-^{14}C-ACETATE INTO LIPIDS OF AORTIC SMOOTH MUSCLE CELL CULTURES GROWN WITH SUPPLEMENTARY VITAMINS TO THOSE GROWN WITHOUT SUPPLEMENTARY VITAMINS

LIPID CLASS	Culture time (weeks)		
	4	5	7
Total Lipid	1.79	2.79	0.85
Phospholipid	1.02	2.59	0.90
Sterol + Diglyceride	2.38	3.36	1.07
Free Fatty Acids	6.30	3.31	1.56
Triglycerides	1.96	2.43	0.65
Cholesterol Esters	4.11	2.11	0.51

TABLE 4

INCORPORATION AND DISTRIBUTION OF LIPID PRECURSORS AMONG THE LIPID CLASSES IN PRIMARY CULTURES OF AORTIC SMOOTH MUSCLE CELLS

LIPID CLASS	1-^{14}C-acetate		U-^{14}C-D-glucose		1-^{14}C-oleic acid	
	DPM ^{14}C/µg prot /5 hr	%	DPM ^{14}C/µg prot /5 hr	%	DPM ^{14}C/µg prot /5 hr	%
Total Lipid	275.8 ±17.5	100	9.0 ± 1.0	100	171.8 ±14.6	100
PL	170.2 ± 13.7	61.52 ± 2.60	4.3 ± 0.6	45.84 ± 1.81	132.1 ±11.3	75.47 ± 1.10
S + DG	60.7 ± 6.9	21.82 ± 2.13	0.6 ± 0.1	7.83 ± 1.72	3.5 ±0.3	1.64 ± 0.09
FFA	5.1 ± 0.6	1.95 ± 0.28	0.2 ± 0.1	1.23 ± 0.59	—	—
TG	36.67 ± 3.2	13.49 ± 1.01	3.8 ± 0.3	44.07 ± 1.49	28.7 ±2.5	22.37 ± 1.10
CE	3.2 ± 0.4	1.19 ± 0.13	0.1 ± 0.01	0.9 ± 0.15	0.4 ±0.04	0.52 ± 0.11
# Observations	12	12	15	15	6	23
Label Concentration	1.87 µCi/ml		1.56 µCi/ml		0.31 µCi/ml	

TABLE 5

INCORPORATION AND DISTRIBUTION OF LIPID PRECURSORS 1-^{14}C-ACETATE AND ^{32}P-PHOSPHORIC ACID INTO PHOSPHOLIPIDS OF CULTURED AORTIC SMOOTH MUSCLE CELLS

Phospholipid	1-^{14}C-acetate		^{32}P-phosphoric acid
	Incorporation (DPM ^{14}C/µg prot/5 hr)	% Distribution	% Distribution
Lysophosphatidyl-choline	6.9 ± 3.4	5.93 ± 1.97	5.38 ± 1.47
Sphingomyelin	5.9 ± 0.8	6.19 ± 0.96	1.85 ± 0.20
Phosphatidyl-choline	50.7 ± 8.9	48.90 ± 5.37	65.14 ± 1.47
Phosphatidyl serine Phosphatidylinositol	15.2 ± 2.9	17.39 ± 3.20	23.94 ± 0.92
Phosphatidyl-ethanolamine	17.8 ± 1.7	21.59 ± 3.80	5.08 ± 0.50
Number of Observations (Number of animals)	11 (6)	10 (5)	21 (3)
Label Concentration	1.87 µCi/ml		6.25 µCi/ml

II. Effect of Different Sera on Lipid Biosynthesis

To study the influence of different sera on the lipid biosynthesis in cultured aortic smooth muscle cells, four-week old primary cultures (grown continuously in medium containing 10% calf serum) were incubated for 48 hours in medium containing three different sera (10% normolipemic human serum (NLHS); 10% hyperlipemic human serum (HLHS); 10% pig serum). After 48 hours, the culture media were replaced by fresh medium and the appropriate sera, and 1-^{14}C-acetate (1.87 μCi/ml) and ^{32}P-phosphoric acid (6.25 μCi/ml) were added to the cultures.

After 5 hours incubation the cells were harvested and the lipids extracted and analyzed as described previously. The results of this study are shown in Tables 6-12 and Figures 4 and 5.

Appendix A shows the polyacrylamide gel lipoprotein electrophoresis of the normolipemic and hyperlipemic human sera used in these experiments.

In Table 6, the incorporation of 1-^{14}C-acetate into total lipids and the five lipid classes for each of the serum treatments is shown. There are significant differences: (1) Incorporation into total lipids is lease for the 10% calf serum, and greatest for the 10% hyperlipemic human serum. Both pig and normolipemic human serum-treated cultures had incorporations into total lipids intermediate between the calf and hyperlipemic human serum; (2) The stimulation of acetate incorporation for the three test sera is distributed among the lipid classes, PL, FFA, TG and CE; (3) Incorporation of acetate into the S + DG fraction is least for hyperlipemic serum, and greatest for the calf

serum treated cultures, with the results of the other treatments intermediate between these. These findings suggest a suppression of the synthesis of this (S + DG) fraction by the three test sera; (4) The greatest relative increases in acetate incorporation occurred in the cholesterol ester fraction. Cultures incubated in 10% pig serum incorporated into CE 1.8 times more ^{14}C-acetate as did the control cultures incubated into 10% calf serum; 10% NLHS and 10% HLHS resulted in increases of acetate incorporation into CE of 4 and 5.9 times, respectively. (5) Acetate incorporation into free fatty acids was stimulated to a small and consistent degree by all three test sera. (6) 1-^{14}C-acetate and ^{32}P-phosphoric acid incorporation into the individual phospholipids was measured. The results are shown in Tables 9-12 and Figures 4 and 5. As previously noted, incorporation of acetate into total phospholipids was stimulated by all three test sera, with values ranging from 1.7 times more ^{14}C-acetate incorporated into cells incubated in 10% pig serum than into the control cells incubated in 10% calf serum, to 2.3 times for 10% HLHS.

This increase in total phospholipid synthesis is reflected in all the phospholipid classes and appears to be relatively evenly distributed over all the classes, with slightly more in sphingomyelin, phosphatidylcholine and phosphatidylethanolamine. Lysophosphatidylcholine shows proportionately the least increase in acetate incorporation.

In comparing the results for the two human sera, there appears to be little difference with respect to acetate incorporation into the

phospholipids. The stimulation of acetate incorporation into the PL classes in cultures incubated in human sera is significant when compared with the control (calf) values; however, it is almost uniform for both human sera. On close examination, lysophosphatidylcholine synthesis may be slightly suppressed and phosphatidyethanolamine synthesis slightly enhanced by hyperlipemic human serum relative to normolipemic human serum. These differences are probably not significant.

As shown in Tables 11 and 12 and Figures 4 and 5, distribution of ^{14}C and ^{32}P among the phospholipid classes is similar for all the three serum treatments; one possible exception is with pig serum. In this case, lysophosphatidylcholine synthesis appears to be enhanced with a corresponding decrease in phosphatidylcholine synthesis.

Incorporation of both ^{14}C-acetate and ^{32}P-phosphoric acid into sphingomyelin was very consistent and similar for all three test sera, accounting for 6 and 4 percent of the incorporation into total phospholipids, respectively. No differences were seen due to the serum treatments.

Fig 4

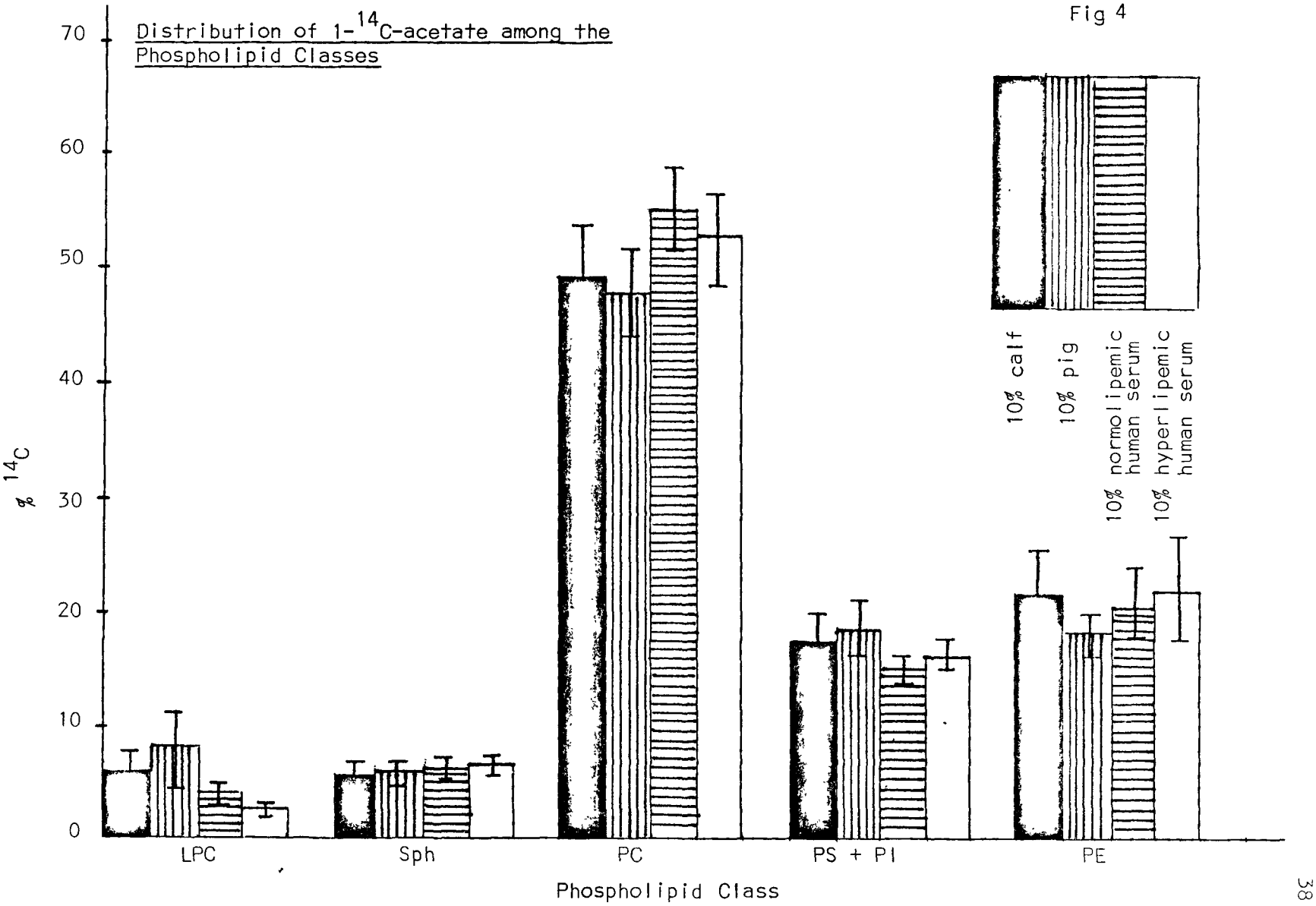

Distribution of 1-14C-acetate among the Phospholipid Classes
% 14C
70
60
50
40
30
20
10
0
LPC
Sph
PC
PS + PI
PE
Phospholipid Class
10% calf
10% pig
10% normolipemic human serum
10% hyperlipemic human serum

Figure 5

Distribution of ^{32}P-phosphoric acid among the Phospholipid Classes

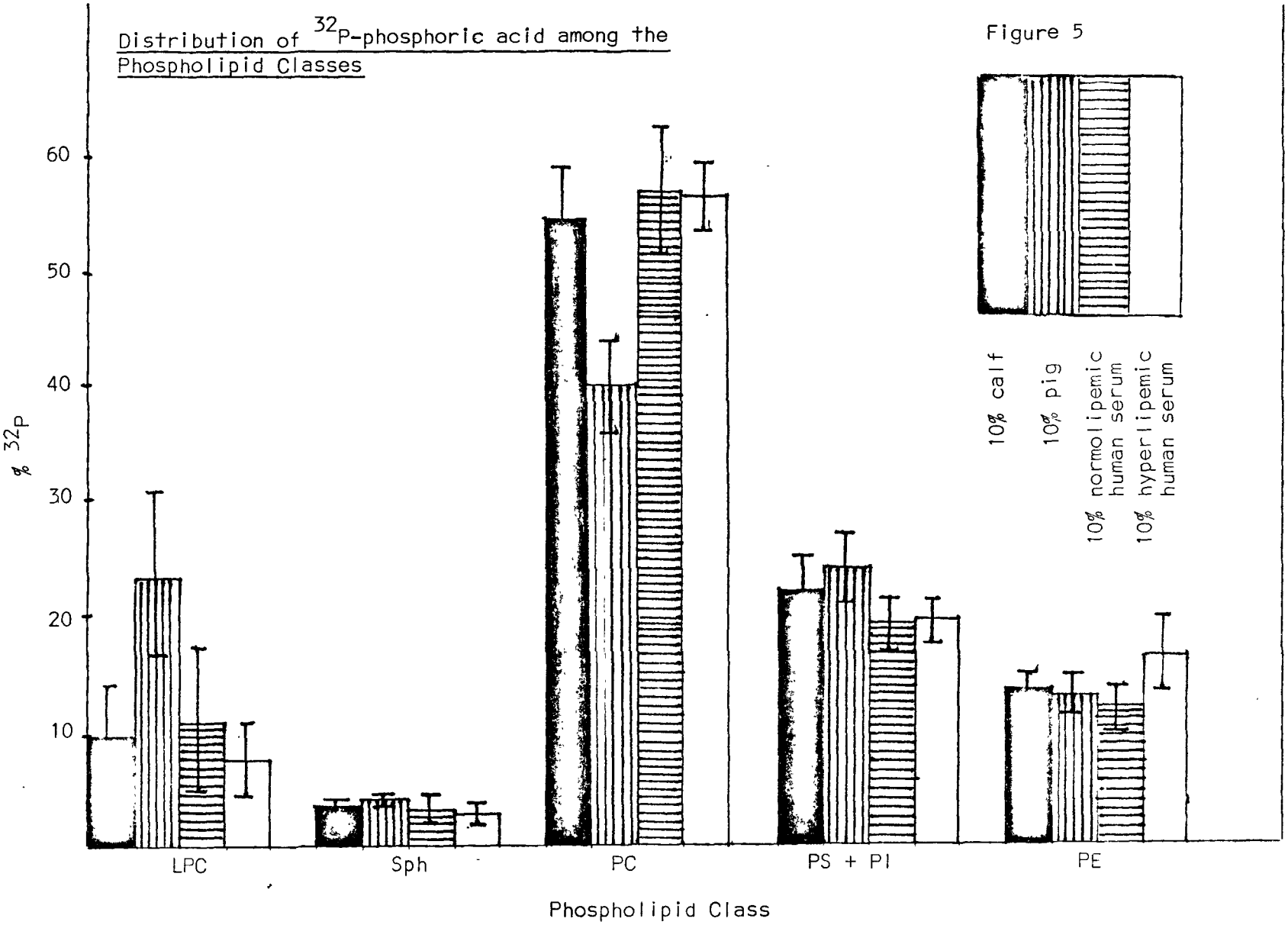


TABLE 6

EFFECT OF SERUM: INCORPORATION OF 1-^{14}C-ACETATE INTO THE LIPID CLASSES OF AORTIC SMOOTH MUSCLE CELLS GROWN IN CULTURE (DPM ^{14}C/µg protein/5 hr incubation)

LIPID CLASS	TYPE OF SERUM			
	10% CALF SERUM	10% PIG SERUM	10% NORMOLIPEMIC HUMAN SERUM	10% HYPERLIPEMIC HUMAN SERUM
Total lipid	262.5 ± 18.8	402.4 ± 40.0	454.6 ± 37.3	547.1 ± 63.4
Phospholipid	159.9 ± 14.4	270.6 ± 44.3	310.6 ± 34.5	369.2 ± 44.1
Cholesterol + Diglyceride	60.7 ± 6.5	55.5 ± 7.9	50.2 ± 9.9	40.9 ± 7.1
Free fatty acids	5.0 ± 0.6	7.3 ± 1.3	6.4 ± 0.8	7.8 ± 0.7
Triglycerides	33.9 ± 3.8	63.6 ± 12.7	70.8 ± 5.9	110.8 ± 13.4
Cholesterol esters	3.0 ± 0.5	5.4 ± 1.0	12.2 ± 1.7	17.9 ± 1.7
Number of Observations	13	4	14	15

TABLE 7

EFFECT OF SERUM: PERCENTAGE DISTRIBUTION OF 1-^{14}C-ACETATE AMONG THE LIPID CLASSES IN CULTURED AORTIC SMOOTH MUSCLE CELLS

LIPID CLASS	TYPE OF SERUM			
	10% CALF SERUM	10% PIG SERUM	10% NORMOLIPEMIC HUMAN SERUM	10% HYPERLIPEMIC HUMAN SERUM
Phospholipids	60.11 ± 2.71	60.60 ± 6.24	67.03 ± 3.22	67.84 ± 1.52
Cholesterol + Diglyceride	22.23 ± 1.81	14.08 ± 2.19	12.95 ± 2.43	6.76 ± 0.85
Free fatty acids	2.15 ± 0.27	1.85 ± 0.83	1.73 ± 0.25	1.68 ± 0.18
Triglycerides	13.29 ± 1.04	16.10 ± 3.47	15.75 ± 0.91	20.94 ± 1.23
Cholesterol esters	1.19 ± 0.12	1.36 ± 0.27	2.55 ± 0.31	3.39 ± 0.16
Number of Observations	14	4	14	16

TABLE 8

EFFECT OF VARIOUS SERA ON INCORPORATION OF 1-^{14}C-ACETATE INTO LIPIDS BY CULTURED SMOOTH MUSCLE CELLS.

