

THE UNIVERSITY OF MANITOBA

INVESTIGATION INTO THE UTILIZATION OF ELECTRON MICROSCOPY IN THE
PRENATAL DIAGNOSIS OF GENETIC DISEASE

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANATOMY

WINNIPEG, MANITOBA

JUNE, 1976

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ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to all who have given assistance in the preparation and completion of this work.

The author wishes to express special appreciation to Dr. D. M. Cox for his invaluable assistance, advice, criticism, and particularly his friendship. All of these were crucial in the completion of this thesis.

The author would also wish to express his appreciation to Dr. K. L. Moore, Chairman of the Department of Anatomy, for the privilege of studying in this department and for his accessibility when advice was needed.

The author would like to thank Dr. C. R. Braekevelt for his advice and criticisms which aided in the completion of this work.

The author also wishes to thank Dr. J. Hoogstraten for providing access to the Zeiss 9AS2 electron microscope, and also for his advice.

The author wishes to thank the Children's Hospital Research Foundation of Winnipeg, Inc. for financial assistance available to me through Dr. D. M. Cox and Dr. J. Hoogstraten.

TABLE OF CONTENTS

CHAPTER		PAGE
I	INTRODUCTION.....	1
II	REVIEW OF THE LITERATURE.....	7
	Cell Culture.....	7
	Methods in the Preparation of Cultured Cells for Electron Microscopy.....	14
	Ultrastructure of Cultured Normal Human Fibroblasts.....	14
	Ultrastructure of Cultured Diseased Human Fibroblasts.....	17
	Summary.....	28
III	MATERIALS AND METHODS.....	30
	Cell Lines.....	30
	Culture Medium and Physical Facilities for Culturing.....	35
	Cell Culture Schedule.....	38
	Fixation and Embedding for Electron Microscopy.....	39
	Sectioning, Staining and Electron Microscopy.....	40
IV	OBSERVATIONS.....	41
	Normal Cell Lines.....	41
	Diseased Cell Lines.....	82
V	DISCUSSION.....	169
VI	SUMMARY.....	183
VII	BIBLIOGRAPHY.....	184

ABSTRACT

The work present in this thesis was concerned with the investigation into the possible use of electron microscopy in the detection of genetic diseases in cultured amniotic fluid fibroblasts. It was demonstrated that cultured skin fibroblasts and cultured amniotic fluid fibroblasts, derived from individuals affected with Tay-Sachs' disease, Fabry's disease and metachromatic leukodystrophy, all have distinctive abnormalities which distinguished the cell lines from each other and from normal control lines. Distinct ultrastructural abnormalities were also observed in cultured skin fibroblasts derived from patients affected with Duchenne's muscular dystrophy, I-cell disease and Pompe's disease. No cultured amniotic fluid fibroblasts were available for examination for these three disease states. Examination of cell lines derived from individuals affected with Lesch-Nyhan disease, infantile cystinosis and maple syrup urine disease were ultrastructurally indistinguishable from normal cultured skin fibroblast lines. Cell lines derived from patients affected with cystic fibrosis produced conflicting results.

The ability to distinguish certain disease cell lines from normal controls indicated that electron microscopy may have potential use in the detection of disease, particularly in the case of prenatal diagnosis.

CHAPTER ONE

INTRODUCTION

The management of families at high risk for a genetic disease has been greatly facilitated by the introduction of techniques for the prenatal detection of disease in the fetus. The establishment of prenatal diagnosis arose from two major developments: the first being the general acceptance that amniocentesis is a relatively safe procedure for obtaining amniotic fluid specimens for diagnostic study; the second being the realization that a small proportion of the cells suspended in the amniotic fluid have the potential to proliferate in vitro and that these cultured amniotic fluid cells could be studied by sophisticated cytogenetic and biochemical methods (Fuchs and Cederqvist, 1973; Mellman, 1971; Mellman and Cristofalo, 1972; Nadler and Gerbie, 1970; Priest, 1972).

Amniocentesis was first documented as a technique for the removal of amniotic fluid by Lamb1 (1881). This first amniotic tap resulted in the loss of the twin fetuses by spontaneous abortion, thus indicating that great caution must be executed before amniotic fluid can be safely removed from the pregnant female. Menees et al (1930) used transabdominal amniocentesis in the radiographic analysis of fetuses, but the technique fell into disuse until the early 1950's. Thereupon, amniocentesis was again employed successfully in over 10,000 cases in the management of erythroblastosis fetalis (Liley, 1965). However, it has only been in the past decade that amniocentesis has been widely used to obtain samples of amniotic fluid for the detection of a large number of different genetic disorders (Becker and Seegmiller, 1975). During this period of time, it

has become generally accepted that transvaginal amniocentesis carries appreciable risk to both the mother and fetus, and that the transabdominal approach is to be preferred (Emery, 1974). Recent large surveys of the effects of transabdominal amniocentesis on morbidity and mortality have confirmed the technique can be carried out with minimal risk to the mother and fetus (Culliton, 1975).

The cells present in the amniotic fluid were first used in the prenatal diagnosis of genetic disease by Fuchs and Riis (1956), who demonstrated that it was possible to detect the sex chromatin and thus infer the sex of the fetus. Four years following this demonstration, Riis and Fuchs (1960) used the determination of the fetal sex as a means of preventative management of the X-linked genetic disorders, Duchenne's muscular dystrophy and hemophilia. Significant advances in the field of prenatal diagnosis were made when Steele and Breg (1966) demonstrated that amniotic fluid cells could be grown in vitro. This provided access to abundance of cellular material which had direct diagnostic relevance to the fetus. It allowed cultivation of sufficient numbers of amniotic fluid cells so that complete analysis of the karyotype of the fetus could be made (Steele and Breg, 1966; Thiede et al, 1966) and, in the following year, Jacobson and Barter (1967) diagnosed the first chromosomal abnormality using cultivated amniotic fluid cells. Shortly after, Nadler (1968) demonstrated that biochemical methods could be utilized in the analysis of cultured amniotic fluid cells. Utilizing these biochemical techniques, it is now possible to detect several score of biochemical genetic disorders in utero (Becker and Seegmiller, 1975).

Prenatal diagnosis involves the investigation of disease in the yet unborn fetus. In addition to the examination of amniotic fluid, methods

such as fetoscopy, amniography and ultrasound have also been used for this purpose. To date, the majority of investigations have utilized the amniotic fluid. The amniotic fluid obtained by amniocentesis has two major components both of which have been used in the prenatal diagnosis of genetic disease: the fluid constituents and the amniotic fluid cells (Huisjes, 1973; Uhlendorf, 1970).

Since the precise origin, composition and distribution of the amniotic fluid is not established, and the biochemical analysis of the fluid to date has shown large variations in the results (Potter and Craig, 1975; Becker and Seegmiller, 1975), great reservations concerning the accuracy of prenatal diagnosis utilizing the fluid components exist (Fuchs and Cederqvist, 1973; Nadler and Burton, 1973). The specific source of the amniotic fluid cells is not conclusively understood. It is rationalized that the amniotic fluid cells are derived from the epithelium of the fetus, from the surrounding amniotic membranes, and also from sources such as the fetal respiratory system, the fetal gastrointestinal tract, and the fetal genitourinary system. Some reports have substantiated these as sources of amniotic fluid cells (Van Leeuwen et al, 1965; Steele and Breg, 1966; Votta et al, 1968) and it has been demonstrated that the majority of these cells are of fetal origin (Kaback and Howell, 1973). The use of uncultured amniotic fluid cells has proved successful for the identification of the sex chromosome status of the fetus by the examination of X and Y chromatin (Rook et al, 1971), but is generally unreliable for the diagnosis of biochemical defects since a large proportion of the cells are non-viable and give rise to unstable and inadequate enzyme levels (Fuchs and Cederqvist, 1973; Whalstrom, 1974). It has been the relatively recent ability to culture amniotic fluid cells, with cytogenetic

and biochemical analysis of the resulting cell population, that has made it possible to provide a highly reliable diagnosis for a number of genetic diseases early in pregnancy.

Contemporary diagnostic procedures are restricted to the identification of aberrations in the chromosomal complement of the cells, and to biochemical measurement of changes in the enzymatic functioning in the cells (Epstein, 1975). Cytogenetic diagnosis using cultured amniotic fluid cells is virtually limited to the detection of Down's syndrome in fetuses of pregnant women over the age of 35, women who have previously borne children with a chromosome aberration, or women whose families have a history of chromosomal aberration. Of the potential number of diseases which have a genetic component, chromosomal alterations represent a distinct minority. Similarly, of the over 3,000 genetic diseases now recognized (McKusick, 1975), only several score can be prenatally detected by biochemical analysis of the cultured amniotic fluid cells. Thus, currently, relatively few genetic diseases can be prenatally detected using cytogenetic and biochemical methods.

One disease state has been documented in which morphological studies by electron microscopy have been used in the diagnosis in utero. Hug et al, originally in 1970 and subsequently on several more occasions, have consistently detected abnormalities in the ultrastructural appearance of amniotic fluid cells, later confirmed to be affected with Pompe's disease. The consistent appearance of abnormal cytoplasmic bodies in the cells obtained from the amniotic fluid enabled the successful diagnosis of this disease (Hug et al, 1970; Hug et al, 1972; Hug et al, 1973).

The potential of electron microscopy in prenatal diagnosis as demonstrated by Hug et al (1970) remains unexploited. Thus a further possi-

bility exists for prenatal diagnosis - the investigation of the ultrastructure of cultured amniotic fluid cells. This lack of information concerning the use of electron microscopy in prenatal diagnosis has led to the development of the work presented in this thesis.

In order to determine if ultrastructural examination of amniotic fluid fibroblast cells could be used successfully in a large number of genetic diseases, it would be necessary to show that diseased amniotic fluid cells can be consistently distinguished from normal amniotic fluid cells. Owing to both the physical impossibility of obtaining sufficient samples of diseased amniotic fluid fibroblasts due to the relative infrequency of appropriate genetic disease states, and, more importantly, the ethical restriction of obtaining amniotic fluids for diseases for which no methods for prenatal diagnosis yet exist, this study could not directly study sufficient numbers of amniotic fluid fibroblast specimens to conclusively demonstrate this point. Thus this work was designed to assess the potential for utilizing electron microscopy in prenatal diagnosis in a less direct fashion: use being made of human fibroblasts derived from postnatal skin biopsies as the major source of information, and supplementation of this information with results obtainable from the few amniotic fluid fibroblast samples available.

Three specific questions were addressed. Firstly, is it possible to distinguish normal postnatal cultured skin fibroblasts from diseased postnatal cultured skin fibroblasts? Secondly, is it possible to distinguish normal cultured amniotic fluid fibroblasts from diseased cultured amniotic fluid fibroblasts? Thirdly, if abnormalities are evident in diseased postnatal cultured skin fibroblasts, are these similar to those observed in cultured amniotic fluid fibroblasts for the same disease?

The resolution of these questions should provide sufficient information to evaluate the potential for the use of electron microscopy in the prenatal diagnosis of genetic disease.

CHAPTER TWO

REVIEW OF THE LITERATURE

CELL CULTURES

The primary purpose of human cell culture is to provide environmental conditions under which cells derived from an individual can be maintained outside of the donor (Parker, 1961). To allow the particular cell or cell population to grow and proliferate, the specific metabolic requirements of the cells must be satisfied by the constituents of the culture medium. Theoretically, this extracellular environment should closely match the physiologic conditions found in the living host. It should provide a balanced salt solution, abundance of nutrients, proper temperature, and control of pH (Rowan, 1973).

Three culturing methods exist which have been developed to allow the maintenance of living mammalian cells in vitro. Organ culture is concerned with maintenance of the structural architecture of the tissue when it is placed in vitro; tissue culture permits the migration of cells from a primary fragment of tissue onto the surface of the culture vessel and is not directed towards maintenance of the structural integrity of the tissue fragment; cell culture is concerned with the disruption of the primary tissue fragment into single cells or clumps of cells which are then cultured (Paul, 1970; Priest, 1972). With both tissue and cell culture, the cells derived from the specimen are capable of long term survival in vitro. Extensive reviews of the establishment of these methods and techniques of human tissue and cell culture (Paul, 1970; Pollack, 1973; Parker, 1961; Priest, 1972), as well as complete compila-

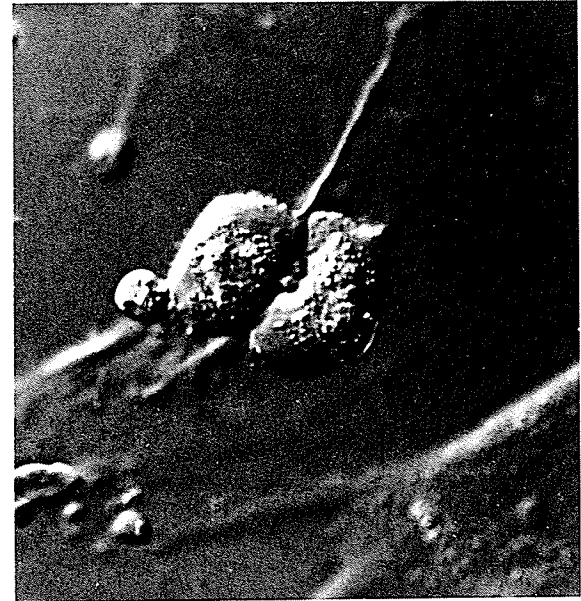
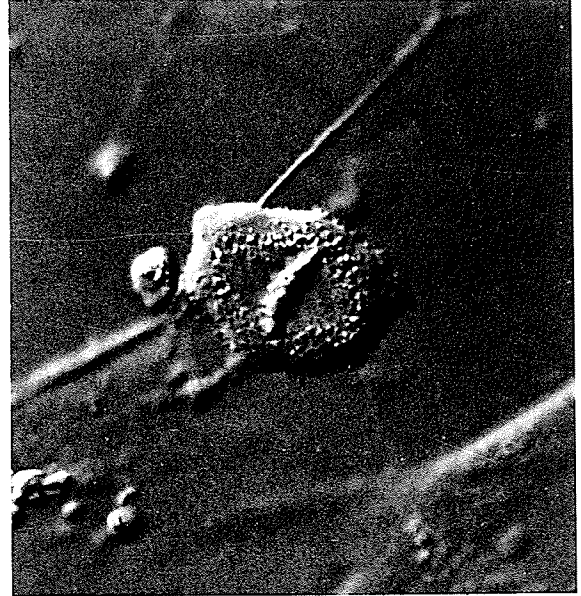
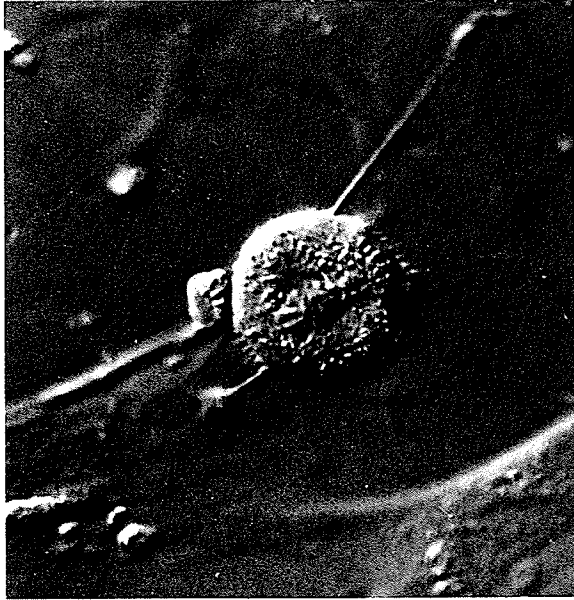
tions of all works relevant to the development of cell and tissue culture and their use (Murray, 1953; Murray 1965a; Murray, 1965b; Cesvet, 1970; Cesvet, 1971; Cesvet, 1973a; Cesvet, 1973b) have been published. Therefore, no detailed review of the history and theory of culturing techniques will be presented in this thesis.

Human cell cultures are started either by disruption of the tissue specimen into single cells, or by the migration of cells from the small pieces of tissue after placing them in a culture vessel with culture medium (Paul, 1970). A tissue specimen, also termed a primary explant, or an amniotic fluid sample when cultured, is termed a primary cell culture (Paul, 1970). Several cell types are capable of growing from this primary cell culture but the cell type which rapidly predominates and extends to fill the culture vessel is morphologically similar to the classic fibroblast (Priest, 1972). These fibroblasts adhere to the bottom of the culture vessel where they undergo growth and proliferate by mitotic division. A single cultured fibroblast cell in mitosis is shown in Figure 1. The rate of division is affected by the accumulation of toxins in the media, medium depletion, and limitations in the size of the culture vessel (Paul, 1970). Proliferation of these fibroblasts can be reinitiated by the transfer, termed a passage, of a sample of the cell line to a new culture dish.

The cells are removed from the bottom of the dish, either by mechanical scraping or by the application of digestive enzymes, usually trypsin (Paul, 1970). The resulting cell suspension can then be distributed to new culture vessels. The cell population can be maintained in this manner for approximately fifty generations before senescence (Hayflick, 1974).

Figure 1. Series of light micrographs taken at different stages of mitosis of cultured human fibroblasts. Nomarski interference microscopy. From Gray's Anatomy, 35th edition. Edited by R. Warwick and P. L. Williams. Longman, Edinburgh, 1973.

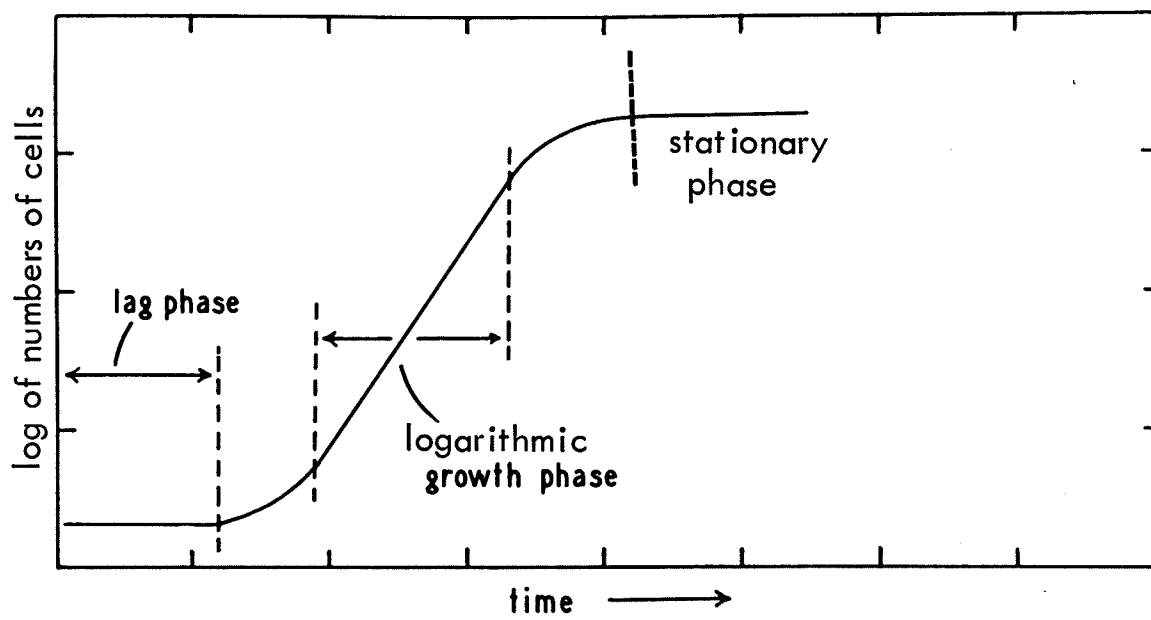
X 1,500



Once passaged from the primary culture, a cell population is called a cell line. Cell lines can be frozen at any point in time and stored at -70°C . until they are needed.

The growth dynamics which can be observed in a cell population are illustrated in Figure 2. Three phases are evident: the lag phase, the logarithmic phase, and the stationary phase (Paul, 1970). Lag phase immediately follows transference of the cell population to a fresh culture dish. It is a period during which there is no increase in the number of cells in the cell population. Lag phase results from a number of factors, among which one is that it may represent a period of readaptation to the new culture surroundings. The cell population then proceeds to increase in number in a logarithmic fashion. Following this logarithmic phase, the cell population enters the stationary phase, with cell multiplication virtually ceased. The cessation of proliferation may be related to contact between cells in the culture vessel (contact inhibition), or may result from medium depletion, or the accumulation of toxins in the cells (Paul, 1970). Many types of media are used in the culture of human fibroblast lines but the effects of the various media on the behaviour of the cells in culture are not well understood. However, it is known that alterations of single components of a medium can dramatically change the metabolic activity of cells in culture (Ryan et al, 1972), be it a change in the serum concentration (Treadwell et al, 1963; Douglas et al, 1976), a change in pH (Wood, 1975), alterations in the concentration of free sugars (Johnson and Schwartz, 1976), or alteration in the concentration of amino acids (Fischer, 1948).

Figure 2. Diagrammatic representation of cell growth pattern. Logarithms of cell numbers plotted against time in culture. Adapted from W. Burrows, Textbook of Microbiology, 12th Edition. W. R. Saunders, Philadelphia, 1973.



METHODS IN THE PREPARATION OF CULTURED CELLS FOR ELECTRON MICROSCOPY

Two major directions have been taken in the processing of cultured cells for electron microscopy. One has been the removal of the cultured cells from the culture vessel, with the processing of the resulting cell numbers - usually in the form of a pellet (Robbins and Jentzsch, 1966; Lucky et al, 1975). The second method which has been utilized has been the fixation of the cultured cells in situ in the culture vessel (Eppig et al, 1976; Chang, 1971; Brinkley et al, 1967).

ULTRASTRUCTURE OF CULTURED NORMAL HUMAN FIBROBLASTS

The characteristics and behaviour of the cultured human fibroblast as seen from the viewpoint of structural morphology is not well understood. This is due to the fact that few studies have been published on the electron microscopic appearance of cultured human fibroblasts - either those derived from skin biopsies or those derived from amniotic fluids.

Some studies have attempted to document the appearance of normal cultured fibroblasts and their response to changes in the culture milieu. Robbins et al (1971) studied seven normal human fibroblast lines derived from skin biopsies at passage numbers from 1 to 22, and reported the consistent accumulation of lysosomes as the passage number was increased. They also noted that, at later passages, the lysosomes started to undergo degenerative changes.

Maciera-Coelho et al (1971) also reported on the ultrastructure of normal cultured skin fibroblasts of unknown passage number in the stationary phase. They reported an increase in the number of autophagocytic

vacuoles as the cells remained in the stationary phase. They also performed acid phosphatase activity levels of the cells which indicate lysosomal function and concluded that the vacuoles were lysosomes. It was also noted that medium changes would produce a sharp decrease in the number of autophagocytic vacuoles. Comings and Okada (1970) studied the difference, in a single cell line, between the logarithmic and stationary phases of the cell population. The fibroblasts were derived from a normal male child and examined at unknown passage numbers. The cells were removed from the culture vessel with trypsin and spun into a pellet which was then fixed for electron microscopy. Comparison between the cells in logarithmic growth and at the stationary phase showed that the endoplasmic reticulum was dilated as the cells passed to the stationary phase, polyribosomal configurations disappeared, and an increase in the amount of microfilaments was seen. They also observed that there was an increase in the number of autophagocytic bodies as the cell lines progressed into confluency.

Lipetz and Cristofalo (1972) studied human fibroblasts ranging from the 15th passage to the 56th passage. They found an increase in the number of autophagocytic bodies, changes in the mitochondrial structure, and an increase in the invagination of the nucleus as the passage number increased. They suggested that the congestive engorgement of lysosomes plays an important role in the cell populations' decrease in activity at later passage number. It was also reported that, at later cell passages, there was an increase in the presence of microfilaments, and an increase in the prominence of the cisternal system of the cell.

Conrad et al (1972), as part of the investigation of diseased fibroblasts, studied the appearance of normal cultured fibroblasts at 7, 21

and 35 days in culture passages from the second to the eighth. It was found that cells in the logarithmic phase were indistinguishable from cultured cells in the stationary phase.

Lie et al (1973) reported on the electron microscopy of five skin fibroblast lines. Some were studied at the 7th passage but others were studied at unspecified passages. They were concerned with the effects of pH on the ultrastructure of human fibroblasts. They were able to demonstrate that an increase in pH could increase the number of lysosomes in the cells. They suggested that this increase in cytoplasmic bodies may result from an inhibition of lysosomal function as the pH was increased.

Lucky et al (1975) studied fibroblast lines at the eighth or ninth passage at periods from one hour to eight days after culture. They demonstrated that pelleting of cells for electron microscopy following removal of the fibroblasts from the culture vessel by scraping or trypsinization prior to fixation caused marked alterations in the cellular structure. They also reported an increase in the number of microfilaments, lysosomes, and increase in the dilation of the cisternal system as the cells passed from the logarithmic phase to the stationary phase.

Douglas et al (1976) reported on the fine structure of normal human fibroblasts. They reported the presence of well-developed Golgi complexes, extensive areas of rough endoplasmic reticulum, and many free ribosomes in the cytoplasm. By changing the concentration of specific components of the medium, they were able to dramatically alter the ultrastructure of the fibroblasts, such that Golgi complexes were rarely observed, cisternae in the rough endoplasmic reticulum were reduced in number, and the number of free ribosomes in the cytoplasm was reduced.

These eight reports represent the total number of published studies

that have been done specifically on the normal morphology of fibroblasts in culture. Despite considerable differences in the design and execution of the studies, certain important conclusions can be drawn:

i) there is apparently an increase in the number of cytoplasmic bodies as the cells pass into confluency from the logarithmic phase to the stationary phase; ii) at higher passages, above 25, there is also an increase in the number of cytoplasmic bodies; iii) cells maintained at the stationary phase show an increase in microfilaments and an increase in the lobulation of the nucleus as the cells remain in culture (Comings et al, 1970; Lipetz and Cristofalo, 1972; Maciera-Coelho et al, 1971; Lucky et al, 1975; Conrad et al, 1972; Douglas et al, 1976; Robbins et al, 1971; Lie et al, 1973).

Only one study has been reported on the ultrastructure of cells from cultured amniotic fluids. It reported the presence of "actively growing cells displaying fibroblast-like characteristics" (Bartman, 1971).

ULTRASTRUCTURAL EXAMINATION OF DISEASED CULTURED FIBROBLASTS

Electron microscopy of cultured human fibroblasts has been undertaken for a number of genetic diseases. In several of these diseases, an abnormal ultrastructure has been described and, in a few instances, the ultrastructural examination has proved to be diagnostically significant (Hug et al, 1973). An alphabetized summary of the published observations for each of these diseases is presented hereafter.

Austin's Disease

A single cell line, established from a patient with Austin's disease, could not ultrastructurally be distinguished from normal cell lines (Lyon et al, 1973).

Batten's Disease

The enzyme defect which causes this disease is unknown. Lyon et al (1973) were not able to detect any ultrastructural abnormality in a cell line derived from a patient with Batten's disease.

Kamensky et al (1973) studied cultured fibroblasts derived from two individuals affected with Batten's disease. They found these two cell lines indistinguishable from the normal lines studied by electron microscopy at the same time.

β -methylcrotonylglycinuria

The enzyme deficiency in this disease is apparently β -methylcrotonyl CoA carboxylase (McKusick, 1975). A single cell line from a patient affected with β -methylcrotonyluria was ultrastructurally studied by McLean and Stewart (1974). Small mitochondrial inclusions were observed in this cell line which were considered to be characteristic for the disease.

Two heterozygotic cell lines for the disease were found to be indistinguishable from the normal cell lines.

Chondroitin-4-sulfate mucopolysaccharidosis

The cell line derived from a patient affected with chondroitin-4-sulfate mucopolysaccharidosis when studied by Kamensky et al (1973) was seen to contain large lysosomes filled with lamellated membranes. The

line was easily distinguished from normals but resembled the Sanfilippo's disease - Type B cell line also studied by them.

Cystic fibrosis

The enzyme defect which produces cystic fibrosis is unknown (McKusick, 1975). Tenconi et al (1972) examined two fibroblast lines by electron microscopy which were obtained from individuals known to be affected with cystic fibrosis. These cell lines were found to be similar to the normal cell lines studied.

Four cell lines derived from patients affected with cystic fibrosis studied by Bartman et al (1970) were found to differ from four normal control cell lines only in the number of cytoplasmic bodies observed. The cells from patients contained larger and more numerous lysosomes than control cells.

Fabry's Disease

McLean and Stewart (1974) reported on the ultrastructure of fibroblasts obtained from two patients affected with Fabry's disease. The disease is due to a deficiency of ceramide trihexosidase (McKusick, 1975). The cells in both cell lines were observed to contain inclusions surrounded by a single membrane and were presumed to be lysosomal in origin.

Three Fabry's disease cell lines ultrastructurally studied by Kamensky et al (1973) known to be derived from individuals affected with Fabry's disease were each found to contain typical cytoplasmic inclusion bodies. These bodies were limited by a single membrane which surrounded stacks of circularly disposed lamellae with alternating dark and light bands with a regular period between 40 and 50 Å. A single heterozygote

cell line ultrastructurally examined was found to have similar bodies at a much reduced frequency.

Galactosemia

Galactosemia is due to a deficiency in the enzyme galactose 1-phosphate uridyl transferase. Miller et al (1968) examined a single fibroblast cells derived from an individual affected with galactosemia and were unable to determine any ultrastructural differences between that cell line and the single normal cell line grown under normal condition.

Gaucher's Disease

This condition is due to a deficiency in the enzyme β -glucosidase (McKusick, 1975). Only one report on a single cell line derived from a patient affected with Gaucher's disease has been published (Kamensky et al, 1973). They reported that no ultrastructural difference from normal cells was apparent.

Hunter's Disease

This disease is due to a deficiency in the enzyme iduronic acid sulfatase (Becker and Seegmiller, 1975). Lyon et al (1973) studied three cell lines derived from patients affected with Hunter's disease and found each to be ultrastructurally indistinguishable from the normal control cell lines.

In direct contrast to the results presented by Lyon et al (1973), fibroblast cell lines obtained from two patients with Hunter's disease were found to be different from normal by Bartman et al (1970) but had a similar morphology to fibroblast lines from Hurler patients. Both

diseased states showed the presence of large numbers of membrane bound vacuolated, cytoplasmic bodies.

Duckett et al (1969) reported the appearance of abnormal cytoplasmic bodies bound by single or double membranes in cultured fibroblasts from both individuals affected with Hunter's syndrome and individuals heterozygotic for the disease. In addition, they reported abnormalities in the mitochondria present in these cultured fibroblasts.

Hurler's Syndrome

This disease is due to a deficiency in the enzyme α -L-iduronidase (Becker and Seegmiller, 1975). Several reports have been published by Kamensky et al (1973) on the ultrastructure of human fibroblast cell lines from patients affected with Hurler's disease. A cell line studied by Bartman et al (1970) showed that the cytoplasm was filled with bodies which contained a variable amount of slightly opaque flocculent material. It was possible to distinguish normal cells from Hurler cells by the presence of these bodies.

Conrad et al (1972), in an extensive survey of three fibroblastic cell lines derived from patients affected with Hurler's disease, reported the presence of large numbers of cytoplasmic vacuoles. By incubating the Hurler's cells with colloidal gold they were able to demonstrate that the gold would localize in the bodies and thus speculation was made that these cytoplasmic bodies were secondary lysosomes.

Hug et al (1973) reported that a single cell line from a patient affected with Hurler's disease contained electron lucent vacuoles enclosing some feathery or granular material. This fibroblast line was distinguishable in its ultrastructural appearance from normal cell lines.

In contrast to these observations, Lyon et al (1972) were unable to detect any ultrastructural difference between two Hurler's cell lines and the normal control cell lines they studied.

I-Cell Disease

I-cell disease, also termed mucopolipidosis II, can be determined prenatally by the decreased activity of a large number of lysosomal enzymes (Becker and Seegmiller, 1975).

Aula et al (1975) cultured fibroblasts derived from a fetus affected with I-cell disease and found abnormal inclusions in all the cells examined. No normal lines were studied.

Terashima et al (1975) studied a single line derived from a patient affected with I-cell disease and found numerous pleomorphic inclusions in the cells. No normal lines were studied.

Tondeur et al (1971) in the ultrastructural study of a single cell line derived from an individual affected with I-cell disease found the fibroblasts filled with numerous cytoplasmic inclusions. They concluded that the disease was easily distinguished by electron microscopy.

Hanai et al (1971) in the study of cultured fibroblasts derived from two patients affected with I-cell disease reported that the cells contained numerous inclusion bodies of various size and shapes.

Examination of fibroblasts grown from an amniotic fluid from a single case of I-cell disease also reported the presence of these cytoplasmic inclusions (Aula et al, 1975).

Krabbe's Disease

Krabbe's disease is due to a deficiency in the enzyme galactocerebroside β -galactosidase (McKusick, 1975). Lyon et al (1973) reported that no ultrastructural distinction could be made between normal fibroblasts and fibroblasts derived from a patient affected with Krabbe's disease.

Lipofuscinosis

Kamensky et al (1973) studied the ultrastructure of cultured fibroblasts derived from a patient with lipofuscinosis and reported that no abnormality was evident in this cell line.

Metachromatic Leukodystrophy

Metachromatic leukodystrophy is due to a deficiency of the enzyme arylsulfatase A (McKusick, 1975). Two cell lines from patients with metachromatic leukodystrophy were studied by Hug et al (1970) and were found to have membrane bound cellular inclusions which were not observed in the normal control lines. However, after further study of another patient from whom a cell line had been grown, Hug et al (1973) stated that "the appearance of these inclusions cannot easily be distinguished from similar fibroblastic inclusions of other lysosomal diseases and of "normal" controls."

Lyon et al (1973) reported that three cell lines from patients with metachromatic leukodystrophy showed ultrastructural abnormalities in the cytoplasm that made it possible to distinguish them from normal cell lines. They observed the cytoplasm contained membrane limited bodies filled with a fine granular material.

Rutsaert et al (1973) were unable to detect any abnormality in four cell lines grown under normal conditions.

Kamensky et al (1973) in a study of one cell line were also unable to detect any differences from normal cells (Kamensky et al, 1973).

Mucopolipidosis III

The enzyme defect in mucopolipidosis III is believed to be a glycosyl transferase deficiency (McKusick, 1975). Four cell lines derived from patients with mucopolipidosis III were examined by electron microscopy and these lines were found to be distinguishable from the normal lines studied by the presence of numerous inclusions (Taylor et al, 1973).

A single cell line studied by Stein et al (1974) also revealed abnormalities in mucopolipidosis III. They reported the presence of many abnormal inclusion bodies in the fibroblasts, the most abundant being single membrane-limited vacuoles, and also reported that round concentrically arranged lamellar membrane-filled bodies were often present.