(RATIO OF INCORPORATION OF 1-^{14}C-ACETATE (SERUM) TEST / INCORPORATION CONTROL (CALF) SERUM)

LIPID CLASS	TYPE OF SERUM		
	10% PIG SERUM	10% NORMOLIPEMIC HUMAN SERUM	10% HYPERLIPEMIC HUMAN SERUM
Total lipids	1.53	1.73	2.08
Phospholipids	1.69	1.94	2.30
Cholesterol + Diglyceride	0.91	0.82	0.67
Free Fatty Acids	1.45	1.28	1.56
Triglycerides	1.87	2.08	3.27
Cholesterol esters	1.76	4.00	5.87

TABLE 9

EFFECT OF VARIOUS SERA ON INCORPORATION OF ^{14}C-ACETATE INTO PHOSPHOLIPIDS BY CULTURED SMOOTH MUSCLE CELLS

RATIO OF INCORPORATION OF 1-^{14}C-ACETATE TEST (SERUM)/ INCORPORATION IN CALF SERUM

PHOSPHOLIPID CLASS	TYPE OF SERUM		
	10% PIG SERUM	10% NORMOLIPEMIC HUMAN SERUM	10% HYPERLIPEMIC HUMAN SERUM
Lysophosphatidylcholine	1.71	1.44	0.99
Sphingomyelin	1.76	2.44	2.37
Phosphatidylcholine	1.42	2.35	2.30
Phosphatidylserine + Phosphatidylinositol	1.78	1.83	1.97
Phosphatidylethanolamine	1.56	2.46	3.10

TABLE 10

EFFECT OF SERUM: INCORPORATION OF 1-^{14}C-ACETATE INTO THE PHOSPHOLIPID CLASSES OF AORTIC SMOOTH MUSCLE CELLS GROWN IN CULTURE (DPM ^{14}C/μg protein/5 hr incubation)

PHOSPHOLIPID CLASS	TYPE OF SERUM			
	10% CALF SERUM	10% PIG SERUM	10% NORMOLIPEMIC HUMAN SERUM	10% HYPERLIPEMIC HUMAN SERUM
Lysophosphatidylcholine	6.9 ± 3.4	11.8 ± 3.4	10.0 ± 4.2	6.9 ± 2.4
Sphingomyelin	5.6 ± 0.9	10.0 ± 2.6	13.8 ± 1.9	13.4 ± 1.4
Phosphatidylcholine	52.4 ± 9.4	74.7 ± 15.3	123.6 ± 17.6	120.6 ± 16.5
Phosphatidylserine + Phosphatidylinositol	17.7 ± 3.7	31.5 ± 11.9	32.4 ± 3.5	34.9 ± 3.0
Phosphatidylethanolamine	17.8 ± 1.7	27.9 ± 4.0	43.9 ± 8.3	55.3 ± 18.1
Number of Observations	10	4	12	13

TABLE 11

EFFECT OF SERUM: DISTRIBUTION OF 1-^{14}C-ACETATE AMONG THE PHOSPHOLIPID CLASSES IN CULTURED AORTIC SMOOTH MUSCLE CELLS (%)

PHOSPHOLIPID CLASS	TYPE OF SERUM			
	10% CALF SERUM	10% PIG SERUM	10% NORMOLIPEMIC HUMAN SERUM	10% HYPERLIPEMIC HUMAN SERUM
Lysophosphatidylcholine	5.93 ± 1.97	8.55 ± 3.40	4.04 ± 1.52	2.73 ± 0.88
Sphingomyelin	6.19 ± 0.96	6.24 ± 0.25	6.47 ± 0.80	6.60 ± 1.11
Phosphatidylcholine	48.90 ± 5.37	48.10 ± 3.92	54.93 ± 3.97	52.94 ± 4.16
Phosphatidylserine + Phosphatidylinositol	17.39 ± 3.20	18.52 ± 2.57	14.48 ± 0.90	15.94 ± 1.22
Phosphatidylethanolamine	21.59 ± 3.80	18.53 ± 1.16	20.08 ± 3.67	21.74 ± 4.68
Number of Observations	10	4	12	13

TABLE 12

EFFECT OF SERUM: DISTRIBUTION OF ^{32}P-PHOSPHORIC ACID AMONG THE PHOSPHOLIPID CLASSES IN CULTURED AORTIC SMOOTH MUSCLE CELLS (%)

PHOSPHOLIPID CLASS	TYPE OF SERUM			
	10% CALF SERUM	10% PIG SERUM	10% NORMOLIPEMIC HUMAN SERUM	10% HYPERLIPEMIC HUMAN SERUM
Lysophosphatidylcholine	9.80 ± 3.67	22.74 ± 7.13	11.24 ± 6.04	7.97 ± 2.95
Sphingomyelin	3.89 ± 1.17	4.26 ± 0.41	3.48 ± 0.81	3.22 ± 1.22
Phosphatidylcholine	54.81 ± 3.78	40.41 ± 4.16	57.20 ± 5.16	57.34 ± 3.24
Phosphatidylserine + Phosphatidylinositol	22.66 ± 2.57	24.32 ± 2.72	19.55 ± 2.15	19.81 ± 1.56
Phosphatidylethanolamine	8.85 ± 1.01	8.26 ± 1.32	7.70 ± 0.93	11.91 ± 3.18
Number of Observations	10	4	12	13

III. Dose-Response Study

A series of experiments using the three sera, calf, normolipemic human and hyperlipemic human, were performed to determine whether the effects of the sera on lipid biosynthesis in the cultured cells were dependent on the serum concentration in the medium. In each of four experiments, 12 cultures from one animal were grown for 4 weeks in 10% calf serum. At four weeks each culture was treated for 48 hours with a different serum, representing one of the twelve combinations of sera from calf, normolipemic human and hyperlipemic human (See Table 13).

Radioactive lipid precursors (1-^{14}C-acetate 1.87 μCi/ml and ^{32}P-phosphoric acid 6.25 μCi/ml) were then added to each culture flask as outlined previously, and the cells were incubated for 5 hours at 37°C in a 5 % CO_2/air atmosphere. After 5 hours the cells were harvested and analyzed for ^{14}C-acetate incorporation into neutral lipids and phospholipids and ^{32}P-phosphoric acid incorporation into phospholipids, as described earlier.

The results of these experiments are shown in Tables 13-17. To determine if there were any overall differences between serum treatments which were statistically significant, the data was analyzed using a two-way analysis of variance. Whenever statistically significant differences were found, the reasons were elicited by a study of the mean responses to each of the twelve sera.

No significant differences among the twelve serum treatments were found for ^{14}C-acetate incorporation into total lipids, total phospholipids or free fatty acids. However, significant differences among the

serum treatments were found for ^{14}C-acetate incorporation into sterols + diglycerides, triglycerides, and cholesterol esters.

The most striking differences for acetate incorporation into sterols + diglycerides were found between normolipemic and hyperlipemic human sera ($P < 0.001$). Normolipemic human serum appeared to have no effect when added to calf serum (48.28 DPM ^{14}C/µg prot/5 hr for calf serum versus 55.05 DPM ^{14}C/µg prot/5 hr for the calf + normolipemic human serum combinations), whereas the addition of hyperlipemic human serum to calf serum resulted in a marked reduction in acetate incorporated into S + DG (48.28 DPM ^{14}C/µg prot/5 hrs for calf serum versus 10.72 DPM ^{14}C/µg prot/5 hr for the calf + hyperlipemic human serum combinations). The magnitude of this reduction was similar for all proportions of HLHS, thus indicating that even low concentrations of HLHS are capable of reducing acetate incorporation into S + DG.

The difference between NLHS and HLHS was confirmed by the progressive reduction in the amount of 1-^{14}C-acetate incorporated into S + DG, as the amount of added HLHS increased in the mixtures of HLHS and NLHS.

These results support the observations mentioned earlier that 10% HLHS inhibited S + DG to a greater extent than 10% NLHS. This response of the aortic smooth muscle cells also appears to be dependent on the concentration of HLHS in the medium.

Incorporation of acetate into triglycerides was affected in a similar manner by both NLHS and HLHS, in that an increase in the amount of human serum resulted in a pronounced and progressive increase in the

amount of acetate incorporated ($P < 0.001$). This is shown in Fig. 6. The same result was seen for acetate incorporation into CE ($P < 0.001$), as shown in Fig. 7; both NLHS and HLHS result in small but steady increase in acetate incorporation into CE ($P< 0.001$).

The results found with these experiments are not identical to those found in the previous study. As in the earlier experiments, acetate incorporation into triglycerides and cholesterol esters was stimulated by both human sera compared to the control (calf) values; however, in the previous study, the degree of stimulation was consistently greater with HLHS, compared to NLHS.

The observations from these two studies do not necessarily conflict. As in the previous study, incorporation of acetate into cholesterol esters was greater in the presence of HLHS (12 vs 15). However, this increase is small, but consistent; this, and also the fact that the data are derived from only three experiments, may account for the failure to reach statistical significance. In the previous study, with data from fourteen experiments, the small consistent increase in acetate incorporation into cholesterol esters (12(NLHS) vs 18 (HLHS)) was statistically significant ($P< 0.001$).

In these dose-response experiments, as with the previous study, both human sera resulted in an increase in the amount of acetate incorporated into triglycerides. However, the earlier experiments showed that the extent of stimulation was greater for HLHS-treated cells, compared to NLHS-treated cells. The apparent discrepancy may be due to the large difference in the number of experiments in each study; no

significant differences in acetate incorporation into triglycerides were found with the dose-response data, derived from three experiments, whereas the earlier study, with fourteen experiments, showed that HLHS stimulated acetate incorporation into triglycerides to a greater extent than did NLHS ($P< 0.01$).

The percentage distribution of the 1-^{14}C-acetate incorporated into the various lipid fractions were also analyzed statistically as outlined above. The results obtained were similar to those found for acetate incorporation into the lipid fractions, expressed per microgram of cell protein; however, in percentage distribution data, changes in one fraction will necessarily result in changes in one or more of the remaining fractions.

The percentage of acetate incorporated into the S + DG fraction appeared to be significantly decreased ($P< 0.001$) by the addition of HLHS to calf serum (23.7 % for calf serum versus 5.6 % for calf + HLHS), whereas the addition of NLHS to calf serum appeared to have little effect (23.7 % for calf serum versus 19.5 % for calf + NLHS). This difference between NLHS and HLHS was again confirmed by the progressive reduction in the percentage of acetate incorporated into S + DG in the mixtures of NLHS and HLHS, as the amount of added HLHS was increased.

The reduction in the percentage of acetate incorporated into S + DG in cells treated with HLHS was reflected in an increase in the percentage of acetate incorporated into phospholipids. As stated earlier, the amount of phospholipid synthesized was not significantly affected by the type of serum used. However, the reduction in the S + DG fraction

resulted in the percentage distribution of the acetate among the lipid classes to change.

As with S + DG, the differences ($P < 0.001$) observed for the phospholipid fraction were mainly between NLHS and HLHS. The addition of NLHS to calf serum appeared to have little effect (59.6 % for calf versus 60.5 % for calf + NLHS), whereas the addition of HLHS to calf serum resulted in a significant increase in the percentage of acetate incorporated into PL's (59.6 % for calf versus 70.4 % for calf + HLHS). Again, the difference between NLHS and HLHS was confirmed by the sequential increase in the percentage of acetate incorporated into PL's in the mixtures of NLHS and HLHS, as the amount of added HLHS was increased.

Both HLHS and NLHS appear to have a similar effect on the percentage of acetate incorporated into triglycerides (TG) and cholesterol esters (CE), as shown in Figures 8 and 9, in that an increase in the amount of human serum resulted in a small and steady increase in percentage of acetate incorporated into each fraction ($P < 0.001$). These results are consistent with the previous study (Chapter III, Section II).

There were no statistically significant differences among the twelve sera with regards to the percentage of acetate incorporated into free fatty acids; this, too, is consistent with the previous study.

1-^{14}C-acetate and ^{32}P-phosphoric acid incorporation into the five individual phospholipid classes (LPC, Sph, PC, PS + PI, PE) were analyzed as outlined above. No statistically significant differences were found in the percentage distribution of incorporated label for the twelve sera.

However, some significant differences were found for ^{14}C-acetate

incorporation (expressed as DPM $^{14}C/\mu g$ prot/5 hr) into PC and PS + PI. For both of these fractions NLHS and HLHS appeared to have a similar effect, as shown in Figures 10 and 11, in that an increase in the amount of added human serum resulted in a small but steady increase in the amount of acetate incorporated into these fractions ($P < 0.01$). No significant differences were found for LPC, Sph, or PE.

These results are generally consistent with the previous study. However, in that study,acetate incorporation into PE was also stimulated by the human sera, in addition to PC and PS + PI.

TABLE 13

DOSE RESPONSE:

Incorporation of 1-^{14}C-Acetate into Lipids in Primary Aortic Smooth Muscle Cell Cultures (DPM ^{14}C/µg Protein/5 hr)

SERUM TREATMENT		Total Lipids	Phospho-Lipids	Sterols and Di-glyceride	Free Fatty Acids	Triglycer-ide	Cholesterol Esters
Calf (C) + Normolipemic Human Serum (NLHS)	10% C	204.4	123.9	48.3	6.7	19.6	1.8
	7.5% C 2.5% NLHS	178.6	115.0	33.5	5.3	22.5	2.5
	5% C 5% NLHS	240.1	247.8	46.4	5.7	36.2	4.1
	2.5% C 7.5% NLHS	249.2	158.6	49.8	4.6	41.8	6.7
	10% NLHS	340.8	180.4	90.6	7.3	50.5	12.0
Calf (C) + Hyperlipemic Human Serum (HLHS)	7.5% C 2.5% HLHS	140.1	101.1	12.1	4.0	20.5	2.5
	5% C 5% HLHS	154.8	106.6	9.4	3.3	31.3	3.9
	2.5% C 7.5% HLHS	168.5	126.3	9.6	3.8	33.7	5.4
	10% HLHS	418.7	333.9	11.8	7.0	49.8	15.6
Normolipemic Human Serum (NLHS) + Hyperlipemic Human Serum (HLHS)	7.5% NLHS 2.5% HLHS	403.7	236.8	64.1	12.6	76.1	14.0
	5% NLHS 5% HLHS	268.7	175.4	24.3	4.4	56.1	8.8
	2.5% NLHS 7.5% HLHS	195.4	139.4	13.3	3.9	33.0	5.4

Averaged over 3 experiments

TABLE 14

DOSE RESPONSE:

Percentage Distribution of 1-^{14}C-Acetate Among the Lipid Classes in Primary Aortic Smooth Muscle Cell Cultures

SERUM TREATMENT		Phospholipids	Sterols + Diglyceride	Free Fatty Acids	Triglyceride	Cholesterol Esters
Calf (C) + Normolipemic Human Serum (NLHS)	10% C	59.57	23.73	3.39	12.17	1.06
	7.5% C 2.5% NLHS	63.91	18.71	2.84	13.03	1.50
	5% C 5% NLHS	64.28	16.84	2.67	14.54	1.66
	2.5% C 7.5% NLHS	60.70	17.87	2.74	16.43	2.50
	10% NLHS	53.10	24.55	2.76	16.23	3.34
Calf (C) + Hyperlipemic Human Serum (HLHS)	7.5% C 2.5% HLHS	72.88	7.71	3.08	14.50	1.83
	5% C 5% HLHS	71.00	5.22	2.52	18.85	2.25
	2.5% C 7.5% HLHS	68.75	4.84	3.82	19.33	3.01
	10% HLHS	69.01	4.53	2.29	20.59	3.51
Normolipemic Human Serum (NLHS) + Hyperlipemic Human Serum (HLHS)	7.5% NLHS 2.5% HLHS	59.90	14.18	2.83	19.60	3.47
	5% NLHS 5% HLHS	62.50	8.49	2.34	23.51	3.23
	2.5% NLHS 7.5% HLHS	71.42	5.91	2.38	17.51	2.77

Averaged over 4 experiments

TABLE 15

DOSE RESPONSE: Incorporation of 1-^{14}C-Acetate into the Phospholipids in Primary Aortic Smooth Muscle Cell Cultures (DPM ^{14}C/µg Protein/5 hr)

SERUM TREATMENT		Lysophosphatidyl-choline	Sphingo-myelin	Phosphatidyl-choline	Phosphatidyl-serine + Phosphatidyl-inositol	Phosphatidyl-ethanolamine
Calf (C) + Normolipemic Human Serum (NLHS)	10% C	3.2	3.2	10.8	5.1	17.0
	7.5% C 2.5% NLHS	1.2	4.1	16.2	5.0	25.3
	5% C 5% NLHS	1.4	5.5	35.5	8.6	16.5
	2.5% C 7.5% NLHS	1.5	5.2	54.5	12.7	17.9
	10% NLHS	2.2	10.5	70.9	15.3	20.4
Calf (C) + Hyperlipemic Human serum (HLHS)	7.5% C 2.5% HLHS	1.8	4.2	30.2	5.8	11.1
	5% C 5% HLHS	1.4	7.5	24.6	10.6	11.7
	2.5% C 7.5% HLHS	1.6	4.6	31.3	7.8	9.8
	10% HLHS	2.4	12.6	41.8	24.3	139.4
Normolipemic Human serum (NLHS) + Hyperlipemic Human serum (HLHS)	7.5% NLHS 2.5% HLHS	3.6	12.1	79.8	19.8	22.0
	5% NLHS 5% HLHS	2.2	21.5	57.3	25.0	18.9
	2.5% NLHS 7.5% HLHS	2.5	7.0	47.1	11.8	16.1

Averaged over 2 experiments

TABLE 16

DOSE RESPONSE:

Percentage Distribution of 1-^{14}C-Acetate among the Phospholipids in Primary Aortic Smooth Muscle Cell Cultures

SERUM TREATMENT		Lysophosphatidylcholine	Sphingomyelin	Phosphatidylcholine	Phosphatidylserine + Phosphatidylinositol	Phosphatidylethanolamine
Calf (C) + Normolipemic Human Serum (NLHS)	10% C	7.18	8.65	30.18	13.02	40.98
	7.5% C 2.5% NLHS	2.63	8.45	33.05	9.93	45.94
	5% C 5% NLHS	2.02	8.56	50.93	12.13	26.38
	2.5% C 7.5% NLHS	1.61	7.23	58.43	13.56	19.17
	10% NLHS	1.79	8.51	59.47	12.85	17.39
Calf (C) + Hyperlipemic Human Serum (HLHS)	7.5% C 2.5% HLHS	3.26	9.05	63.60	11.96	22.58
	5% C 5% HLHS	12.74	13.25	42.63	17.97	23.43
	2.5% C 7.5% HLHS	2.84	8.31	56.67	14.25	17.94
	10% HLHS	1.32	10.30	27.37	16.95	43.86
Normolipemic Human Serum (NLHS) + Hyperlipemic Human Serum (HLHS)	7.5% NLHS 2.5% HLHS	2.32	8.88	59.08	13.56	16.15
	5% NLHS 5% HLHS	1.79	15.81	46.00	18.33	18.06
	2.5% NLHS 7.5% HLHS	2.94	8.02	55.85	14.17	19.03

Averaged over 2 experiments

DOSE RESPONSE:

TABLE 17

Percentage Distribution of ^{32}P-Phosphoric Acid among the Phospholipids in Primary Aortic Smooth Muscle Cell Cultures

SERUM TREATMENT		Lysophosphatidylcholine	Sphingomyelin	Phosphatidylcholine	Phosphatidylserine + Phosphatidylinositol	Phosphatidylethanolamine
Calf (C) + Normolipemic Human Serum (NLHS)	10% C	15.37	8.97	54.54	14.24	6.89
	7.5% C 2.5% NLHS	6.30	5.16	65.76	16.11	6.67
	5% C 5% NLHS	3.28	3.28	68.38	16.93	8.26
	2.5% C 7.5% NLHS	2.02	1.80	70.96	16.98	8.27
	10% C	3.15	3.44	68.90	17.24	7.27
Calf (C) + Hyperlipemic Human Serum (HLHS)	7.5% C 2.5% HLHS	6.49	3.86	65.33	17.45	6.69
	5% C 5% HLHS	3.44	8.26	63.22	16.06	5.13
	2.5% C 7.5% HLHS	3.84	2.46	69.39	17.68	6.63
	10% HLHS	4.88	9.52	53.64	15.48	17.08
Normolipemic Human Serum (NLHS) + Hyperlipemic Human Serum (HLHS)	7.5% NLHS 2.5% HLHS	4.61	3.40	66.78	17.22	7.01
	5% NLHS 5% HLHS	4.03	10.10	58.89	17.32	5.92
	2.5% NLHS 7.5% HLHS	8.35	2.03	67.01	15.28	6.84