Mucopolipidosis IV

Kohn et al (1975) reported that cytoplasmic bodies were present in both biopsy specimens from a patient affected with Mucopolipidosis IV and the cells from an amniotic fluid culture (Kohn et al, 1975).

Niemann-Pick Disease - Type A

The enzyme sphingomyelinase is deficient in Niemann-Pick disease - Type A (McKusick, 1975). Ultrastructural examination of a single cell line obtained from a patient with Niemann-Pick Type A disease by Kamensky et al (1973) showed that an abnormal number of cytoplasmic bodies resem-

bling wavy lamellar bodies distinguished this line from normal cell lines studied.

Niemann-Pick Disease - Type C

Kamensky et al (1973) reported that cultured fibroblasts derived from a patient affected with Niemann-Pick disease when examined ultra-structurally contained irregular lamellar cytoplasmic bodies. These bodies were not observed in any of the normal lines studied.

Niemann-Pick Disease - Type D

One human skin fibroblast cell line studied from a patient affected with Niemann-Pick disease Type D was found to be indistinguishable ultra-structurally from the normal cell line studied at the same time (Vethamany et al, 1972).

Pompe's Disease

Hug et al (1970, 1971, 1972, 1974) ultrastructurally studied cultured fibroblasts from patients affected with Pompe's disease and consistently noted the presence of membrane surrounded accumulations of glycogen. These abnormal bodies were not observed in any other disease state studied by them nor in any normal line.

Hug et al (1972) have also detected similar bodies in amniotic fluid cells, both uncultured and cultured.

Sanfilippo's Disease - Type A

Sanfilippo disease A is due to a deficiency in the enzyme heparan sulfate sulfatase (McKusick, 1975). Ultrastructural study of a single cell line obtained from a patient affected with the disease did not reveal any ultrastructural difference from normal cultured fibroblasts (Kamensky et al, 1973).

Sanfilippo's Disease - Type B

Sanfilippo's disease B is probably due to a deficiency of the enzyme N-acetyl-alpha-D-glucosaminidase (McKusick, 1975). Kamensky et al (1973) in the ultrastructural examination of a single cell line from a patient affected with Sanfilippo's disease - Type B found that the fibroblasts showed large lysosomes which contained a reticular matrix with little electron dense material. They considered this line to be easily distinguishable from normal fibroblast lines.

Unspecified Triglyceride Storage Disease

Miranda et al (1976) examined cultured fibroblasts derived from an individual affected with an unspecified triglyceride storage disorder. They reported that all the fibroblasts showed the presence of a marked increase in lipid containing bodies and many abnormal lysosomal structures.

Wolman's Disease

Wolman's disease is apparently due to a deficiency in an acid lipase (McKusick, 1975). Lyon et al (1973) studied the fine structure of a cell line derived from a patient affected with Wolman's disease and consistently found abnormal cytoplasmic bodies. These bodies were limited by a single

membrane and contained small clear vesicles. They were not able to find these bodies in any normal fibroblast lines nor in any other disease state studied.

SUMMARY

It is apparent from this documentation of the literature concerning investigations on the ultrastructure of cultured fibroblasts from patients with a genetic disease that only scanty and sometimes contradictory information exists. In light of this, consideration of major points which produced some of these contradictions is appropriate.

One major contradiction concerned the difference of appearance between cells processed in the logarithmic phase and the stationary phase. It was suggested by the majority of investigators that more cytoplasmic bodies were evident in cells examined at the stationary phase than at the logarithmic phase. Conrad et al (1972) directly contradicted this observation. It is obvious from the reported studies on cells at different growth phases that great caution must be exercised to assure that each cell population examined is at the same growth phase.

A second problem which may affect the appearance of the cells was that Lipetz and Cristofalo (1972) and Robbins et al (1971) both reported that cells of passages above number 25 showed an increase in cytoplasmic bodies. Other reports did not record the passage number of the cell lines studied but did report the appearance of large numbers of bodies in the cells after extended periods in culture. As a result of this, it is obvious that the passages studied for each cell line should be closely matched.

A third problem evident from the literature was the difference in observations in a number of the diseases studied by more than one investigator. In several of the investigations, a different medium was used to culture the cells. This suggests that it is imperative to assure that

the cells are all cultured under conditions as similar as possible.

CHAPTER THREE

MATERIALS AND METHODS

CELL LINES

Normal Skin Fibroblast Cell Lines

A total of fifteen normal cultured human fibroblast cell lines were used in this study. Three were purchased as monolayers from the Mutant Human Cell Repository, Montreal, Quebec. These three lines were derived from phenotypically normal donors and had been frozen at passage numbers 6, 6, and 10 respectively. The remaining twelve lines were obtained by primary explant of postmortem skin biopsy specimens taken from phenotypically normal donors. The donors comprised eight males and four females, and ranged in age from 22 to 76. This material was made available by the Department of Pathology, Deer Lodge Hospital, Winnipeg, Manitoba. These skin biopsies were established in culture as explants using standard culturing procedures (Parker, 1961). After removal from the donor, the biopsy specimen was placed in a sterile container with tissue culture medium and transferred to the cell culture facilities at the Division of Genetics, Department of Paediatrics, Children's Centre, Health Sciences Centre, Winnipeg, Manitoba. Under sterile conditions, the specimen was transferred to a 60 mm Falcon plastic petri dish. The tissue was then minced with sterile forceps and scissors into approximately 1 mm³ fragments. These fragments were then placed, five to a dish, into other 60 mm plastic petri dishes. Several dishes were established for each biopsy specimen. A single drop of medium was placed on each tissue fragment and the dishes were incubated at 37.5°C. This

allowed initial adherence of the fragments to the bottom of the petri dish. Four ml of tissue culture medium was then slowly added to these dishes, care being taken not to dislodge the tissue fragments, and the dishes were returned to the incubator. The fibroblasts, which migrated from the tissue fragments between 8 and 14 days after explant, were trypsinized and transferred to other 60 mm dishes. They were cultured thereafter for at least four passages.

Normal Amniotic Fluid Fibroblast Lines

Three normal amniotic fluid fibroblast lines were purchased as monolayers from the Mutant Human Cell Repository, Montreal. These three lines were derived from amniotic fluids taken for non-medical reasons. The three lines had been frozen at passage numbers 3,3, and 8 respectively. The Department of Obstetrics and Gynecology, Womens' Centre, Health Sciences Centre, Winnipeg, Manitoba kindly provided fourteen amniotic fluid specimens taken for non-medical reasons which were cultured in accordance with established procedures (Cox et al, 1974). Amniotic fluid was drawn from the patients under sterile conditions and transferred to sterile containers which were then brought to the Division of Genetics. One ml of amniotic fluid was pipetted to a 60 mm dish and 3 ml of tissue culture medium was added to the dish. Several dishes were established for each amniotic fluid sample. The dishes were incubated at 37.5°C. The fibroblasts from each of these primary specimens were then trypsinized and transferred to separate 60 mm dishes and cultured thereafter for at least four passages.

Skin Fibroblast Lines from Known Homozygotes for a Genetic Disease

Fibroblast lines derived from individuals confirmed by clinical and/or biochemical studies to be homozygotes for a single gene defect were purchased from either the Mutant Human Cell Repository, Montreal or the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, New Jersey. All cell lines used in this study have been confirmed by the Cell Banks concerned to be derived from donors affected with a specific genetic disease, either by biochemical methods or clinical diagnosis. In some cases, confirmation by both methods was possible.

These cell lines are listed in Table I. The genetic diseases utilized in this study were Duchenne's muscular dystrophy, Fabry's disease, Tay-Sachs disease, infantile cystinosis, metachromatic leukodystrophy, I-cell disease, Pompe's disease, Lesch-Nyhan syndrome, maple syrup urine disease, cystic fibrosis. Efforts were made to obtain at least five cell lines for each disease under study; however, the Cell Banks were not able to fulfill this requirement for all the disease states.

Skin Fibroblast Lines from Known Heterozygotes for a Genetic Disease

Efforts were made to purchase from the two Cell Banks five heterozygotic cell lines for each of the ten diseases under study. However, only a limited number of the heterozygotic cell lines requested were available through the Cell Banks. These are listed in Table II.

Amniotic Fluid Fibroblast Lines from Known Homozygotes for a Genetic Disease

Amniotic fluid fibroblast cell lines from known homozygotes for the

TABLE I
SKIN FIBROBLAST CELL LINES FROM CONFIRMED HOMOZYGOTES
FOR A GENETIC DISEASE

<u>Disease</u>	<u>Cell Line Designation</u>	<u>Passage Number, from Explant, Examined</u>
Muscular dystrophy (Duchenne type)	DY	6,7,8,9,10
	WG348	6,7,8,9
	WG433	4,5,6,7
	WG448	5,6,7,8
Fabry's disease	WG330	?,6,7,8,9
	SM	?,7,8,9,10
	JF	6,7,8,9
	WG386	?,6,7,8,9
	RC	11,12,13,14
	GM107	7,8,9,10
Tay-Sachs disease	HD	7,8,9,10
	SIM	12,13,14,15
	GM527	5,6,7,8,9
	GM77	9,10,11,12
	GM221	6,7,8,9
	KS	7,8,9,10
Infantile cystinosis	PF11	7,8,9,10
Metachromatic Leukodystrophy	WG476	19,20,21,22
	WG477	11,12,13,14
	WG478	11,12,13,14
	WG457	10,11,12,13
	BL	7,8,9,10
I-cell disease	MT	7,8,9,10
Pompe's disease	LAD	28,29,30
Lesch-Nyhan syndrome	GL	12,13,14,15
Maple syrup urine disease	EC	9,10,11
Cystic fibrosis	CF	10,11,12,13
	MB	6,7,8,9
	RR	7,8,9,10
	KL	7,8,9,10
	RL	6,7,8,9

TABLE II
SKIN FIBROBLAST CELL LINES AVAILABLE FROM CONFIRMED HETEROZYGOTES
FOR A GENETIC DISEASE

<u>Heterozygotic for:</u>	<u>Cell Line</u>	<u>Passage Number, from Explant, Examined</u>
Fabry's disease	GM1070 WOR	10,11,12,13 7,8,9,10
Tay-Sachs disease	GM1108 GM1109	9,10,11 11,12,13,14
Metachromatic leukodystrophy	WG479 GM132 GM200	9,10,11,12 8,9,10 6,7,8,9
I-cell disease	GM80	7,8,9,10

ten diseases under study were sought from the Cell Banks, but only five could be provided by them. Communication with over fifty genetic laboratories in North America was able to bring in only three more amniotic fluid fibroblast cell lines. The amniotic fluid lines available for study are listed in Table III.

CULTURE MEDIUM AND PHYSICAL FACILITIES FOR CULTURING

McCoy's 5a (modified) medium supplemented with 10% fetal calf serum and 5% human cord serum with added antibiotics was used exclusively as the culture medium throughout this study.

The powdered medium was purchased from Schwarz Bioresearch, Orangeburg, New York. The exact composition of this medium is listed in Table IV. 71.9 grams of powdered medium was dissolved in 5 liters of deionized water. To this solution was added 590 ml of fetal calf serum, purchased from Microbiological Associates, Bethesda, Maryland, and 295 ml of human cord serum made available by the Department of Obstetrics and Gynecology, Womens' Centre. In addition, 50 ml of a solution of 10 gms of streptomycin and 10,000,000 International Units of penicillin in one liter of deionized water was added. Eleven gms of NaHCO_3 were also added. The medium was then adjusted to pH 7.3 with 1 N NaOH, sterilized by Millipore filtration, and tested for sterility.

The trypsin solution used for removal of cells from the culture vessels was applied at a concentration of 0.05%. The solution was made by the addition to 4700 ml of deionized water of 250 ml of saline D concentrate, 2.5 gms of powdered 1:300 trypsin concentrate purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio, 5.0 gms of glucose, and 0.65 gms of NaHCO_3 . 50 ml of the stock antibiotic solution used in the

TABLE III

AMNIOTIC FLUID FIBROBLAST CELL LINES
AVAILABLE FROM CONFIRMED HOMOZYGOTES
FOR A SPECIFIC GENETIC DISEASE

<u>Disease</u>	<u>Cell Line</u>	<u>Passage Number, from Explant, Examined</u>
Fabry's disease	GM636	10,11,12,13
Tay-Sachs disease	A61	6,7,8,9
	A77	6,7,8,9
	A41	6,7,8,9
	TS8SD	not specified
Cystinosis	AFC2BNJ	6,7,8
	GM804	5,6,7,8
Metachromatic leukodystrophy	AML	not specified

TABLE IV
COMPOSITION OF McCOY'S 5A (MODIFIED) CELL CULTURE MEDIUM

	mg/liter		mg/liter
L-alanine	13.4	p-aminobenzoic acid	1.0
L-arginine-HCL	42.1	ascorbic acid	0.5
L-asparagine-H ₂ O	45.03	d-biotin	0.2
L-aspartic acid	20.0	d-pantothenate-Ca	0.2
L-cysteine-HCL-H ₂ O	35.1	Choline chloride	5.0
L-glutamic acid	22.1	Folic acid	10.0
L-glutamine	219.2	i-inositol	36.0
L-histidine-HCL-H ₂ O	21.0	Nicotinic acid	0.5
L-hydroxyproline	20.0	Nicotinamide	0.5
L-isoleucine	39.4	Pyridoxal-HCL	0.5
L-leucine	39.4	Pyridoxine-HCL	0.5
L-lysine-HCL	36.5	Riboflavin	0.2
L-methionine	14.9	Thiamine-HCL	0.2
L-phenylalanine	16.5	Vitamin B ₁₂	2.0
L-proline	17.3	Bacto-peptone	600.0
L-serine	26.3	Sodium chloride	6460.0
L-threonine	17.9	Potassium chloride	400.0
L-tryptophan	3.1	Calcium chloride (anhydrous)	200.0
L-tyrosine	18.1	Magnesium sulfate (anhydrous)	97.67
L-valine	17.6	Monobasic sodium phosphate-H ₂ O	140.0
Glutathione	0.5	Dextrose	4000.0
Phenol Red	2.5		

preparation of the McCoy's medium was also added. The saline D concentrate used was made by the addition of 160.0 gms of NaCl, 8.0 gms of KCl, 0.9 gms of Na_2HPO_4 and 0.024 gms of phenol red to one liter of deionized water. The trypsin solution was then neutralized to pH 7.3 with 1 N NaOH and sterilized by Millipore filtration.

The incubator used for the study was a Napco water jacketed incubator set at 37.5°C . with a 100% humid atmosphere of 5% CO_2 in air.

CELL CULTURE SCHEDULE

Each fibroblast cell line studied by electron microscopy was cultivated under conditions of culture which were kept as constant as possible. From the primary specimen, either a skin biopsy, amniotic fluid sample, or a monolayer supplied by the Cell Banks, the first trypsinization was transferred into a single dish which was allowed to grow to confluency. At least three dishes had been established from each skin biopsy and amniotic fluid sample; this ensured that at least three separate dishes for each normal control line were independently carried through the cell culture schedule. This first passage ensured that adequate growth was initially achieved. Trypsinization of this dish was then performed and between 2.0×10^5 and 4.0×10^5 cells from the resulting cell suspension were transferred into two separate 60 mm dishes containing 3.5 ml of McCoy's 5a (modified) medium and the dishes were incubated at 37.5°C . One of these dishes would, after the designated period of time, be processed for electron microscopy; the second would be allowed to grow to confluency and then be passaged again into two more dishes. This procedure was continued until four consecutive passages were available for examination for each cell line.

For the purpose of comparison and control, normal cells were grown throughout the entire period of the study. Also, efforts were made to ensure that normal lines and diseased cell lines were grown at the same time in the same incubator. However, owing to the logistics of receiving cell lines over large distances and various times, this was not always possible.

The dishes were initially examined for growth by light microscopy on the 4th day. On day 5, the medium was changed and 4.0 ml of fresh medium was added. 24 hours later, the dishes were re-examined by light microscopy to ensure the monolayers were not confluent and then processed for electron microscopy.

FIXATION AND EMBEDDING FOR ELECTRON MICROSCOPY

Each non-confluent monolayer of fibroblasts to be examined by electron microscopy was processed in situ in the petri dish after 6 days in culture. The cells were fixed with 5% gluteraldehyde in 0.1 M Sorensen's buffer for one hour (Gomeri, 1955), washed at pH 7.3 in a solution of 5% sucrose in 0.1 M Sorensen's buffer for 30 minutes, and postfixed for 30 minutes in 1% osmium tetroxide in 0.1M Sorensen's buffer. Each petri dish was then passed through two minute washes of 35%, 50%, 75%, 90%, 100% ethanol and 100% methanol. The petri dishes were then filled with araldite to a depth of approximately 0.5 cm and placed in an oven at 60°C for 24 hours. At the end of the 24 hour polymerization period, the araldite was separated from the dish. The cells and a surface film of the plastic dish remained in the araldite sheet. Small segments of the araldite, approximately 0.25 cm² were mounted in araldite on wooden supports with the cell side facing outwards. These wooden blocks were placed in an oven

at 60°C for 24 hours to polymerize the araldite.

SECTIONING, STAINING AND ELECTRON MICROSCOPY

Sections were cut in the horizontal face of the monolayer with glass knives on a Reichert Om U2 ultramicrotome. The sections were collected from the water boat on uncoated 400 mesh copper grids. The grids were stained with a 1.5% solution of lead citrate in water for 5 minutes and counterstained with a 7% solution of uranyl acetate in water for 90 minutes (Venable and Coggeshall, 1965; Hayat, 1970).

Sections were viewed on a Zeiss 9AS2 electron microscope in the Department of Pathology, Children's Centre, Health Sciences Centre, Winnipeg, Manitoba.

A minimum of 150 cells or parts of cells were examined for each passage. In the majority of cases, many more than the 150 cells were examined.

CHAPTER FOUR

OBSERVATIONS

NORMAL CELL LINES

Ultrastructural Appearance of Normal Cultured Human Skin Fibroblasts

Fifteen normal cell lines were examined. At least four consecutive passages were studied for each fibroblast line; the total number of passages studied for the fifteen cell lines was in excess of 70. No ultrastructural differences were noticed between those cell lines which had been frozen, and those grown directly from skin biopsies and not frozen. Examples of normal cultured fibroblasts are seen in Figure 3 through to Figure 10.

The fibroblasts grown in culture were all of low passage number; the highest passage studied was the eighteenth. The cells were normally seen in a spindle-shape, with lengths commonly between 40 micrometers and 60 micrometers. The width of the spindle-shaped fibroblasts usually varied between 6 micrometers and 12 micrometers. The overall shape of the cells, however, did vary from the more predominant spindle-shapes to virtual circular shapes. This pattern of gross morphological shape is probably related to the demonstrable behaviour of cells in culture which has shown that as cells approach the mitotic stage of the cell cycle they round up and become virtually spherical (Rajaraman et al, 1974). Cells sectioned in this study confirm this observation since those with a circular configuration always showed distinct chromosomes indicating that they were in mitosis.

Figure 3. Normal human cultured fibroblasts. Shown are short segments of rough endoplasmic reticulum(RER) and several lipid storage bodies(LIP). This number of lipid storage bodies was not observed in subsequent passages. Also shown is a nucleolus(NUCL).

X 17,300

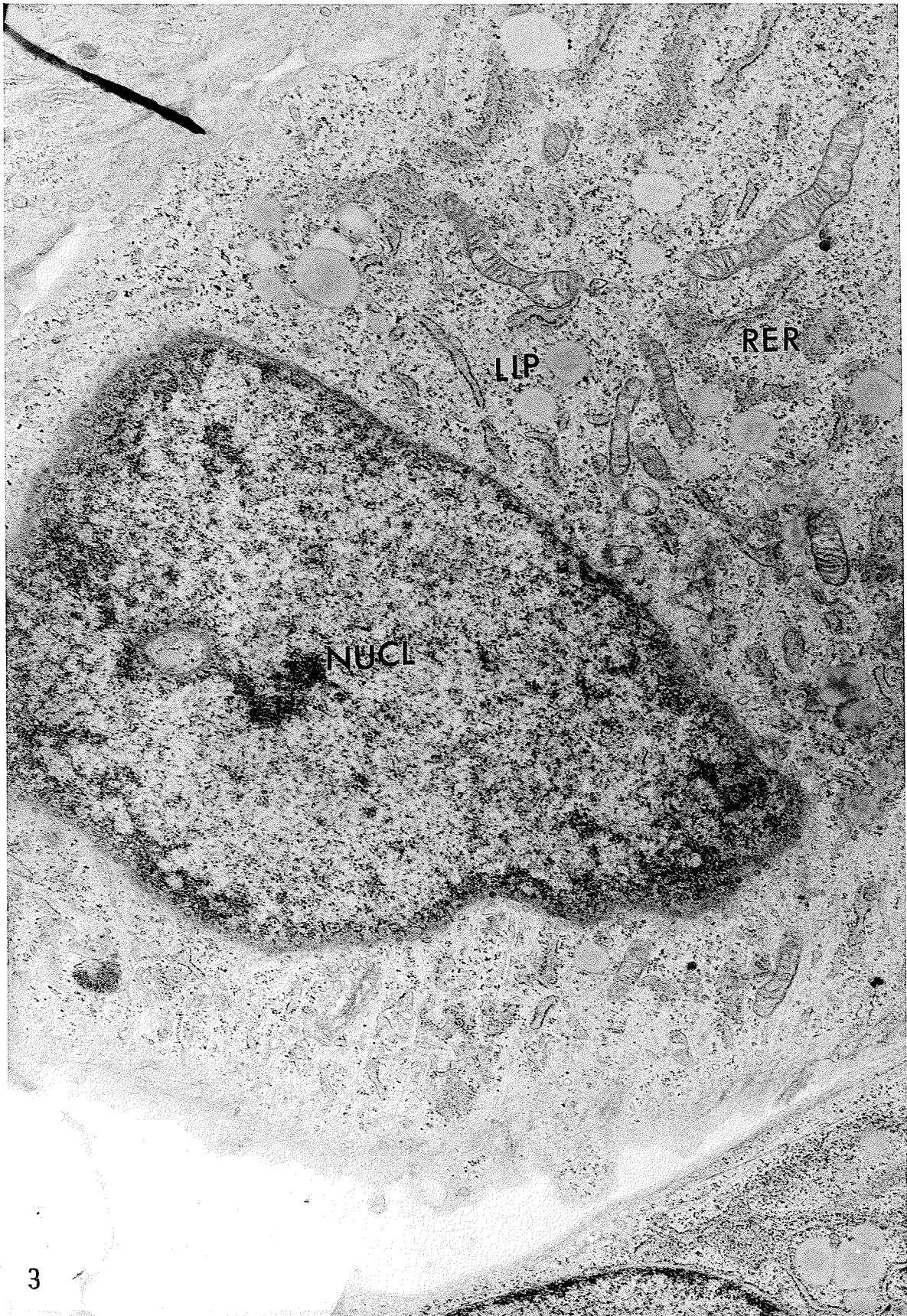


Figure 4. Normal human cultured fibroblasts. Note absence of unique cytoplasmic bodies. Also shown are vacuoles (VAC), lipid storage bodies (LIP), and rough endoplasmic reticulum (RER).

X 10,900

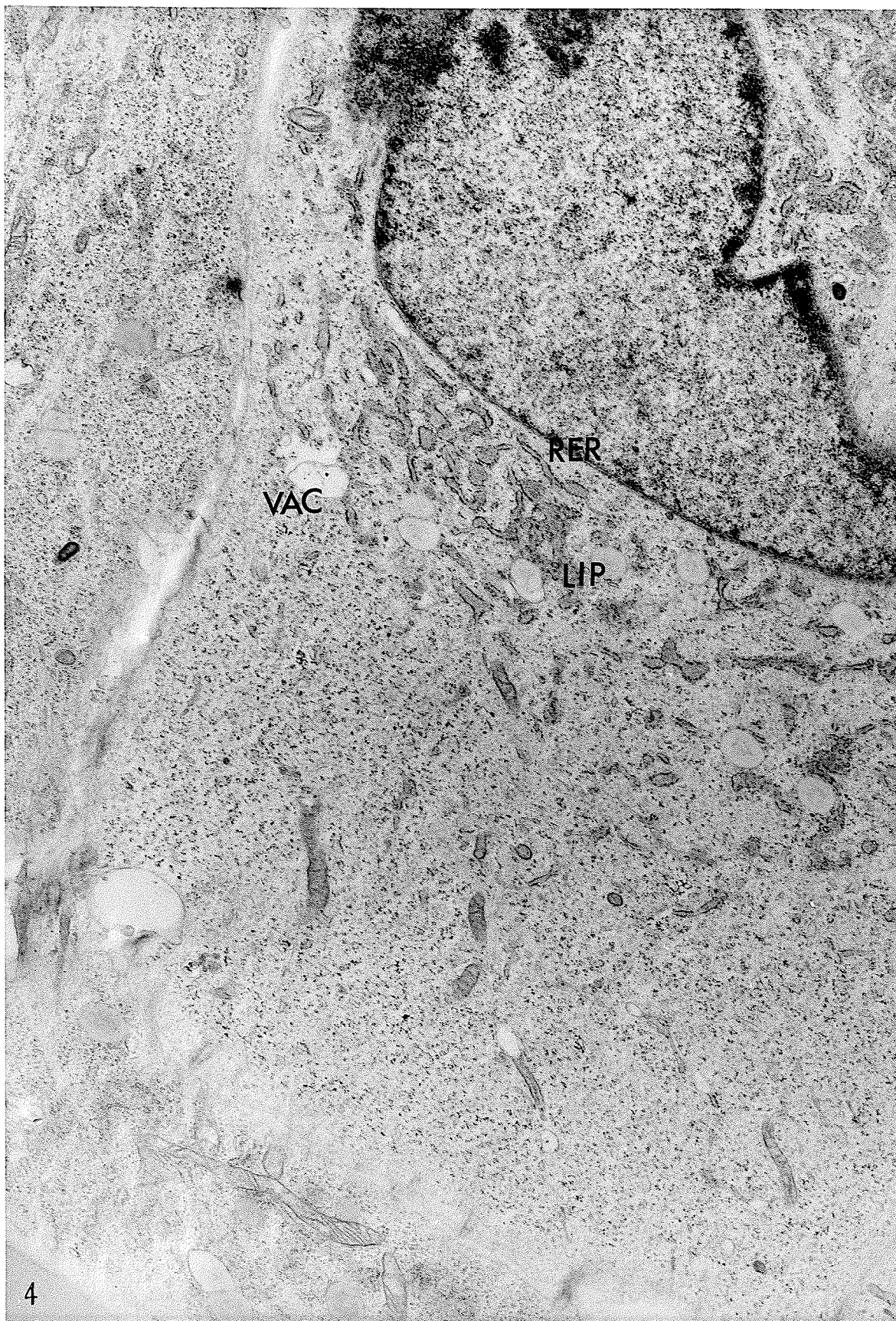


Figure 5. Normal human cultured fibroblasts. Note a well developed and extensive Golgi complex(GOL). Not separation between cells(SEP).

X 16,500

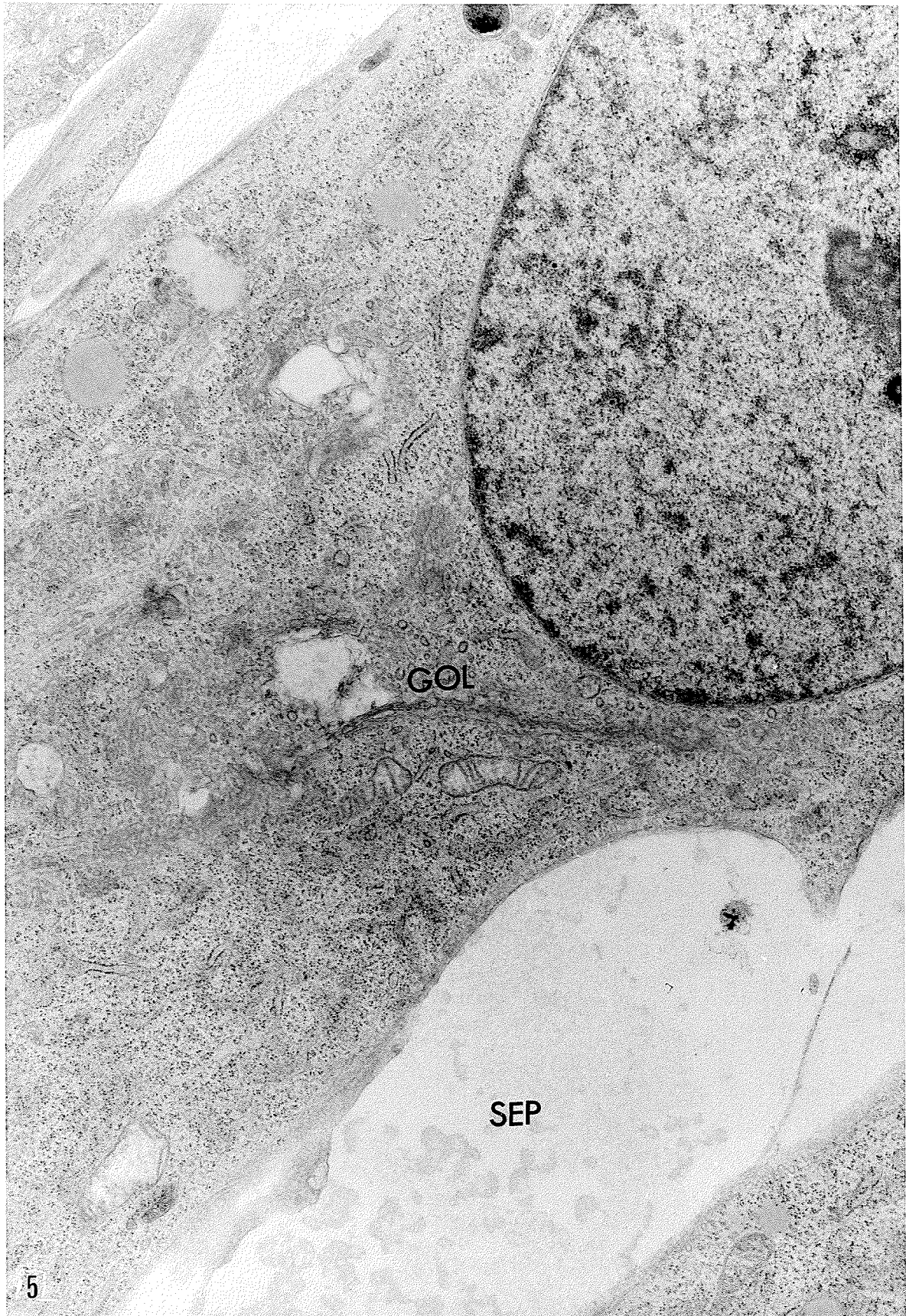


Figure 6. Normal human cultured fibroblasts. Note appearance of filaments surrounding periphery of cells(FIL). Note also the appearance of several lipid storage bodies in these normal cells(LIP).

X 9,200

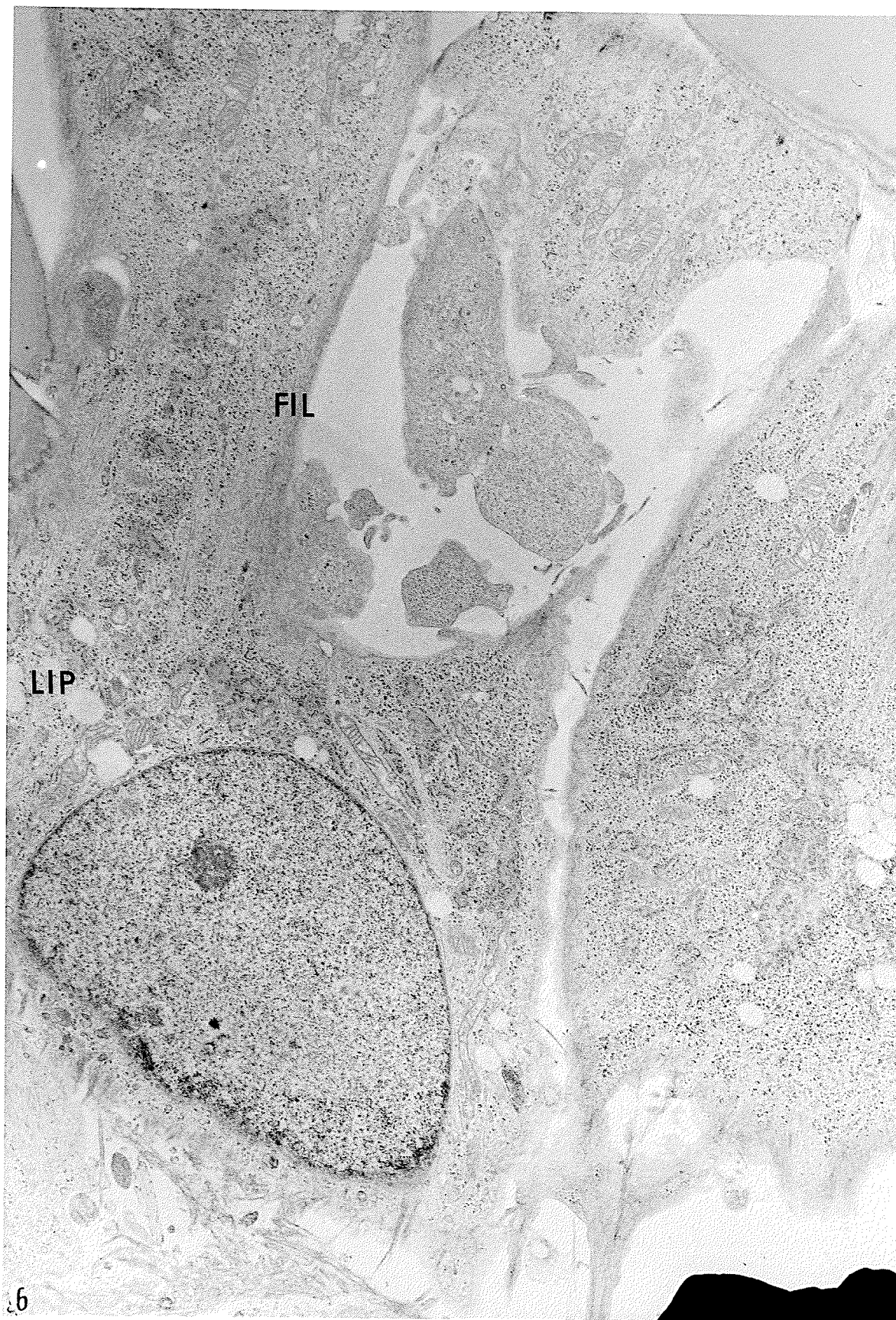


Figure 7. Human cultured normal fibroblasts. Shown are abundance of filaments surrounding periphery of cells(FIL), and a number of lysosomes(LYS), and lipid storage bodies(LIP). Also shown is an area of pinocytosis(PIN).