Averaged over 2 experiments

Figure 6

Effect of HLHS on Incorporation of 1-^{14}C-acetate into Triglycerides

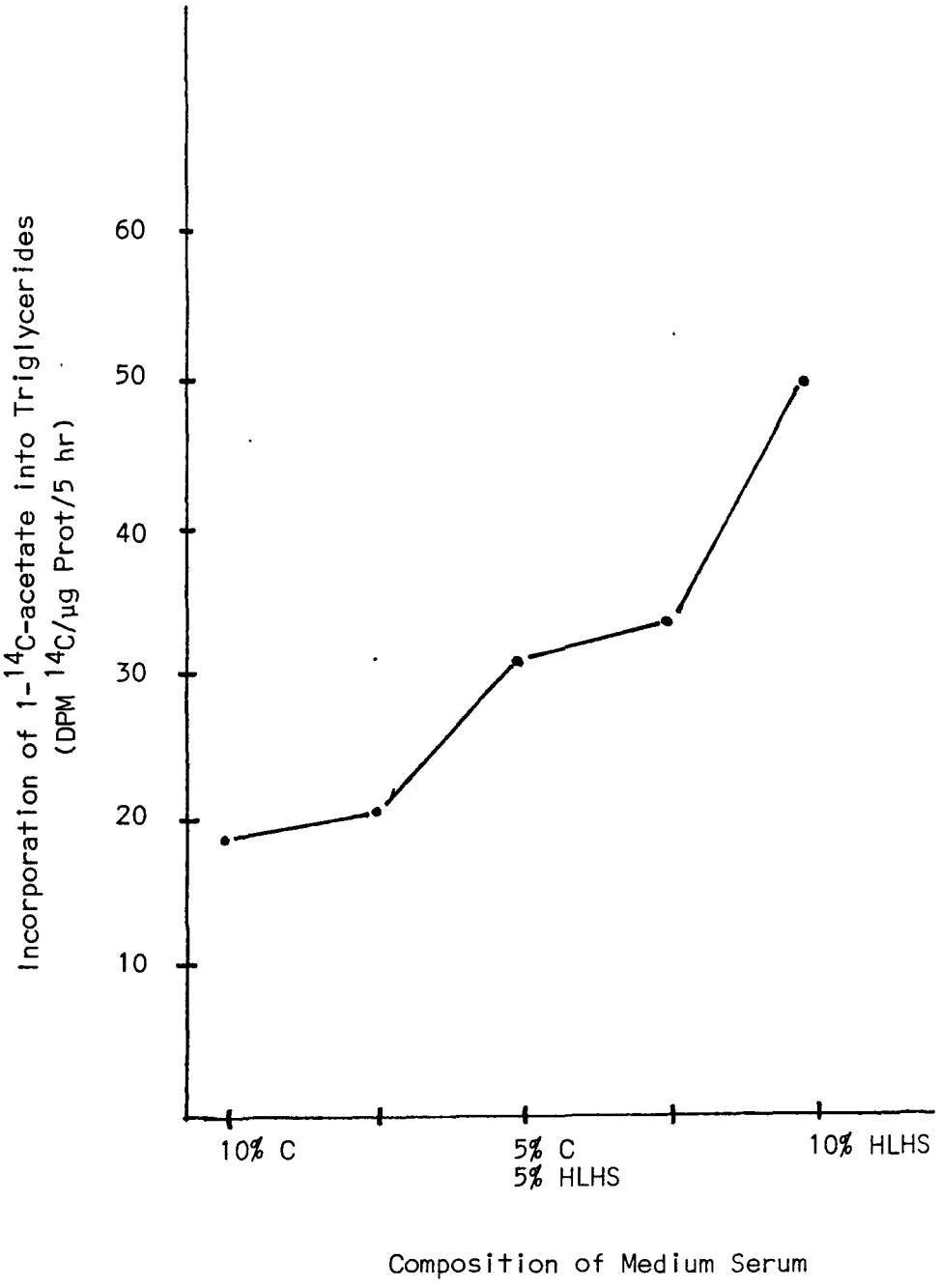


Figure 7

Effect of HLHS on Incorporation of 1-^{14}C-acetate into Cholesterol Esters

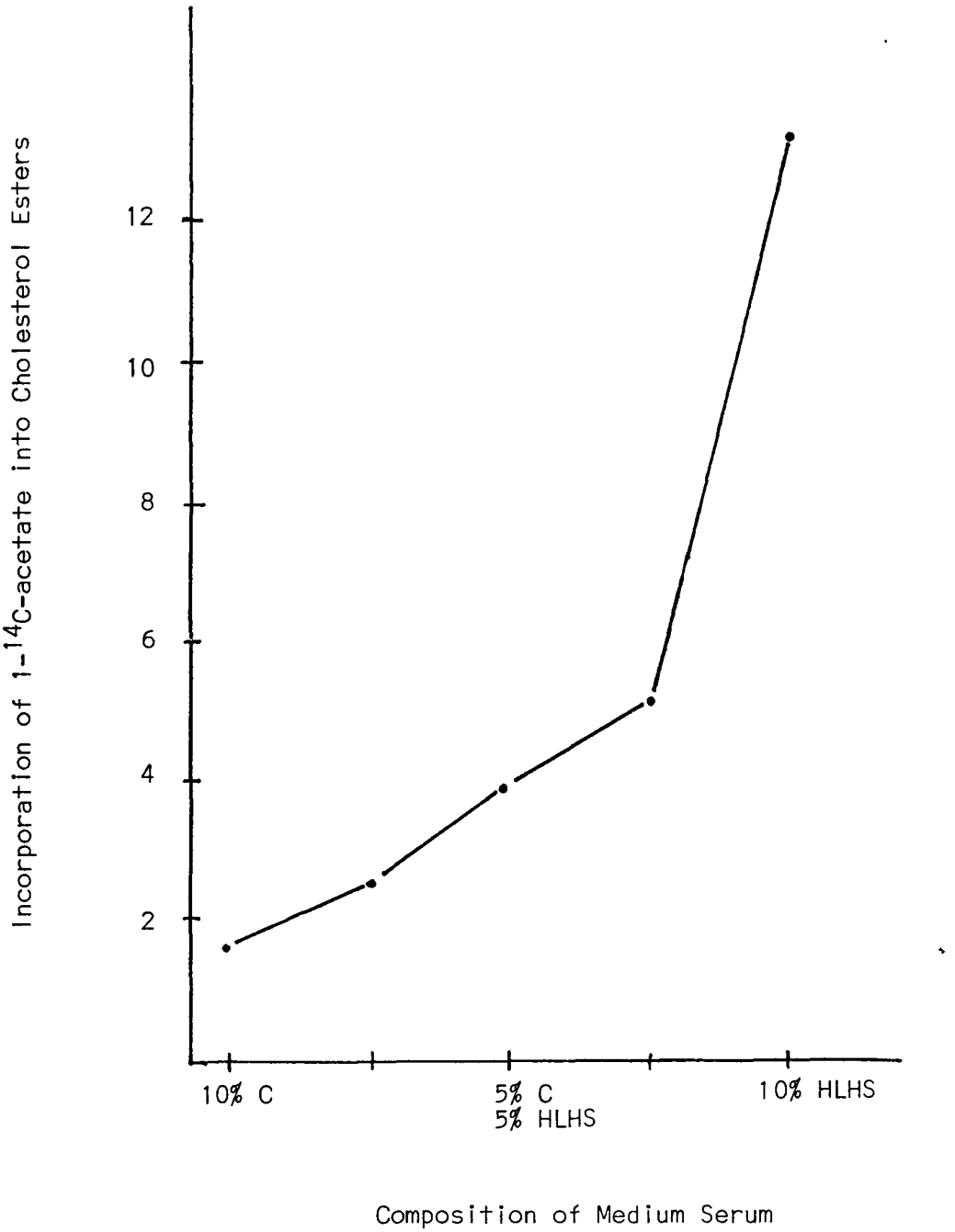


Figure 8

Effect of HLHS on Percentage of Total 1-^{14}C-acetate Incorporated into Triglycerides

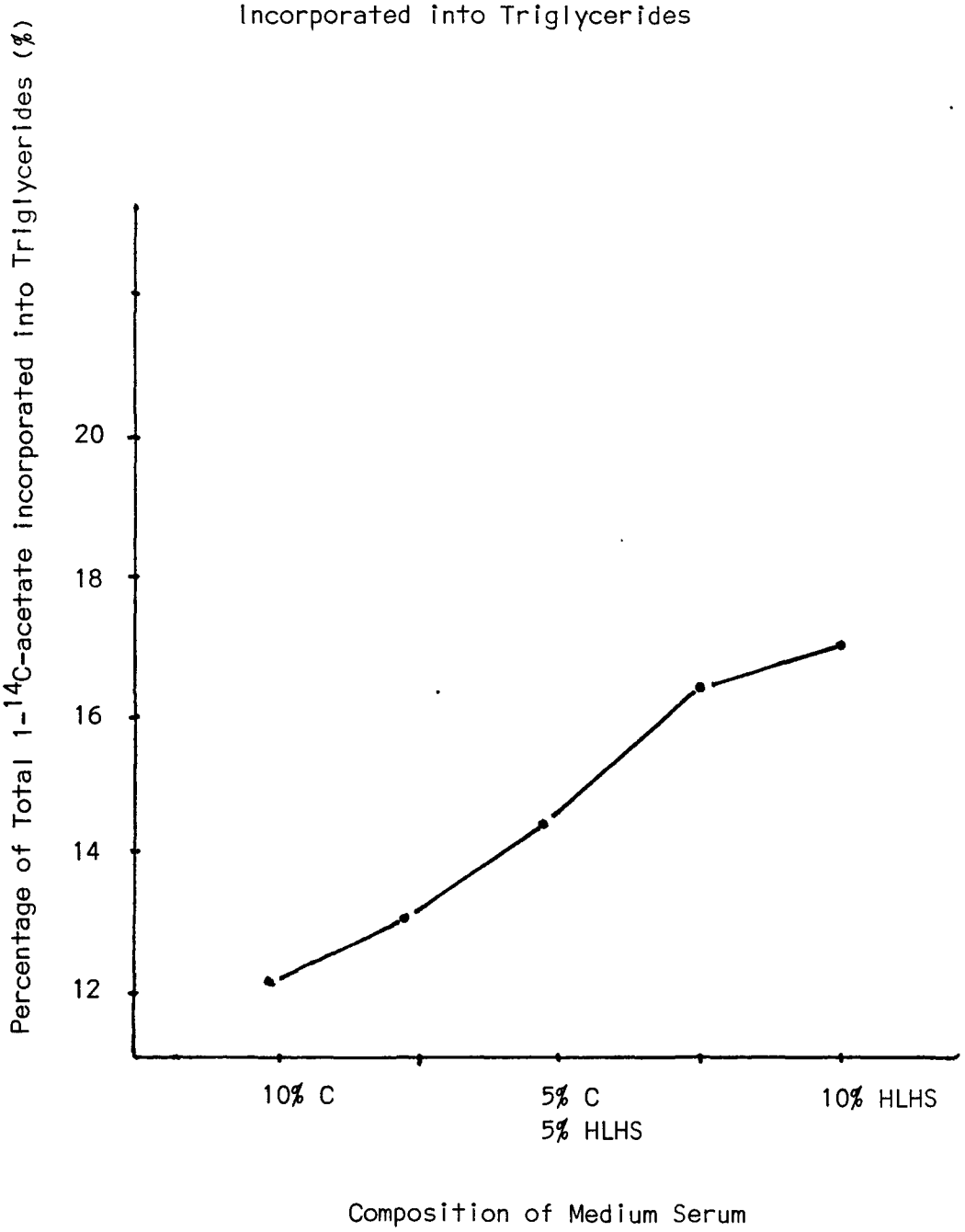


Figure 9

Effect of HLHS on Percentage of Total 1-^{14}C-acetate Incorporated into Cholesterol Esters

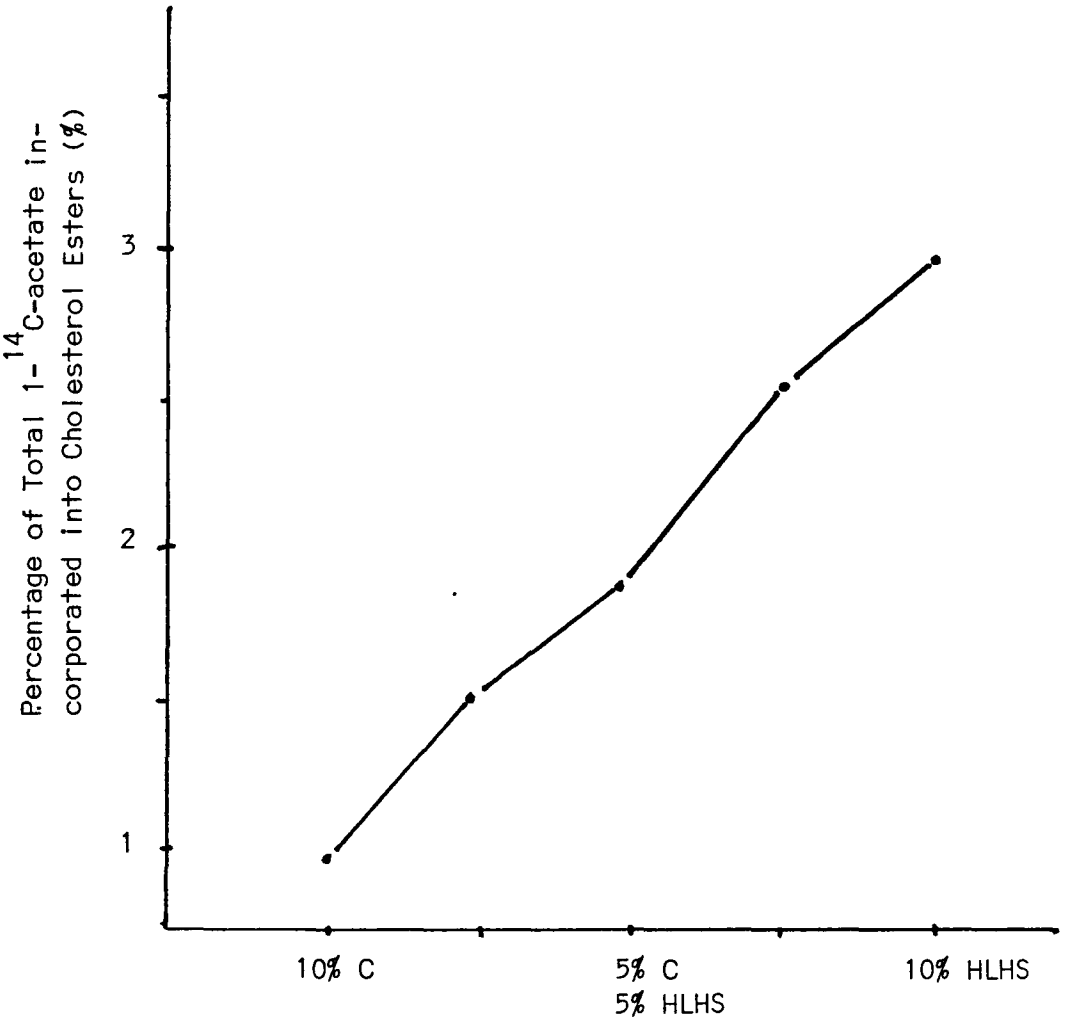


Figure 10

Effect of HLHS on Incorporation of 1-^{14}C-acetate into Phosphatidylcholine

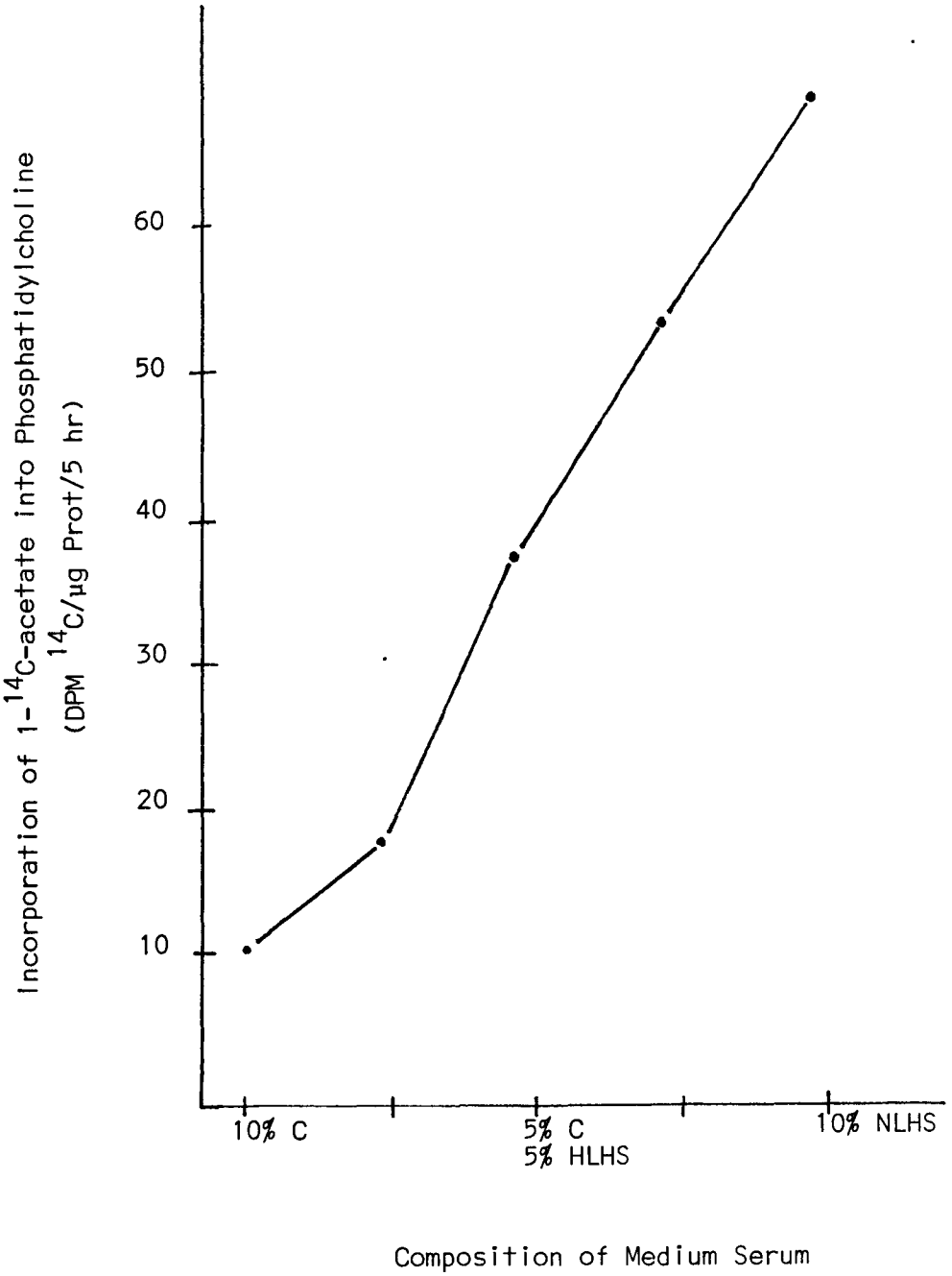
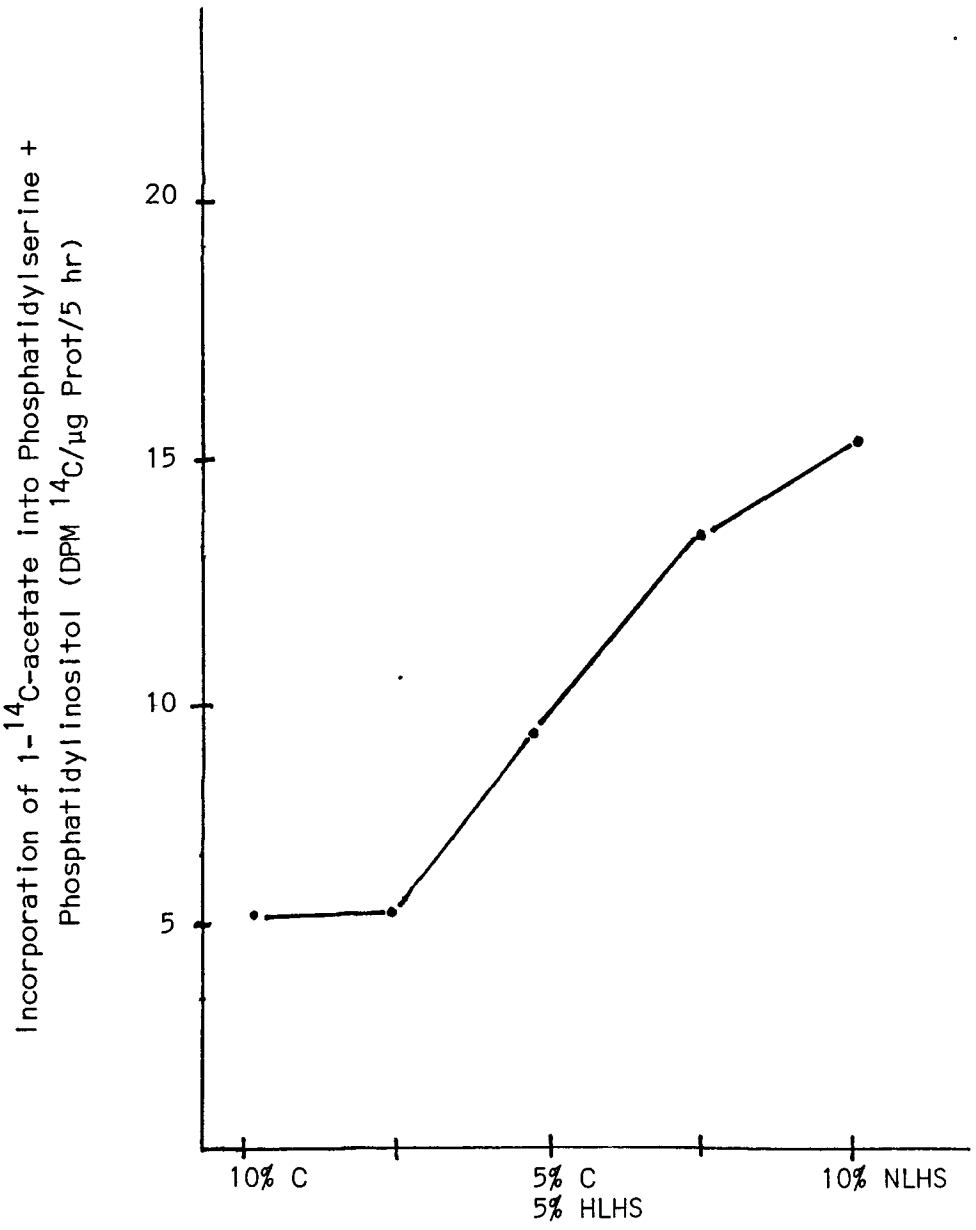


Figure 11

Effect of HLHS on Incorporation of 1-^{14}C-acetate into Phosphatidylserine + Phosphatidylinositol



Ultrastructure of Smooth Muscle Cells

"Normal" Cell Morphology

1) Growth

The morphology of the cultured smooth muscle cells was examined at various culture times, using scanning electron microscopy.