X 16,700

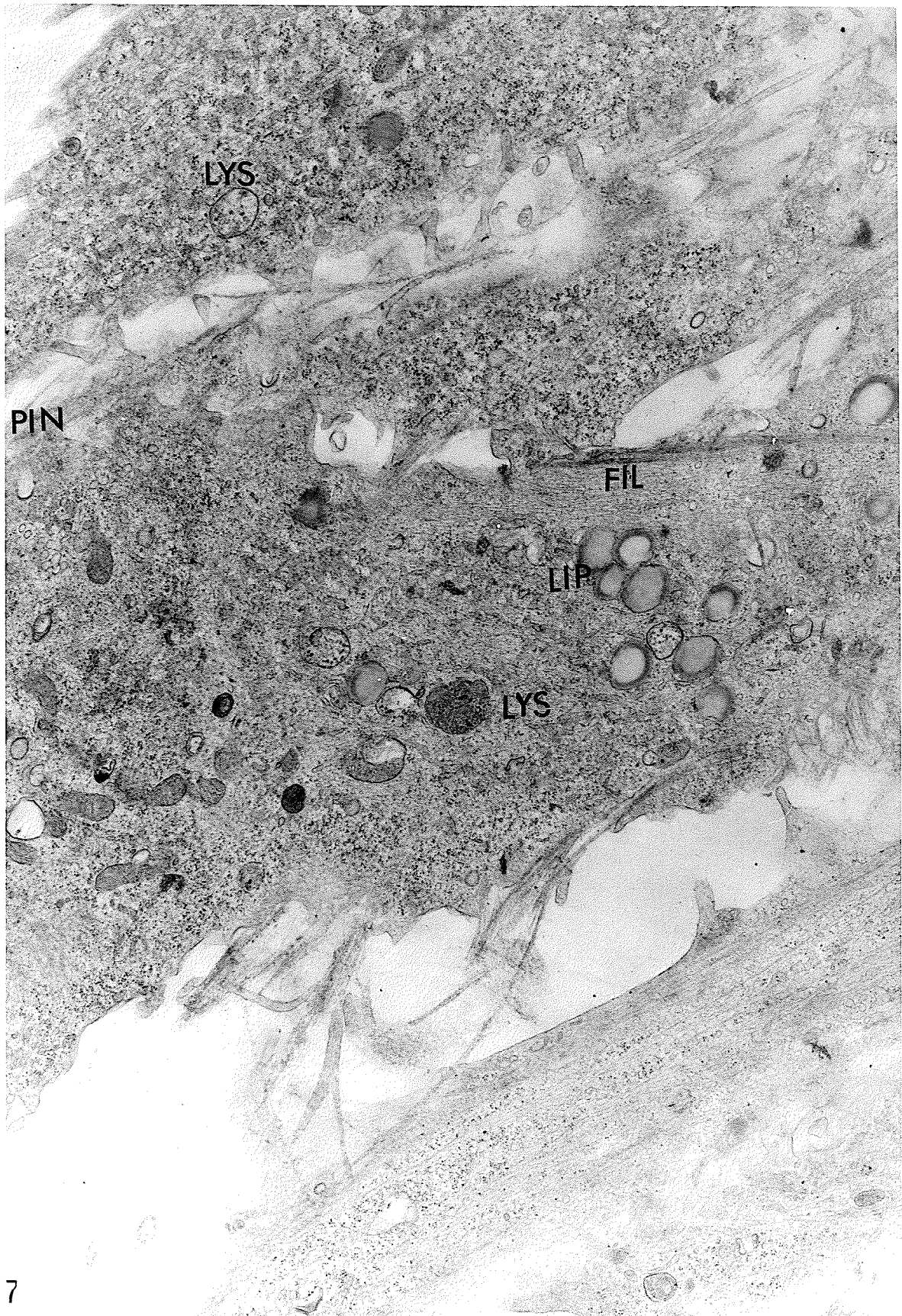


Figure 8. Human cultured normal fibroblasts. Shown are numerous mitochondria(MIT), some are in a swollen configuration. This swollen appearance was uncommon and was not observed in all the passages of any cell line, but was observed to occur rarely in all the lines examined. Also shown is an area of rough endoplasmic reticulum(RER), and an area of pinocytosis(PIN).



Figure 9. Normal human cultured fibroblast. Shown are nuclear membranes(NUC), well developed Golgi complex(GOL), and area of rough endoplasmic reticulum(RER), and lysosomes(LYS).

X 68,000

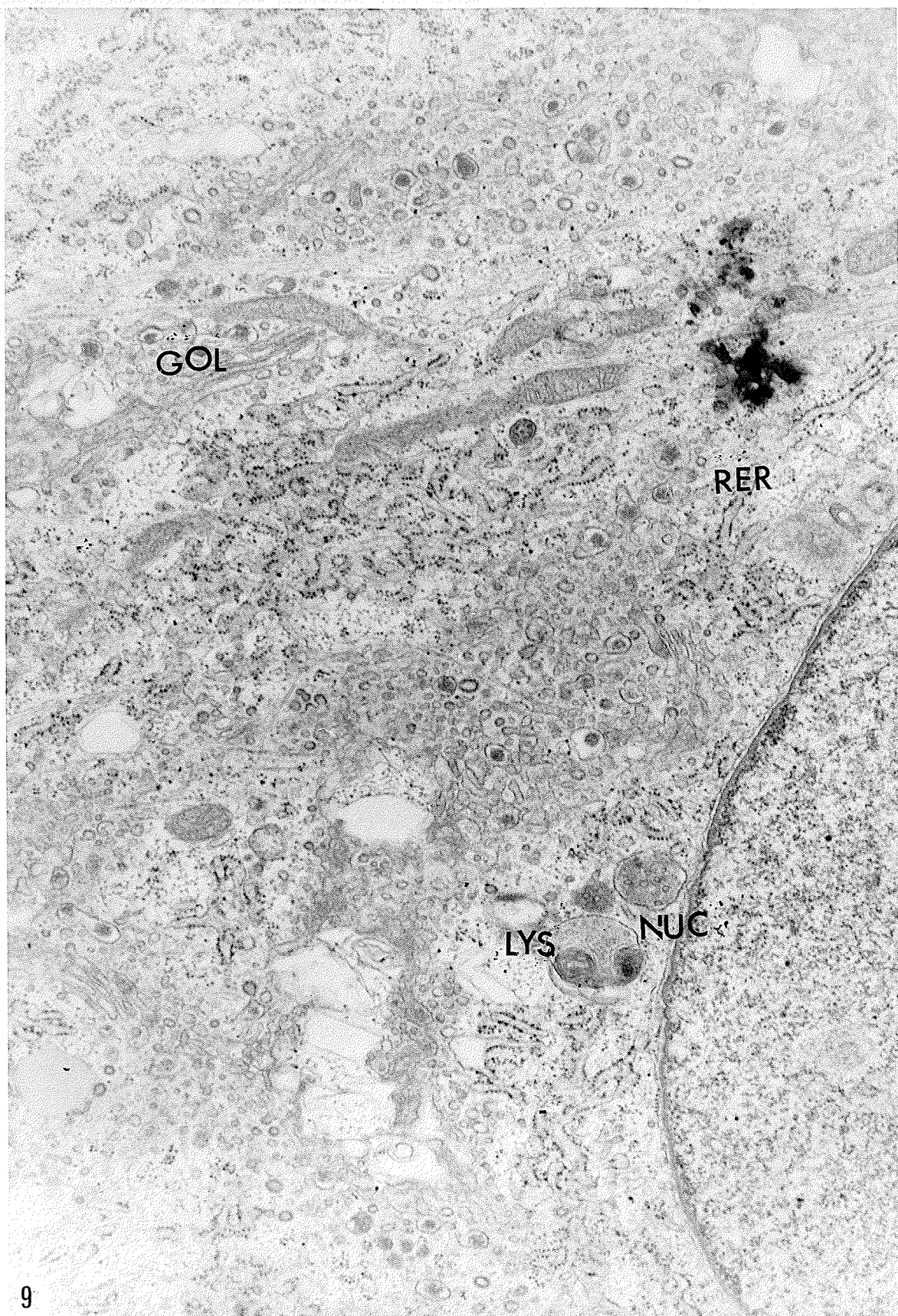
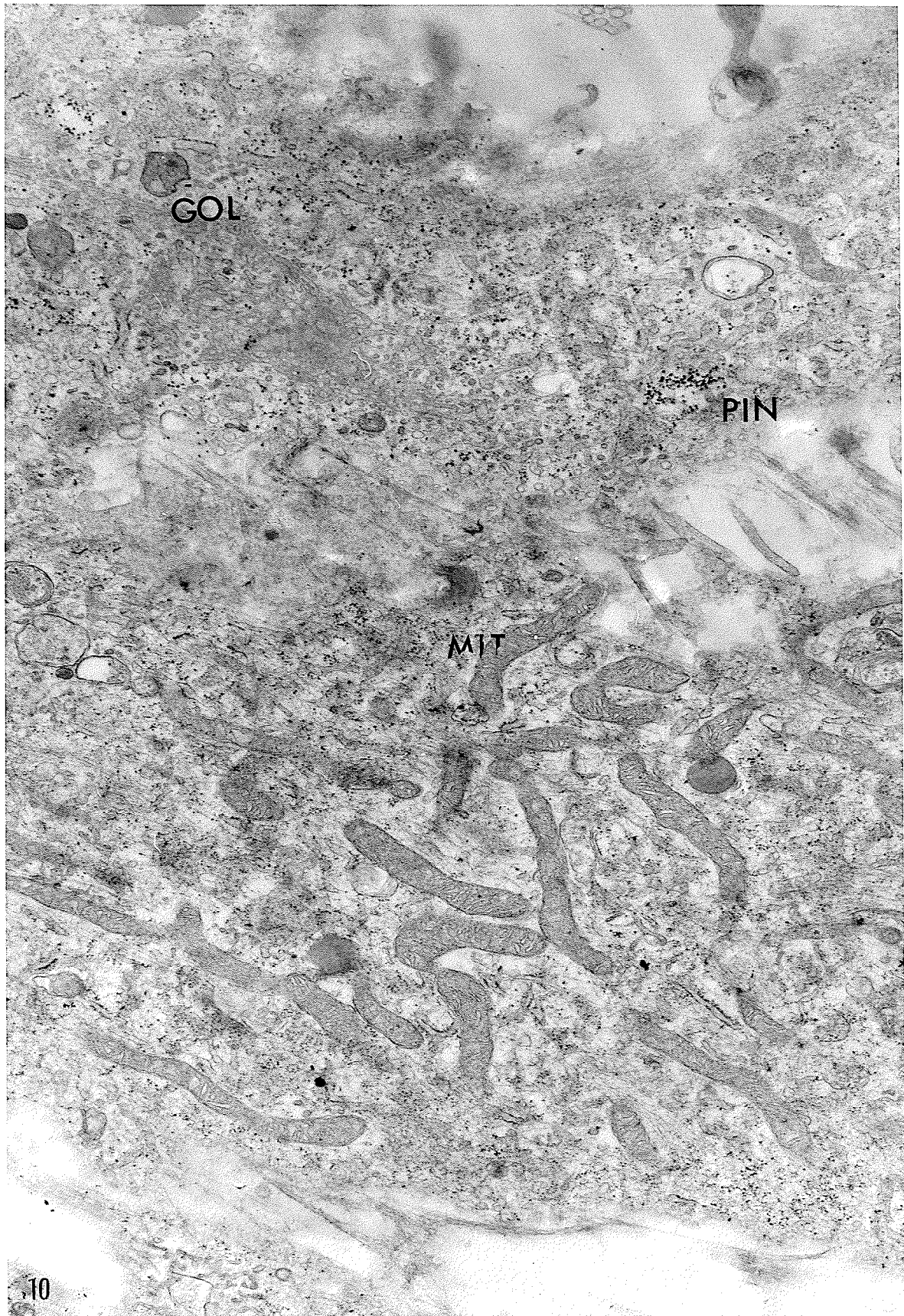


Figure 10. Normal human cultured fibroblasts. Shown are abundant mitochondria(MIT), area of well developed Golgi complex(GOL), and area of pinocytosis(PIN).

X 19,100



The Nucleus

The nucleus of a cultured fibroblast in an average cell section occupied between 25% to 30% of the cell. The shape of the nucleus generally followed that of the cell itself; spindle-shaped cells having spindle-shaped nuclei, while rounded cells had round nuclei. The nucleus in a section of a single cell rarely showed any lobulation or indentations and was generally smooth in contour.

The chromatin in the nucleus was usually very disperse and was condensed only as a thin rim intimately associated with the interior of the nuclear envelope. Figures 3, 4, 5 demonstrate this observation. In a number of cells, in all likelihood those approaching mitosis, condensation of the chromatin was apparent and, in several cell sections, discrete chromosomes were encountered. Figure 25 shows this point.

Generally only one nucleolus was evident in a single section of a cultured fibroblast. Figure 3 shows a cell with this single nucleolus. However, occasionally up to six nucleoli were observed in a single cell section.

The fine structure of the nuclear membrane conformed to the classical descriptions (Toner and Carr, 1968; Fawcett, 1969; DeRobertis et al, 1975). Two membranes separated by a narrow intramembranous gap were usually observed. Nuclear pores were commonly noted. The areas of the nucleus associated with the nuclear pores usually showed no evidence of chromatin condensation; thus gaps in the condensed chromatin rim occurred at the nuclear pores. Figures 8 and 9 clearly demonstrate these points.

It was unusual to observe inclusions in the nuclei of the cells examined in this study.

The Cytoplasm

The cytoplasm of a fibroblast in the proliferative stage of growth was observed to contain the organelles normally seen in an active cell. The organelles commonly seen were ribosomes, smooth and rough endoplasmic reticulum, Golgi complexes, lysosomes, mitochondria, microtubules, microfilaments. Occasionally sections would show the presence of centrioles in the fibroblast. The majority of these organelles were found in close association with the nucleus but were, on occasion, observed at a distance from the nucleus. However, ribosomes, microtubules and microfilaments were usually evenly distributed throughout the cells.

Ribosomes

The majority of the ribosomes observed in the cultured fibroblasts were found either in small ribosomal complexes of between 4 and 8 ribosomes, or as free ribosomes. Less frequently the ribosomes were found either in spiral formations or in association with the endoplasmic reticulum. The polyribosomal complexes and the free ribosomes were usually seen to be evenly distributed throughout the cell.

The Endoplasmic Reticulum

Little endoplasmic reticulum was normally observed in the cultured fibroblasts. The endoplasmic reticulum which was observed was usually composed of short segments of rough endoplasmic reticulum randomly distributed near the nucleus. Figures 3, 4, 8 and 9 show these short segments of rough endoplasmic reticulum. These segments were normally found in the perinuclear region of the cell and were generally oriented to run with the long axis of the cell. Small amounts of smooth endo-

plasmic reticulum were also observed which followed the same distribution and orientation patterns as observed for the rough endoplasmic reticulum. The diameter of the endoplasmic reticulum was usually about 50 nanometers but was observed to distend to eight to ten times this width on occasions. Only rarely were large concentrations of endoplasmic reticulum observed.

Golgi Complexes

The Golgi apparatus of the cultured fibroblast was observed to be extensive and well developed. In some cell sections, areas of the Golgi complex could be seen to extend almost one third of the cell's length. The Golgi complex was generally found to run with the long axis of the cell. Small membrane bound translucent bodies were normally found in close association with the Golgi complex. Figures 5, 9 and 10 show these well developed Golgi systems.

Lysosomes

In the normal fibroblast in the proliferative stage of growth, few lysosomes were observed. Figures 7 and 9 show representative lysosomal bodies. In a single cell section rarely would more than five lysosomal resembling bodies be present. Normally the bodies described here as lysosomes would measure between one and three micrometers and would vary in density from almost translucent to dense. However, occasionally larger lysosomes were seen. The criterion for being termed a lysosome was the presence of a single limiting membrane (Dingle, 1974). The identification of lysosomes was made on the basis of morphological criteria alone. No histochemical stains for lysosomal enzymes were performed.

Mitochondria

Mitochondria were very abundant in the fibroblasts. Some cell sections contained in excess of twenty. Figure 10 shows the appearance of mitochondria in fibroblasts. The majority of the mitochondria were found in close association with the nucleus of the cell and were normally oriented to run with the long axis of the fibroblast. Some mitochondria had lengths in excess of 20 micrometers with diameters of only 0.5 micrometers. The shape of the mitochondria generally mimicked that of the cell; mitochondria of cells in mitosis were usually rounded, while the mitochondria of spindle-shaped fibroblasts were elongated.

Occasionally bizarre mitochondrial shapes were observed but these were not seen on the subsequent passage of the same cell line and thus were not considered to be related to the genotype of the cell. Figure 8 demonstrates this point.

Mitochondrial granules were observed very infrequently.

Microtubules

Microtubules were extensively distributed throughout all the fibroblasts studied. Cells in mitosis were noted to contain large numbers of microtubules associated with the condensed chromosomes.

Microfilaments

Microfilaments were abundant in all the fibroblasts studied. They were normally found in bundles which were associated with the cell membrane. They appeared to run in two directions. One series of microfilaments associated with the cell membrane ran parallel to the membrane; a second grouping of microfilaments projected at angles into the cyto-

plasm and also outside the cell membrane, and passed through the microfilaments which ran parallel to the membranes. Figures 6 and 7 demonstrate the presence of microfilaments in cultured human fibroblasts.

Single or small bundles of microfilaments were also seen. These ran throughout the cytoplasm of the cell and appeared to attach to the cell membrane.

Other Cytoplasmic Bodies

Other cytoplasmic bodies were observed in the normal fibroblast. These included the occasional myelin figure, lipid storage bodies, vacuoles, multivesicular bodies, free glycogen, and other unique structures. These various bodies were only rarely observed. However, they did appear with a frequency which indicated that their occurrence was normal in an active cultured fibroblast. They were not consistently seen from passage to passage of a single cell line but appeared randomly throughout the cells examined. Figures 3, 4, 6 and 7 demonstrate several of these cytoplasmic bodies.

The Cell Membrane

The cell membrane was rarely clearly defined. It was normally obscured by the abundant number of microfilaments found at the periphery of the cell.

Pinocytosis was very evident in the cells and large areas of the cell contained pinocytotic vesicles. In the areas where pinocytosis was seen, the microfilaments were usually absent. Figures 7 and 10 show areas of pinocytosis. Occasional evidence of phagocytotic activity was observed.

Since the cells were not grown to the confluent monolayer stage, cell to cell contact was not extensive. When cell to cell contact was observed, it was usually an interdigitation at the microfilaments surrounding the periphery of the cells. When the membranes met, an interdigitation of the membranes occurred. Occasionally desmosomal contacts were seen. Figures 15, 18 and 26 show examples of cellular junctions.

Ultrastructural Appearance of Normal Cultured Amniotic Fluid Fibroblasts

Figure 11 through to Figure 19 show examples of the fine structure of normal amniotic fluid fibroblasts. The ultrastructural appearance was similar to that of the normal cultured skin fibroblasts and thus the previous description of cultured skin fibroblasts is applicable to the cultured amniotic fluid fibroblasts. However, some differences were noted and they are described below.

Free glycogen was much more evident in the amniotic fluid fibroblasts. It was evenly distributed throughout the cell, and consistently appeared from passage to passage of a cell line.

Microfilaments were more evident in the interior of the amniotic fluid fibroblasts than they were in the fibroblasts derived from post-natal skin biopsies.

Pinocytosis was less evident in the amniotic fluid fibroblast.

Vacuoles, lysosomes and lipid storage bodies were in greater evidence in amniotic fluid fibroblasts. It was not uncommon to observe up to five large cytoplasmic vacuoles in the cytoplasm of the cell and up to ten lysosomes were commonly observed in the cultured amniotic fluid fibroblasts. Figures 11 and 18 illustrate this point.

The cells showed evidence of small blebs of membrane on the cell

Figure 11. Normal human cultured amniotic fluid fibroblasts. Shown are portions of eight cells. Note three nuclei showing different distribution of chromatin indicating different stages of activity. Also shown are several cytoplasmic bodies, which morphologically are lysosomes(LYS).

X 11,500

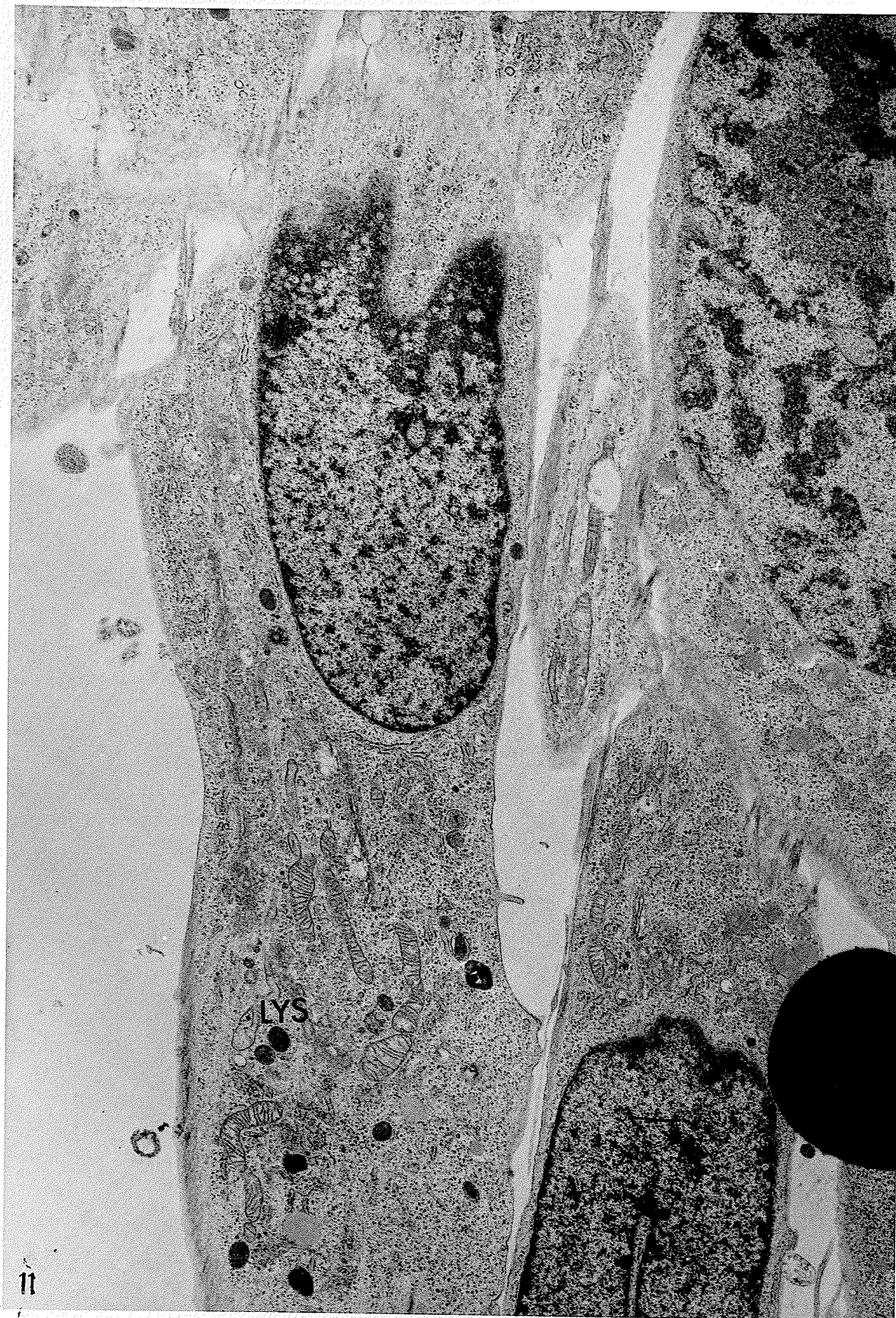


Figure 12. Normal human cultured amniotic fluid fibroblasts. This figure illustrates the relative absence of unique cytoplasmic bodies observed in normal fibroblast cell lines. Note appearance of well developed mitochondria(MIT).

X 21,800



Figure 13. Normal human cultured amniotic fluid fibroblasts. Note cell junction(JUNC), abundance of filaments (FIL), and several swollen mitochondria(MIT). These swollen mitochondria were occasionally observed in both normal and diseased fibroblasts.

X 9,100

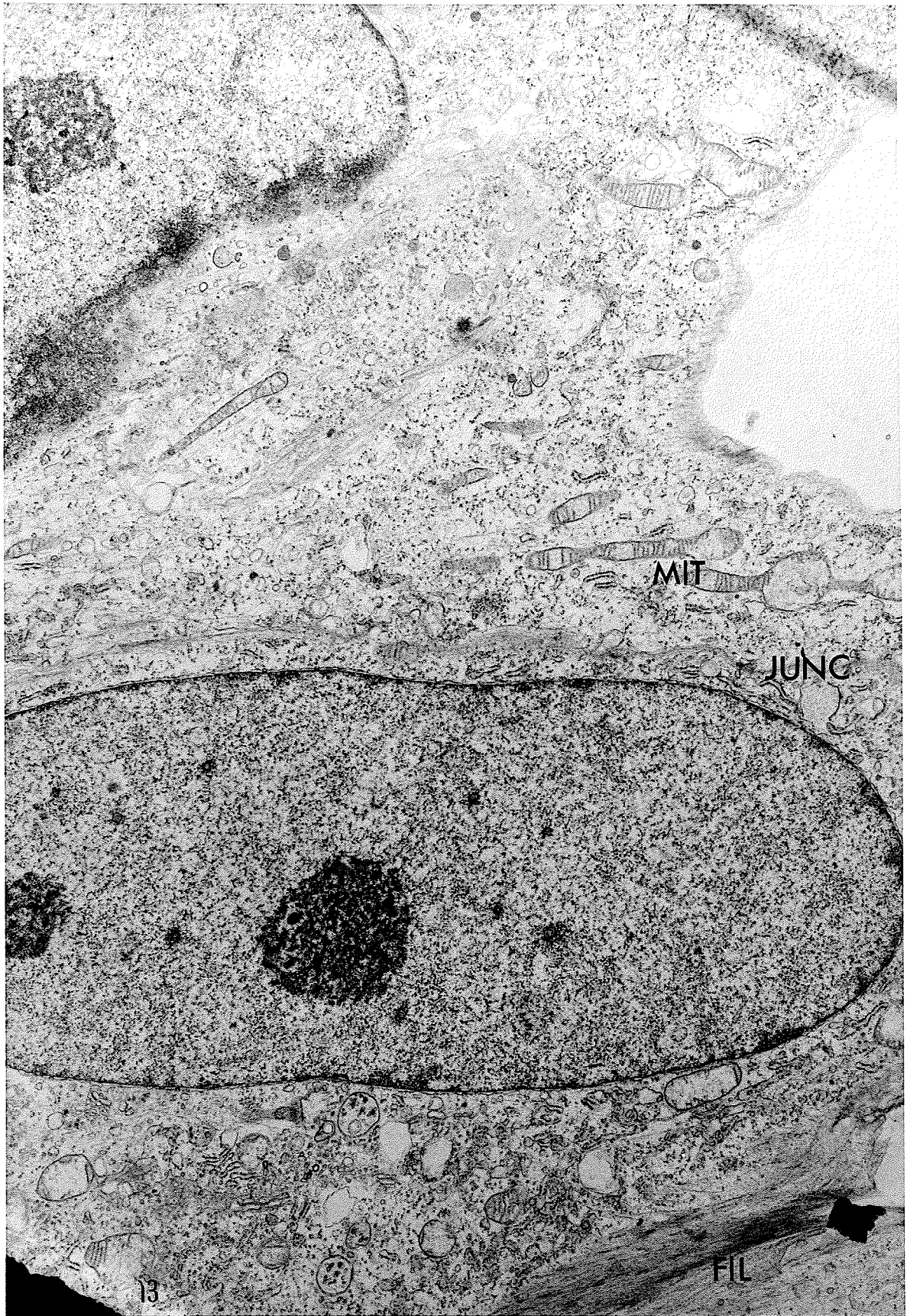


Figure 14. Normal human cultured amniotic fluid fibroblasts.
Note separation between cells(SEP). Also note
relative absence of unique cytoplasmic bodies.

X 18,700

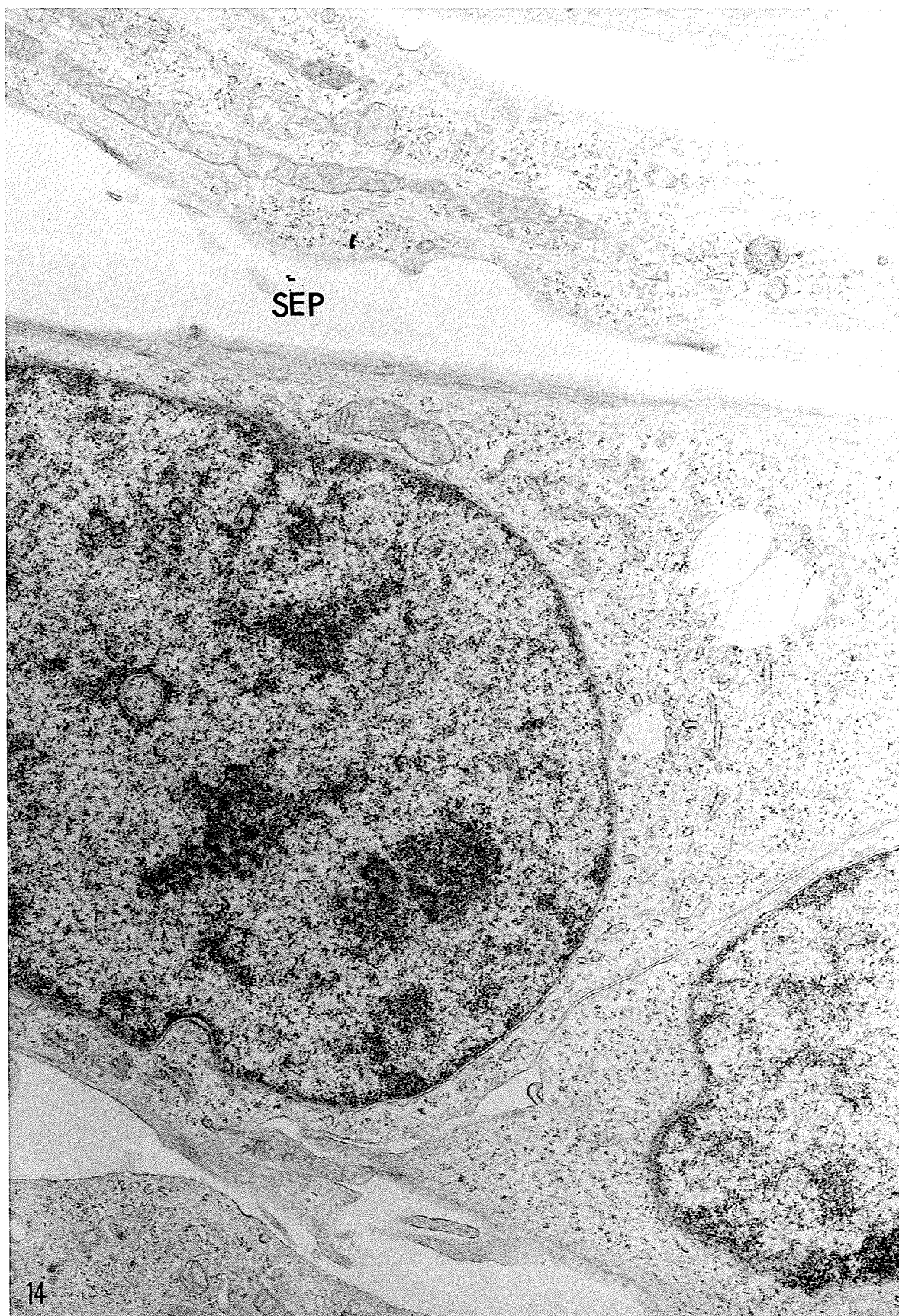


Figure 15. Normal human cultured amniotic fluid fibroblasts.
Note presence of microvilli at cell junction
(JUNC) and at free surface of cells(MV).

X 19,700

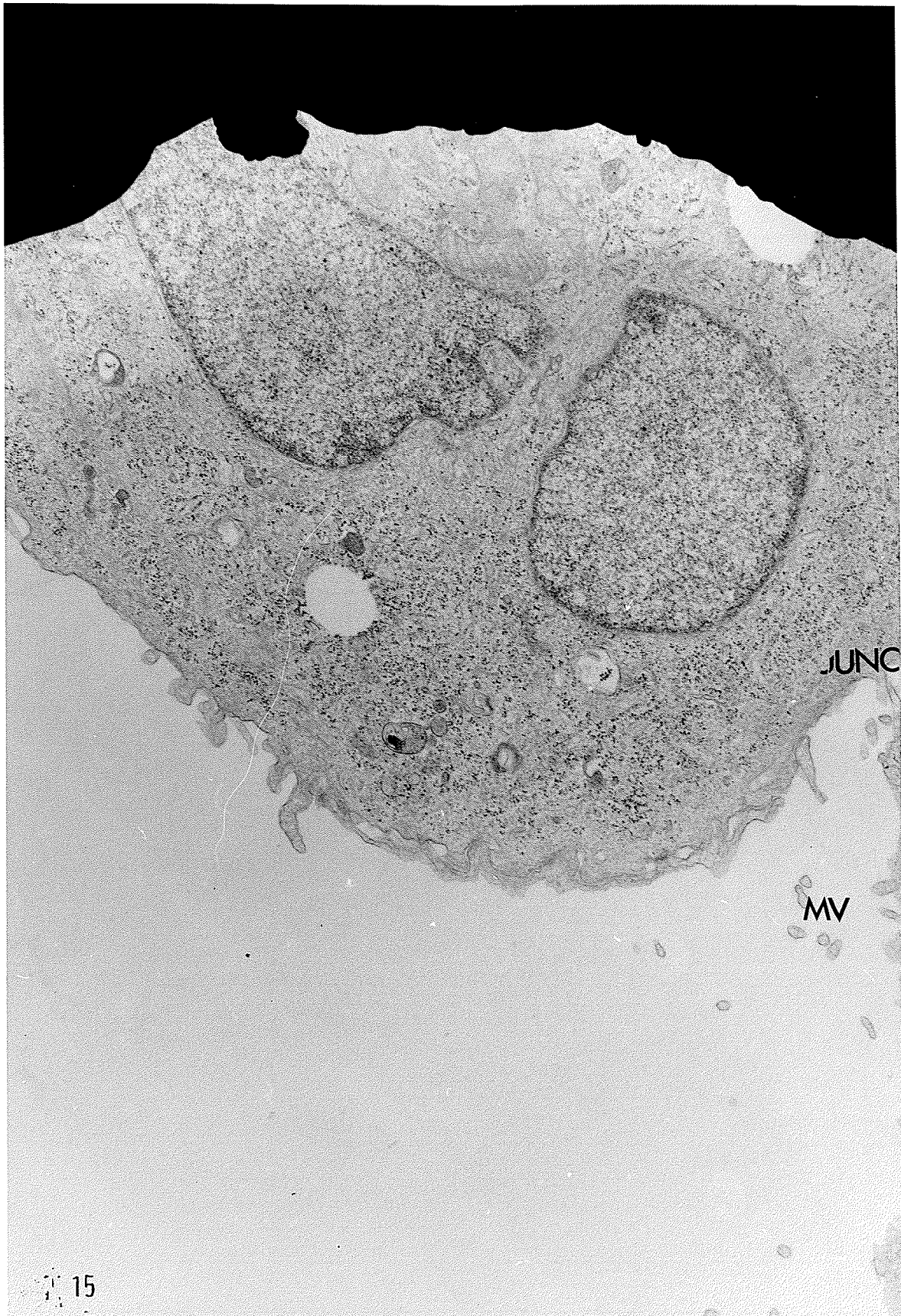


Figure 16. Human cultured normal amniotic fluid fibroblast.
Note lobulated nucleus and microvilli(MV).