One Week

After one week of culture (Fig 12) only a few scattered cells are seen close to the explant. The cells appear flat and have a moderate number of filamentous processes extruding from the cell surface. There are also some long cytoplasmic projections originating mainly from the cell edge remote from the tissue explant.

Two Weeks

At two weeks (Fig 14) the cell density is greater, with some overlap of cells. The cells are more stellate than those grown only for one week, and have cytoplasmic projections on all sides. Subsurface granular bodies can be seen on flat areas of some cells, apparently deforming the cell surface.

Four Weeks

Cell density at four weeks (Fig 15,16) is again increased, with more cellular overlap. The cells have a greater degree of polarity with fewer cytoplasmic extensions or processes. At this stage there is a definite formation of "hills and valleys" due to partial multilayering of the cells. The deeper cells are flatter while those on the surface have more extensions and filamentous processes.

Five Weeks

At five weeks, (Fig 17,18,19) the morphology is essentially similar to that seen at four weeks. The cells have a distinct polarity, (Fig 17) forming confluent cultures. There is considerable cellular overlap with formation of "hills and valleys". Some cells, especially those in the superficial layer of multilayered areas or hills, exhibit a variety of surface processes; some globular, (Fig 14) some short and spiky, and others long, fine and filamentous. (Fig 18) Subsurface globular bodies are also seen. (Fig 19) Cells at the edge of the culture show more surface projections than those closer to the explant.

Transmission Electron Microscopic Features

Transmission electron microscopy was used to examine five week cultures. (Fig 20,21,22) Since the cells were trypsinized prior to processing for electron microscopy, the cells are rounded up (Fig 22) instead of being flat and elongated, as seen by scanning electron microscopy. The cellular surface does show globular projections, (Fig 22) as well as elongate ones filled with filaments. (Fig 21)

The cytoplasm of these cells is very definitely portional, with organelles aligned almost in concentric circles around the nucleus. The nucleus itself is centrally located (Fig 22) and is multilobular and indented (Fig 20) in outline, containing one or more dense nucleoli. (Fig 20,22)

Adjacent to the nucleus, small golgi and smooth surface reticular elements are prominent. (Fig 23) These are frequently associated with numerous dense lysosomal and residual bodies. (Fig 23) Lipid droplets, short profiles of rough endoplasmic reticulum (RER) and

cultures of small vesicles are also seen in this area.(Fig 23,20) There are also a few mitochondria, but most of the mitochondria are arranged in large numbers adjacent to these regions in a circular fashion around the nucleus.(Fig 22,20) Lipid droplets of variable size and osmophilia,(Fig 20) frequently associated with lysosomal bodies, are interspersed.

Elongated extensive profiles of RER are present, in some cells particularly(Fig 22,24) in the area surrounding the mitochondria. The peripheral cytoplasm of these cells is relatively sparse in organelles.(Fig 22,21) Myofilaments are numerous peripherally, frequently ending in dense bodies internal to the plasma membrane.(Fig 21) Myofilaments are also prominent through all areas of the cytoplasm,(Fig 21,20,23,24) but tend to lack definite orientation.(Fig 20,23) Dense bodies are frequently seen in the internal cytoplasm as well.(Fig 24,25)

Hyperlipemic Serum-Treated Aortic Smooth Muscle Cell Cultures - Ultrastructural Features.

Cultured aortic smooth muscle cells grown for 5 weeks were treated with 10% hyperlipemic human serum for 48 hours and examined using both scanning and transmission electron microscopy.

The scanning electron micrographs (SEM)(Fig 26,27) show that the cells grow in confluent cultures, with cellular overlap, similar to the appearances seen with the untreated cultures. However, there are numerous morphologic differences.

(1) The treated cells do not show the same degree of polarity, and appear more stellate in shape and not as flattened in outline.

(2) There are many more spiky short surface projectons together with globular surface projections.

(3) HLS-treated cells also exhibit many more degenerate areas than seen in the untreated cell cultures.

Using transmission electronmicroscopy the overall structure of the treated cells was essentially similar to the untreated cells.(Fig 28,29) However, again prominent morphologic differences were evident:

(1) The nuclei of treated cells show less peripheral clumping of chromatin and are more rounded.

(2) Nucleoli are less frequent and less electron dense, as is the nucleoplasm.

(3) There are fewer organelles of all types, and those which are present are poorly developed.

(4) The cytoplasm is less electron dense (probably due to a decreased number of myofilaments). Partitioning of the cytoplasm is not as prominent.

(5) There are many more electron dense lipid droplets scattered in the cytoplasm.

(6) The cell surface is extremely convoluted on many cells and many cells have elongate cytoplasmic extensions, short spiky projections, and large globular projections frequently containing lipid inclusions.

Conclusions

The smooth muscle cell grown in culture cannot be classified as a "normal smooth muscle cell" as seen *in vivo*. They do, however, contain numerous myofilaments and dense bodies which, although they are not ordered, are definitely consistent with the smooth muscle origin of the cells.

The organellar composition of these cells indicates a very active cell metabolically, both in terms of anabolism and catabolism. The large numbers of RER and the prominent golgi apparatus are not necessarily abnormal; on the other hand, the considerable numbers of mitochondria and lysosomes are abnormal and may be present in response to the lipid inclusions. However, they probably are also indicative of cellular degeneration to a certain extent, or at least to altered metabolism.

Figure 12 SEM of cultured aortic smooth muscle cells. Age - 1 week. Magnification 5400X Cytoplasmic extensions - Cx

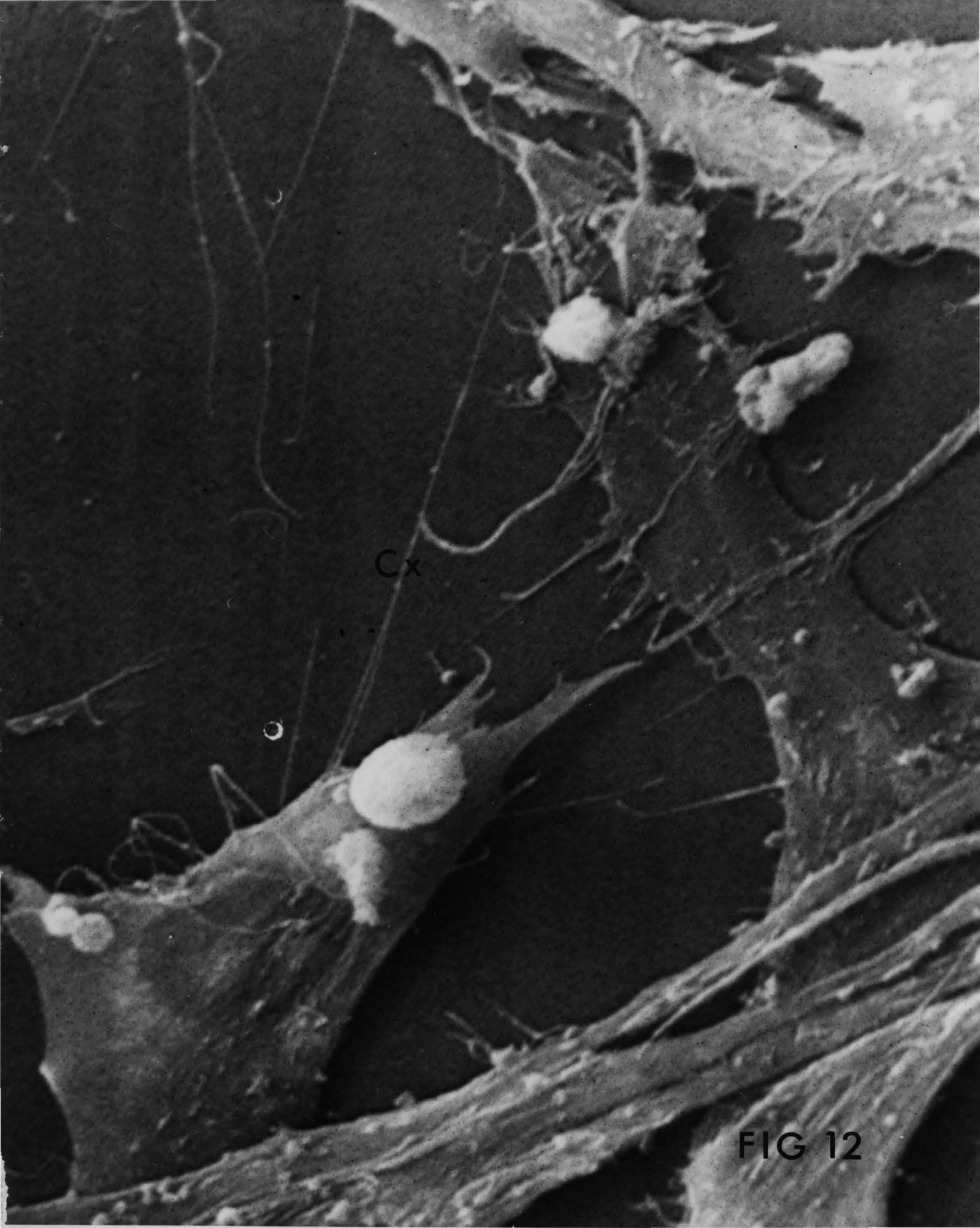


FIG 12

Figure 13 SEM of cultured aortic smooth muscle cells. Age - 2 weeks. Magnification 2160X

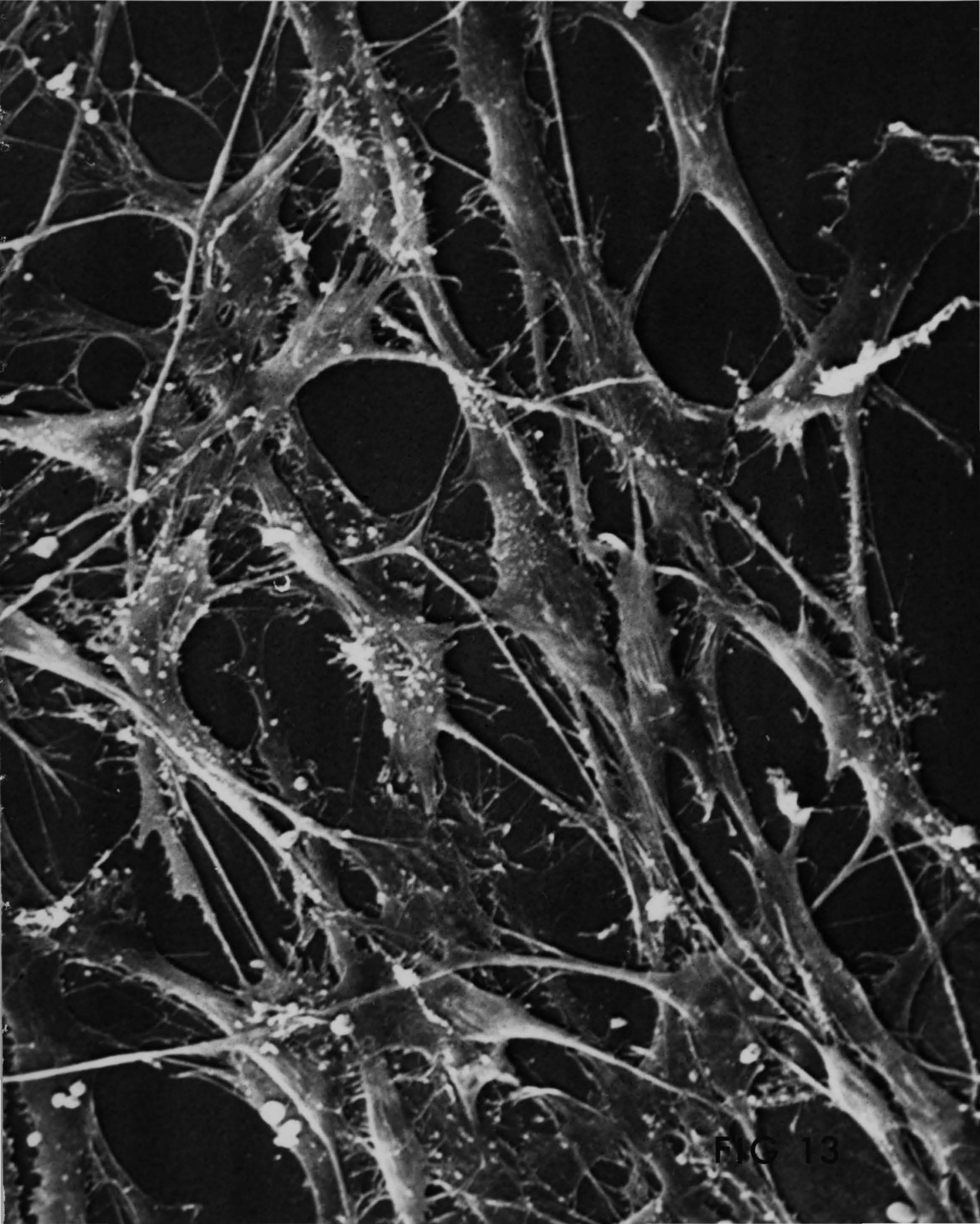


FIG 13

Figure 14

SEM of cultured aortic smooth muscle cells. Age - 2 weeks. Magnification 5400X

Subsurface Granular elements -SG

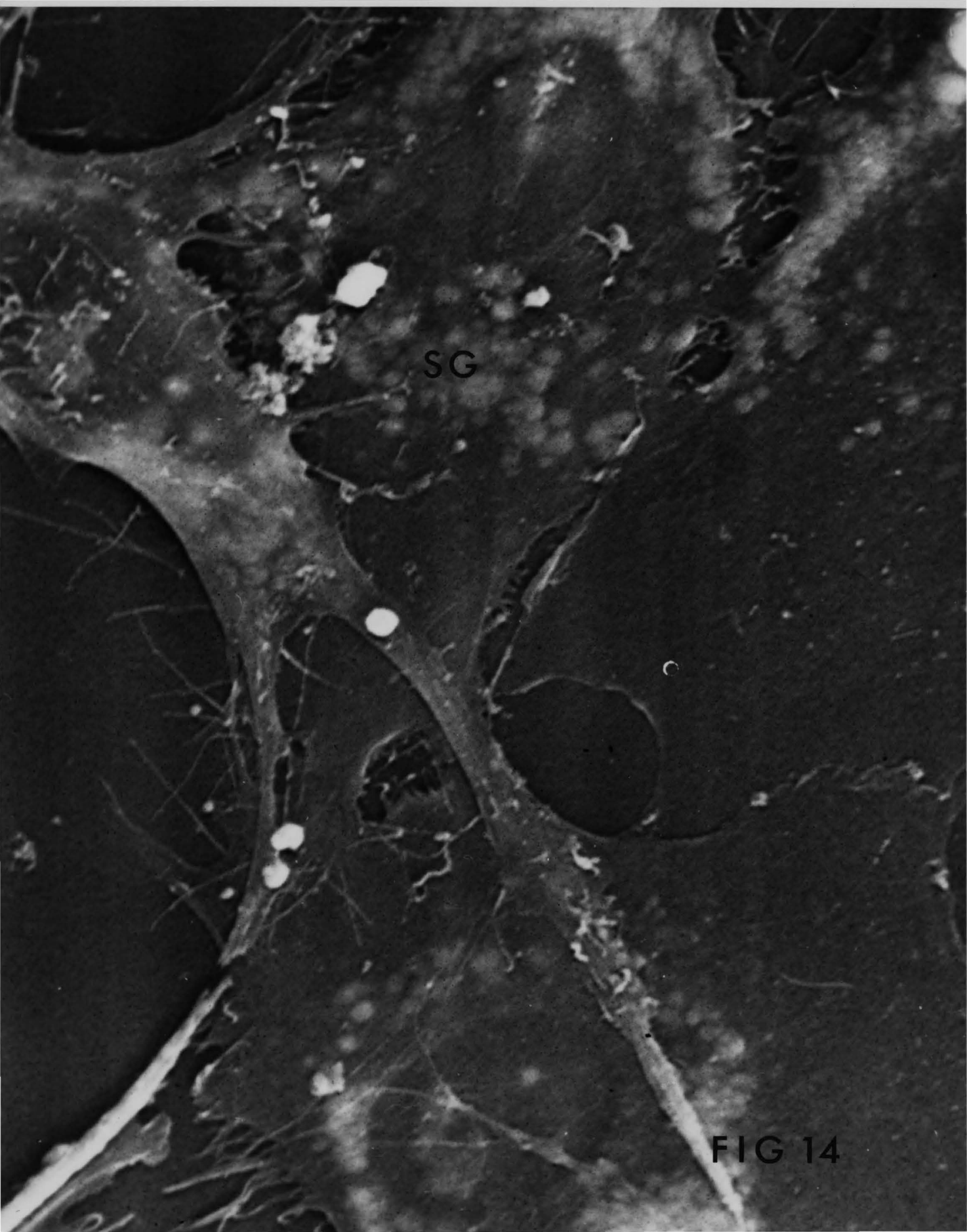


FIG 14

Figure 15 SEM of cultured aortic smooth muscle cells. Age - 4 weeks. Magnification 4800X

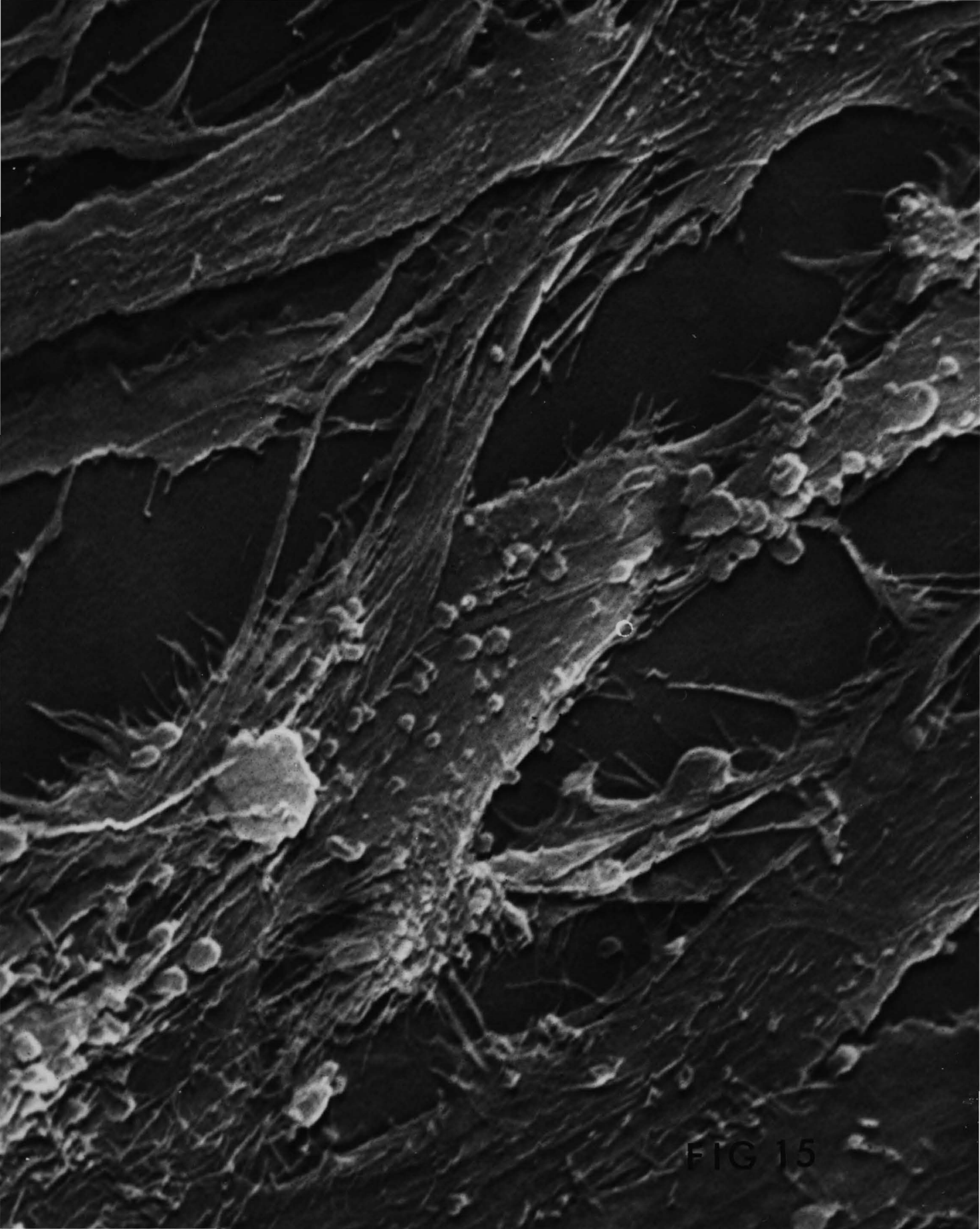


FIG 15

Figure 16 SEM of cultured aortic smooth muscle cells. Age - 4 weeks. Magnification 1200X

Hills - H

Valleys - V

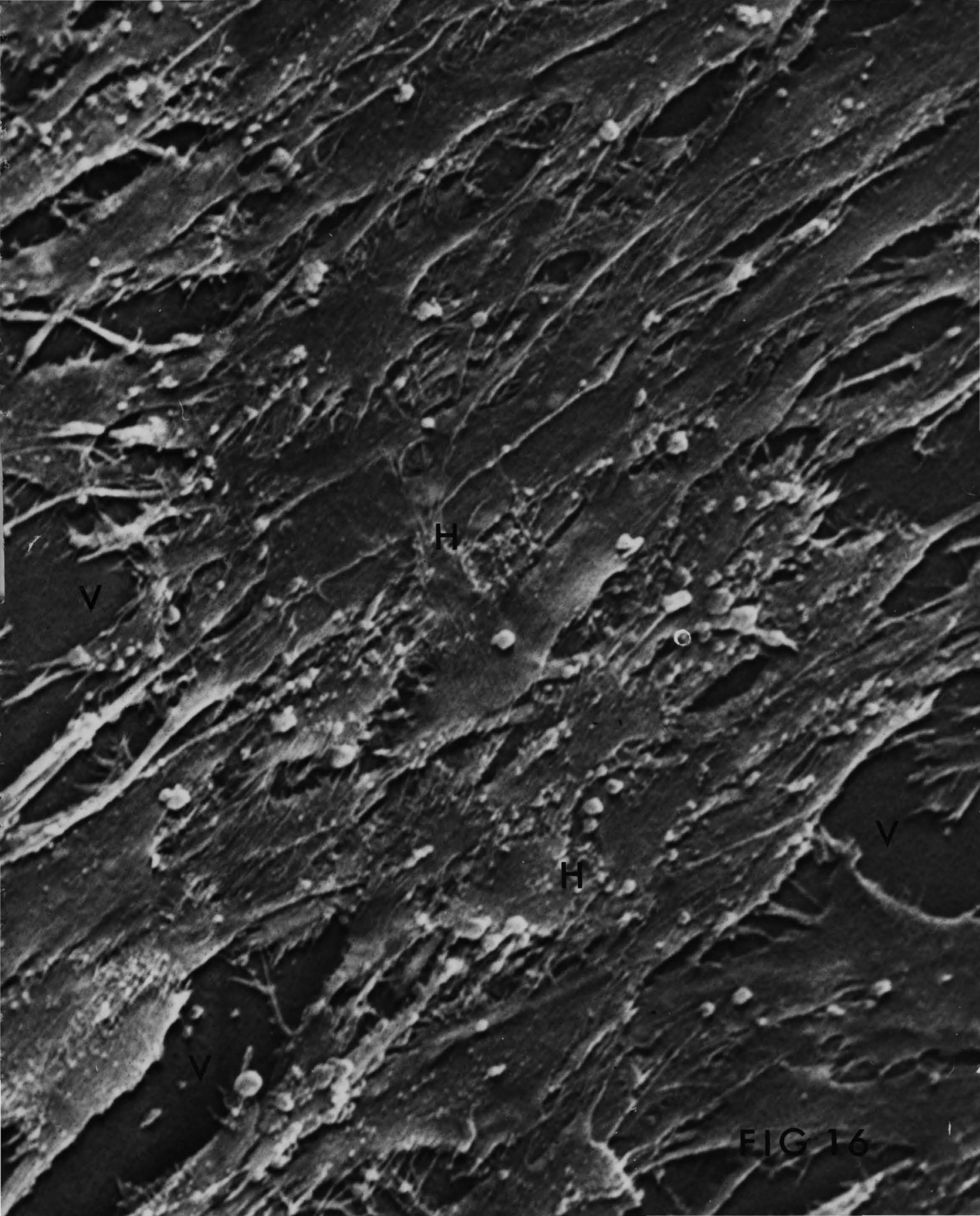


FIG 16

Figure 17 SEM of cultured aortic smooth muscle cells. Age - 5 weeks. Magnification 9000X

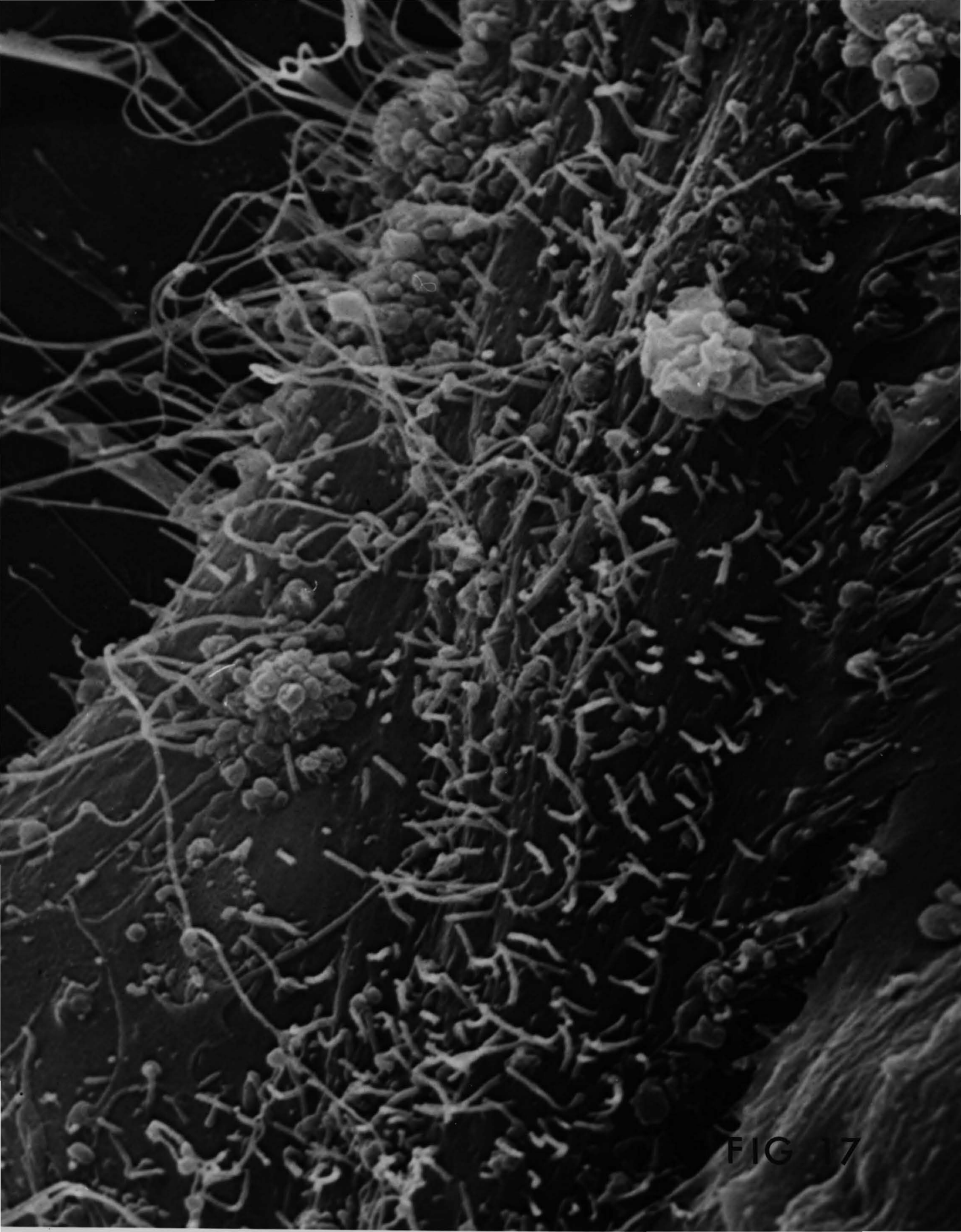


FIG 7

Figure 18

SEM of cultured aortic smooth muscle cells. Age - 5 weeks. Magnification 9000X

Filamentous projections - Fil

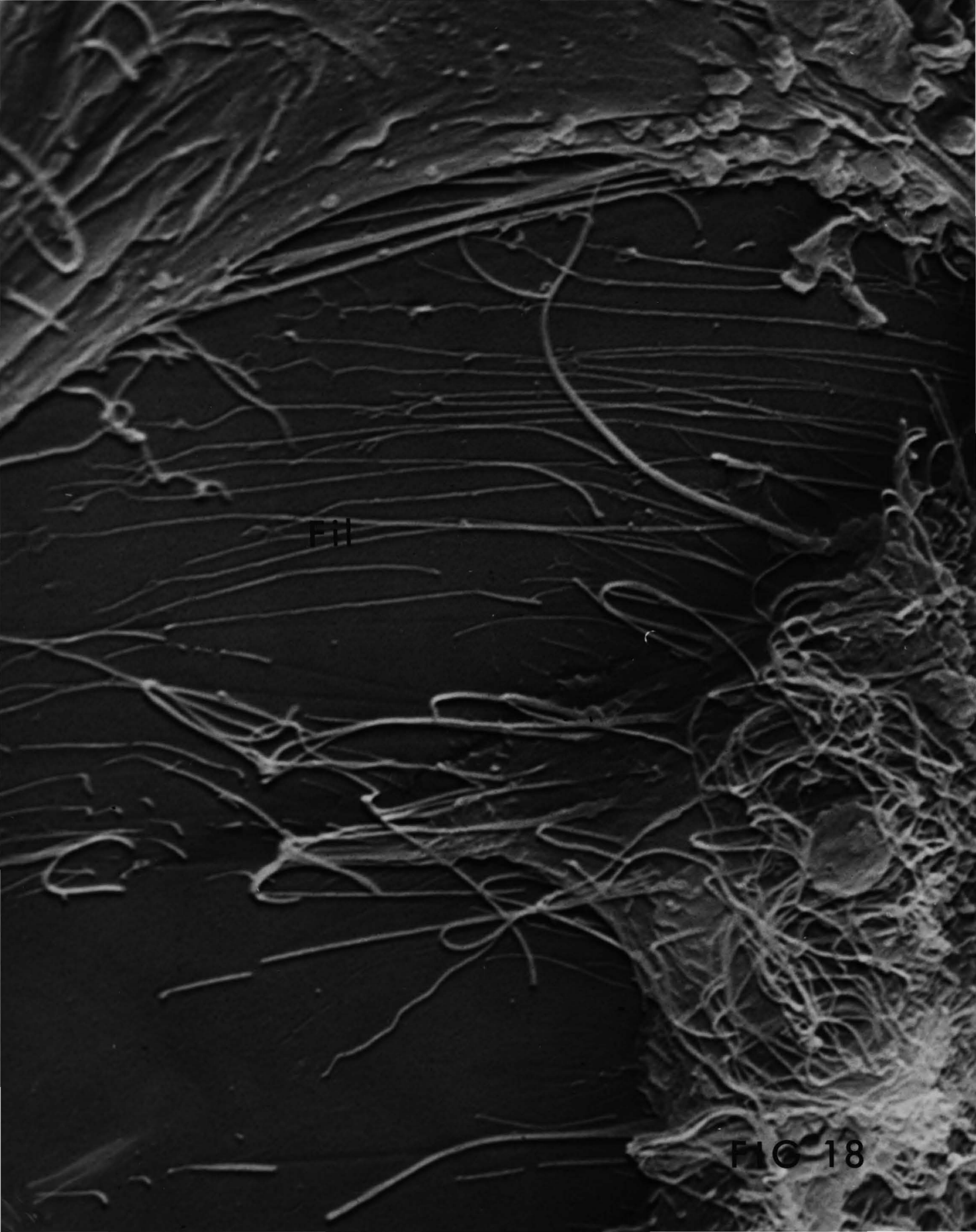


FIG 18

Figure 19

SEM of cultured aortic smooth muscle cells. Age - 5 weeks.

Magnification 9000X

Subsurface globular elements - Su

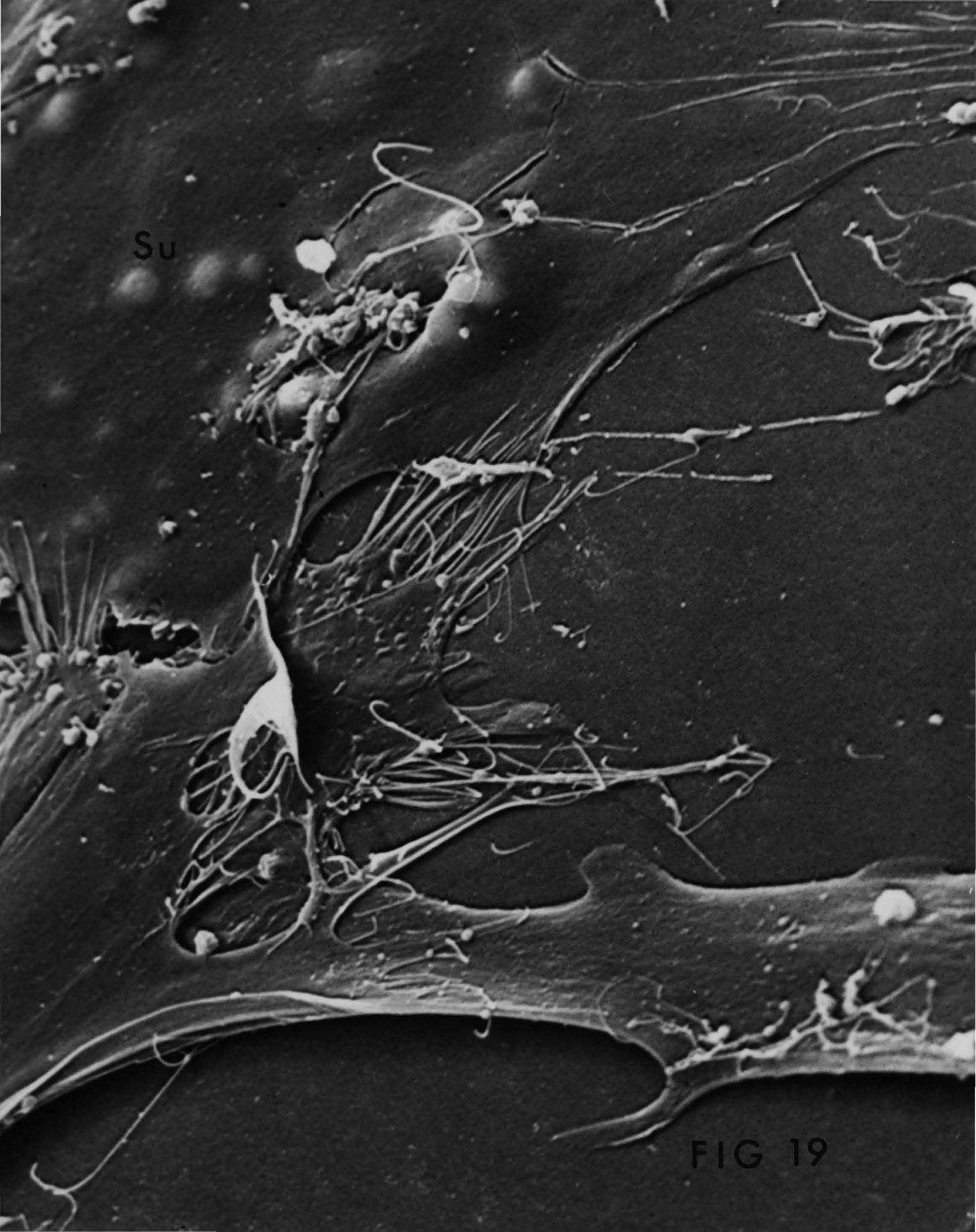


FIG 19

Figure 20

TEM of cultured aortic smooth muscle cells. Age - 5 weeks.
Magnification 7100X
Myofilaments - M
Nucleus - N
Nucleolus - n
Mitochondria - m
Lysosomes - Ly
Lipid - L