X 19,700

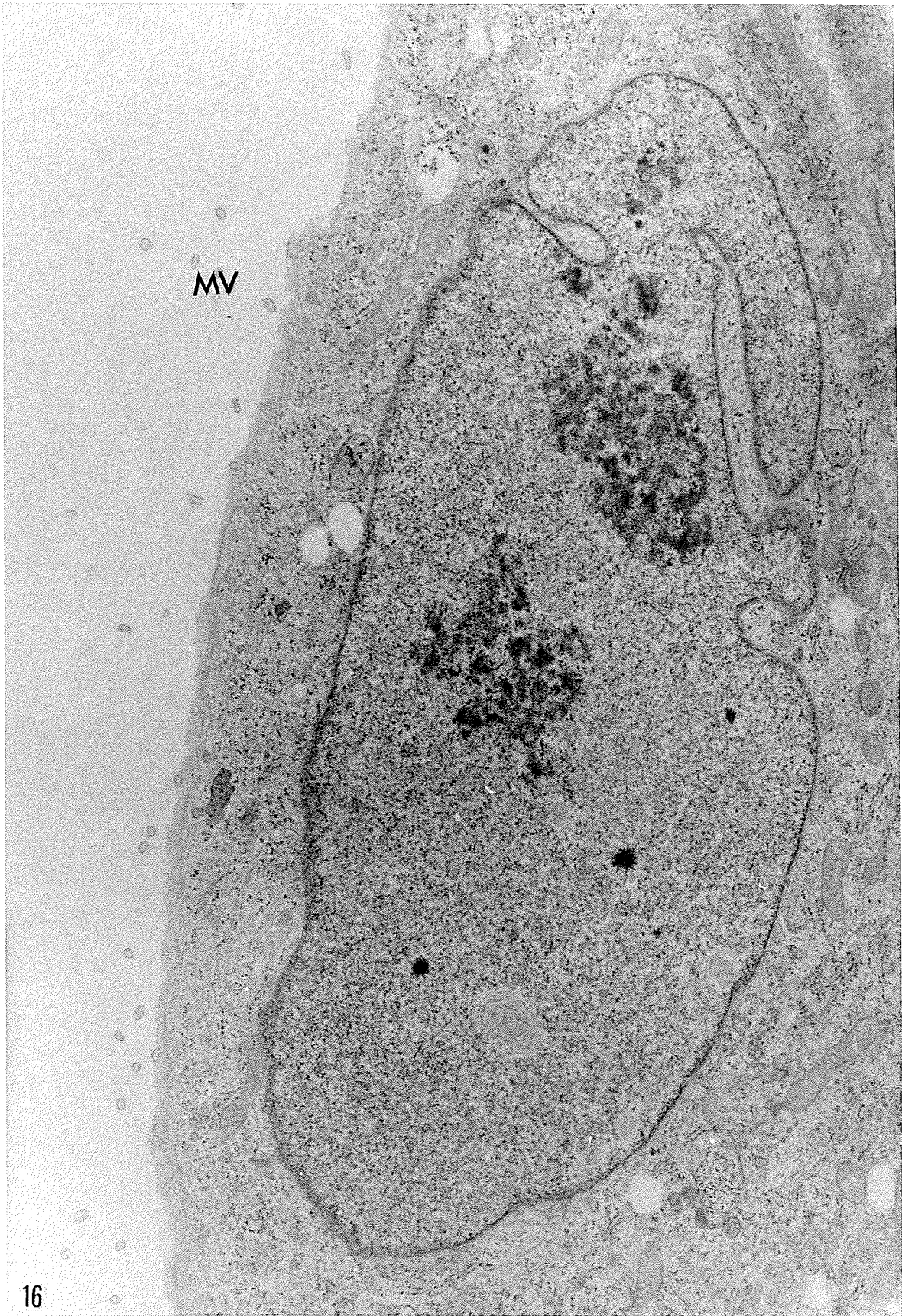


Figure 17. Normal human cultured amniotic fluid fibroblasts. Note well developed Golgi complex(GOL). Also shown is a unique cytoplasmic body(UCB) which was not observed in any other cell studied for this cell line.

X 17,100

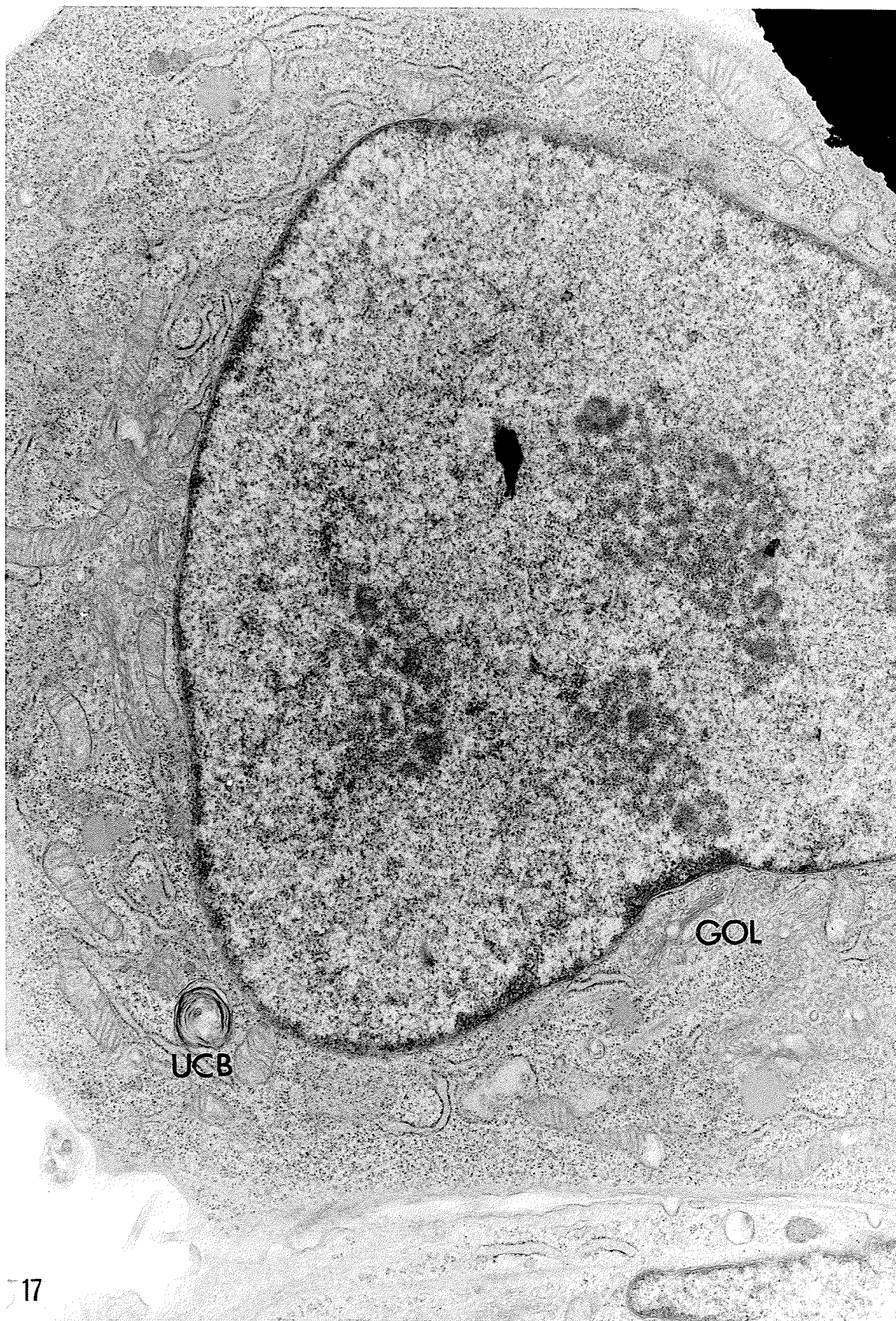


Figure 18. Normal human cultured amniotic fluid fibroblasts. Note cell junction(JUNC) and appearance of several unique cytoplasmic bodies(UCB). These bodies would not appear in subsequent passages of this cell line. Also note several nucleoli in the nucleus.

X 19,000

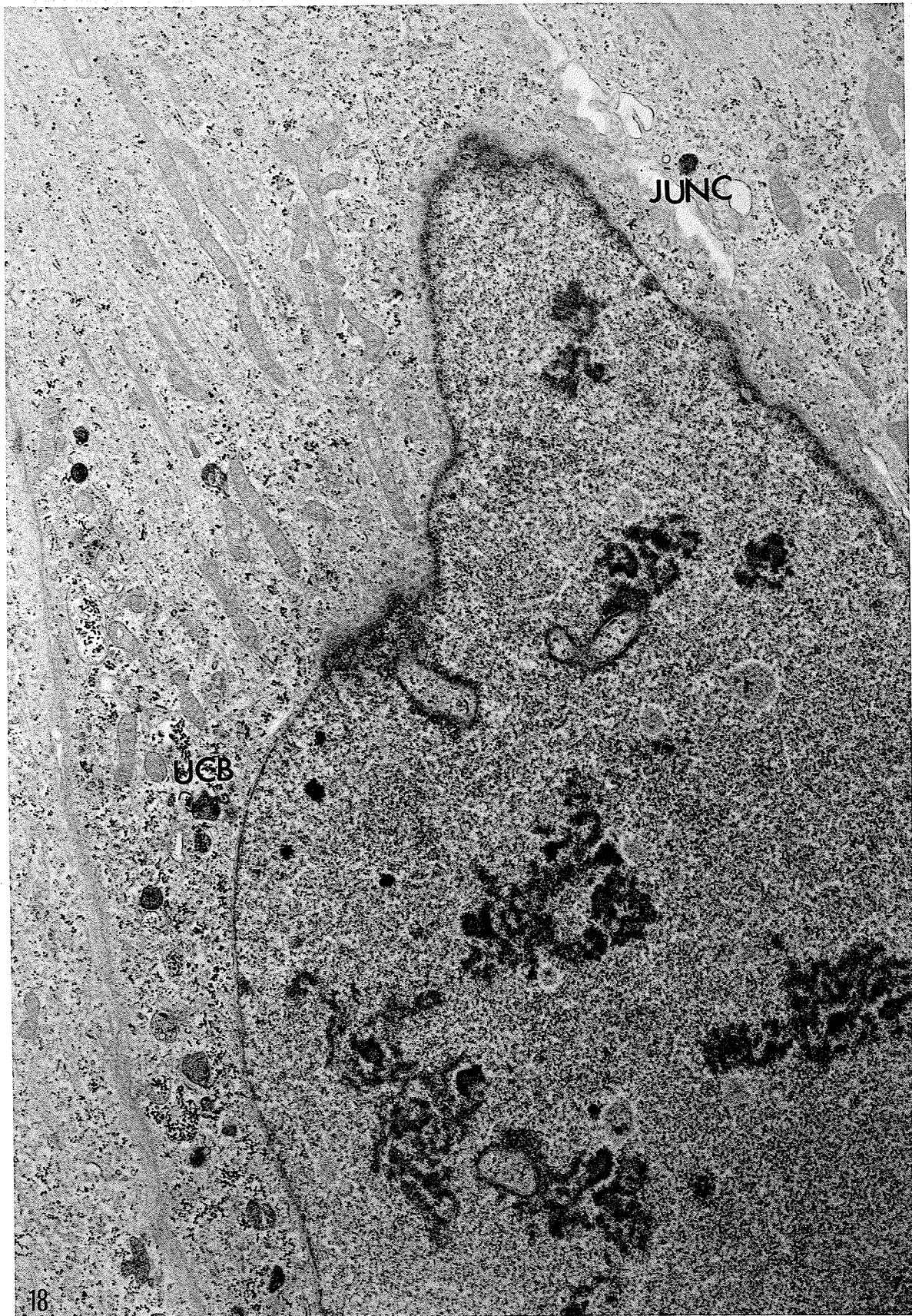
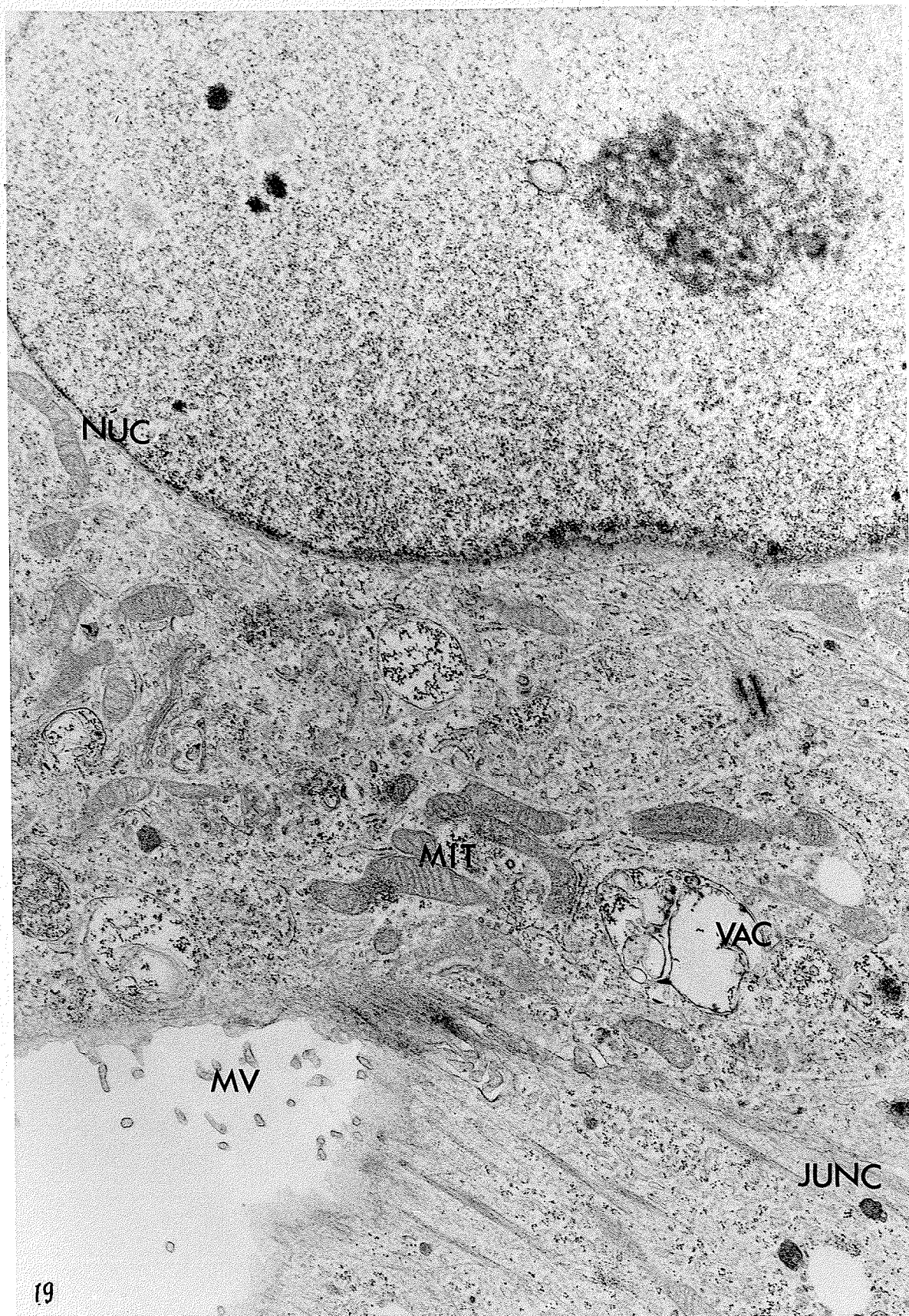


Figure 19. Normal human cultured amniotic fluid fibroblasts. Note appearance of mitochondria(MIT), microvilli (MV), and clearly defined nuclear membranes(NUC). Also shown are several large cytoplasmic vacuoles (VAC) and junction between the two cells(JUNC).

X 15,300



periphery. These blebs resembled microvilli and were frequently observed in the amniotic fluid fibroblasts. Figures 15, 16 and 19 show these microvilli.

The organelles of the amniotic fluid fibroblast seemed to be less centered around the nucleus of the cell.

DISEASED CELL LINES

Ultrastructural Appearance of Cultured Fibroblasts Derived from Patients Affected With Pompe's Disease

Pompe's disease, also termed Type II glycogen storage disease, is transmitted as an autosomal recessive condition (McKusick, 1975). Pompe's disease is apparently due to a deficiency of the enzyme alpha 1,4-glucosidase which is absent in homozygotes for the mutant gene, while heterozygotes have enzyme levels intermediate between that for affected homozygotes and that for the normal controls (Howell, 1972). The enzyme defect results in the alteration of the normal glycogenesis pathway. Prenatal diagnosis has been successfully performed in a number of cases by the determination of abnormal enzyme activity levels in both uncultured and cultured amniotic fluid cells (Howell, 1972).

One cell line, from a case of Pompe's disease biochemically confirmed by the Mutant Cell Repository, Montreal, Quebec, to be deficient for the enzyme alpha 1,4-glucosidase, was examined in this study through three consecutive passages. Figure 20 through to Figure 22 show examples of the ultrastructural appearance of the fibroblasts examined. The general morphology of the cells differed from the normal controls in that there was the consistent appearance of single membrane surrounded bodies

Figure 20. Human cultured fibroblasts biochemically confirmed to be homozygotic for Pompe's disease. Note appearance of numerous abnormal cytoplasmic bodies (ARROWS). These single membrane bound bodies were filled with material resembling β -glycogen. These bodies were observed in subsequent passages of the Pompe's disease fibroblasts. They were not observed in any other cell line studied.

X 15,700



Figure 21. Human cultured fibroblasts biochemically confirmed to be homozygotic for Pompe's disease. Arrows indicate the abnormal cytoplasmic bodies only observed in cells affected with Pompe's disease. These regularly shaped, single membrane bound bodies appeared to be filled with material resembling β -glycogen.

X 41,500

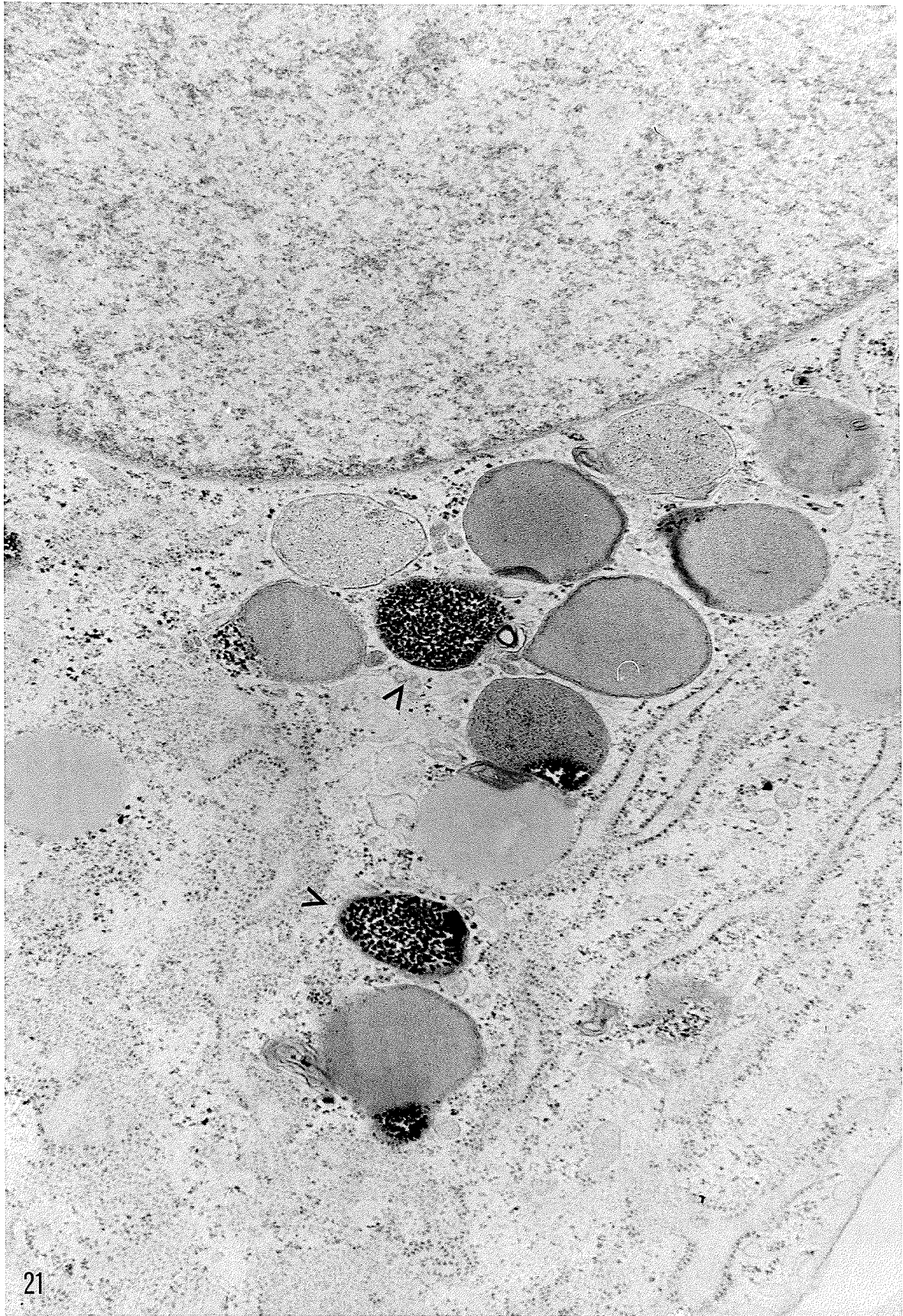
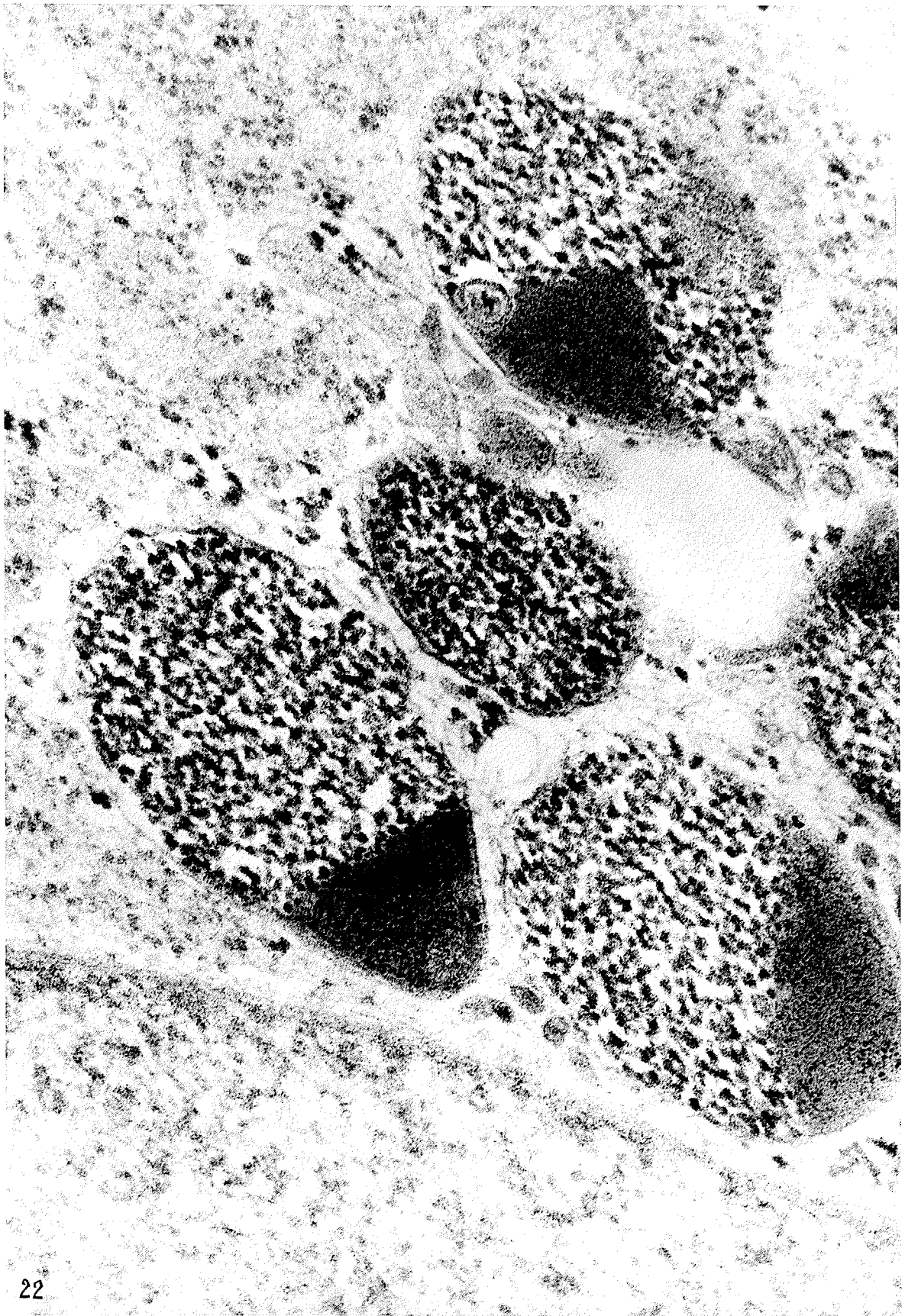


Figure 22. High magnification of abnormal cytoplasmic bodies consistently observed in human cultured fibroblasts biochemically confirmed to be homozygotic for Pompe's disease.

X 104,000



which contained large amounts of particles resembling β -glycogen. In some cell sections up to forty of these abnormal cytoplasmic bodies were observed. These bodies were not seen in any of the normal fibroblast controls nor in any of the other disease states studied. The bodies continued to appear from passage to passage indicating that they were a feature of the cell line.

No heterozygotic fibroblast lines were available for study.

No amniotic fluid fibroblast lines, either homozygotic or heterozygotic for Pompe's disease, were available for study.

Ultrastructural Appearance of Cultured Fibroblasts Derived from Patients Affected With Lesch-Nyhan Syndrome

Lesch-Nyhan syndrome is transmitted as an X-linked recessive condition (McKusick, 1975). The disease is due to deficiency of the enzyme hypoxanthine-guanine phosphoribosyl transferase, which is an enzyme involved in the purine metabolism of the cell (Seegmiller et al, 1967). Both the hemizygous and heterozygous states have been successfully diagnosed in amniotic fluid cells by biochemical methods (DeMars et al, 1969).

One cell line, obtained from the Mutant Cell Repository, known to be derived from an individual diagnosed to have the disease, was examined in this study through four consecutive passages. It was not possible to distinguish this cell line from normal controls by ultrastructural examination.

No heterozygotic fibroblast lines were studied.

No amniotic fluid fibroblast lines, either hemizygotic or heterozygotic for Lesch-Nyhan syndrome, were available for study.

Ultrastructural Appearance of Cultured Fibroblasts Derived from Patients Affected With Maple Syrup Urine Disease

Maple syrup urine disease is transmitted as an autosomal recessive condition (McKusick, 1975). It is apparently due to a deficiency of the enzyme branched chain ketoacid decarboxylase which results in accumulation in the cells of free amino acids, particularly leucine, isoleucine and valine.

Biochemical methods have successfully allowed the prenatal diagnosis of the disease to be made (Becker and Seegmiller, 1975).

One cell line known to be derived from a patient diagnosed to have maple syrup urine disease was obtained from the Mutant Cell Repository in Montreal. It was studied through three passages before being lost to contamination. It was impossible to ultrastructurally distinguish this line from normal control cell lines.

No heterozygotic cell lines from skin biopsies were available for examination.

No amniotic fluid cell lines were available for study.

Ultrastructural Appearance of Cultured Fibroblasts Derived from Patients Affected With Infantile Cystinosis

Infantile cystinosis is transmitted as an autosomal recessive condition. In cultured cells this results in an increase of free cystine in the cells (McKusick, 1975). The specific enzyme defect is unknown. Prenatal diagnosis has been made by the demonstration of increased levels of non protein cystine in the amniotic fluid cells (Schneider et al, 1974).

One cell line from a known case of infantile cystinosis was obtained from the Mutant Cell Repository in Montreal. It was indistinguishable

from the normal control cell lines.

No heterozygotic cell lines were available for study.

Two amniotic fluid cell lines were available for study. One was purchased from the Montreal Mutant Cell Repository; the second was kindly provided by Dr. J. Lockwood, University of California, School of Medicine, San Diego. Both cell lines had a fine structure indistinguishable from normal amniotic fluid fibroblast control lines.

Ultrastructural Appearance of Cultured Fibroblasts Derived from Patients Affected With I-Cell Disease

I-cell disease, also termed mucopolidosis II, is so-called because of the large number of inclusions that are evident in the cells when observed by light microscopy. It is transmitted as an autosomal recessive disease. No specific enzyme defect has been demonstrated for this condition; however, it has been noted that a large number of lysosomal enzymes are strikingly deficient (Leroy and Spranger, 1970). Prenatal diagnosis has been successfully accomplished based on the demonstration of these enzyme deficiencies.

One cell line derived from a patient diagnosed to have I-cell disease was purchased for this study from the Montreal Mutant Cell Repository. It was examined through four consecutive passages and was easily distinguishable from normal control fibroblasts and all other disease states. Numerous cytoplasmic bodies were observed to be present. These are seen in Figure 23 through to Figure 26. These bodies varied in size up to approximately 2.5 micrometers in diameter. They were very pleomorphic in appearance, some being translucent, others electron dense, others were vacuolated, others contained evidence of residual membranes. Some cell sections contained in excess of eighty of these bodies.

Figure 23. Human cultured fibroblasts biochemically confirmed to be homozygotic for I-cell disease. Note that cytoplasm is filled with lysosomes. This excessive accumulation of pleomorphic lysosomes was not observed in any other diseased fibroblasts, nor in any normal control. These bodies were consistently present on other passages examined for this cell line.

X 7,650

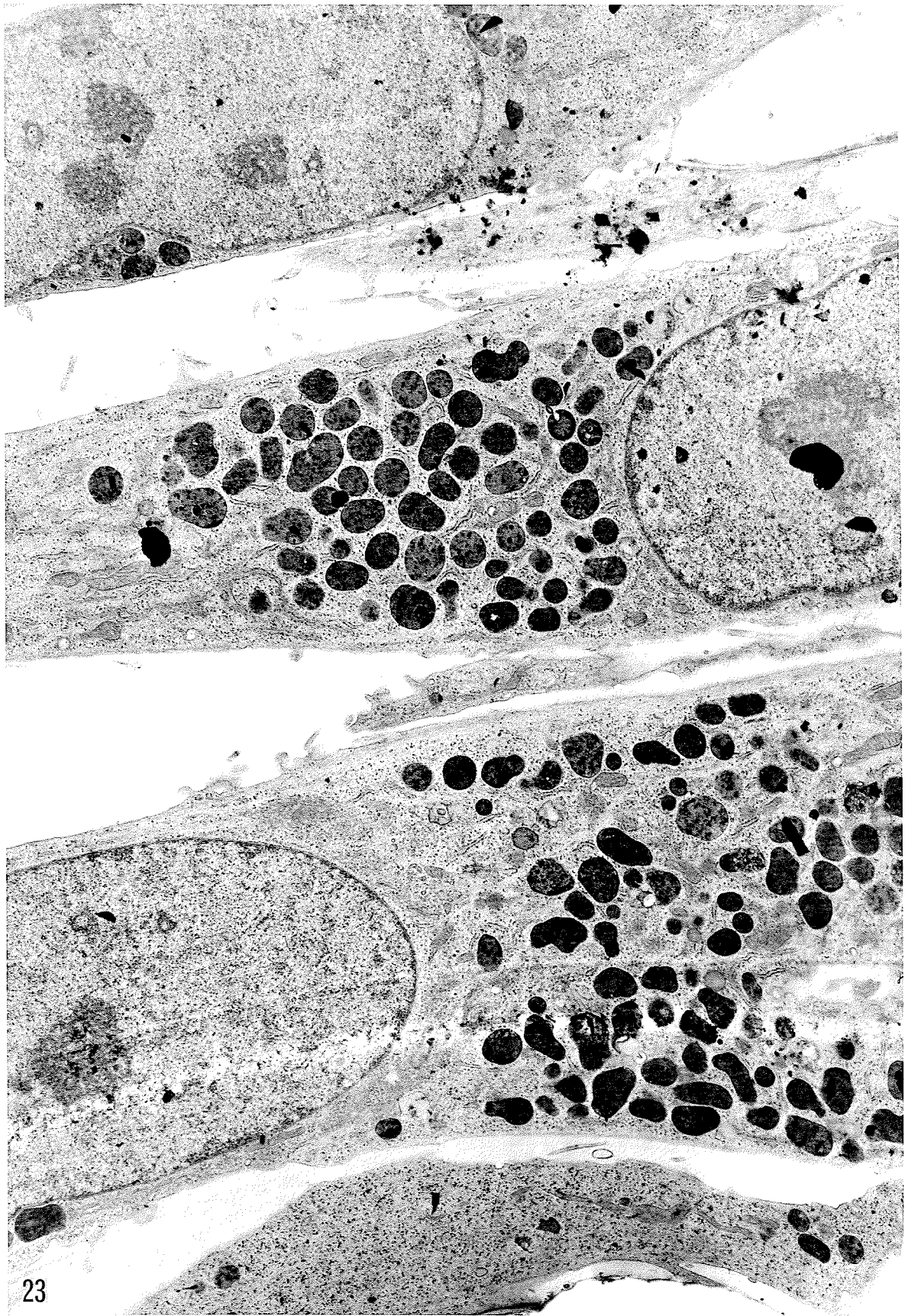


Figure 24. Higher magnification of Figure 23. Note cytoplasm is filled with excessive number of lysosomes.