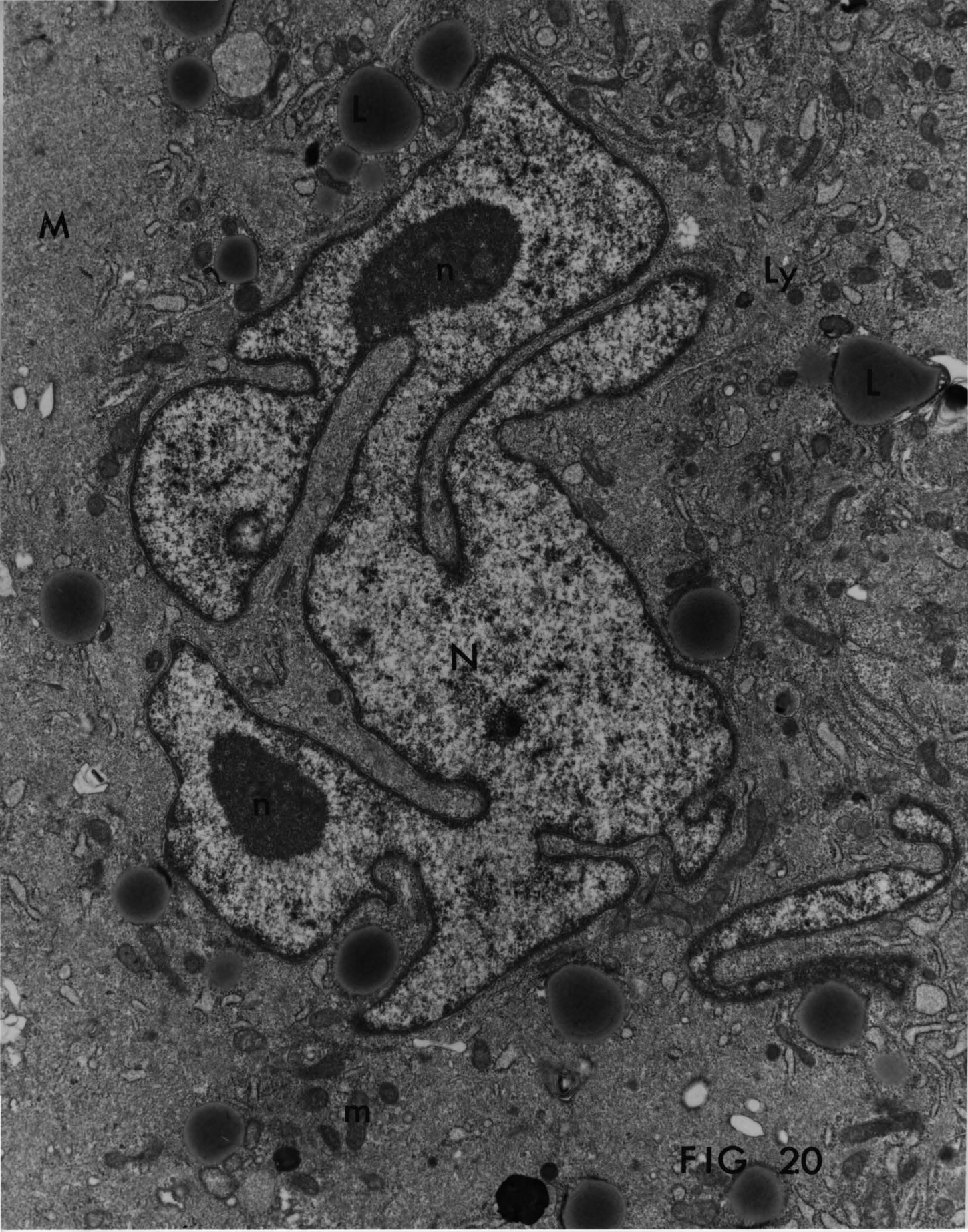


FIG 20

Figure 21

TEM of cultured smooth muscle cells. Age - 5 weeks.
Magnification 9100X
Elongate extensions - Ex
Myofilaments - M
Mitochondria - m

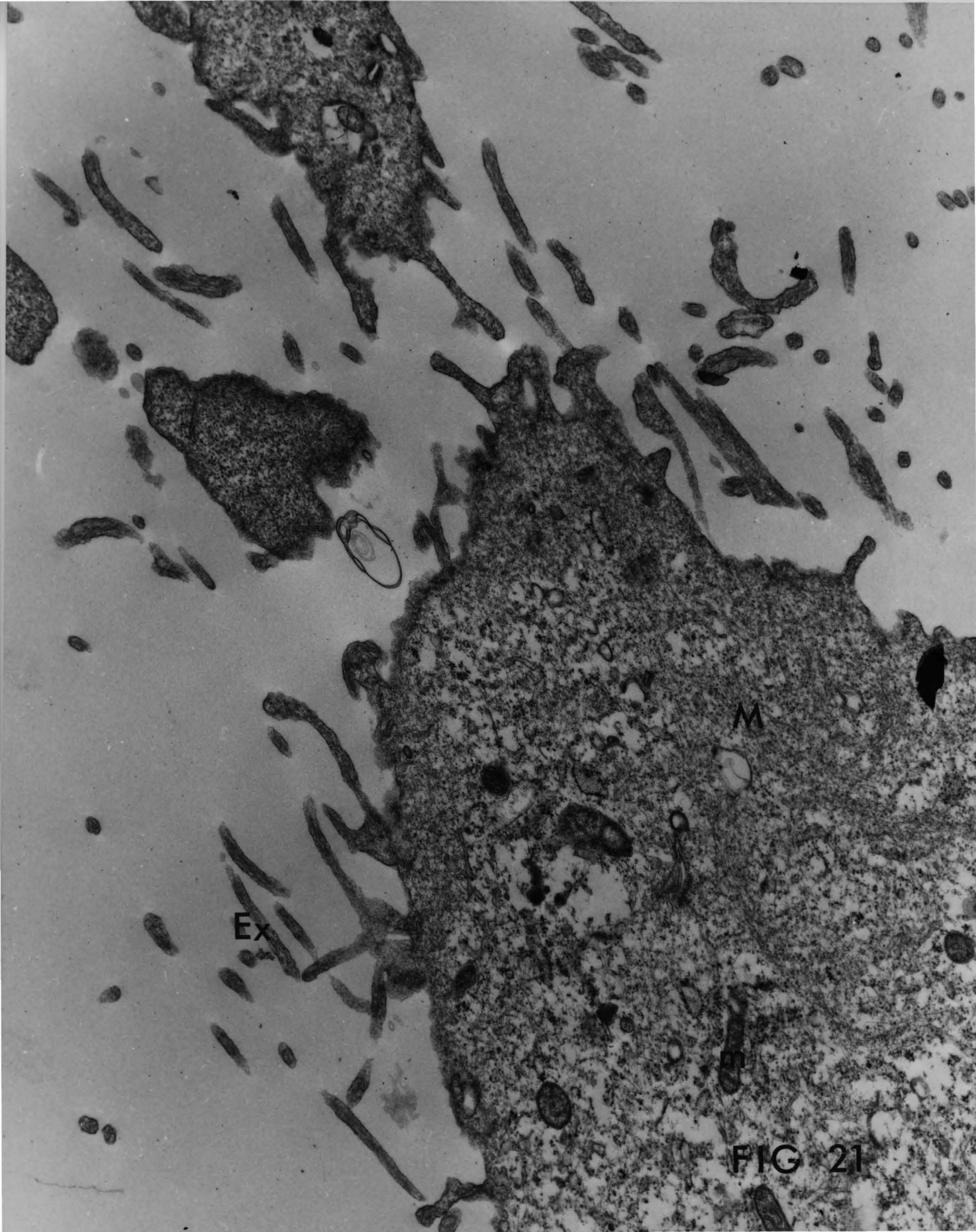


FIG 21

Figure 22

TEM of cultured smooth muscle cells.

Age - 5 weeks.

Magnification 4500X

Globular projections -Gp

Nucleus - N

Nucleolus - n

Lipid - L

Mitochondria - m

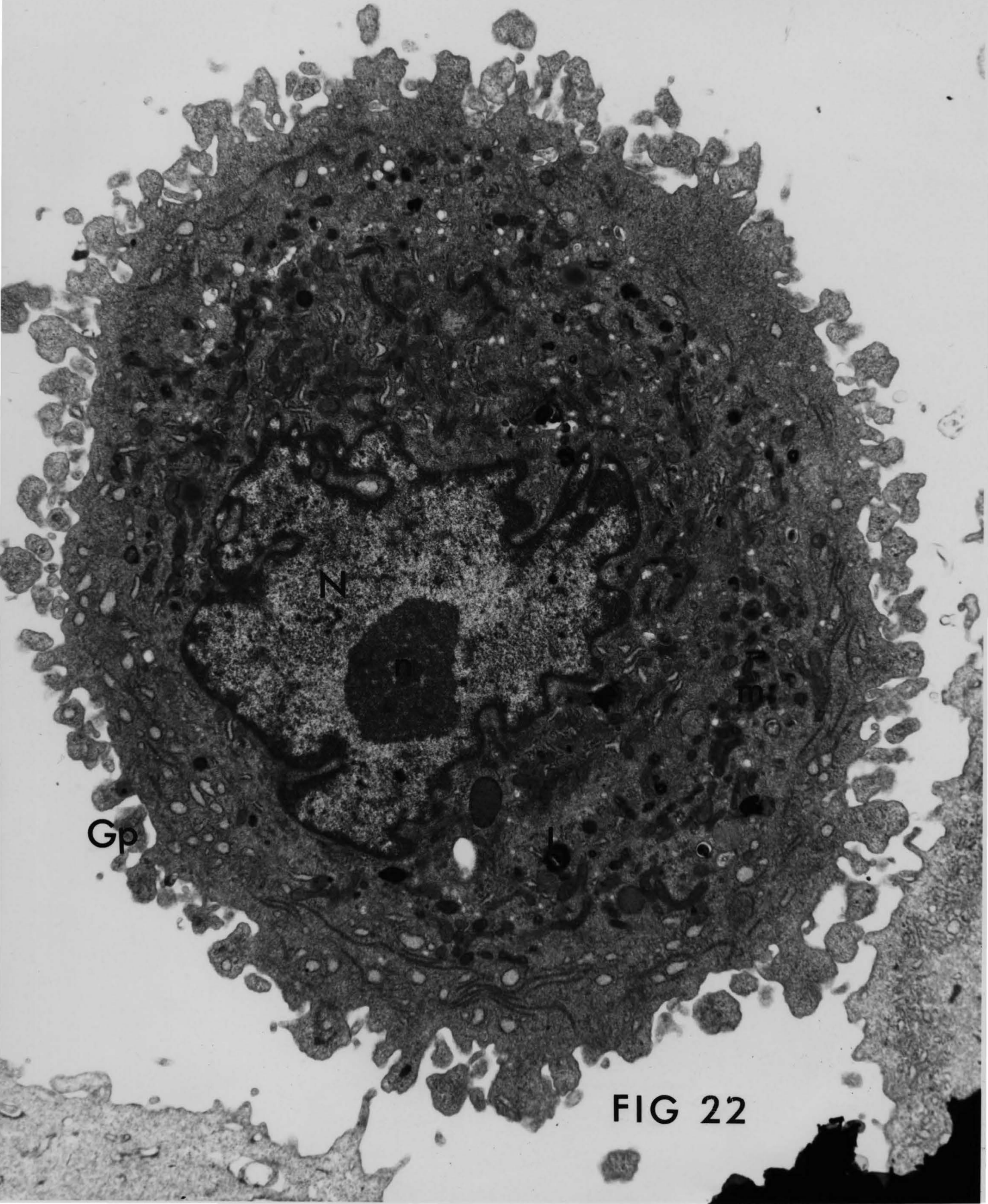


FIG 22

TEM of cultured smooth muscle cells.

Age - 5 weeks.

Figure 23 Magnification 19000X

Rough endoplasmic reticulum - RER

Mitochondria - m

Golgi apparatus - G

Lipid - L

Vesicle - V

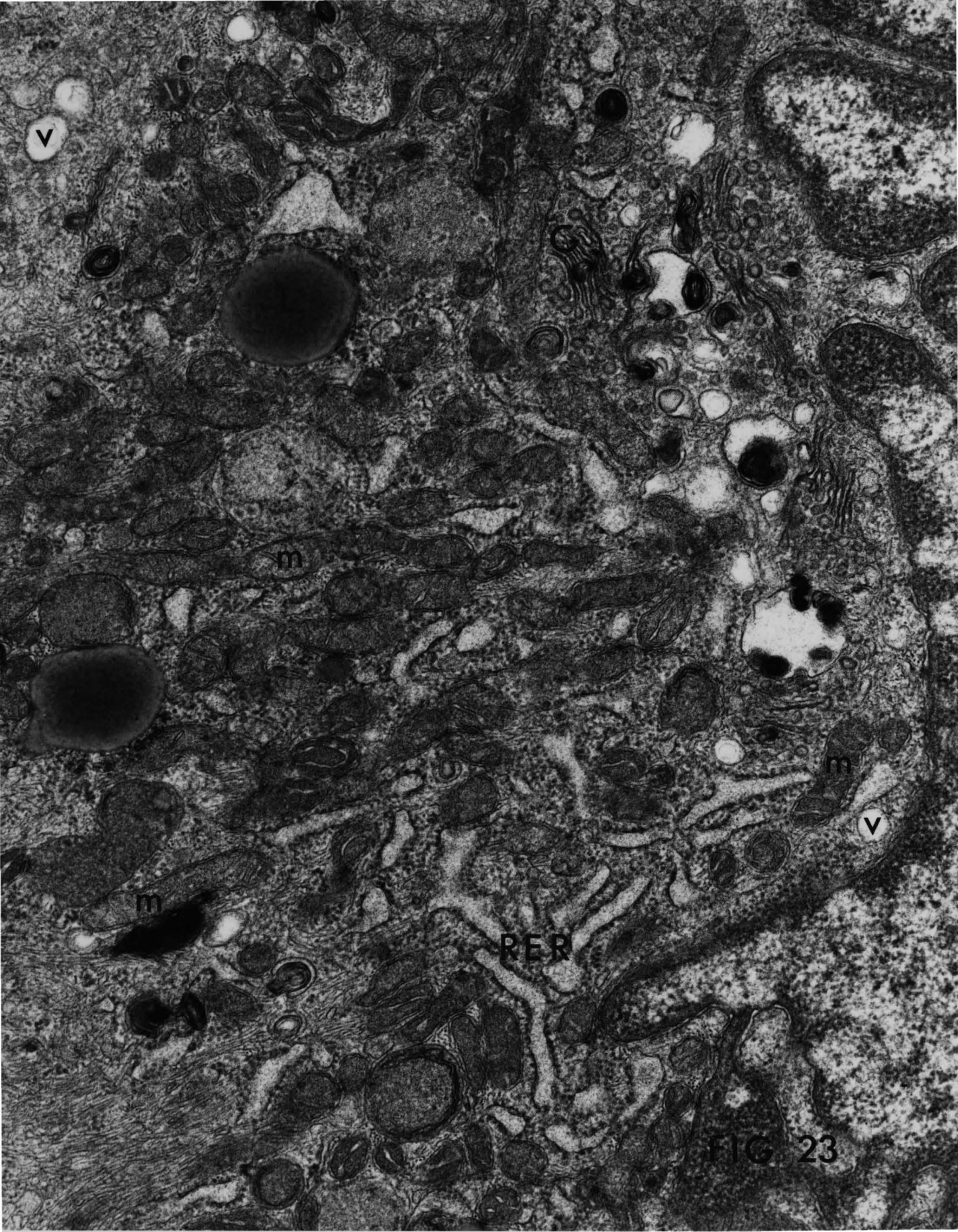


FIG 23

Figure 24

TEM of cultured smooth muscle cells.

Age - 5 weeks.

Magnification 25000X

Dense bodies - DB

Rough endoplasmic reticulum - RER

Myofilaments - M

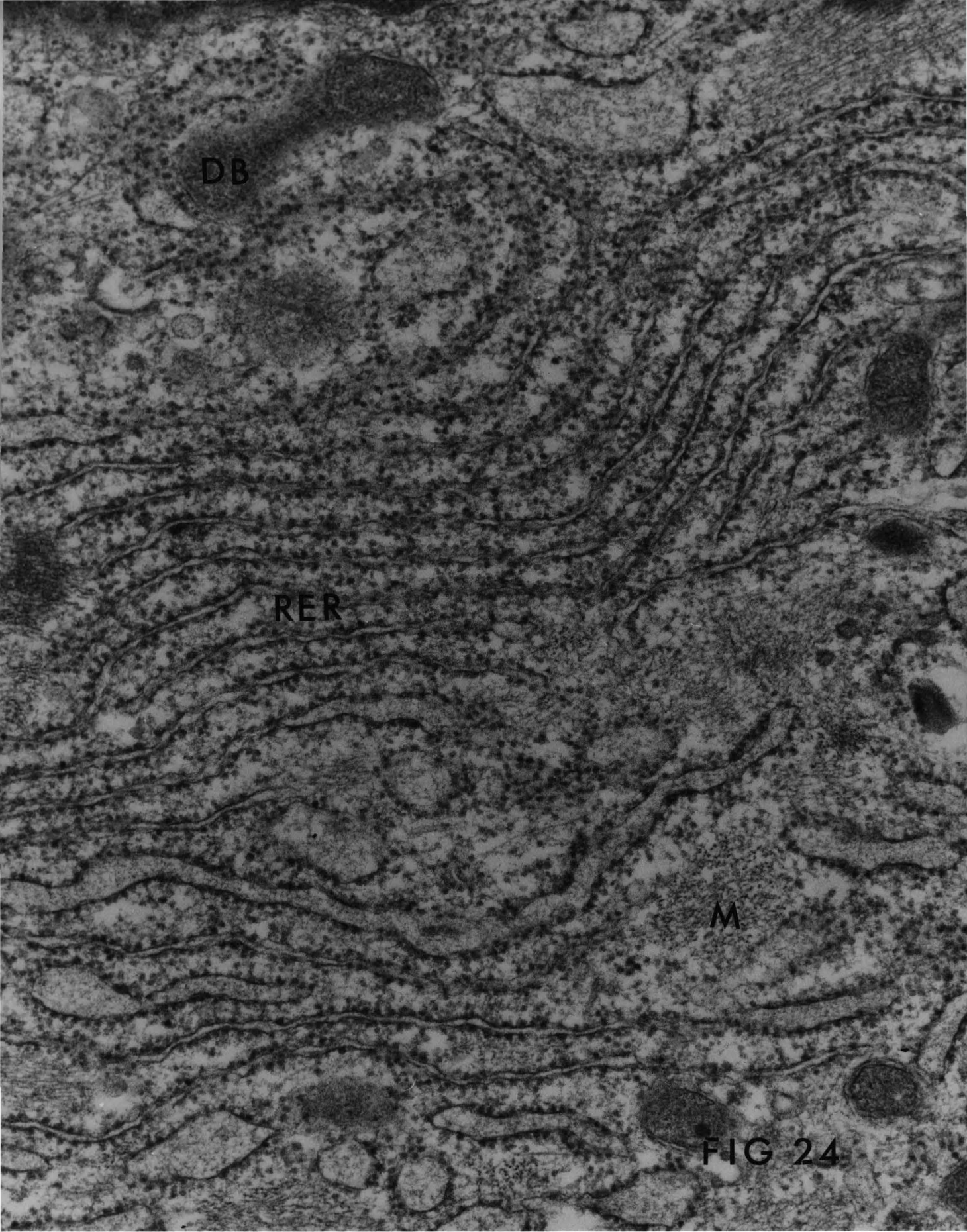


FIG 24

Figure 25

TEM of cultured aortic smooth muscle cells. Age - 5 weeks.
Magnification 25000X
Dense bodies - DB

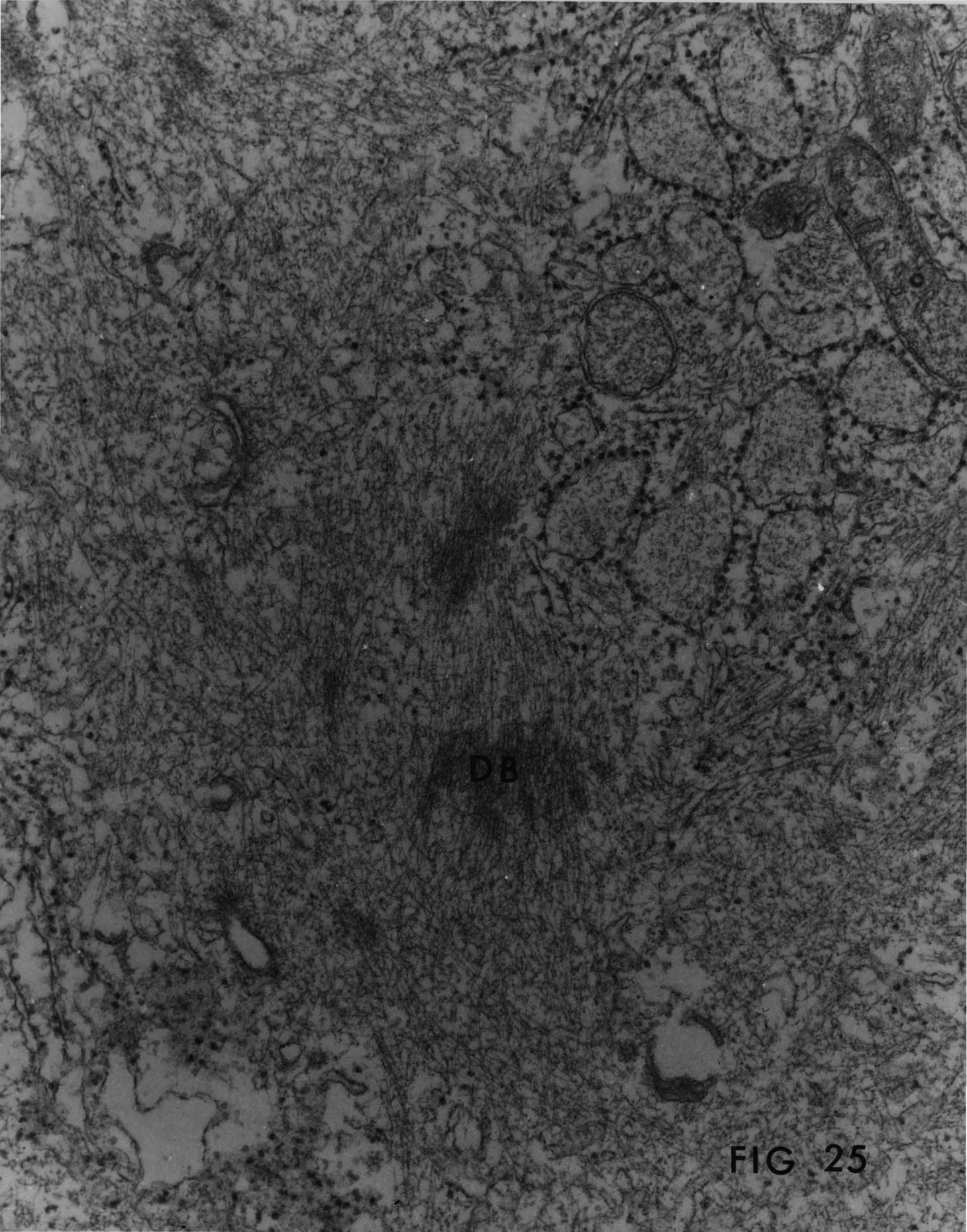


FIG 25

Figure 26

SEM of cultured aortic smooth muscle cells treated with 10% hyperlipemic human serum. Age - 5 weeks. Magnification 10,200X

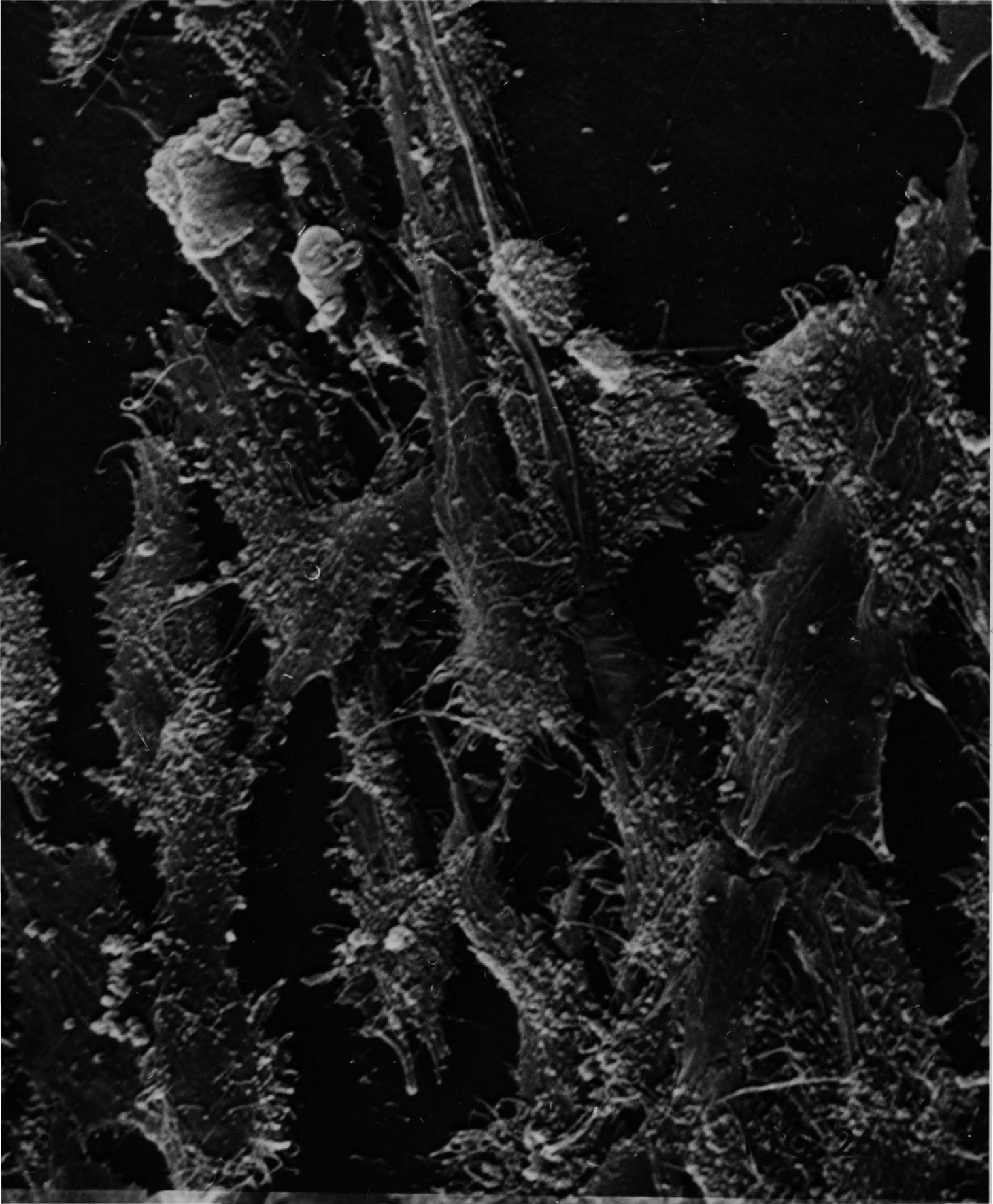


Figure 27

SEM of cultured aortic smooth muscle cells treated with 10% hyperlipemic human serum. Age - 5 weeks. Magnification 21,300X

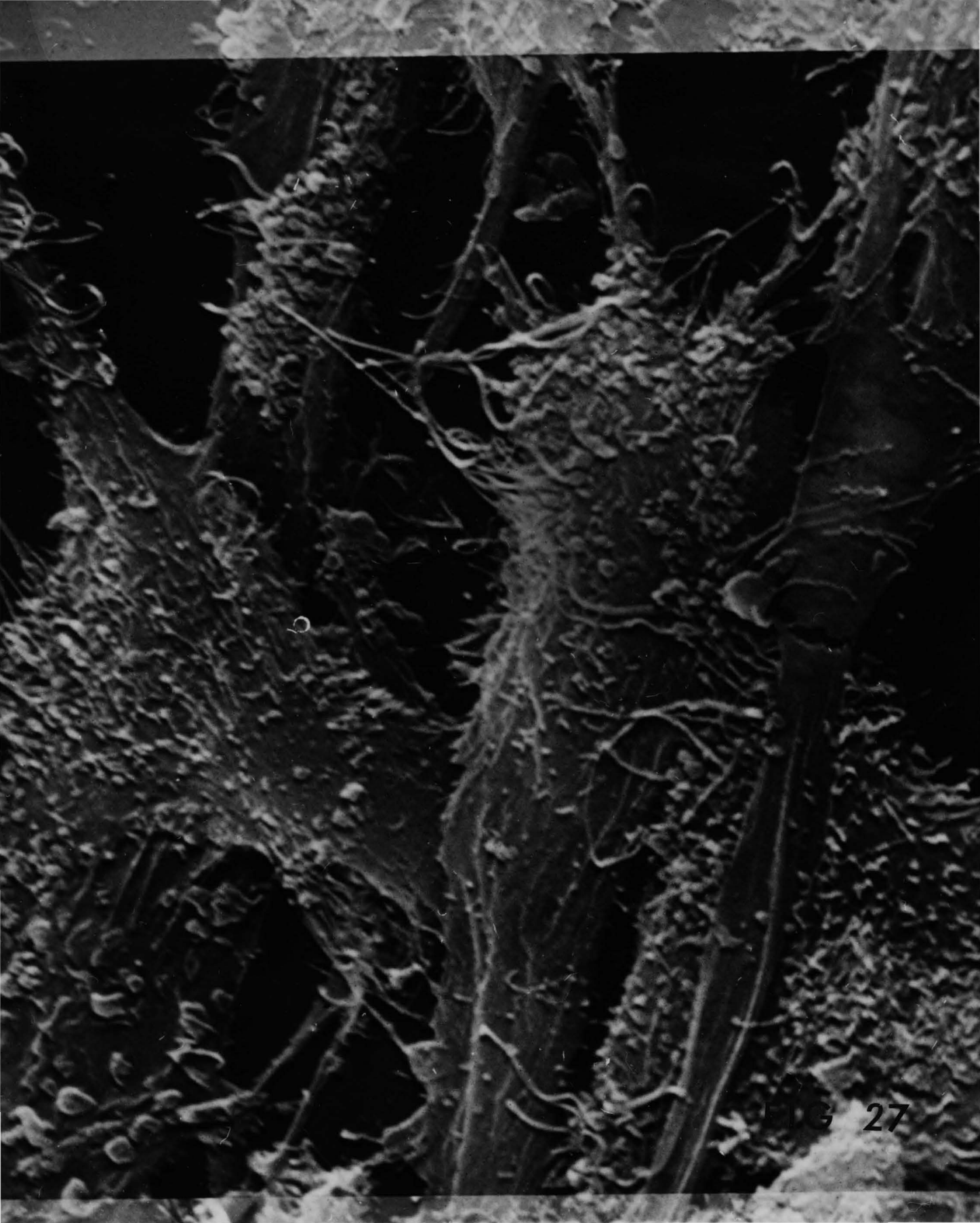


Figure 28

TEM of cultured aortic smooth muscle cells treated with 10% hyperlipemic human serum. Age - 5 weeks.

Magnification 5400X

Mitochondria - m

Lipid - L

Elongate cytoplasmic extensions - ECx

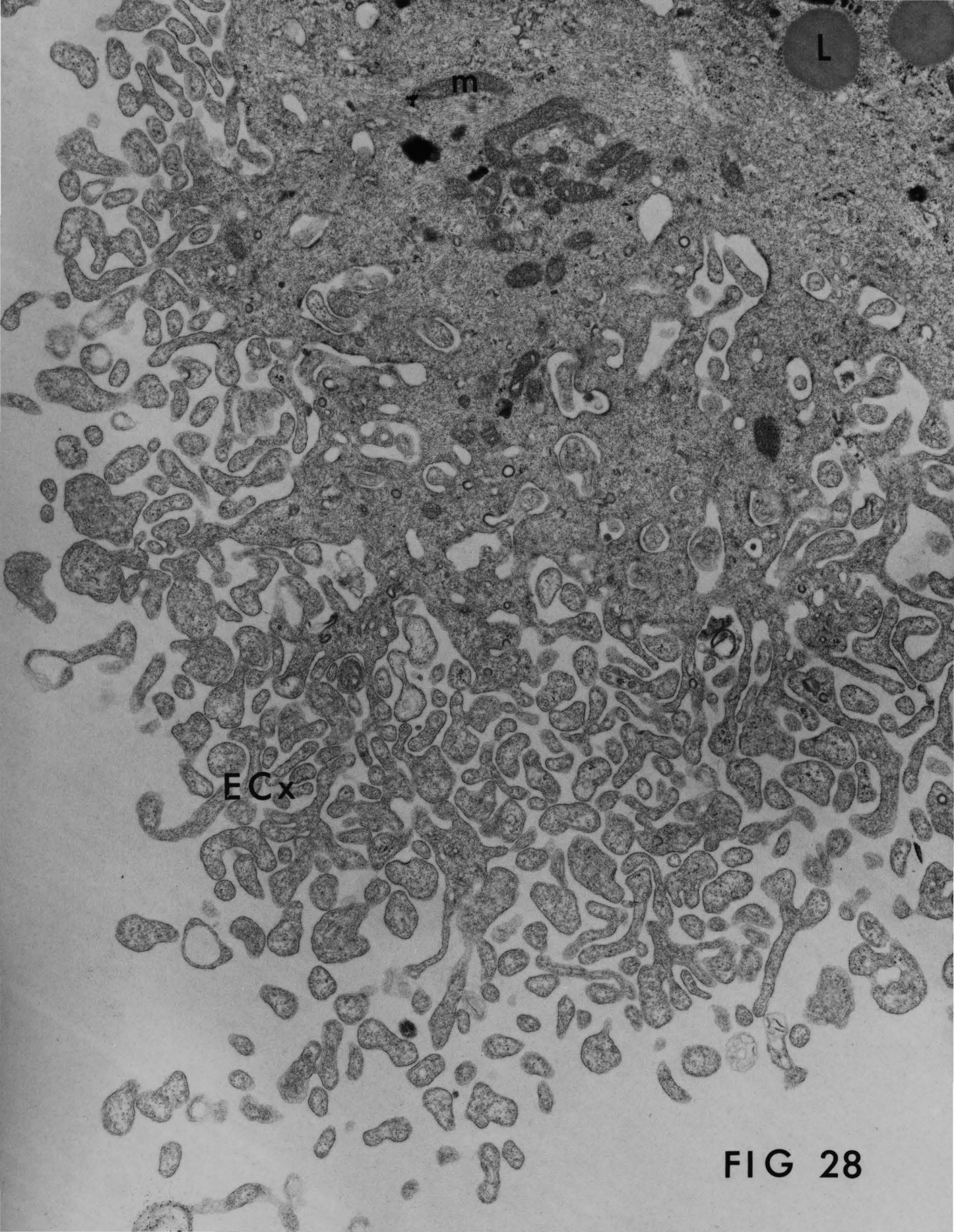


FIG 28

Figure 29

TEM of cultured aortic smooth muscle cells treated with 10% hyperlipemic human serum.

Age - 5 weeks.

Magnification 2160X

Nucleus - N

Lipid - L

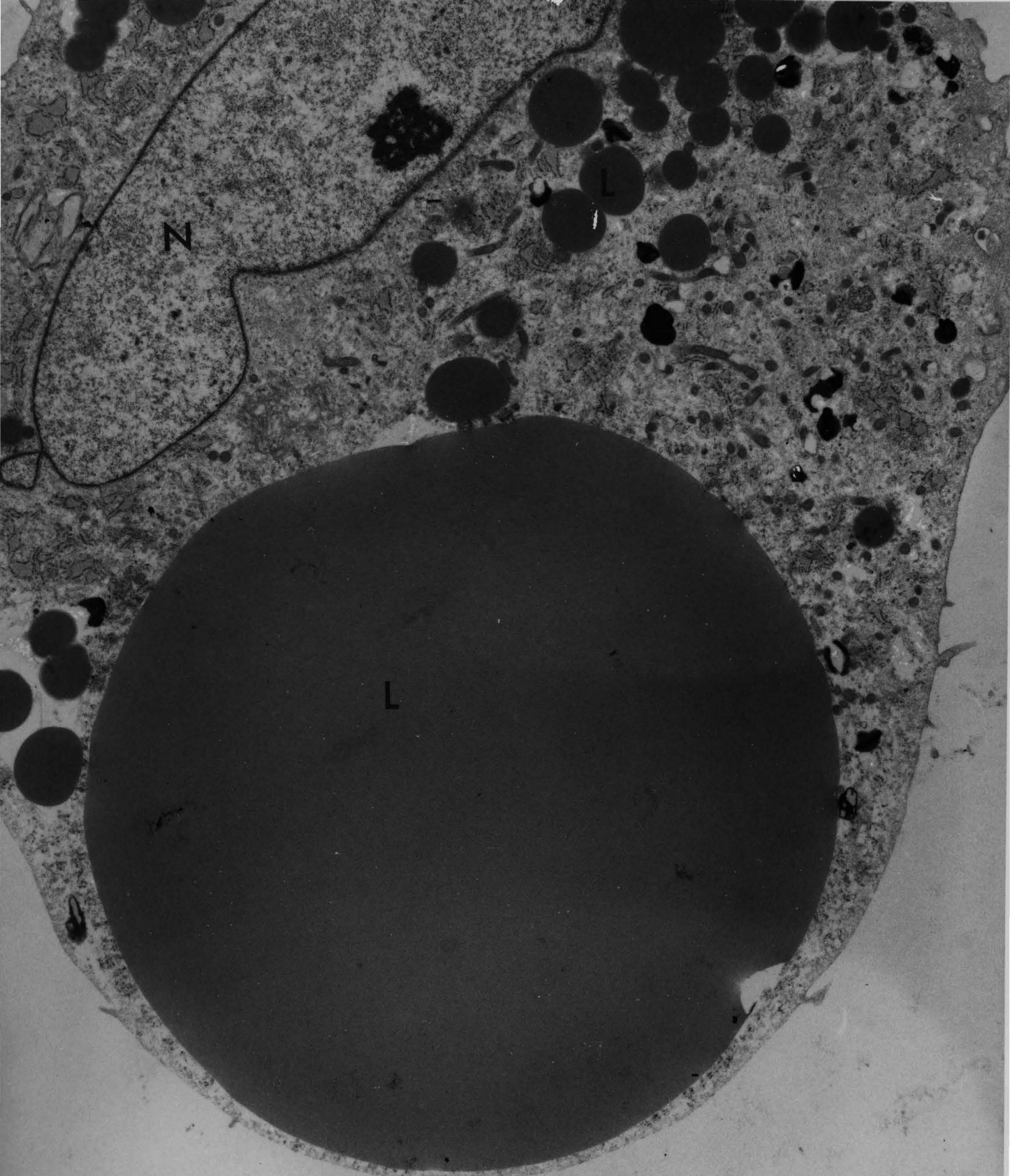


FIG 29

Discussion

(I) Use of Pig Aorta for Biochemical Studies

The domestic pig has been used as an experimental model for the study of atherosclerosis for several reasons.[60] i) Anatomically, the origin and distribution of the coronary arteries of man and pig correspond closely; ii) the atherosclerotic lesions in pigs, occurring either spontaneously or induced, have many characteristics of those seen in man; iii) also, much of the cholesterol is carried in LDL rather than HDL, as in other animals which are usually used; iv) the size of the artery is convenient for biochemical studies; v) the relative inexpense. For these reasons, the pig aorta was chosen as the source of tissue for smooth muscle cell cultures in the experiments. Studies in other laboratories have used a variety of animals as sources for aortic smooth muscle cell cultures, including the rat,[61] monkey,[62] and rabbit.[48] The choice of species for culture studies usually depends upon the experience and preference of each laboratory which will reflect consideration for such features as availability of tissue, costs and species variations in metabolism. However, with the pig as the source of tissue, many of the problems associated with the use of other animals are avoided; for example, large amounts of tissue for replicate studies and various treatments can be obtained from one pig, whereas many rats would have to be sacrificed to supply the same amount of tissue. For these reasons, the pig is considered an appropriate model for the

study of lipid metabolism and atherogenesis.