X 16,200

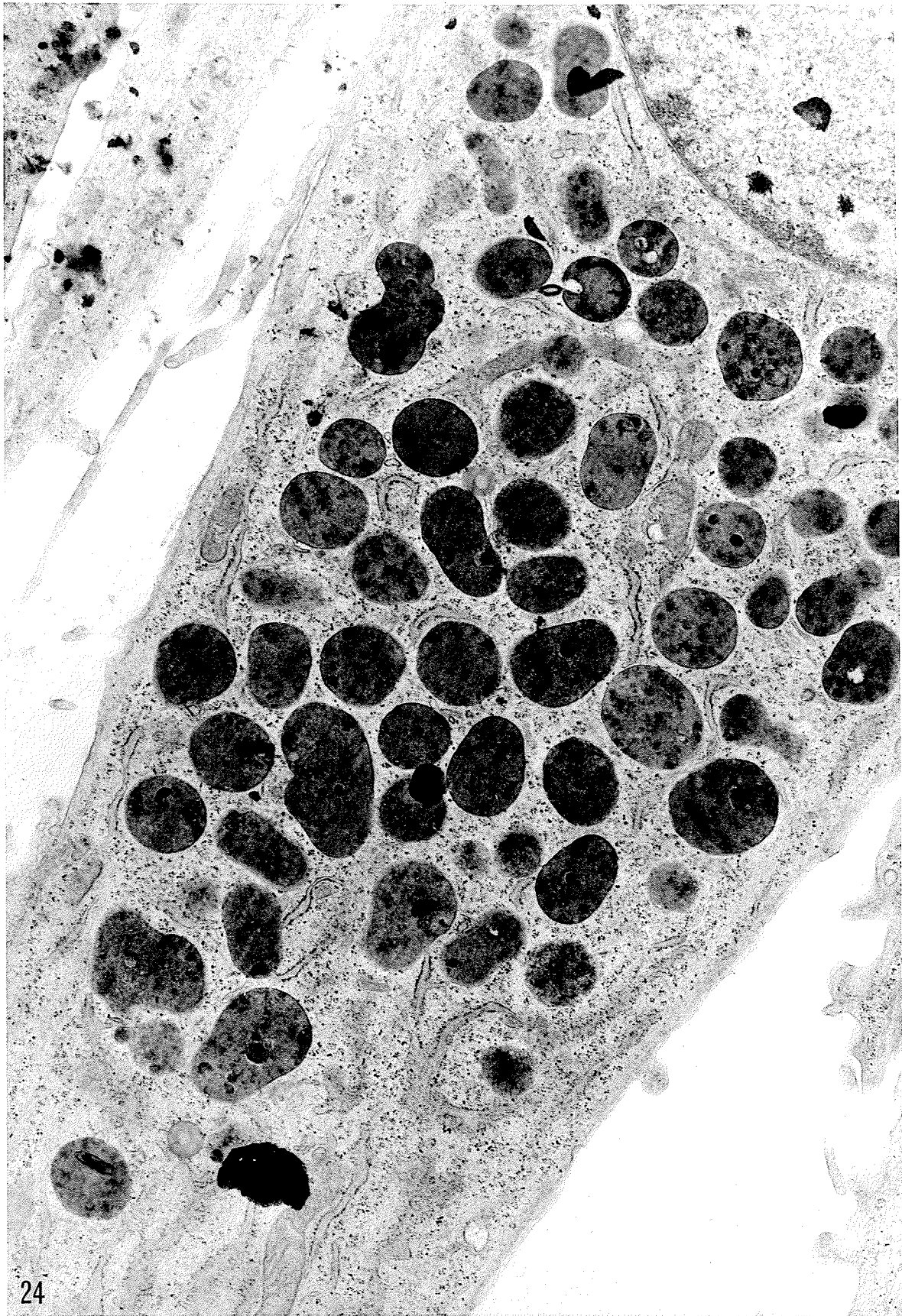


Figure 25. Human cultured fibroblast biochemically confirmed to be homozygotic for I-cell disease. This cell is apparently in mitotic division. Note condensed chromosomes(CHR) and excessive number of lysosomes observed only in cells affected with I-cell disease.

X 11,100

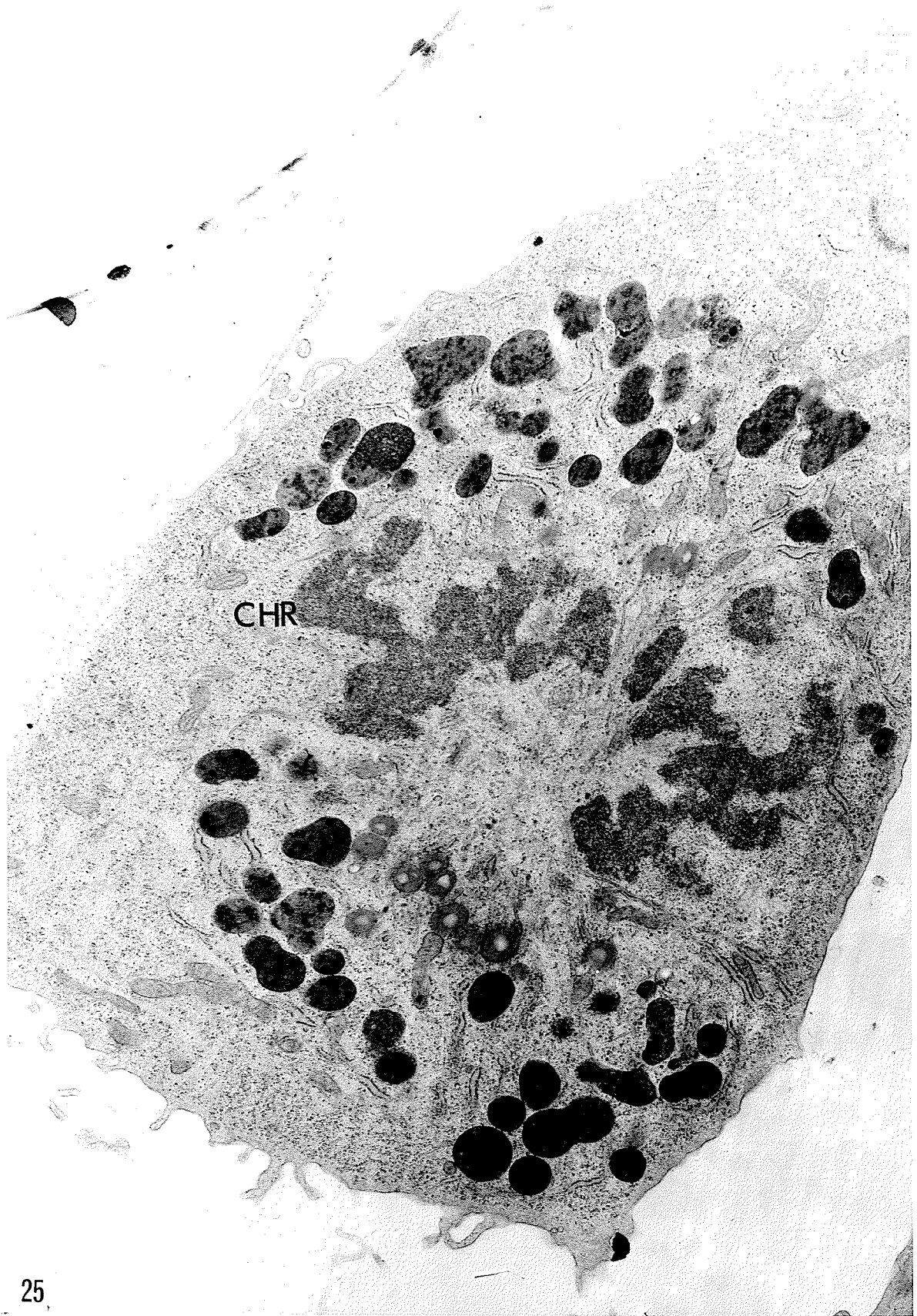
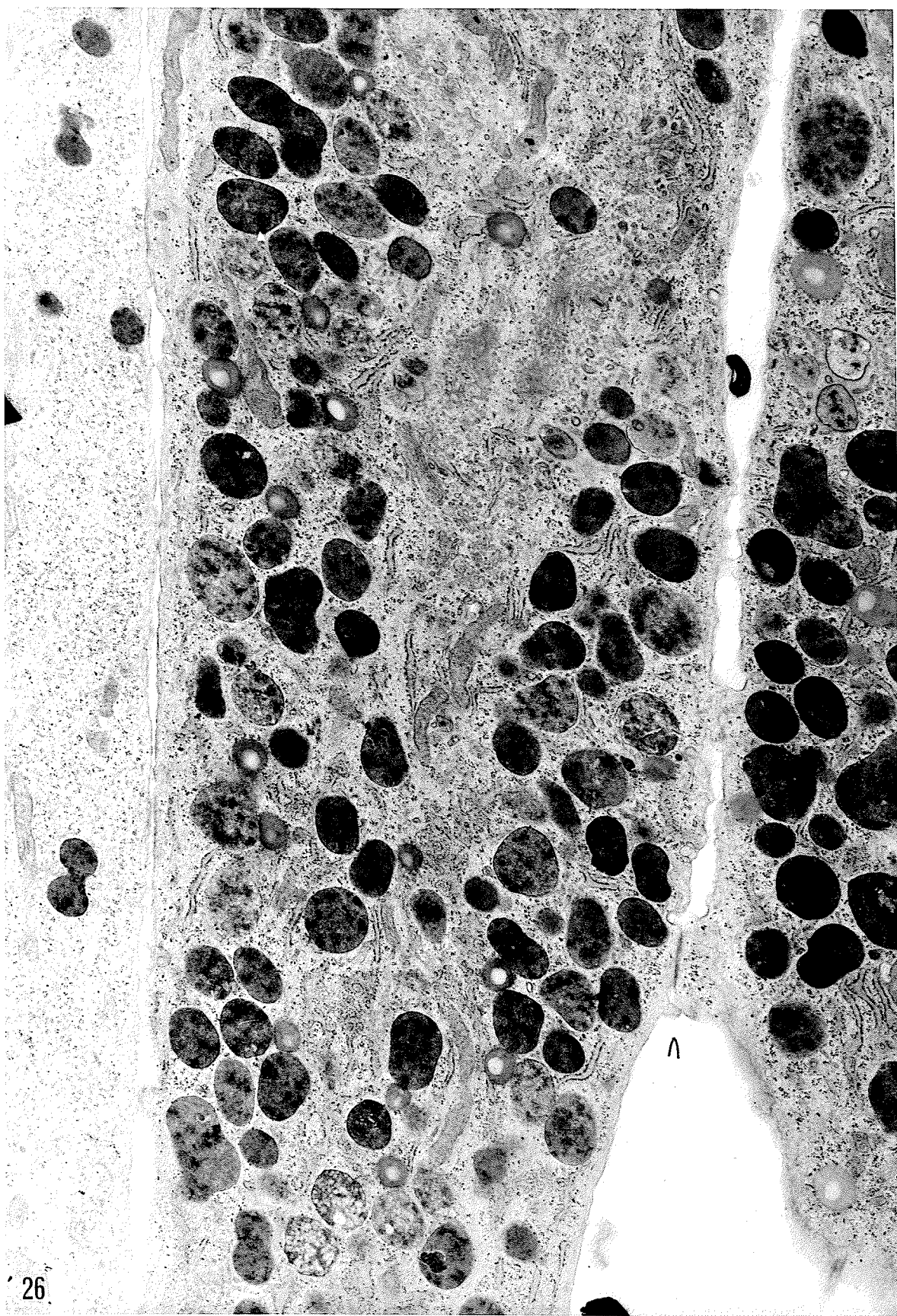


Figure 26. Human cultured fibroblasts biochemically confirmed to be homozygotic for I-cell disease. Note excessive number of lysosomes filling cytoplasm of cells. Arrow shows one of few desmosomal junctions seen in cultured fibroblasts.

X 13,200



One heterozygotic fibroblast line was available for study. It was purchased from the Mutant Cell Repository in Camden. The cells were indistinguishable from normal control fibroblasts.

No amniotic fluid fibroblast lines were available for study.

Ultrastructural Appearance of Cultured Fibroblasts Derived from Patients Affected With Fabry's Disease

Fabry's disease, also termed diffuse angiokeratoma, is transmitted as an X-linked recessive condition (McKusick, 1975). The primary enzyme defect is due to a deficiency of ceramide trihexosidase which results in a disturbance of the glycosphingolipid metabolism of the cell (Sweeley et al, 1972). Prenatal diagnosis of Fabry's disease has been successfully accomplished by biochemical assay of cultured fibroblasts (Brady et al, 1971).

Six cell lines, which were confirmed by the Cell Banks to be hemizygotic for Fabry's disease, were examined in this study for four consecutive passages. Typical cytoplasmic bodies were seen in the cells making them ultrastructurally easily distinguishable from the normal controls and from all other disease states. Examples are seen in Figure 27 through to Figure 29. These bodies are membrane bound, evenly staining, and conspicuous by their number. Up to 40 could be observed in some cell sections. These cytoplasmic bodies resembled the lipid storage bodies seen in other cell lines but the excessive numbers observed in cultured fibroblasts from patients with Fabry's disease made these lines easily distinguishable.

One cell line derived from a skin biopsy from a heterozygote for Fabry's disease was obtained. No abnormality was noted in any of the four passages studied.

Figure 27. Human cultured fibroblasts biochemically confirmed to be homozygotic for Fabry's disease. Note excessive number of cytoplasmic bodies which resemble lipid storage bodies. Accumulation of excessive numbers of these cytoplasmic bodies was seen only in those fibroblasts known to be derived from individuals affected with Fabry's disease.

X 34,500

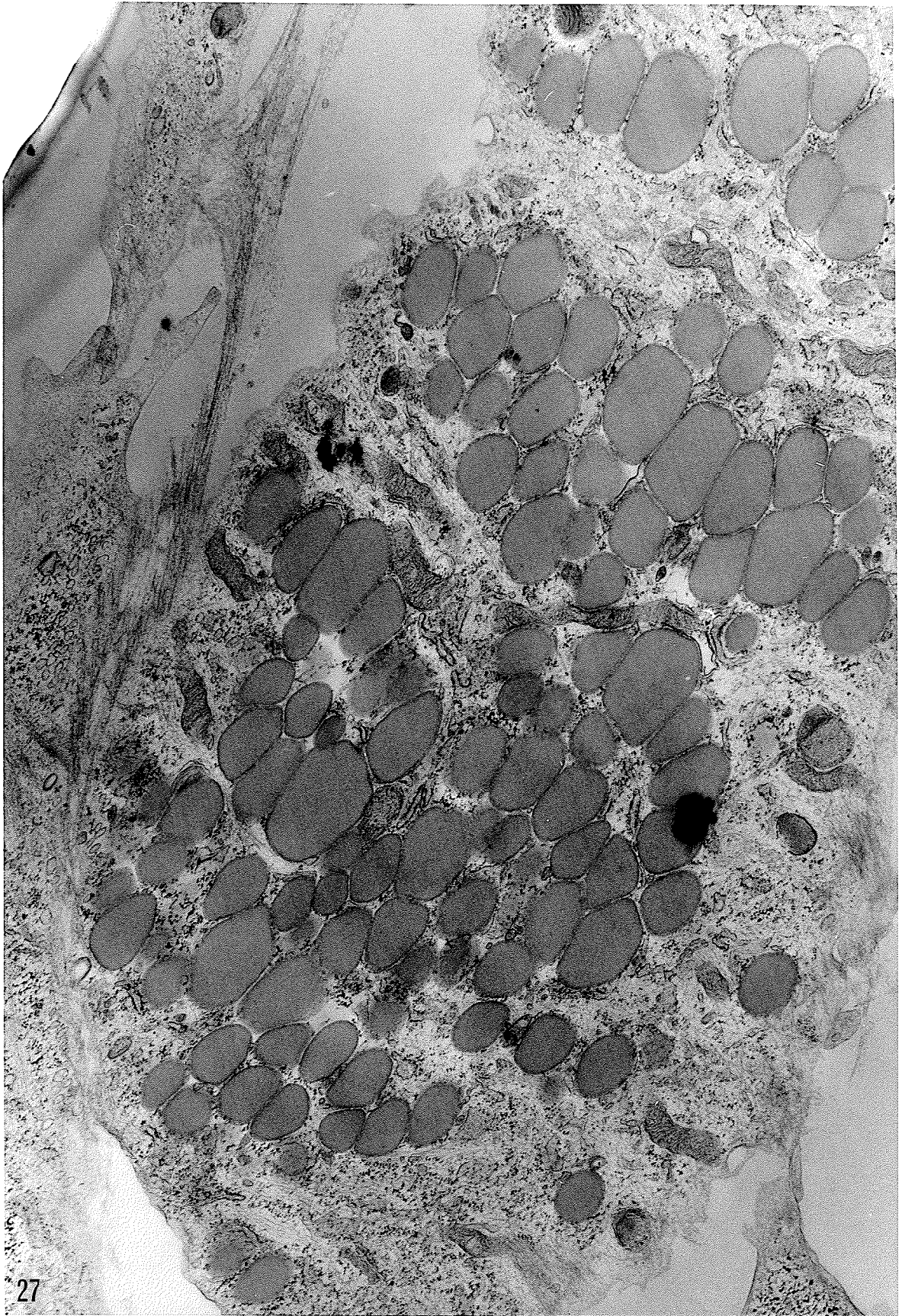


Figure 28. Human cultured fibroblast known to be affected with Fabry's disease. Note excessive number of cytoplasmic bodies which resemble lipid storage bodies. Compare these bodies to those seen in Figure 27.

Difference in staining density was attributed to variation in staining and fixation techniques.

X 16,500

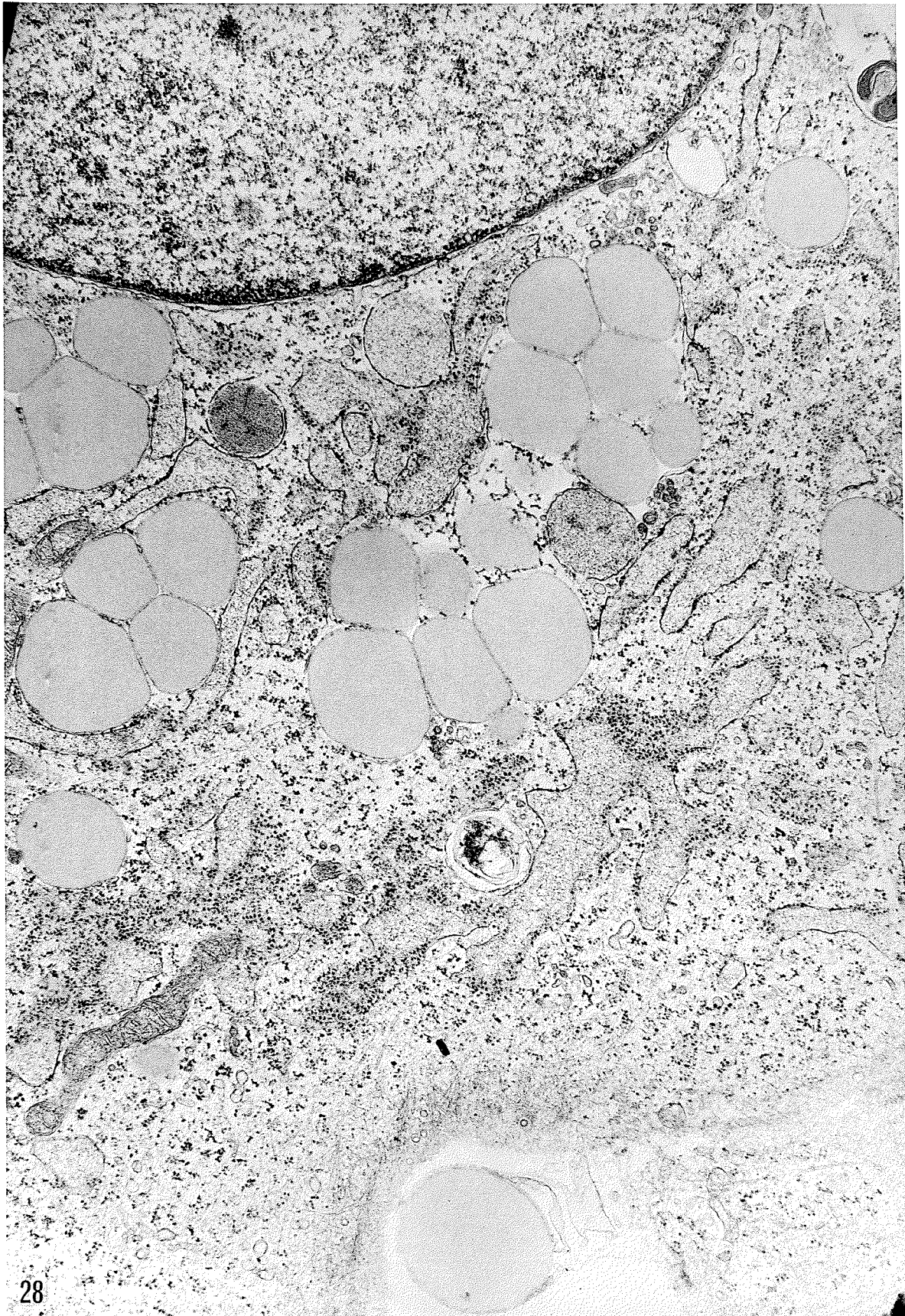
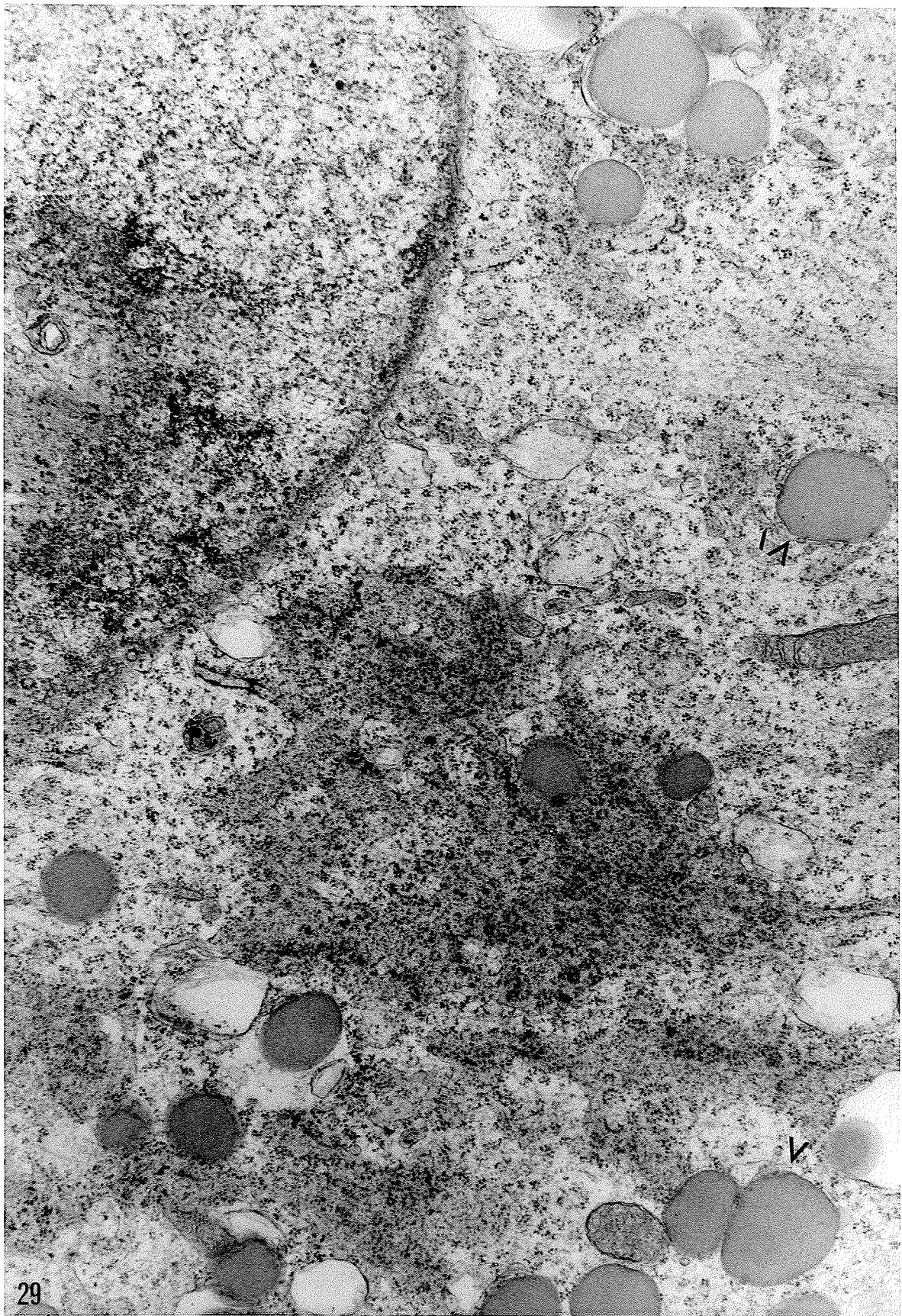


Figure 29. Human cultured fibroblast biochemically confirmed to be affected with Fabry's disease. Note appearance of numerous bodies resembling lipid storage bodies(Arrows).

X 37,800



One amniotic fluid fibroblast line from an amniotic fluid confirmed to be affected with Fabry's disease was available from the Mutant Cell Repository, Camden. This line showed the appearance of large numbers of cytoplasmic bodies similar to those seen in the skin fibroblast lines. Figure 30 and Figure 31 show these bodies in the amniotic fluid fibroblasts. The frequency of the single membrane bound, evenly staining bodies appeared to be slightly less than that observed in adult skin fibroblasts; however, the studies were not designed for quantitative analysis and this observation cannot be stated conclusively.

Ultrastructural Appearance of Cultured Fibroblasts Derived from Patients Affected With Tay-Sachs Disease

Six skin fibroblast lines which had been demonstrated by the Cell Banks to be deficient in the enzyme hexosaminidase A and thus were homozygous for the mutant gene causing Tay-Sachs disease, also termed GM2-gangliosidosis (Sloan et al, 1972), were available for study. Hexosaminidase A deficiency results in disturbance of sphingolipid metabolism and produces an accumulation of GM2 ganglioside in the tissues (Volk et al, 1975). Prenatal diagnosis has been successfully achieved by the demonstration of deficient enzyme activity in amniotic fluid cells (Schneck et al, 1970).

The six lines, purchased from the Mutant Cell Repository in Montreal and the Mutant Cell Repository in Camden, were each studied through four passages. For each of the cell lines, and every passage studied, abnormal cytoplasmic bodies were seen. These bodies were not observed in any of the normal fibroblast lines nor in cell lines from any other disease state. The bodies varied in appearance and number but their presence was considered to be specific for the disease. The bodies

Figure 30. Human cultured amniotic fluid fibroblast biochemically confirmed to be hemizygotic for Fabry's disease. Note excessive number of bodies resembling lipid storage bodies.

X 37,800

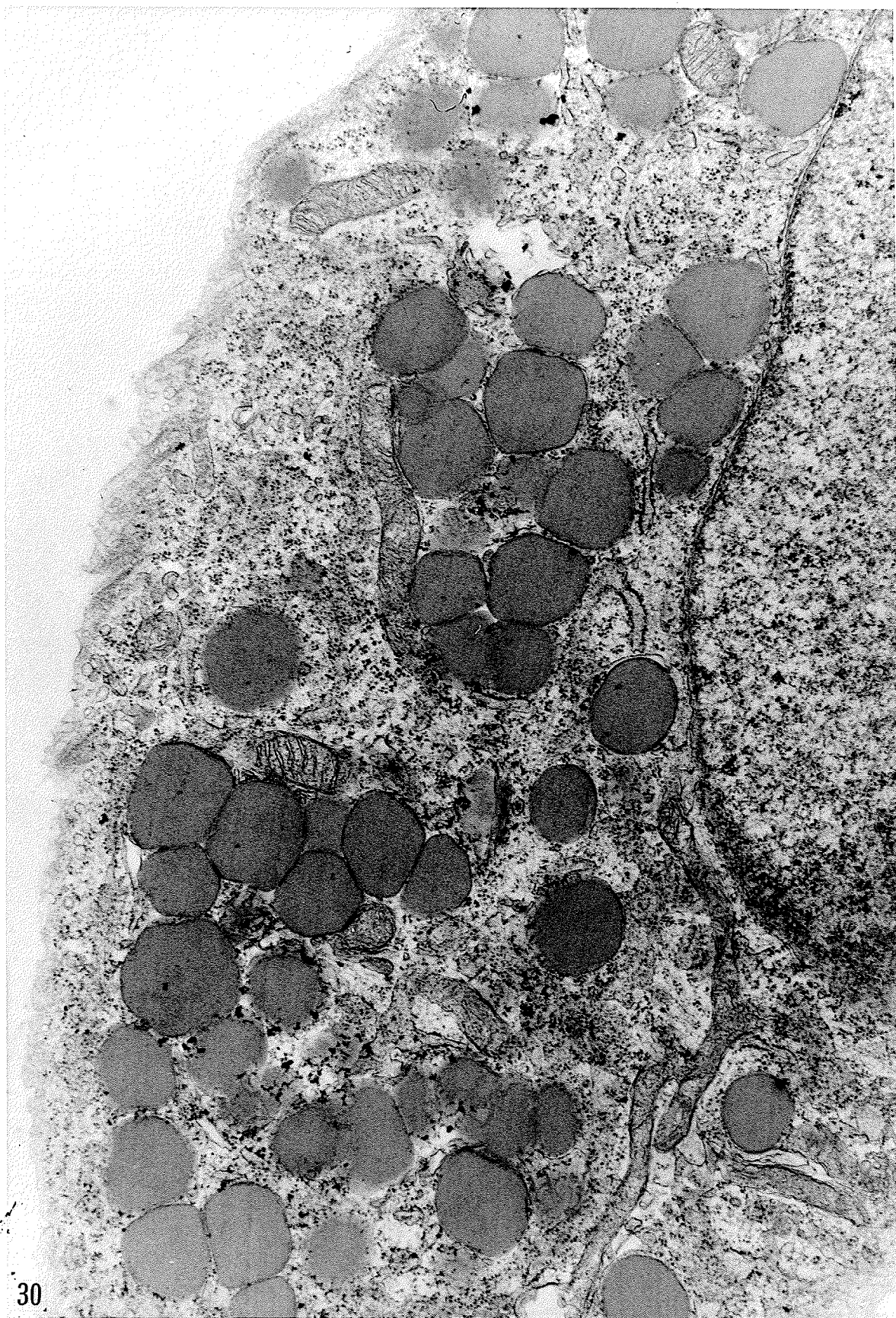
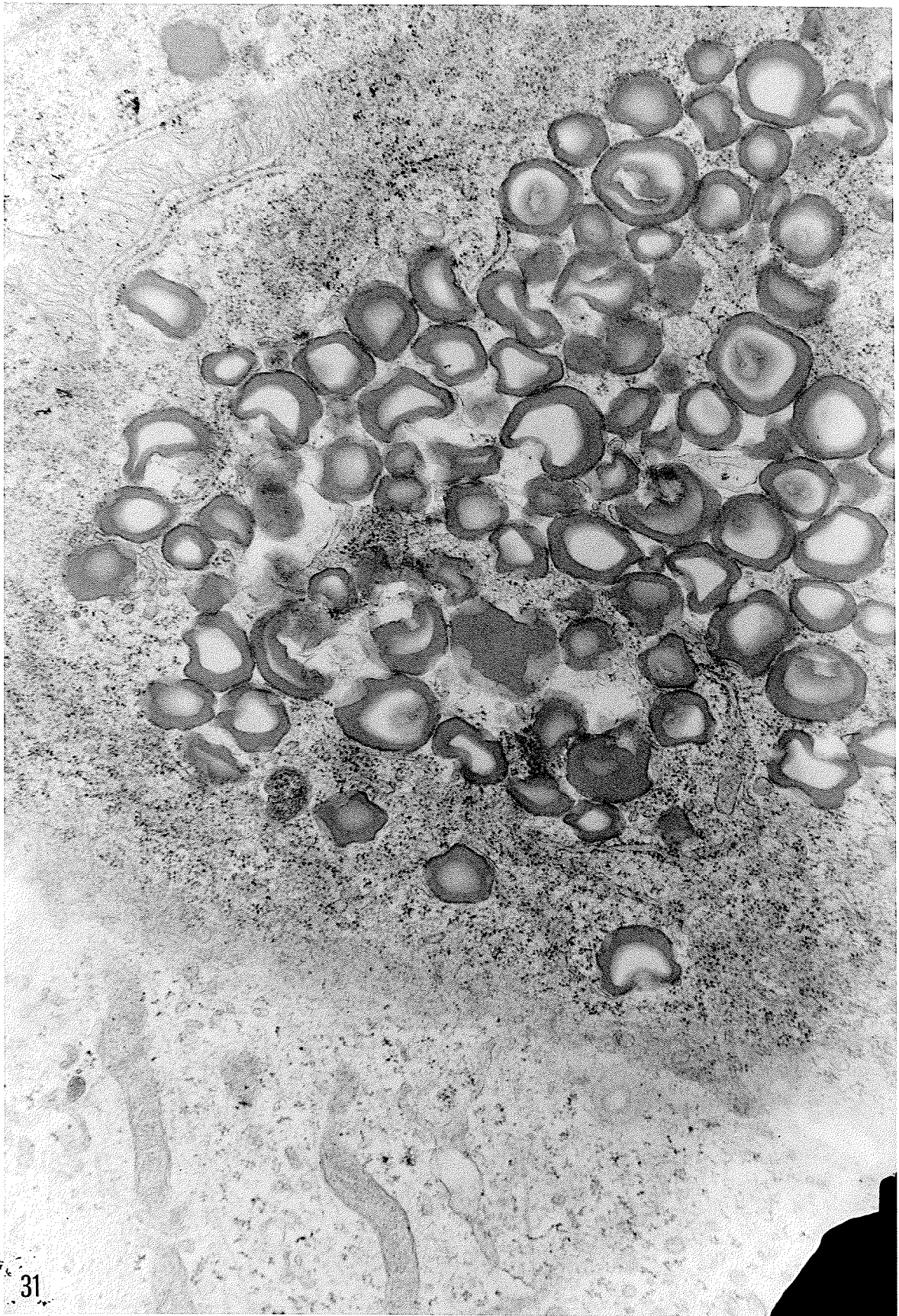


Figure 31. Human cultured amniotic fluid fibroblast biochemically confirmed to be homozygotic for Fabry's disease. Note excessive number of cytoplasmic bodies resembling lipid storage bodies. 'Leached out' centers of these bodies due to faulty processing for electron microscopy.

X 39,000



ranged in size from 0.5 micrometers to 2.0 micrometers and were composed of concentrically wound membranes which surrounded a central core. Figure 32 through to Figure 36 show examples of these abnormal cytoplasmic bodies. Variations among the bodies were seen. These variations ranged from a body which showed the presence of only one or two membranes concentrically arranged through to bodies measuring 2.0 micrometers which were filled with these concentrically arranged membranes. Two cell lines derived from individuals heterozygotic for Tay-Sachs disease were studied. The bodies described and considered to be characteristic for Tay-Sachs disease were not observed nor was any abnormality observed which could distinguish these cells from normal control fibroblasts.

Four cell lines derived from amniotic fluids deficient for hexosaminidase A activity were available for study. Three cell lines were purchased from the Mutant Cell Repository in Montreal; the fourth line was purchased from Dr. J. O'Brien, University of California in San Diego. Bodies similar to those observed in postnatal fibroblast lines from individuals known to be affected with Tay-Sachs disease were also observed in each of the four amniotic fluid cell lines. They varied in size from 0.4 micrometers to 1.8 micrometers. These bodies are shown in Figures 37, 38 and 39. No normal amniotic fluid fibroblasts showed any evidence of these bodies.

Heterozygotic amniotic fluid fibroblast lines were not available for study.

Figure 32. Human cultured fibroblast biochemically confirmed to be homozygotic for Tay-Sachs disease. Arrow indicates abnormal cytoplasmic body observed only in fibroblasts known to be affected with Tay-Sachs disease. These bodies were consistently observed in each passage examined of fibroblasts known to be affected with Tay-Sachs disease.

X 18,700

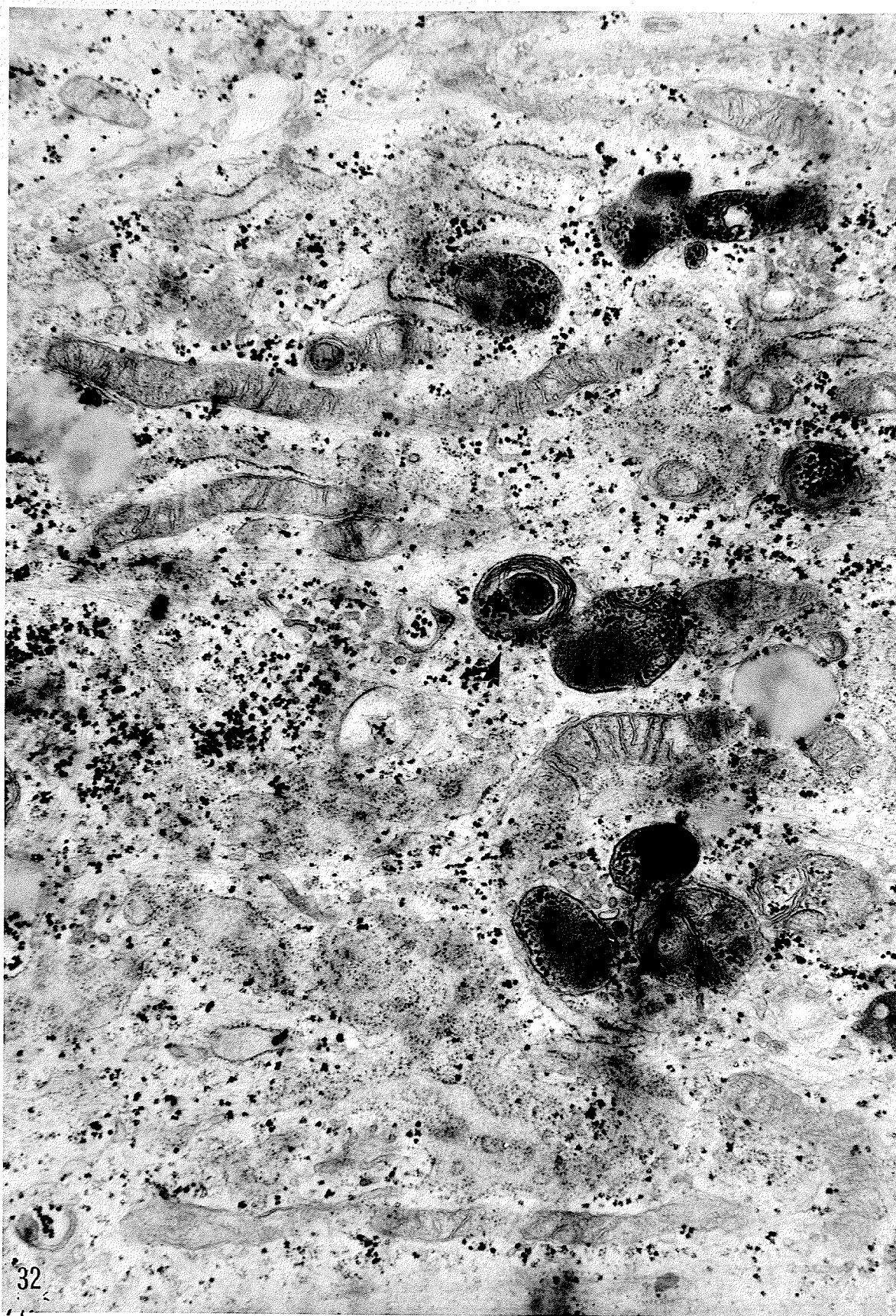


Figure 33. Higher magnification of Figure 32. This type of body (ARROW) was not seen in any cell lines except for lines biochemically confirmed to be homozygotic for Tay-Sachs disease.

X 115,000

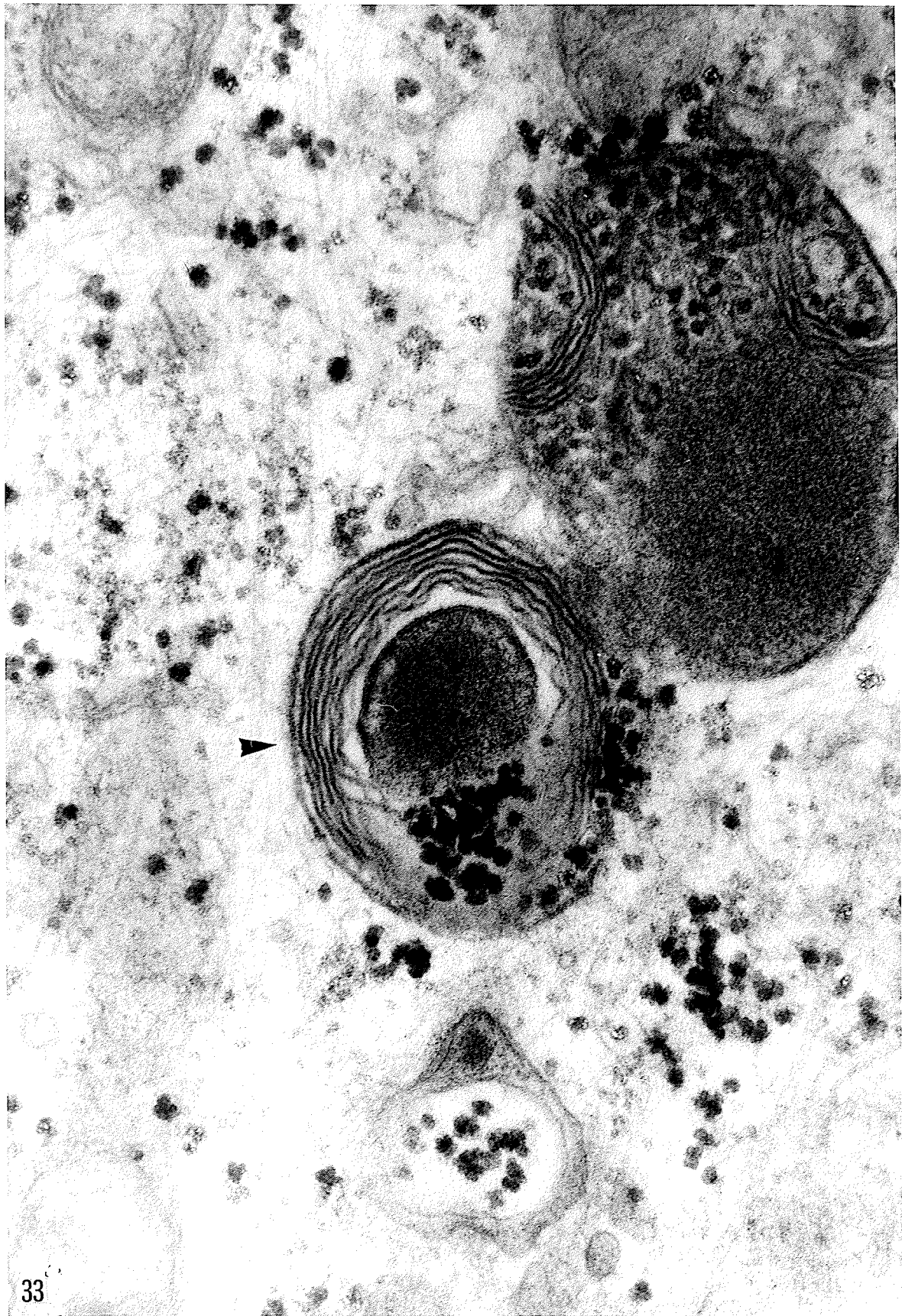


Figure 34. Human cultured fibroblasts biochemically confirmed to be homozygotic for Tay-Sachs disease. Arrows indicate several of the abnormal cytoplasmic bodies found only in cells affected with Tay-Sachs disease.

X 15,700

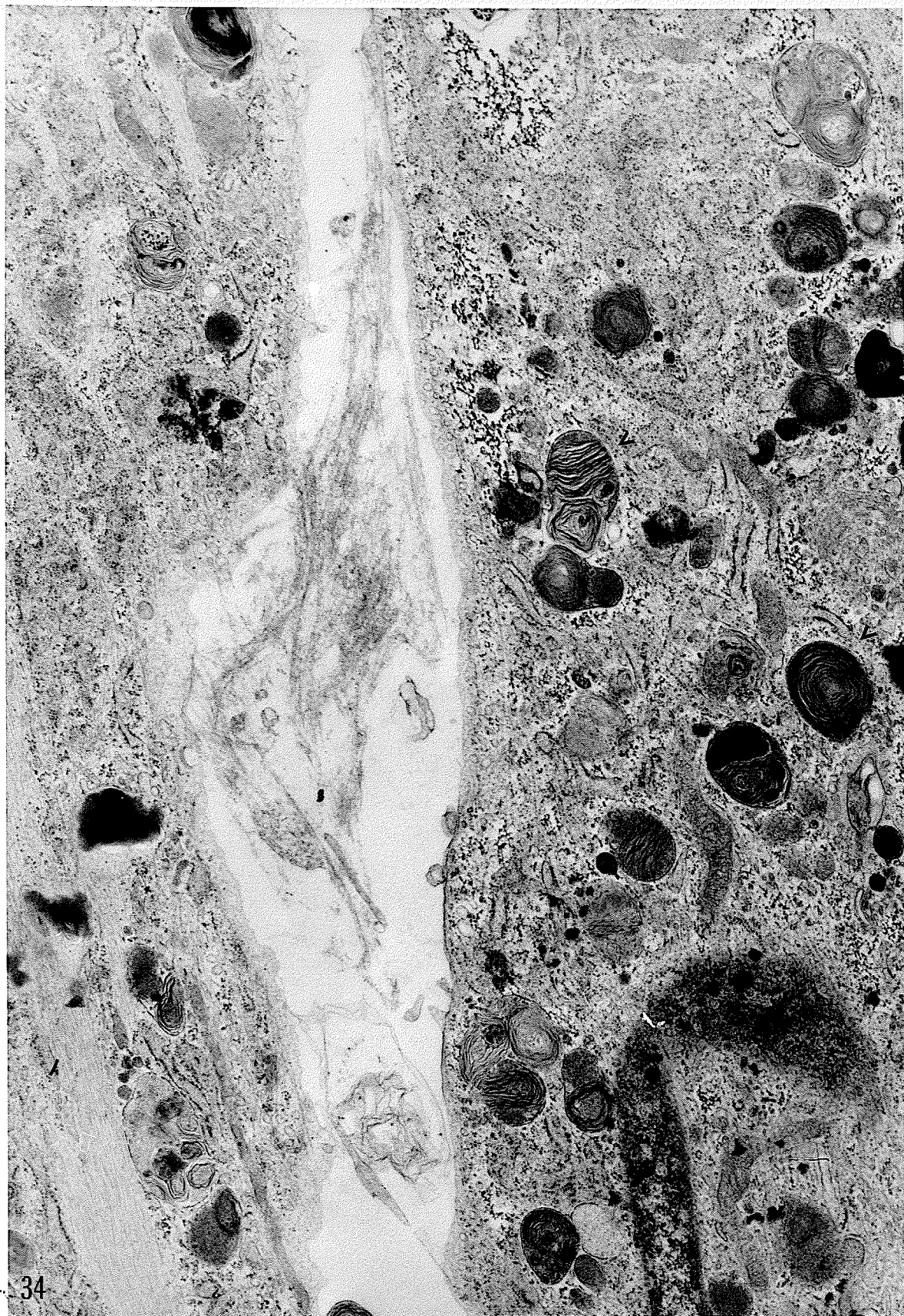


Figure 35. Human cultured fibroblast biochemically confirmed to be homozygotic for Tay-Sachs disease. Note cytoplasm is filled with membrane bound bodies filled with varying amounts of membranous structures. These bodies were observed in all subsequent passages of this cell line.

X 16,500

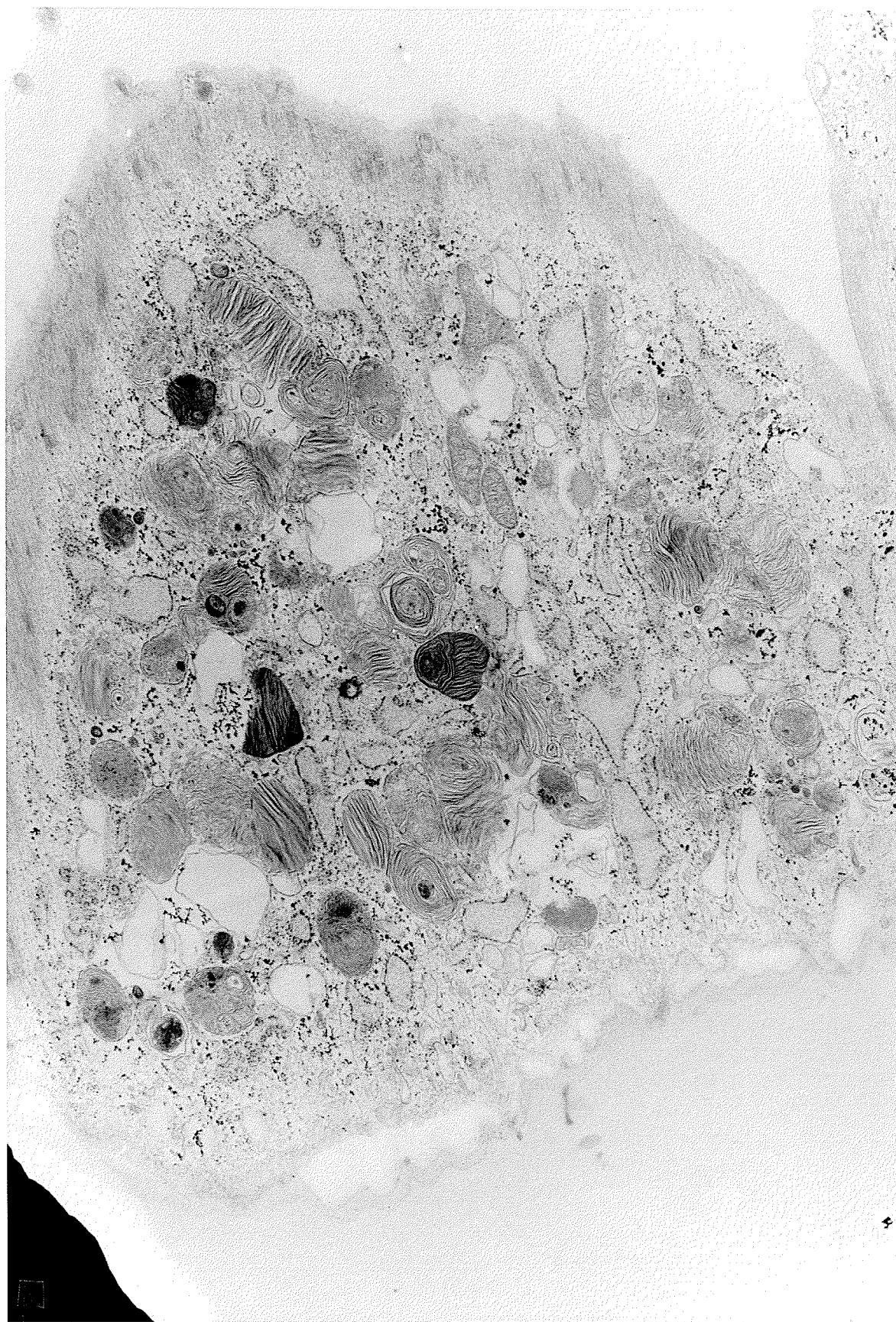


Figure 36. High magnification of two abnormal bodies found only in human cultured fibroblasts biochemically confirmed to be homozygotic for Tay -Sachs disease.

X 115,000

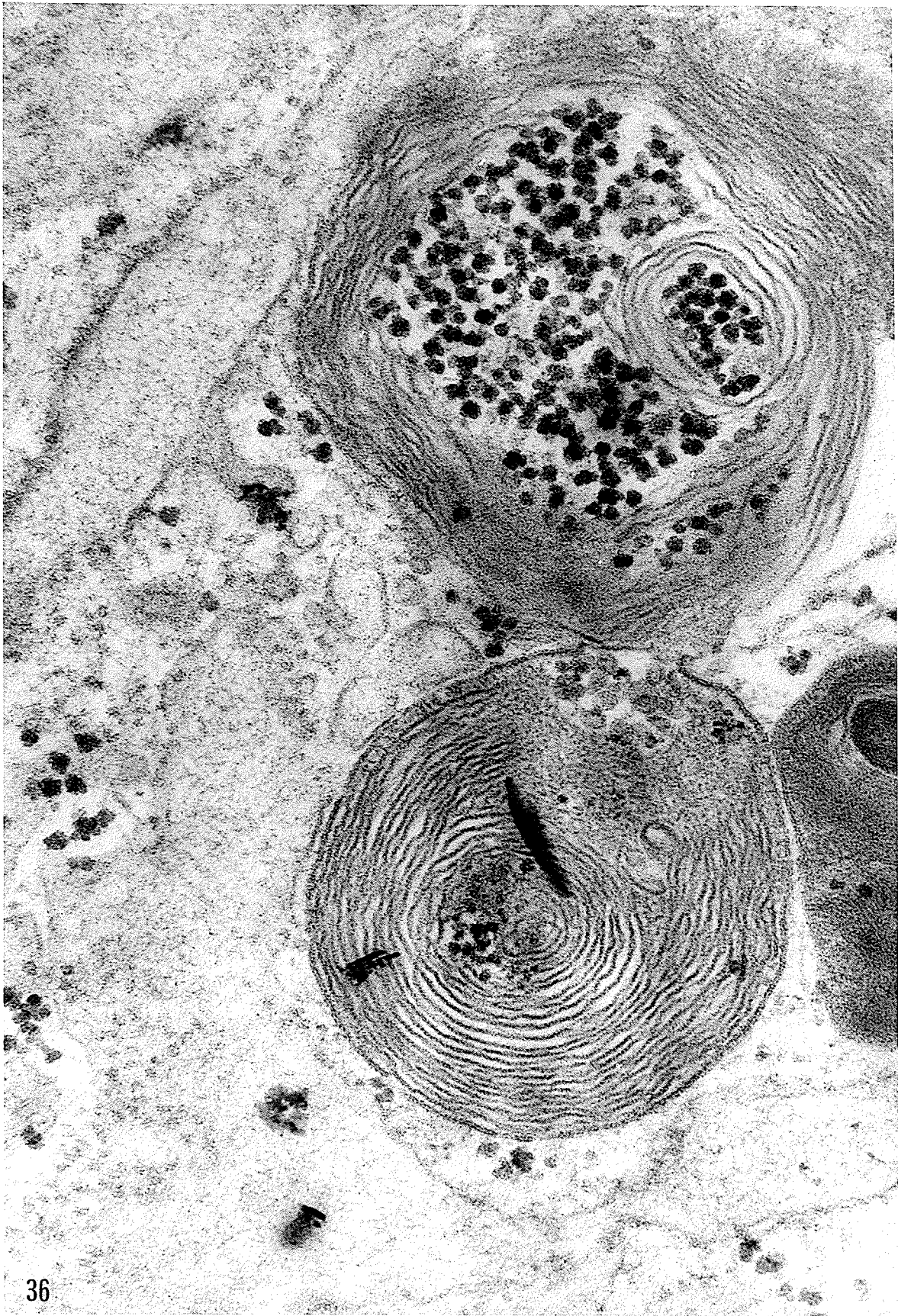


Figure 37. Human cultured amniotic fluid fibroblast biochemically confirmed to be homozygotic for Tay-Sachs disease. Arrow shows abnormal cytoplasmic body found only in cells affected with Tay-Sachs disease.

X 38,000

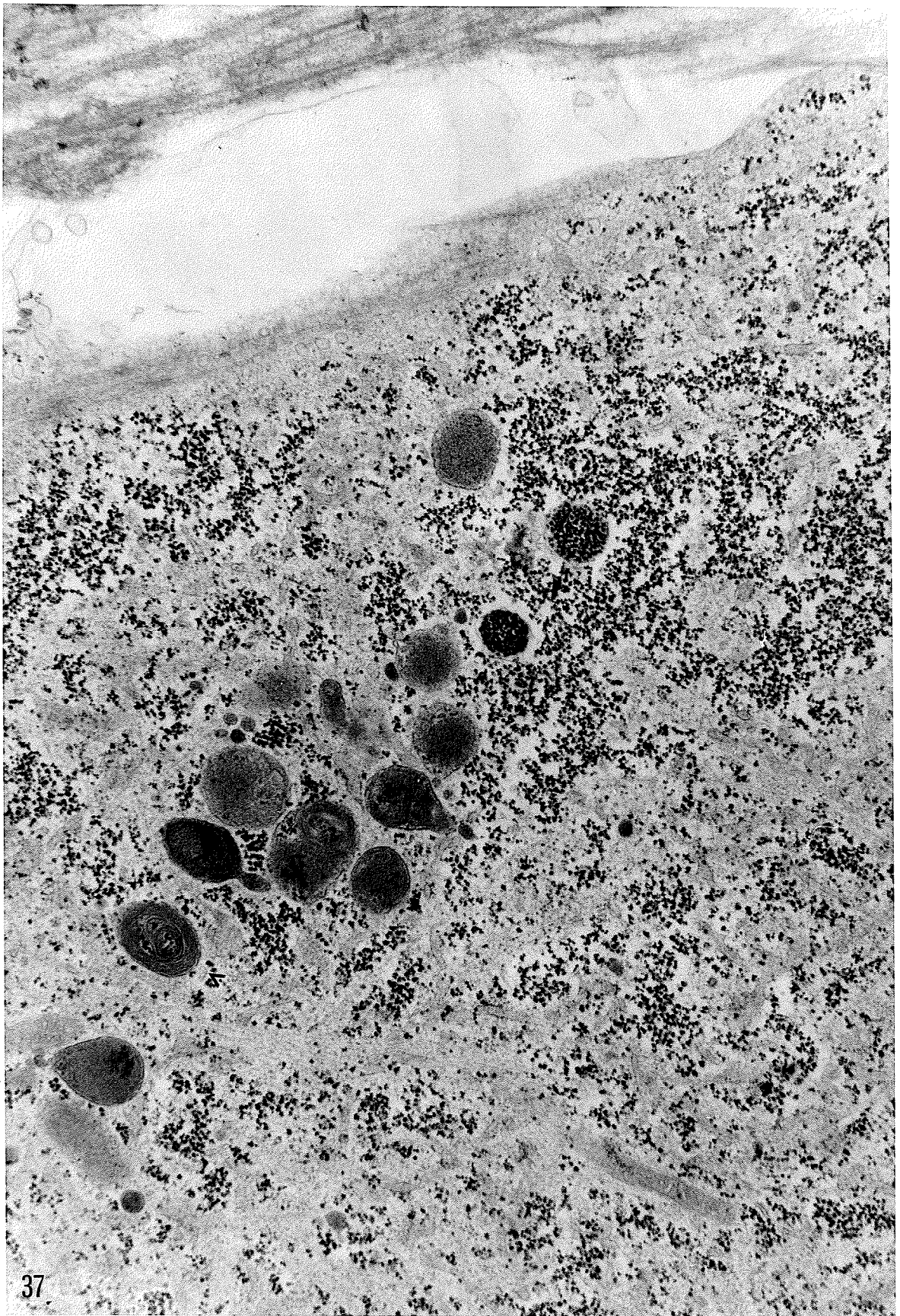


Figure 38. Human cultured amniotic fluid fibroblast. Arrows demonstrate abnormal cytoplasmic bodies found only in cells affected with Tay-Sachs disease.

X 31,000

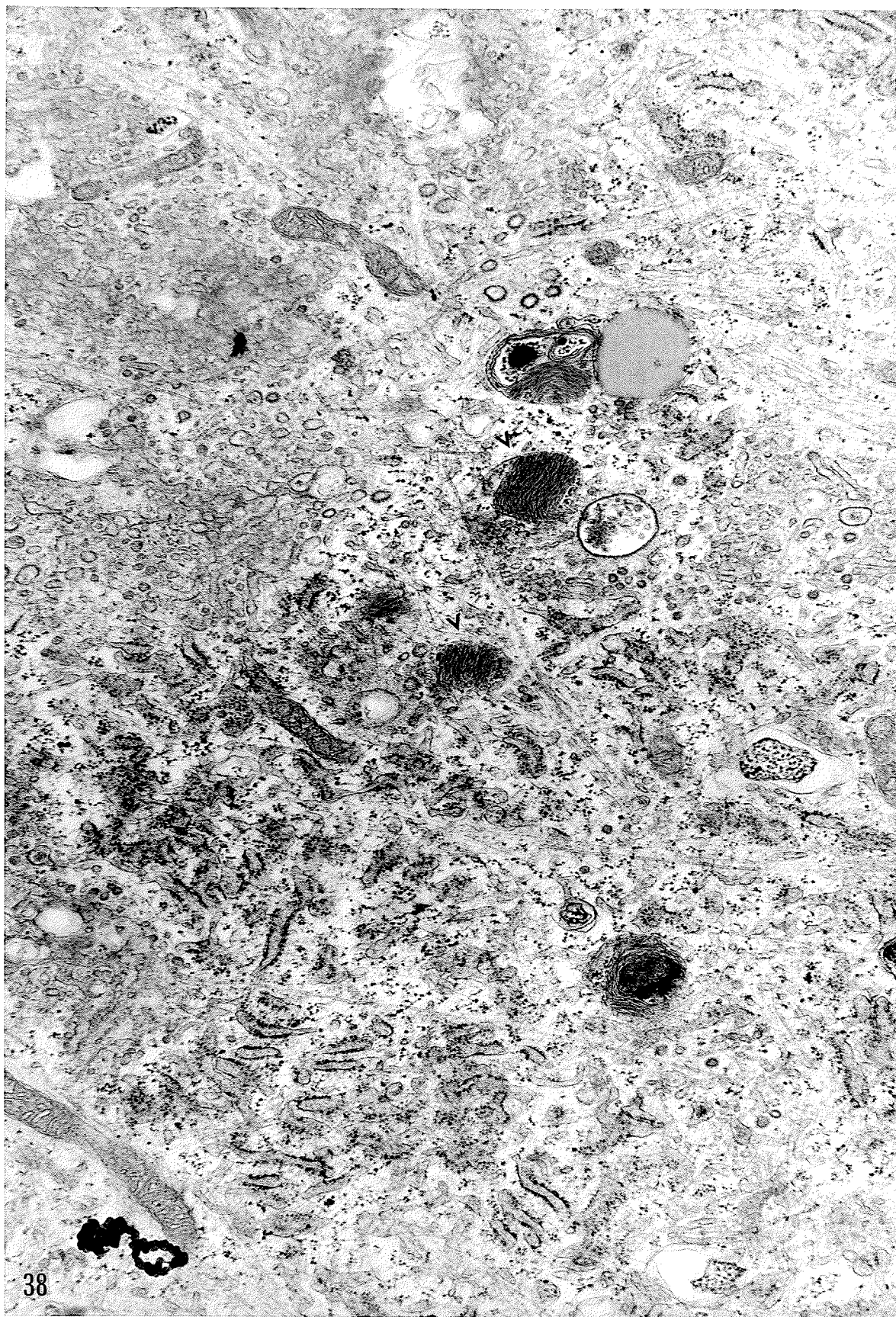
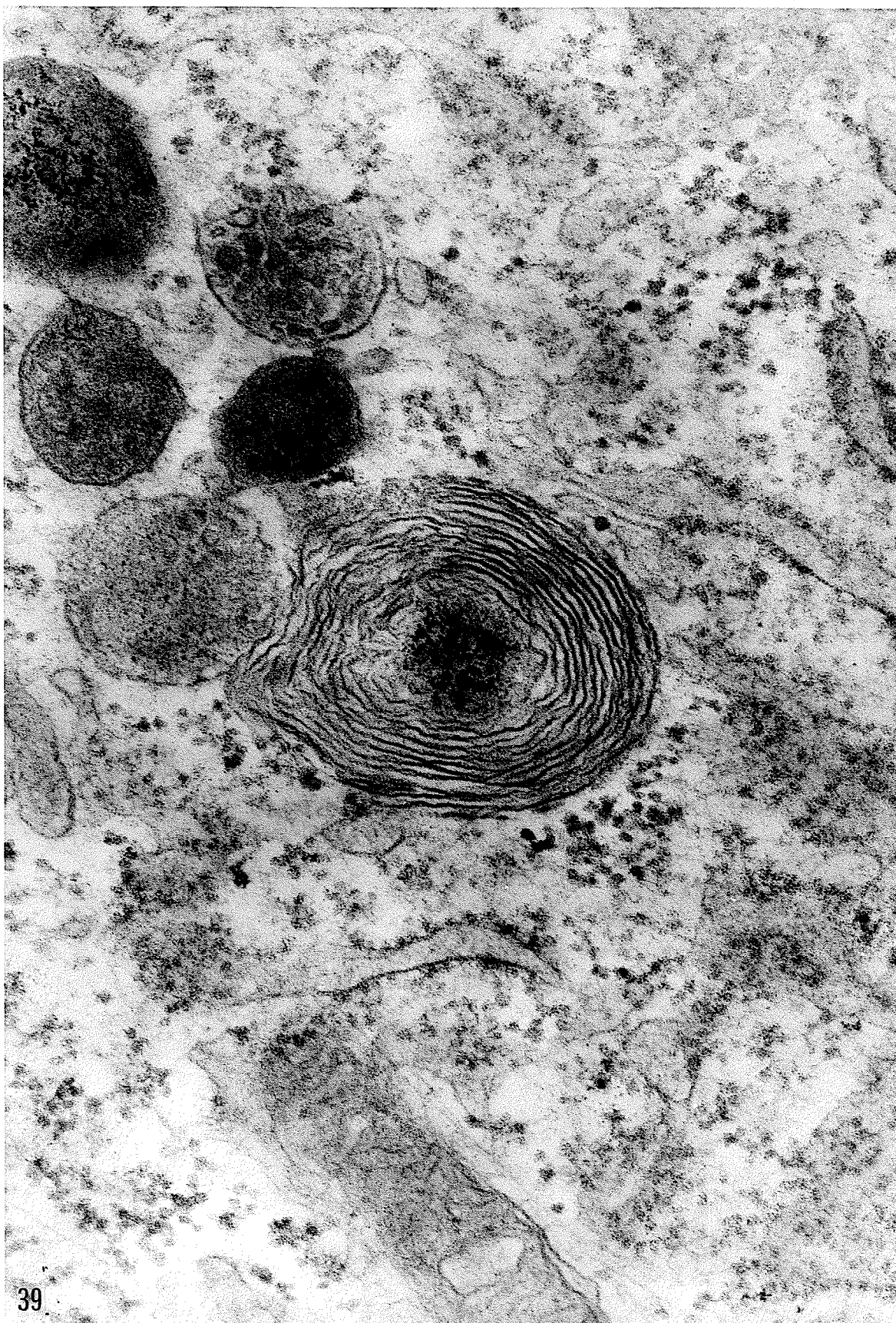


Figure 39. Human cultured amniotic fluid fibroblast biochemically confirmed to be homozygotic for Tay-Sachs disease. Shown is membranous body found only in amniotic fluid fibroblasts known to be affected with Tay-Sachs disease. Compare to bodies shown in Figure 38.

X 100,000



Ultrastructural Appearance of Cultured Fibroblasts Derived From Patients Affected With Metachromatic Leukodystrophy

Metachromatic leukodystrophy is transmitted as an autosomal recessive condition (McKusick, 1975). Two common forms of the disease exist, infantile and adult, both being due to a deficiency of the enzyme arylsulfatase A which affects the metabolic pathways associated with degradation of sulfatides. Prenatal diagnosis of infantile metachromatic leukodystrophy has been successfully performed by biochemical determination of abnormalities in arylsulfatase A (Nadler et al, 1970).

Examination of the five cell lines available for study, all of which were demonstrated by the laboratory from which they were purchased to be deficient in the enzyme arylsulfatase A, showed differences in ultrastructural appearance which distinguished them from normal controls. Figure 40 through to Figure 43 illustrate these cells. Some cell lines contained in excess of sixty cytoplasmic bodies. They were single membrane bound bodies, evenly staining, which, upon high magnification, showed a very granular fine structure. Some of the bodies measured up to four micrometers; however, the average body measured approximately three micrometers. Bodies similar to these were observed in both normal control lines and other disease states but the consistent presence of large numbers of them was observed only in the lines known to be derived from patients affected with metachromatic leukodystrophy.

Examination of three cell lines heterozygous for metachromatic leukodystrophy showed them to be indistinguishable from normal controls.

One amniotic fluid cell line known to be deficient in arylsulfatase A was kindly provided for study by Dr. M. Rattazzi, Buffalo, New York. This line was studied through four passages and showed the presence of

Figure 40. Human cultured fibroblast biochemically confirmed to be homozygotic for metachromatic leukodystrophy. Note presence of excessive number of membrane limited, evenly staining bodies (Arrows).