(2). Comparison of Lipoproteins from Human, Pig and Calf Sera

For satisfactory growth, tissue cultures must be grown in medium containing serum from some animal species. In many cases, the tissue culture is not maintained in its homologous serum, and serum from another species is used instead for a number of reasons including availability, cost, quality control and laboratory experience and preferences. In the experiments described here, the cells are grown continuously for the majority of the culture time in medium containing 10% calf serum. In the experiments designed to study the influence of different sera, the calf serum medium was replaced with medium containing 10% of the test serum for 48 hours prior to the addition of the radioactive lipid precursors.

The lipoproteins from different species are, not surprisingly, not all identical. There are four general classes of human lipoproteins. These are chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). LDL and HDL may also be fractionated into several subclasses. Pig serum contains three major classes of lipoproteins, resembling VLDL, LDL and HDL of human serum, but in smaller amounts; the total lipid present (approximately 450 mg%)[63] is half that found in human serum. Calf serum lipoproteins are also different from both human and pig serum lipoproteins. The composition of the major serum lipoproteins of these three species[64] is shown in Table 18.

TABLE 18

COMPOSITION OF SERUM LIPOPROTEINS

Lipoprotein	Species	Composition (% Total)				
		Protein	PL	TG	CE	C
VLDL	Man	7.7	18.6	49.9	14.9	6.7
LDL	Cow	29.8	16.3	12.8	33.4	7.7
	Man	20.9	22.1	11.2	38.0	9.0
	Pig	17.7	23.9	10.2	39.4	8.9
HDL	Cow	43.5	21.3	4.4	31.3	3.7
	Man	51.9	22.7	8.0	15.0	2.9
	Pig	45.8	22.4	2.0	26.0	3.9

(1971) Comp. Biochem. Physiol. 40B, 489-501
from: Mills, G.L.; Taylor, C.E. The Distribution and Composition of Serum Lipoproteins of 18 Animals.

(3) Effect of Serum on Lipid Biosynthesis

a) Homologous and Heterologous Sera

The data on the acetate incorporation into lipids following treatment with the various sera (Table 6), show that the treatment with pig serum results in the same relative changes in the lipid biosynthesis in the smooth muscle cell cultures as does the human test sera, although the changes produced by the human sera are more pronounced. The effect, namely the stimulation of synthesis of all lipid classes, excepting sterols, which shows an inhibition, is not due to the cholesterol concentration alone, since the concentration of cholesterol in the pig serum (108 mg %) is even lower than that of the calf serum (120 mg%).

There are several possible explanations for these apparent similarities and differences. Basic differences in the serum lipoproteins from the three species could result in different effects on lipid biosynthesis. Also the calf serum lipoproteins may be structurally damaged in the processing and shipment and refreezing of the serum since it is known that lipoproteins are not stable with repeated freezing and thawing. The pig and human sera were not obtained commercially, and were subjected to only one freezing-thawing cycle. The calf serum, on the other hand, was frozen and thawed twice. According to the theory of Goldstein[37] the cells contain specific receptors for LDL; therefore any structural alterations in LDL could alter the interaction of LDL molecules with the receptor, thus making the biologically available concentration of LDL lower than would appear merely from the LDL-cholesterol concentration of the serum.

From the results of this study it appears, however, that the pig serum resembles human serum more closely in its effect on the smooth muscle cells than does calf serum. Homologous serum would appear to be the ideal choice for use in tissue culture studies since it more closely approximates the *in vivo* situation. However, it is notable that these cultured cells behave in a similar way in both human and pig serum.

b) Effect of Human Sera

i) Normolipemic Human Serum

Treatment of cultured aortic smooth muscle cells with normolipemic human serum results in modifications of their lipid metabolism. In these studies, 10% calf serum was chosen as the control serum. Human serum stimulates acetate incorporation into total lipids (173% of control (calf) values). This increased acetate incorporation is distributed among several lipid classes; phospholipids (194% of control); free fatty acids (128% of control); triglycerides (208% of control); cholesterol esters (400% of control); (See Table 8). Cholesterol synthesis is inhibited (82% of control). The increase in phospholipid synthesis is evenly distributed among the phospholipid classes ranging from LPC (144% of control) to PE (246% of control); (See Table 9).

There are several possible reasons for the stimulation of lipid synthesis by human serum. Factor(s) may be present in the serum which could stimulate proliferation of the cells, and therefore stimulate the lipid synthesis necessary for cell division. Lipid synthesis under the control (calf) conditions may be slightly depressed; the addition of human serum, with a higher lipid content may be stimulating the lipid

synthesis of the cells to a more "normal" level. The calf serum, may in some fashion, be deficient in the substances required for optimum growth. Pig serum, which also had a low lipid concentration also stimulated lipid synthesis, which would tend to support this idea.

ii) Hyperlipemic Human Serum

Hyperlipemic human serum (10%) causes the same general changes in the lipid metabolism of cultured aortic smooth muscle cells as does 10% NLHS. However, with HLHS, the stimulation of lipid synthesis (PL, TG,CE) is enhanced to a greater extent than with NLHS, and the inhibition of cholesterol synthesis is more pronounced. Compared to the control values, acetate incorporation into the various lipid classes was as follows: Total lipids, 208%; PL, 230%; FFA, 156%; TG, 327%; CE, 587%; S + DG, 67%. (See Table 8).

Comparing the two human sera, significant differences were found for acetate incorporation; total lipids, phospholipids, cholesterol esters and triglycerides had significantly more acetate incorporated, whereas S + DG synthesis was significantly depressed. The increase in the phospholipid fraction was due to increased PC, PS + PI and PE synthesis.

Sphingomyelin was not affected by HLHS treatment. Sphingomyelin is of interest because it is known to accumulate to a great extent in atherosclerotic arteries, and can account for up to 70% of the phospholipid present in atherosclerotic plaques.[65] However, this study, and others,[57] show that this accumulation of Sph is not due to increased cellular synthesis, but to another mechanism, possibly uptake.

The effect of hyperlipemic serum on all cultures in general has been studied in a number of laboratories. Some of the reported findings are as follows: (1) Hyperlipemic serum causes a proliferation of cultured cells. At the same time, cell death is accelerated;[66] (2) cholesterol ester synthesis is stimulated in cultured human fibroblasts by oxygenated sterols (25-OH-cholesterol, 7-keto-cholesterol, 6-keto-cholesterol).[67] This stimulation is associated with an increase in the activity of the membrane-bound enzyme, fatty acyl CoA - cholesteryl acyltransferase;[68,41] (3) Cholesterol biosynthesis is suppressed by LDL, by means of a mechanism involving the inhibition of HMG CoA-reductase, the rate-limiting enzyme in cholesterol biosynthesis;[42,69] (4) Cells in a stationary growth phase can be stimulated to a second logarithmic growth phase by the addition of hyperlipemic serum. There is also an associated increase in total lipids.[49]

The observations and results reported here are in agreement with many previous studies carried out by other researchers. Data reported here are consistent with other studies on the incorporation of lipid precursors into lipids of cultured cells and various comparable tissues.

Some variation in reported data is to be expected due to the large variety of tissues and tissue cultures from different species, different culturing media and experimental techniques. Tables 19-23 show a sample of other reported data, and how they compare with that reported here.

Table 19 shows the precentage distribution of 1-^{14}C-acetate among

the lipid classes of various cultured cells and tissues. The relative distributions of acetate correspond closely for most of the lipid classes and the trends observed for HLS-treated cells are very similar.

Table 20 shows the percentage distribution of glucose among the lipid fractions. Here it is notable that the pig aorta and the pig aorta cultured smooth muscle cells have very similar ^{14}C-glucose distributions; this implies that the tissue and the cultured cells are metabolizing glucose in the same manner. The distribution of ^{14}C-glucose in the cultured rabbit smooth muscle cells also corresponds closely to that seen for the pig aortic smooth muscle cells.

In Table 21, the percentage distribution of ^{14}C-acetate among the phospholipids of three cultured cell lines is given. In each case, the greatest amount of acetate is incorporated into phosphatidylcholine; however, the data do not agree with respect to the influence of hyperlipemic serum. Percentage distribution of 1-^{14}C-acetate among the phospholipids does not appear to be significantly affected in the rabbit and pig smooth muscle cell cultures; MAF fibroblasts incorporate more acetate into PC and Sph when treated with hyperlipemic serum.

The percentage distribution of 1-^{14}C-oleate among the lipid classes of various tissues and cultured cells is shown in Table 22. The variation is great; however, the percentage distributions for cultured rabbit aortic medial cells and cultured pig aortic medial cells are similar.

Table 23 shows the percentage distribution of ^{32}P-phosphoric acid among the phospholipids of various tissues and cultured cell lines.

Again, there is significant variation among the results.

TABLE 19

PERCENTAGE DISTRIBUTION OF 1-^{14}C-ACETATE AMONG THE LIPID CLASSES OF VARIOUS TISSUES AND CULTURED CELL LINES

Lipid Class	Tissue or Cultured Cell Line						
	L-cells	Rabbit Fibroblasts	Rabbit SMC (Normal Serum)	Rabbit SMC [1] (HLS treated)	Pig Media	Pig SMC [1] (calf serum)	Pig SMC [1] (HLS treated)
PL	71.5	20.9	29.3	69.6	34.0	60.1	67.8
S + DG	6.9	23.0	54.7	9.6	9.3	22.2	6.8
FFA	8.9	17.4	5.1	3.1	9.8	2.2	1.7
TG	11.8	37.2	10.2	15.0	42.0	13.3	20.9
CE	1.0	1.5	0.7	2.7	4.9	1.2	3.4
Reference Number	48	48	48	48	53	this thesis	

[1] SMC - aortic smooth muscle cells (cultured)

TABLE 20

PERCENTAGE DISTRIBUTION OF ^{14}C-GLUCOSE AMONG THE LIPID CLASSES OF VARIOUS TISSUES AND CULTURED CELL LINES

Lipid Class	Tissue or Cultured Cell Line					
	Rabbit SMC [1]	Rabbit aorta	Human media	Human intima	Pig aorta	Pig SMC [1]
PL	52.0	64.0	52.7	34.1	~45	45.8
S + DG	6.1	13.6	5.3	33	~4	7.8
FFA	0.8	0.3	16.1	0.7	<1	1.2
TG	41.0	22.0	23.1	48.7	~49	44.1
CE	0.2	0	0.4	0.5	<1	0.9
Reference Number	48	54	55	55	53	this thesis

[1] SMC - aortic smooth muscle cells

TABLE 21

PERCENTAGE DISTRIBUTION OF 1-^{14}C-ACETATE AMONG THE PHOSPHOLIPIDS IN CULTURED CELLS

Phospholipid Class	Cultured Cell Line					
	Rabbit SMC [1] (normal serum)	Rabbit SMC (HL serum)	MAF fibroblasts (normal serum)	MAF fibroblasts (HL serum)	Pig SMC (normal serum)	Pig SMC (HL serum)
LPC	—	—	0.4	0.2	5.9	2.7
Sph	12.1	14.2	0.5	3.0	6.2	6.6
PC	64.4	63.0	31.0	57.0	48.9	52.9
PS + PI	23.5	23.1	25.4	11.0	17.4	15.9
PE			43.0	27.0	51.6	21.7
Reference Number	48	48	47	47	this thesis	

[1] SMC - cultured aortic smooth muscle cells

TABLE 22

PERCENTAGE DISTRIBUTION OF ^{32}P-PHOSPHORIC ACID AMONG THE PHOSPHOLIPIDS OF VARIOUS TISSUES AND CULTURED CELL LINES

Phospholipid Class	Tissue					
	Rabbit Foam Cells	Rabbit intima-media (normal)	Rabbit intima-media (atherosclerotic)	Rabbit Aortic media (cultured)	Rabbit intima (cultured)	Pig media (aortic) cultured
LPC	6.57	5.98	3.19	0.45	0.4	5.38
Sph	19.84	19.12	3.52	5.20	4.2	1.85
PC	45.33	48.53	59.56	63.8	66.5	65.1
PS + PI	10.38	19.52	27.99	13.7	17.1	23.94
PE	17.88	6.85	5.74	16.9	12.0	5.08
Reference Number	56	57		58		this thesis

TABLE 23

PERCENTAGE DISTRIBUTION OF 1-^{14}C-OLEATE AMONG THE LIPID CLASSES OF VARIOUS TISSUES AND CULTURED CELL LINES

Lipid Class	Tissue Or Cultured Cell Line				
	Rabbit intima (atherosclerotic)	Rabbit media (atherosclerotic)	Rabbit aortic intima (cultured)	Rabbit aortic media (cultured)	Pig aortic media (cultured)
Phospholipid	29.1	39.4	80.2	70.3	75.5
Sterol + Diglyceride	12.6	4.4	2.0	1.9	1.6
Triglyceride	53.1	48.4	24.3	24.1	23.4
Cholesterol Ester	5.1	7.8	3.7	3.7	0.5
Reference Number	59	59	58	58	this thesis

Conclusions

The lipid metabolism of cultured aortic smooth muscle cells can be significantly modified by the serum present in the culturing medium. In these studies it was found that normal human serum at a level of 10 % was generally stimulatory to the synthesis of all lipid classes, with the exception of the sterols. In the case of sterol synthesis, the addition of 10 % human serum was inhibitory. Hyperlipemic human serum was even more effective than normolipemic human serum in stimulating lipid synthesis by cultured smooth muscle cells and at the same time was more potent as an inhibitor of sterol biosynthesis. A further distinction could be made between the 2 types of sera in that in the presence of HLHS, cholesterol ester synthesis increased significantly.

Hyperlipemic serum also causes morphological changes in cultured smooth muscle cells, as observed by electronmicroscopy. Many of the morphological changes are considered to be degenerate in nature.

It would be appropriate at this time to comment briefly on the further utilization of the smooth muscle cell culture model to understanding the responses of cells to many stimuli, including those considered to be atherogenic. The experimental conditions can be carefully monitored to eliminate as many variables as possible.

Smooth muscle cell cultures are of great interest in the study of atherosclerosis because of the role of the smooth muscle cell in atherogenesis *in vivo*. Cultures of smooth muscle cells provide a very simple

model for the study of atherogenic agents on the biochemistry and morphology of arterial cells.

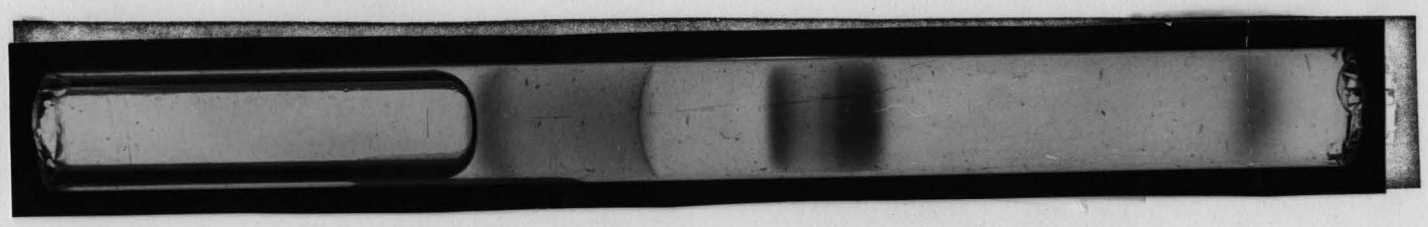
In this study, and other related studies carried out in different laboratories, changes in the biochemistry and morphology of smooth muscle cells in response to hyperlipemic serum have been similar to the changes observed in arterial tissue under conditions of hyperlipemia in vivo. There is always caution to be exercised in interpreting results of metabolic studies performed on cultured cells because it is difficult to evaluate the extent to which an in vitro system parallels conditions in vivo. Of course, there is also the additional problem of possible cell de-differentiation in vitro. Therefore, care must be exercised when one tries to extrapolate in vitro results to the in vivo situation. In view of these difficulties, smooth muscle cell cultures are, nevertheless, very useful in the study of atherogenesis.

The basic biochemical and morphological studies on smooth muscle cell cultures, such as this study, are of utmost importance in the understanding of the nature of these cells. In addition, the usefulness of the smooth muscle cell culture could be dramatically extended, even to the benefit of the developmental scientist. For example, smooth muscle cell cultures could be used as tools for testing anti-atherogenic drugs in order to evaluate the effects of long term drug therapy on smooth muscle cells. The search for agents capable of reversing atherogenesis could be aided by the use of tissue cultures. For example, drugs capable of facilitating the release or degradation of lipids by cells could be examined in smooth muscle cell cultures. The list of uses for

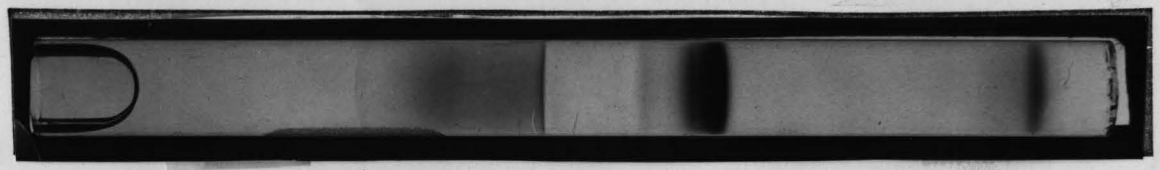
smooth muscle cell cultures could become very long; the possibilities are only limited by the availability of interested researchers, time and money. Atherosclerosis is a very serious medical problem for a large percentage of the population; smooth muscle cell cultures could be used to find part of the solution to this problem.

APPENDIX A POLYACRYLAMIDE GEL LIPOPROTEIN ELECTROPHORESIS OF NORMAL AND HYPERLIPEMIC HUMAN SERA

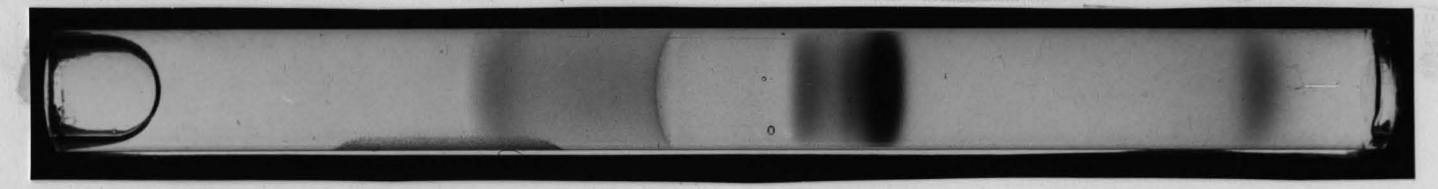
a) Normal Human Serum - 1 cholesterol 210 mg %
triglycerides 198 mg %



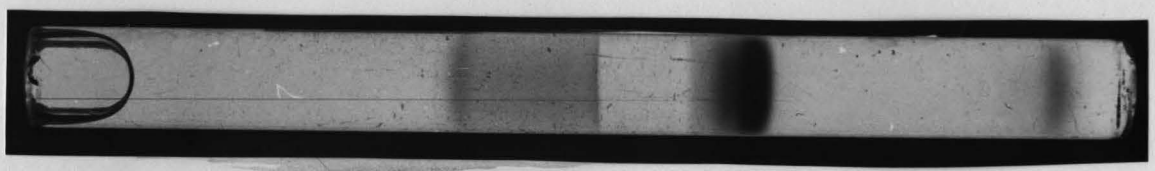
b) Normolipemic Human Serum - 2 cholesterol 180 mg %
triglycerides 90 mg %



c) Hyperlipemic Human Serum - 1 cholesterol 280 mg %
triglycerides 244 mg %



d) Hyperlipemic Human Serum - 2 cholesterol 240 mg %
triglycerides 128 mg %



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