X 35,400

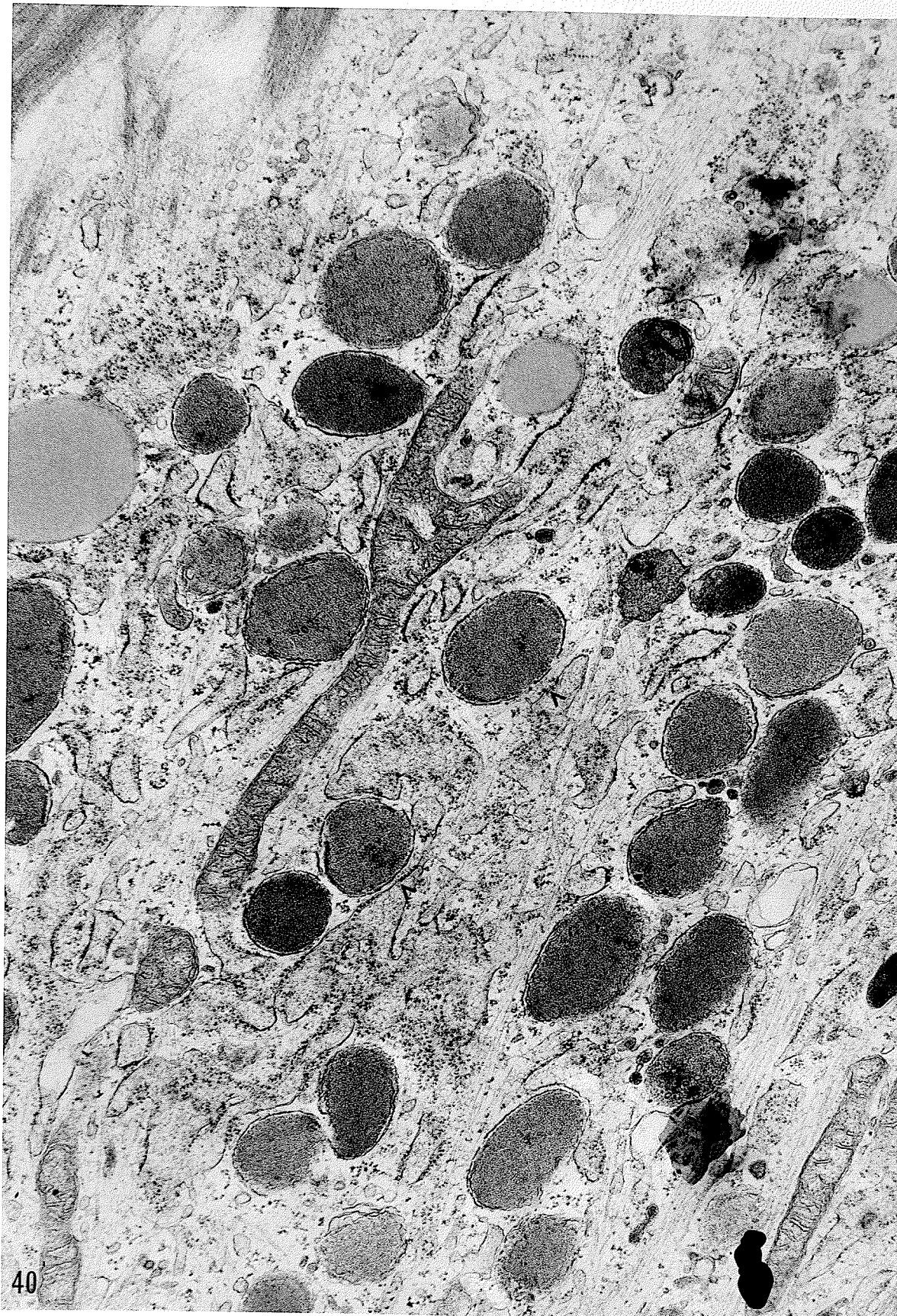


Figure 41. Human cultured fibroblasts biochemically confirmed to be homozygotic for metachromatic leukodystrophy. Note that cytoplasm is filled with evenly staining membrane limited bodies.

X 36,000

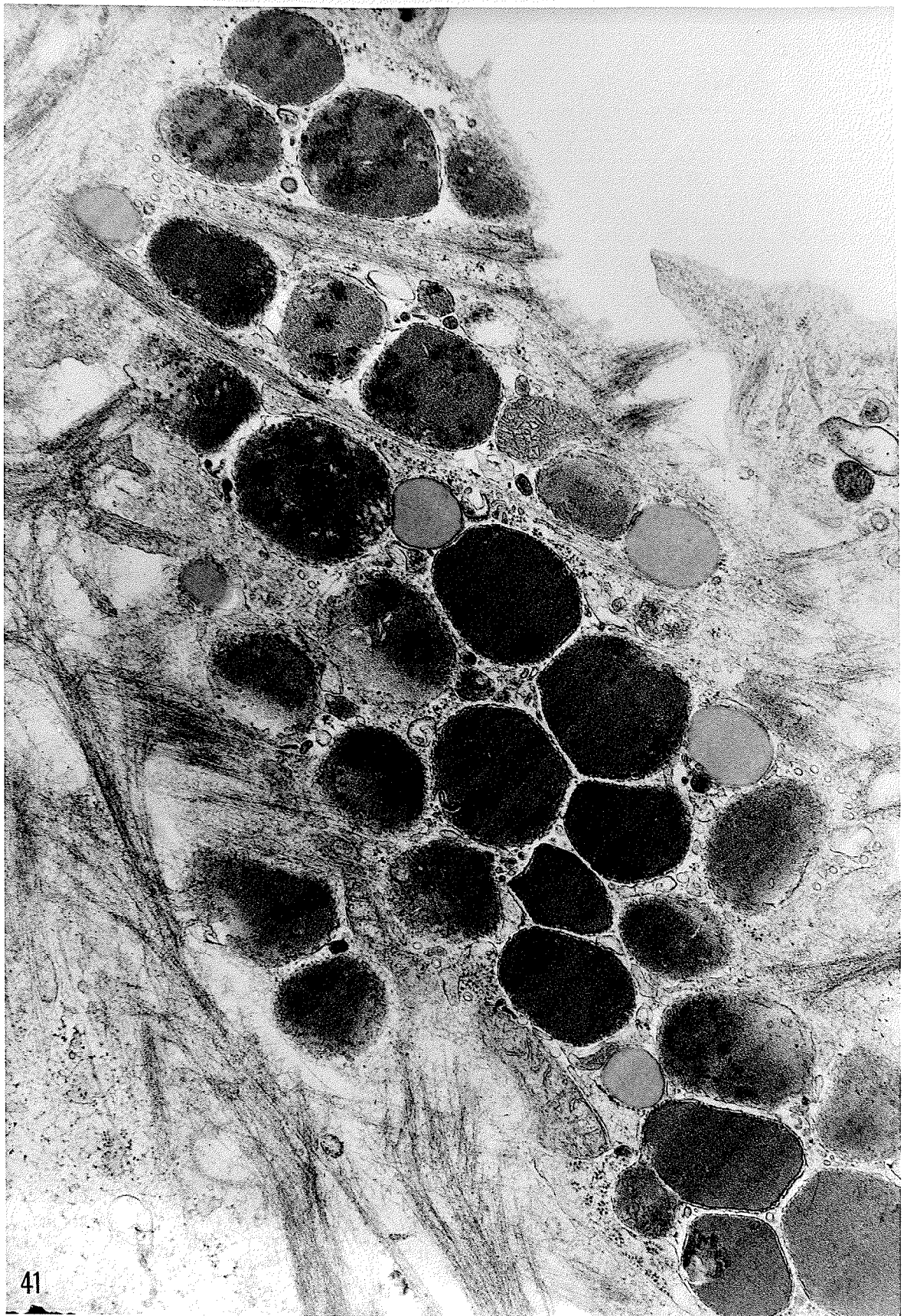


Figure 42. Higher magnification of Figure 41. Note granular appearance of these bodies found in cells affected with metachromatic leukodystrophy.

X 124,000.

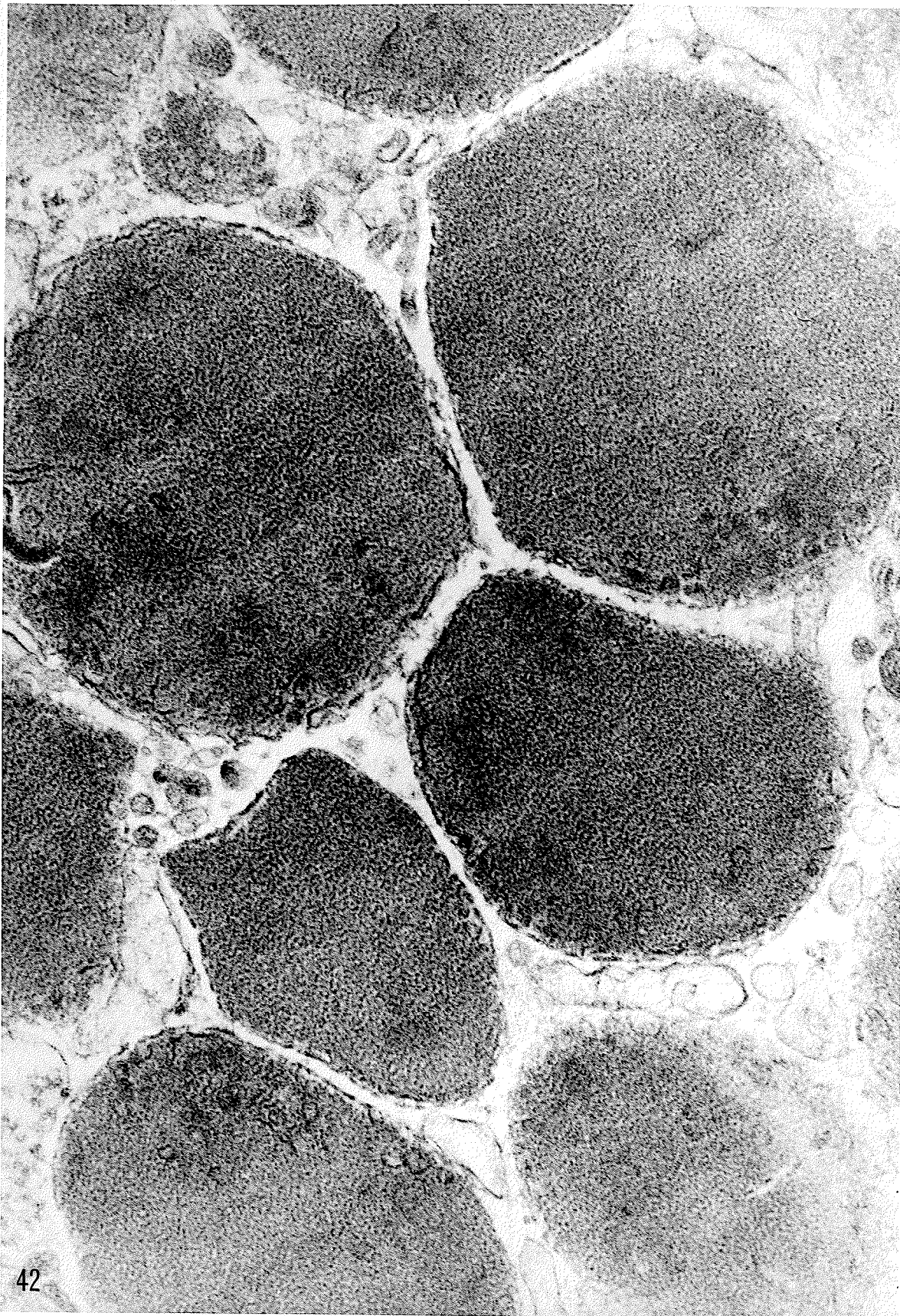


Figure 43. Human cultured fibroblast biochemically confirmed to be homozygotic for metachromatic leukodystrophy. Note excessive number of membrane limited evenly staining bodies (Arrows).

X 28,800

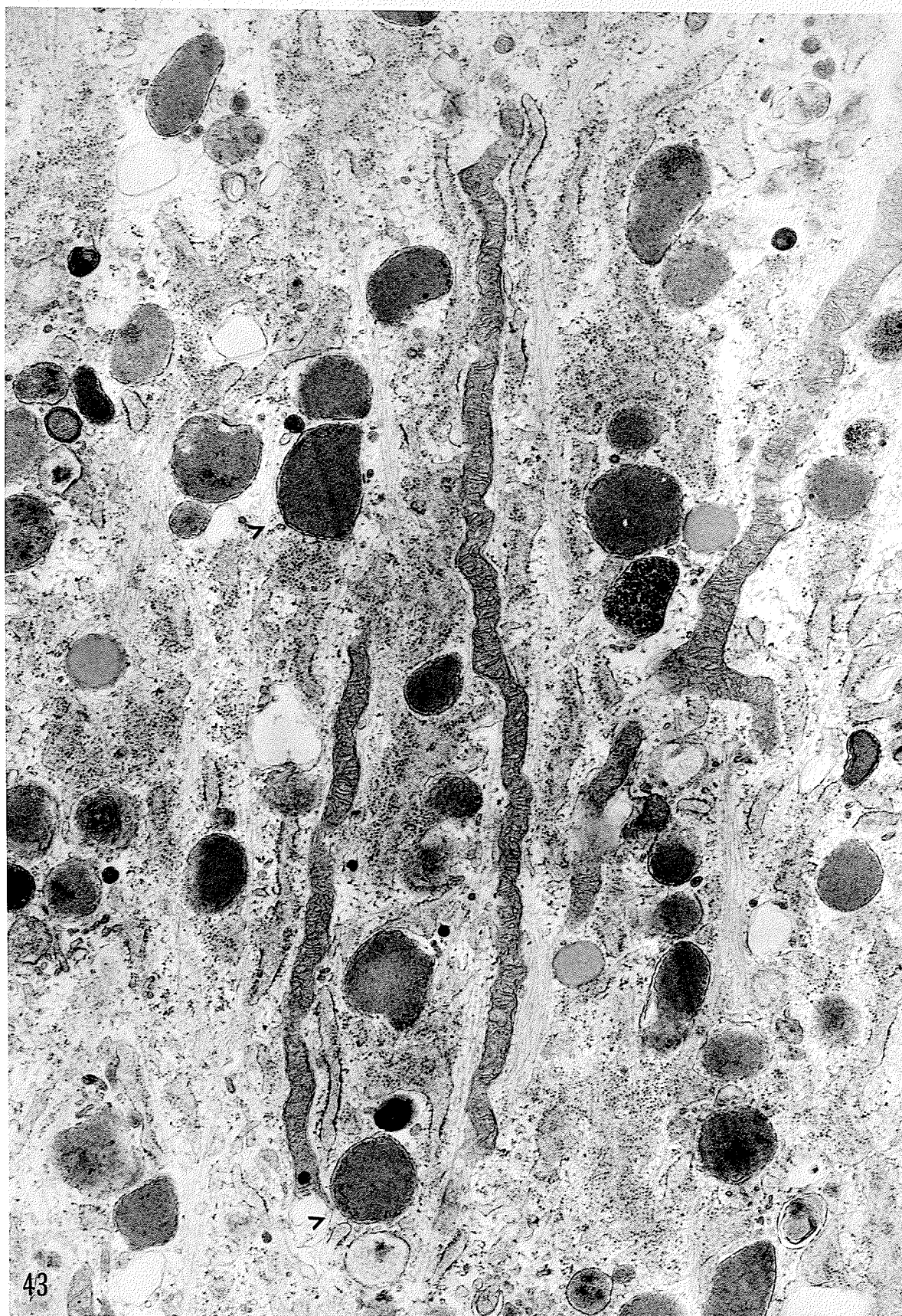


Figure 44. Human cultured amniotic fluid fibroblasts biochemically confirmed to be homozygotic for metachromatic leukodystrophy. Note excessive number of cytoplasmic bodies. Arrows indicate several of these bodies.

X 7,100

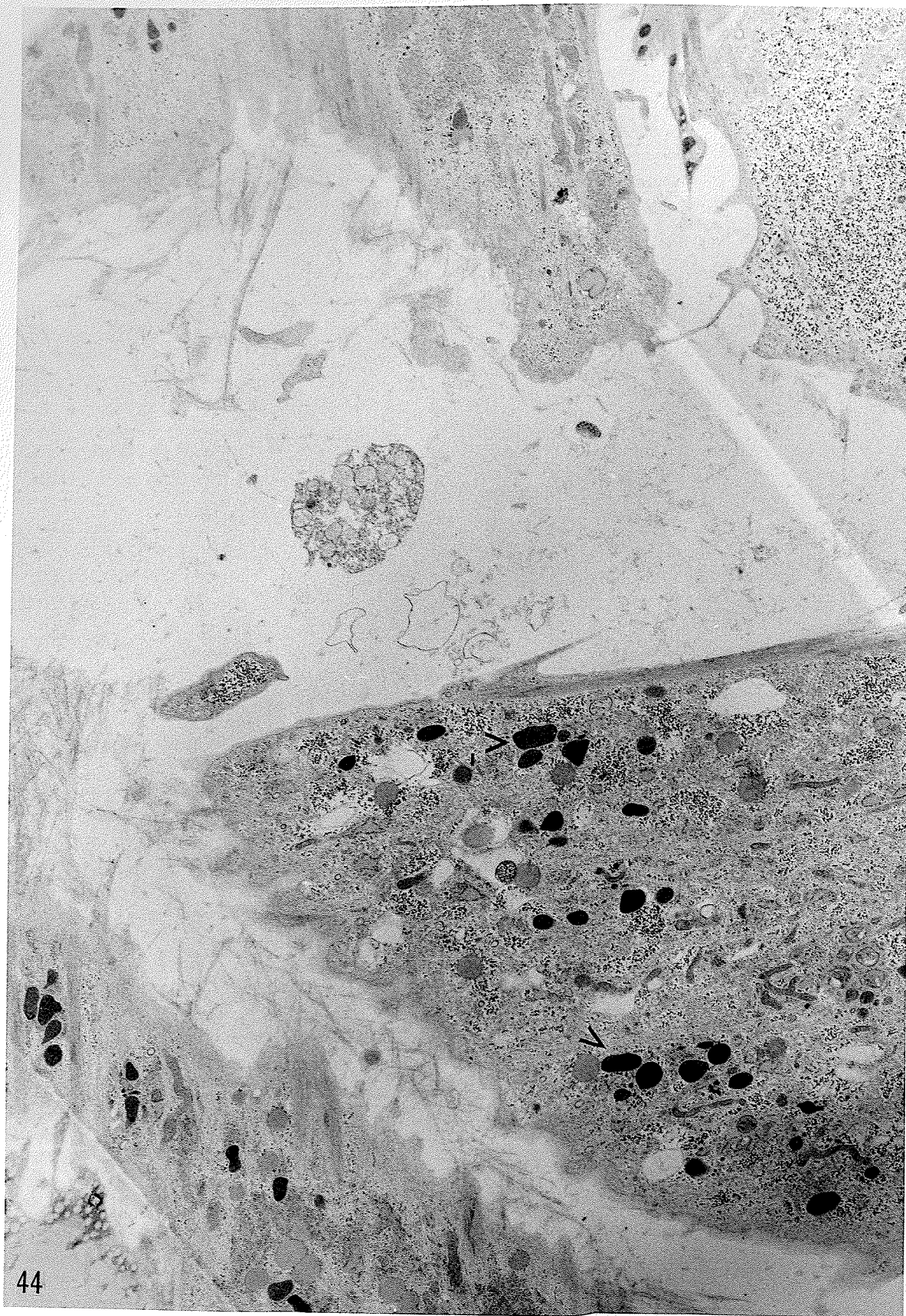


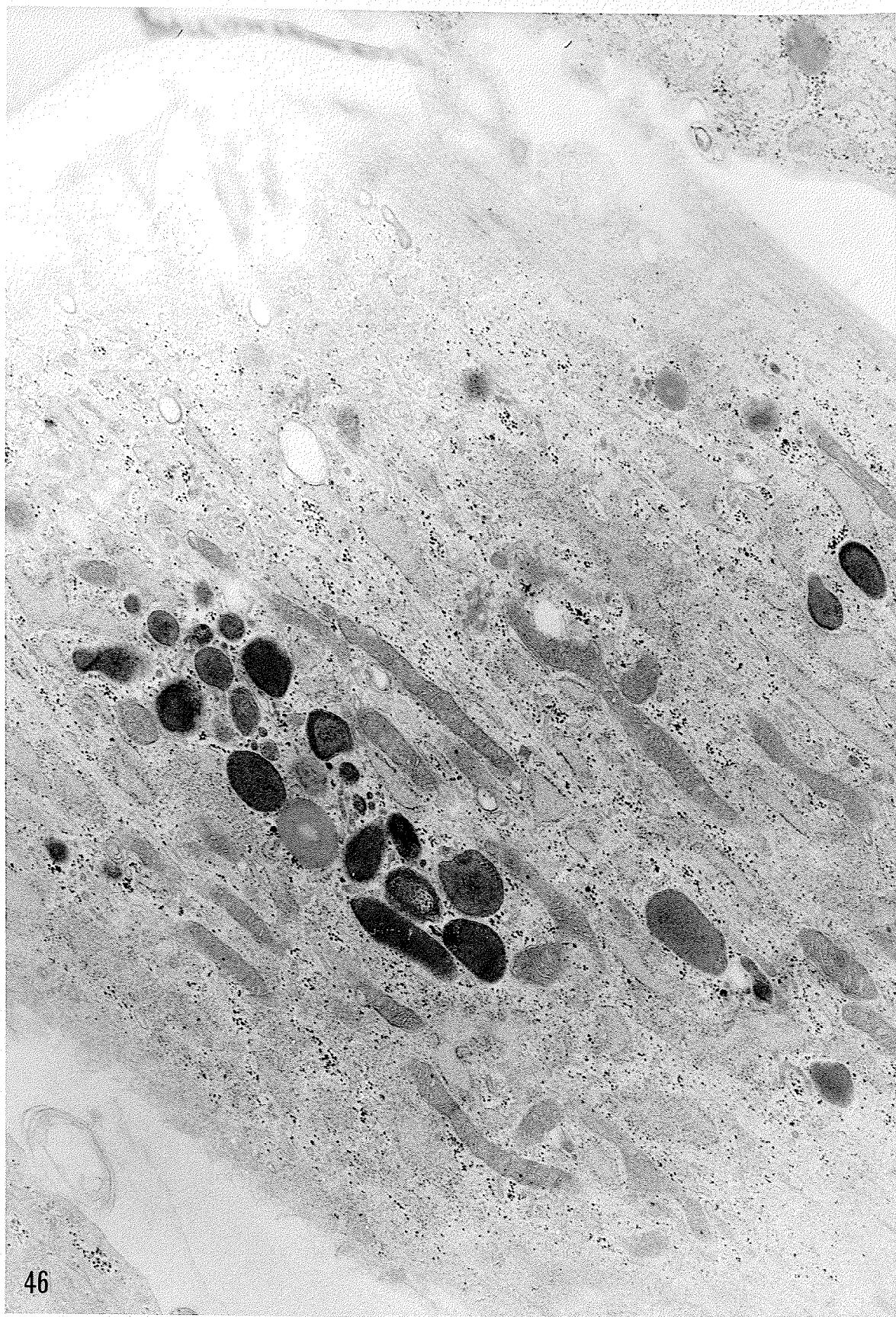
Figure 45. Human cultured amniotic fluid fibroblasts biochemically confirmed to be homozygotic for metachromatic leukodystrophy. Note appearance of numerous cytoplasmic bodies. Similar quantities of these evenly staining, membrane limited bodies were not observed in any other amniotic fluid fibroblast lines.

X 9,750



Figure 46. Human cultured amniotic fluid fibroblasts biochemically confirmed to be homozygotic for metachromatic leukodystrophy. Note accumulation of cytoplasmic bodies.

X 16,700



abnormal quantities of these evenly staining membrane bound bodies which, under higher magnification, showed a granular structure. The bodies were similar to those observed in the affected postnatal skin fibroblast lines. Examples of these bodies are shown in Figure 44 through to Figure 46.

No heterozygotic amniotic fluid fibroblast lines were available for study.

Ultrastructural Appearance of Cultured Fibroblasts Derived From Patients Affected With Muscular Dystrophy

Four postnatal skin fibroblast lines derived from individuals clinically expressing the symptoms characteristic for Duchenne's muscular dystrophy were obtained from the Mutant Cell Repository in Montreal. No biochemical defect is known for the disease which is transmitted as an X-linked recessive condition (McKusick, 1975). Specific prenatal diagnosis of the condition has not yet been achieved.

The four cell lines were examined through four consecutive passages. In each line and at every passage, two abnormal phenomena were evident. Firstly, there was apparently an increased number of lipid storage bodies and lysosomal bodies in all the cell lines. It was, however, not possible to quantitate the number of bodies observed in cells as the study was not designed to this end. Secondly, the consistent appearance of a specific abnormal cytoplasmic body was noted. These bodies, which appeared in Duchenne's muscular dystrophy and not in any normal or other disease state, were membrane bound, relatively evenly staining, and consistently irregular in shape. Higher magnification of these bodies usually showed remnants of membrane in them. The bodies occurred in some

Figure 47. Human cultured fibroblast derived from individual clinically confirmed to be affected with Duchenne's muscular dystrophy. Note increase in number of cytoplasmic bodies (compare to normals). Arrows indicate bodies found only in fibroblasts affected with Duchenne's muscular dystrophy.

X 17,200

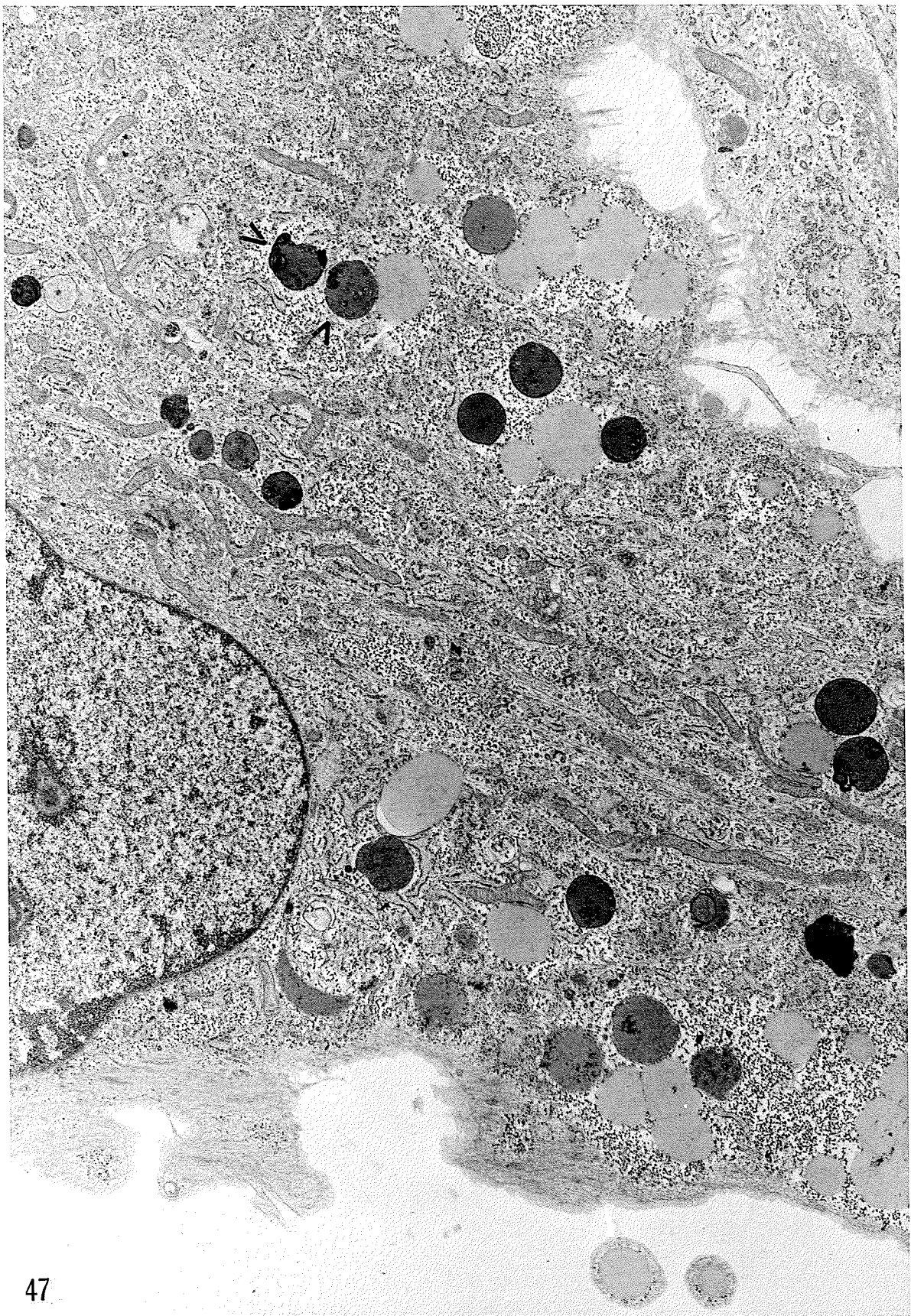


Figure 48. Higher magnification of bodies indicated by Arrows in Figure 47. Note irregular shape and remnants of membrane in these bodies.

X 119,500.

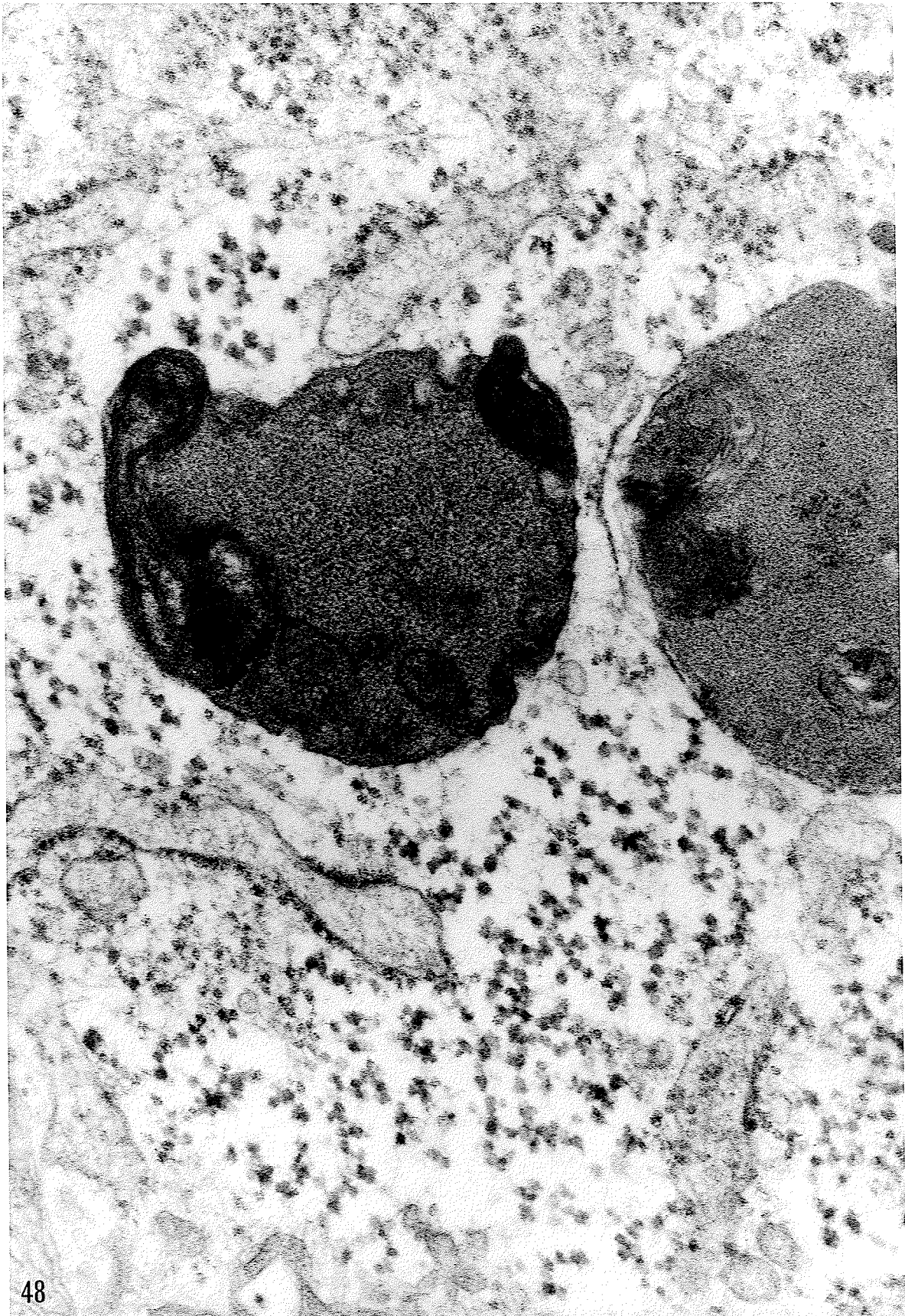


Figure 49. Human cultured fibroblast derived from individual clinically confirmed to be affected with Duchenne's muscular dystrophy. Arrows indicate abnormal cytoplasmic bodies found in fibroblasts affected with Duchenne's muscular dystrophy.

X 34,000



Figure 50. Human cultured fibroblasts derived from individual clinically confirmed to be affected with Duchenne's muscular dystrophy. Arrow indicates abnormal cytoplasmic body found only in fibroblasts affected with Duchenne's muscular dystrophy.

X 40,000

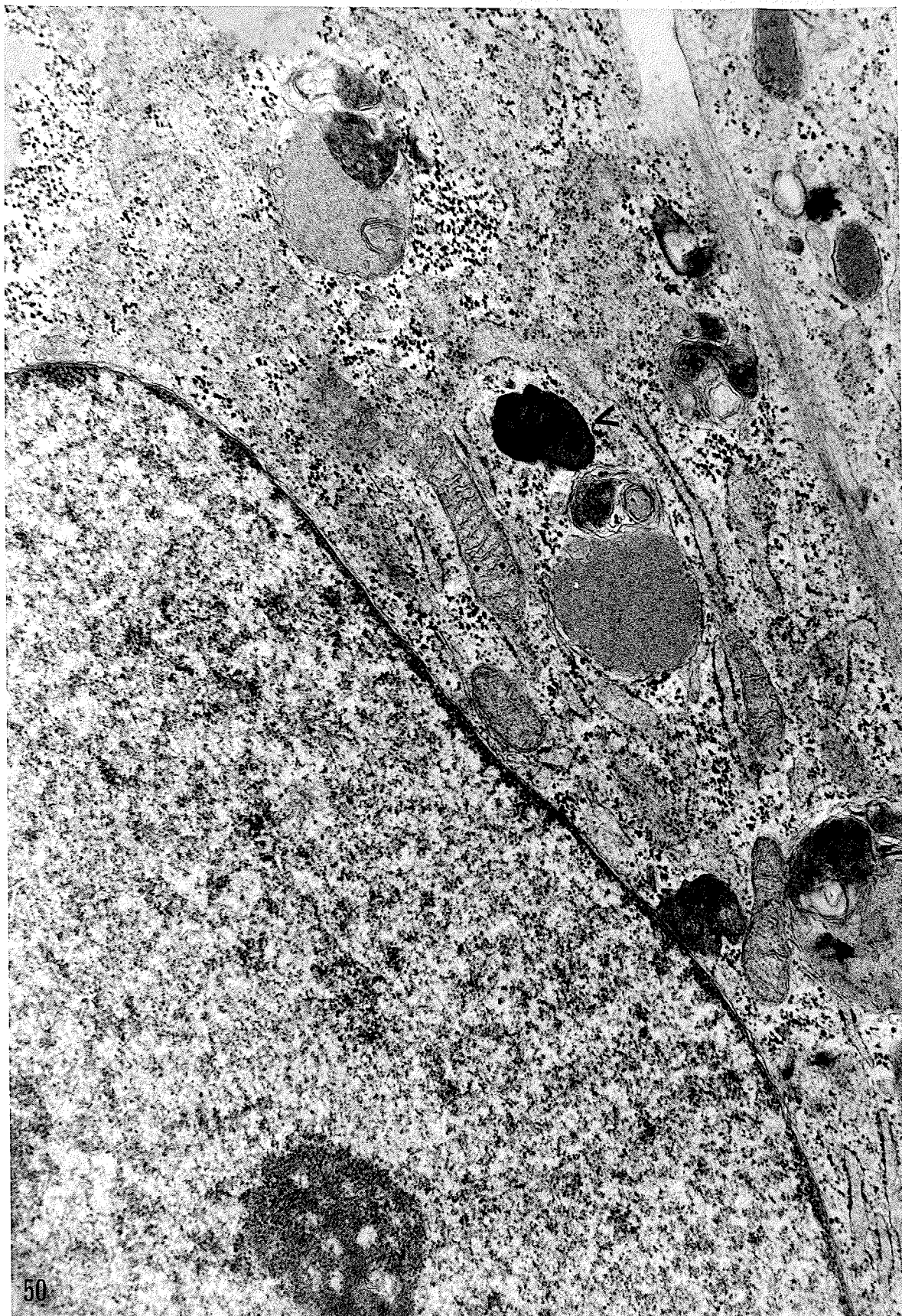


Figure 51. Higher magnification of body marked by arrow in Figure 50. Note irregular shape and evidence of membrane remnants in the body.

X 112,000

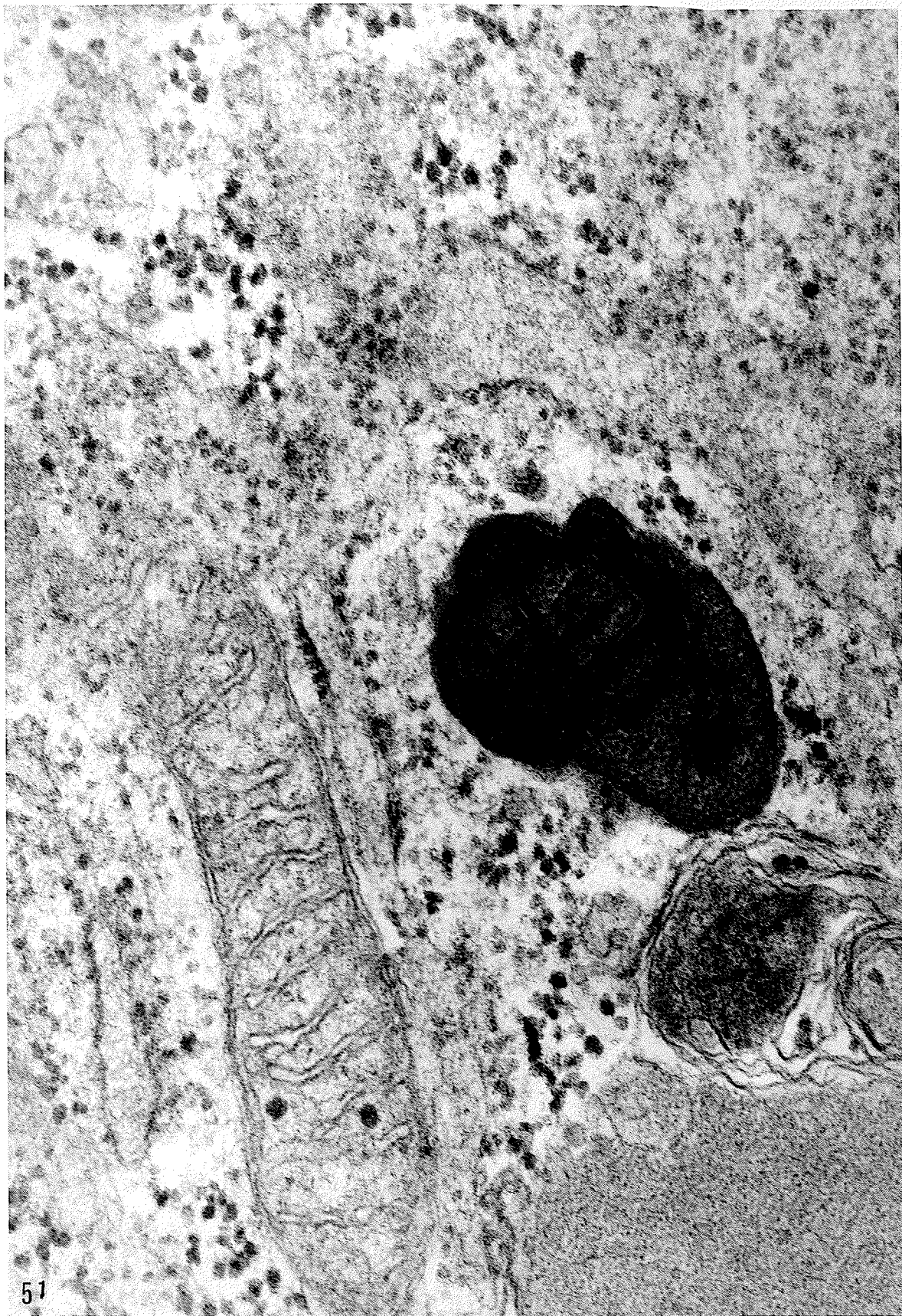


Figure 52. Human cultured fibroblast derived from individual clinically confirmed to be affected with Duchenne's muscular dystrophy. Note numerous bodies, irregularly shaped. The majority of these bodies contain membrane remnants.

X 47,000.

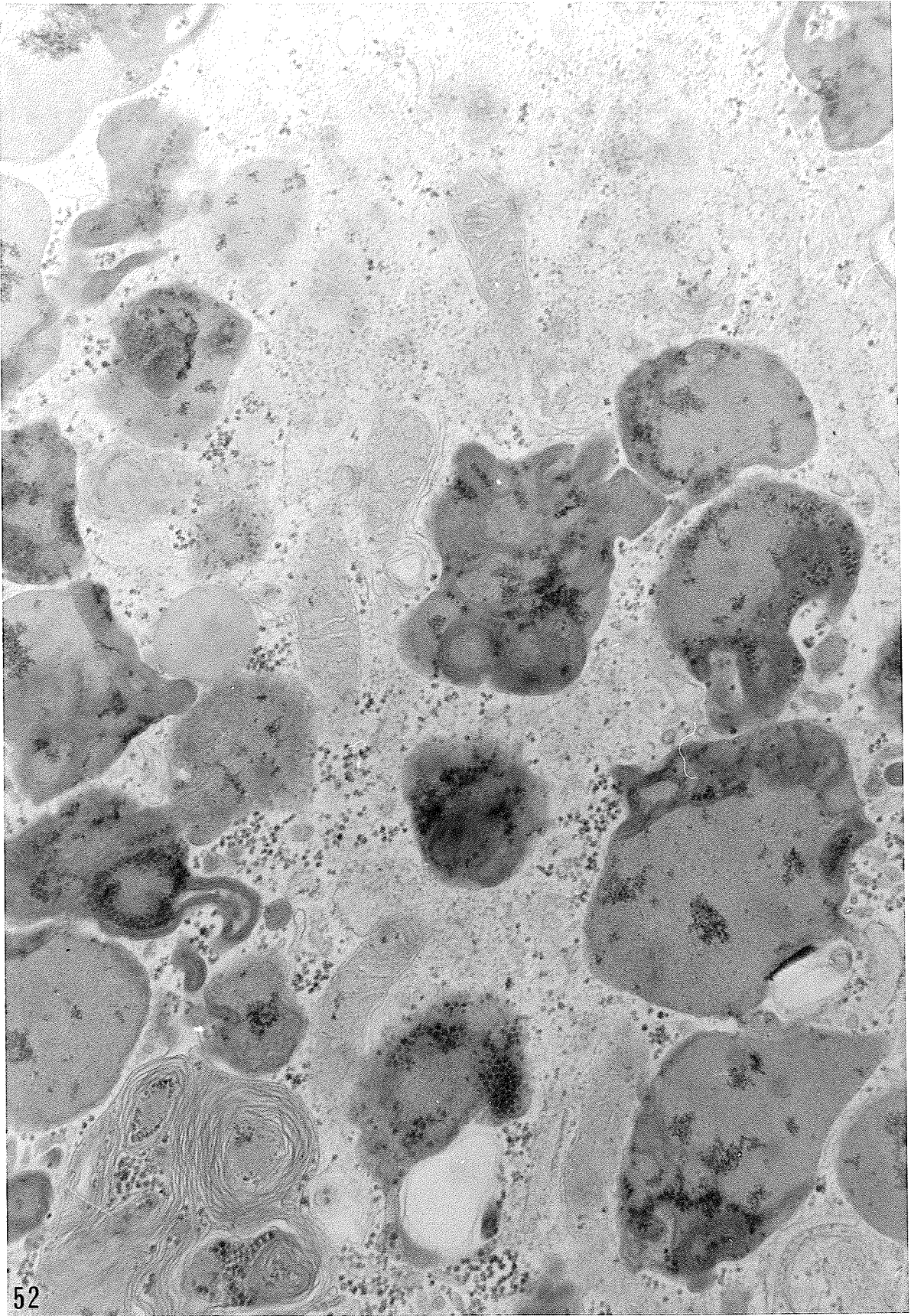


Figure 53. Human cultured fibroblast derived from individual clinically confirmed to be affected with Duchenne's muscular dystrophy. Cytoplasmic bodies of this type (arrow) were observed only in cells affected with Duchenne's muscular dystrophy.

X 109,000



cell sections at a frequency in excess of forty per section. In other sections, only one abnormal body was noted. The bodies ranged in size from 0.5 micrometers to 2.0 micrometers. The smaller bodies were normally observed singly in the cytoplasm while the larger ones were normally noted in groupings. Figure 47 through to Figure 53 show examples of muscular dystrophic cells and the cytoplasmic bodies associated with the disease.

No heterozygotic cell lines were available for study.

No amniotic fluid cell lines were available for study.

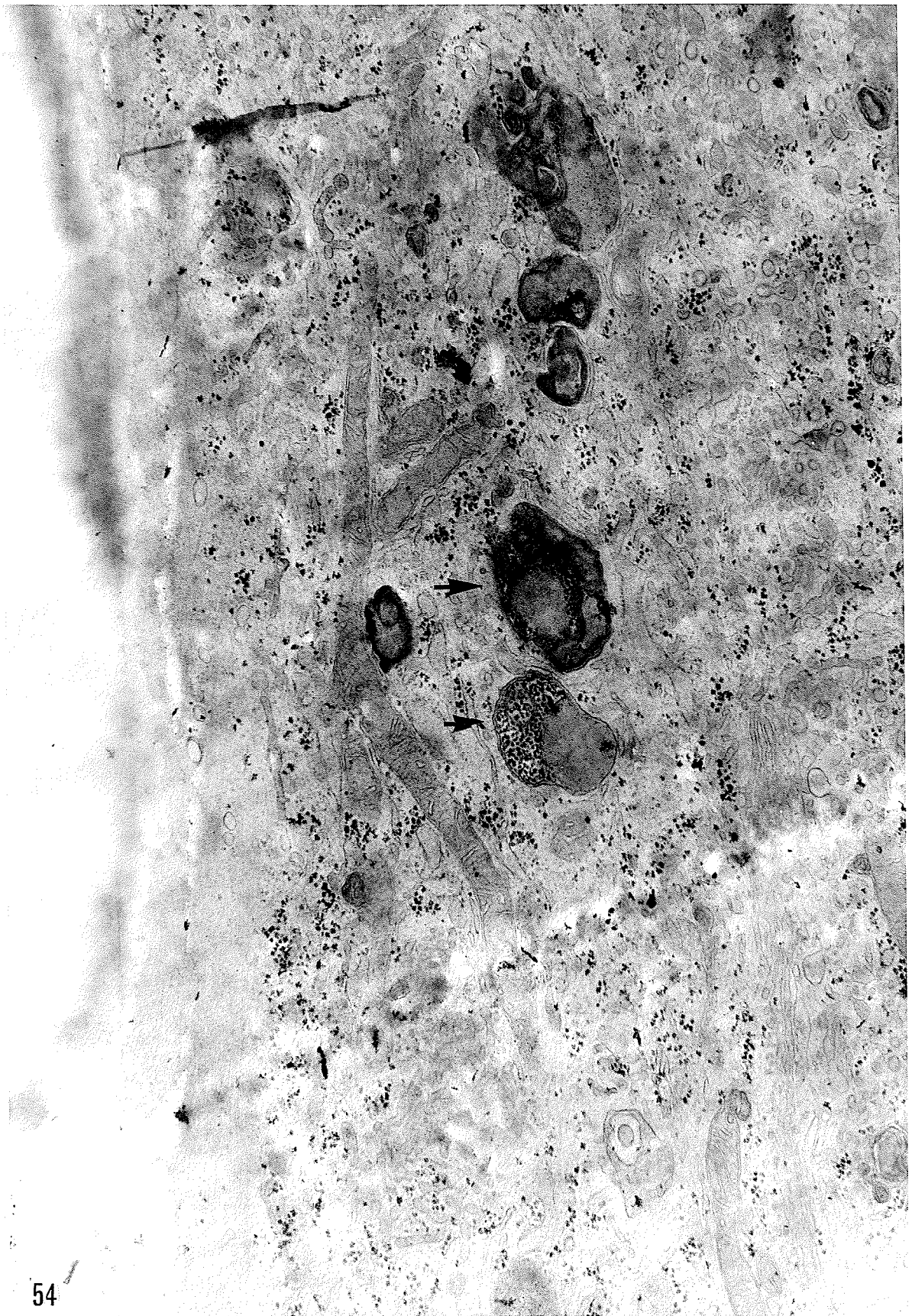
Ultrastructural Appearance of Cultured Fibroblasts Derived From Patients Affected With Cystic Fibrosis

Cystic fibrosis, also known as mucoviscidosis, is transmitted as an autosomal recessive condition. The enzyme defect is unknown. Prenatal diagnosis has not been performed (McKusick, 1975).

Five fibroblast lines derived from patients affected with cystic fibrosis were available for study. Two of the cell lines showed no ultrastructural differences from normal control lines. Two others, each studied through four passages, showed abnormalities which made them distinguishable from normal controls. These bodies were very pleomorphic in appearance and ranged in size from 1.0 micrometers to 3.0 micrometers. They usually showed a double limiting membrane and contained an abundant number of particles resembling β -glycogen and remnants of membranes contained within their limiting membranes. These bodies are shown in Figure 54 and Figure 55. Bodies resembling these were not seen in any normal controls nor in any other disease state. The fifth cell line also showed abnormalities but these differed from the other two cell lines.

Figure 54. Human cultured fibroblast derived from individual clinically confirmed to be affected with cystic fibrosis. Note abnormal cytoplasmic bodies (ARROWS). These bodies were observed in two cell lines affected with cystic fibrosis. Poor fixation and sectioning as observed in this electron micrograph was due to faulty processing by the author.

X 38,000



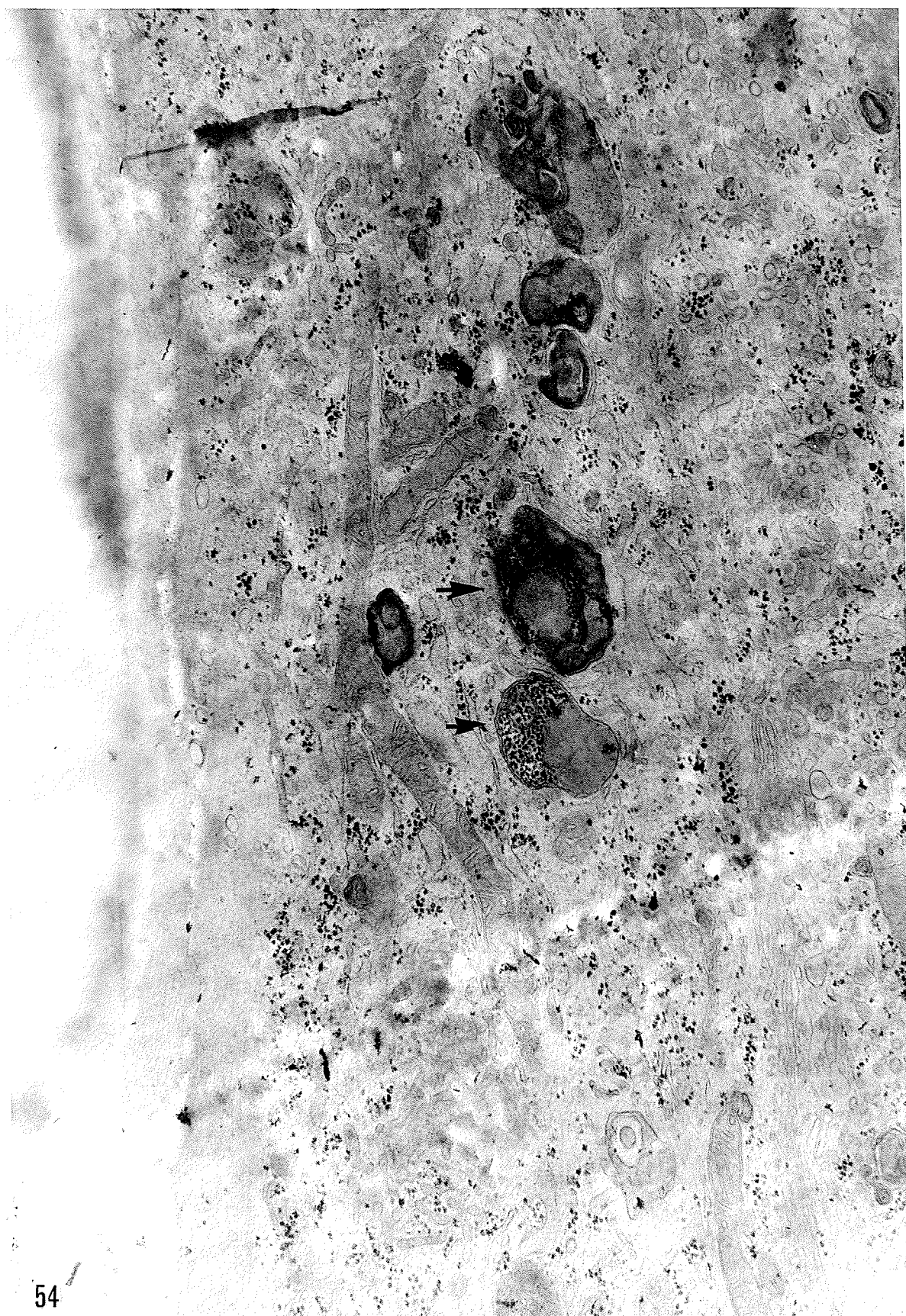
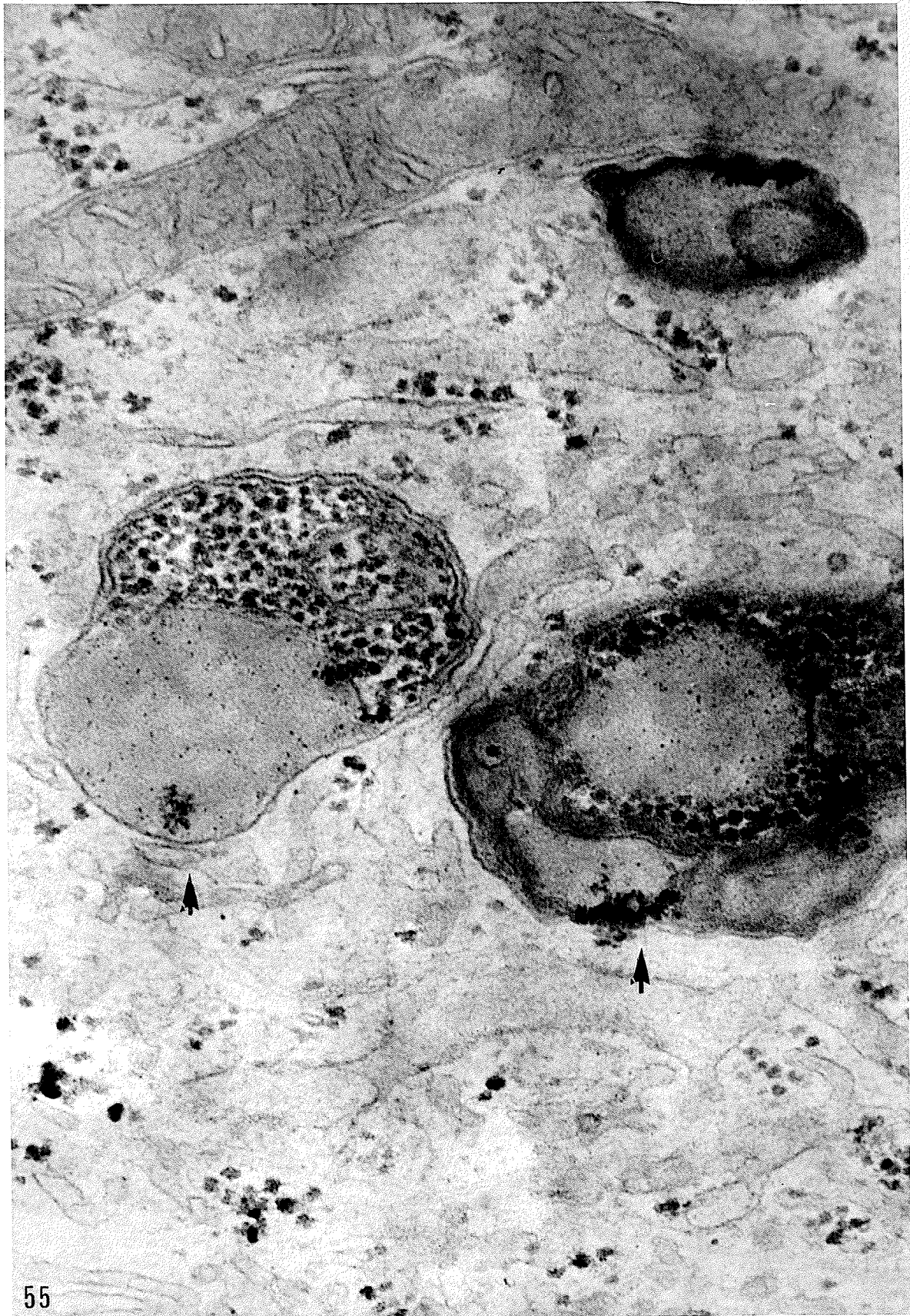


Figure 55. Higher magnification of Figure 54. Arrows indicate abnormal bodies seen in cells affected with cystic fibrosis.

X 100,000



These bodies are seen in Figure 56 and Figure 57. They were not observed in any other cell line examined. The bodies measured between 1.0 micrometers and 3.0 micrometers. They appeared to be composed of a lamellar structure wrapped around itself. They were observed with a frequency of between one and three bodies per cell section.

No heterozygotic lines were available for study.

No amniotic fluid fibroblast lines were available for study.

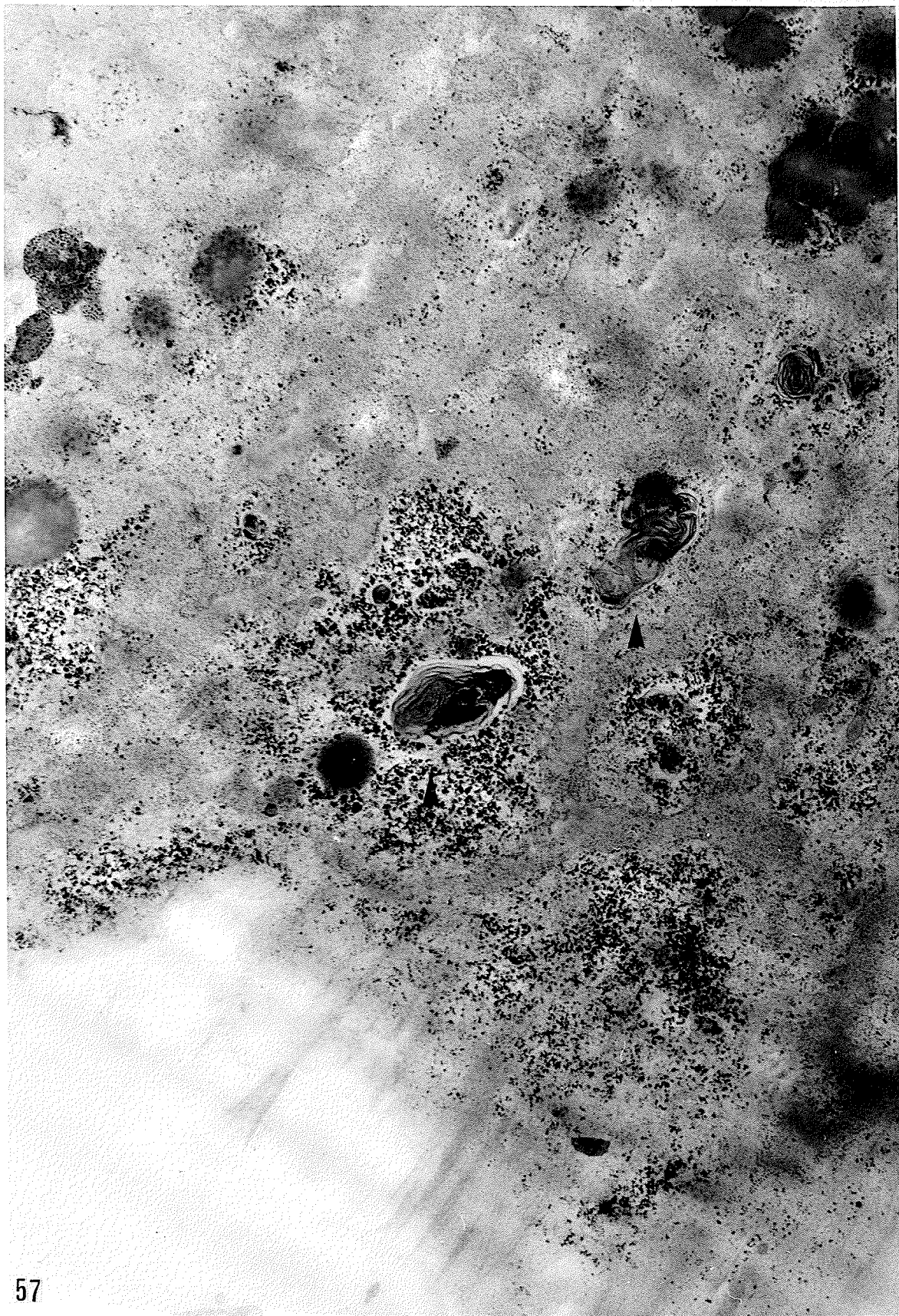
Figure 56. Human cultured fibroblast derived from individual clinically confirmed to be affected with cystic fibrosis. Arrows indicate abnormal cytoplasmic bodies seen only in this cell line. Four other cell lines known to be affected with cystic fibrosis did not show evidence of these abnormal bodies. Compare to Figure 54 and Figure 55.

X 40,000



Figure 57. Human cultured fibroblast derived from individual clinically confirmed to be affected with cystic fibrosis. Arrows indicate abnormal bodies. This cell line is the same as that shown in Figure 56.

X 16,500 :



CHAPTER FIVE

DISCUSSION

Despite the increasing realization that an anomalous fine structure is present in cultured skin fibroblasts for a number of genetic diseases as summarized in Chapter Two, the potential of electron microscopic examination of cultured amniotic cells for prenatal diagnosis of disease remains unexploited. This may be due to the fact that cytoplasmic anomalies have been reported in normal skin fibroblasts in vitro (Lie et al, 1973; Lipetz and Cristofalo, 1972). The anomalies found in normal cultured skin fibroblasts may, however, be due to the variations in culturing and preparative techniques utilized in different laboratories. For example, it has been demonstrated that alkaline pH (Lie et al, 1973), the stage of cell growth (Lucky et al, 1975; Maciera-Coelho et al, 1971), age of cells in culture (Robbins et al, 1971) and mechanical scraping and trypsinization (Lucky et al, 1975) all can produce the appearance of cytoplasmic anomalies in cultured cells. A number of other culturing variations have been associated with the appearance of cytoplasmic organelles (Lie et al, 1973; Lucky et al, 1975), and also the stage of activity of the cell types is known to affect the structure of cells (Ryan et al, 1972; Wood, 1975). It has also been demonstrated that medium changes decrease the occurrence of cytoplasmic bodies (Maciera-Coelho et al, 1971).

As a consequence of the foregoing considerations, in this study only fibroblast cultures of low passage number were used. These cell lines were examined in situ to avoid mechanical distortion, with processing for electron microscopy occurring before the cell monolayer achieved confluency. Fresh media were added to each dish 24 hours prior to processing. By

strict adherence to this protocol, it was hoped to minimize culturally inducible ultrastructural variation. The electron microscopic examination of normal cell lines indicated that the following of this regimen was a success, in that no consistent abnormal bodies were observed through the four passages studied for each normal cell line.

In response to the first question posed in Chapter One, it was necessary to study the ultrastructure of both normal and diseased cultured fibroblasts. Figure 3 through to Figure 10 give confirmation that the fine structure of normal cultured postnatal skin fibroblasts is virtually free of the consistent appearance of anomalous cytoplasmic bodies. However, abnormal bodies were very conspicuous in fibroblasts in seven of the genetic diseases under investigation here. Three other disease states studied were indistinguishable from the normal lines.

In postnatal cultured skin fibroblasts derived from an individual affected with Pompe's disease studied for this thesis, abnormal cytoplasmic bodies specific to this disease state were observed in all the four passages. These bodies were single membrane bound, regular in shape and contained particles resembling β -glycogen. They were not seen in any other disease state nor in any of the normal lines studied. Hug et al (1971, 1973) consistently found that cultured fibroblasts affected with Pompe's disease contained membrane bound bodies which contained material resembling β -glycogen. They considered these bodies diagnostic for the disease. They described the medium the cells were grown in, the number of consecutive passages studied, the cell growth stage, and described comparison of these fibroblasts to normal controls. The results presented here report the presence of similar bodies. The minor difference seen between the bodies described by Hug et al (1973) and the bodies described

here might possibly be due to the difference in culture medium and conditions. Tenconi et al (1972) in the investigation of two cell lines affected with Pompe's disease were not able to distinguish these lines from normal controls. Since they published no information regarding the conditions under which the cells were grown, what stage of cell growth the cells were at, or the culture medium they were grown in, interpretation of their results is impossible.

The single cell line derived from an individual affected with Lesch-Nyhan syndrome was found to be indistinguishable from the normal controls. There are no other works published on the ultrastructure of cultured fibroblasts from patients affected with this disease.

Maple syrup urine disease was studied in a single cell line from a patient known to be affected with the condition. This line was found to be indistinguishable from normal controls. No published reports exist on the ultrastructure of cultured fibroblasts in this disease.

The single fibroblast line studied derived from a patient affected with Infantile cystinosis was found to be indistinguishable from normal cell lines. No electron microscopic studies on cultured fibroblasts have been reported for this disease.

In this study, fibroblasts derived from an individual affected with I-cell disease were examined and the cytoplasm was observed to be filled with pleomorphic inclusions. Several other reports exist on the ultrastructure of cultured fibroblasts in this disease. All have recorded that the cytoplasm of the fibroblasts is filled with inclusions. Aula et al (1975) reported that two types of inclusions were present, an empty vacuole type and a second type surrounded by a single membrane but containing abundant polymorphic material. Terashima et al (1975)

reported the presence of numerous inclusion bodies of pleomorphic structure. Hanai et al (1971) reported that numerous cytoplasmic bodies were present which were surrounded by a unit membrane and contained a pleomorphic material. The electron micrographs published in these reports each differed slightly in the appearance of the cytoplasmic inclusions and also differed from the observations made in this study. However, it is apparent that fibroblasts derived from patients with I-cell disease are easily distinguished by the appearance of the large number of cytoplasmic inclusions.

The six cell lines derived from individuals affected with Fabry's disease showed an accumulation of bodies which resembled the lipid storage bodies seen in both normal controls and other disease states. These fibroblast lines were easily distinguishable from all other lines. Two independent reports on the ultrastructural appearance of cultured fibroblasts from patients affected with Fabry's disease have been published. Kamensky et al (1973) studied three cell lines each at a single passage which were grown to confluency. They reported that typical bodies characteristic for the disease were evident. These bodies were surrounded by a unit membrane and contained stocks of parallel or concentric lamellae. McLean and Stewart (1974) studied two cell lines derived from donors affected with Fabry's disease, each at single passage, which were grown to confluency, but no normals were studied at the same time. They found cytoplasmic bodies which had a crystalline fine structure which they considered characteristic for the disease. The results presented here and in each of the two other published reports differ in the specific morphological abnormality observed.

Six postnatal skin cultured fibroblast lines were studied which were

derived from individuals known to be affected with Tay-Sachs disease. Each cell line, at each of the four passages studied, showed the appearance of specific cytoplasmic bodies which were not observed in any normal controls or any other disease state. No other reports on the ultra-structure of cultured postnatal skin biopsies have been published.

In the five fibroblast lines, derived from individuals affected with infantile metachromatic leukodystrophy, all showed the presence of abnormal numbers of bodies which were limited by a single membrane and contained a granular central core. Bodies similar to these were observed in both normals and other disease states but it was the excessive number of these bodies in every passage which made it easy to distinguish these lines from all others. Of the four published reports on cultured cells from patients affected with metachromatic leukodystrophy, only one observed abnormalities in the cells. Lyon et al (1973) reported the appearance of similar bodies in fibroblast lines from three patients which were studied at confluency for several consecutive passages. They found that single membrane limited bodies, with a fine granular central core were evident. Comparison between the micrographs published by Lyon et al (1973) and the results demonstrated here show striking similarity in morphology of these bodies. In direct contrast, Rutsaert et al (1973) reported no abnormalities in the cell lines they studied for one passage which were grown to confluency. Hug et al (1973) found no consistent abnormality in cultured cells from individuals affected with metachromatic leukodystrophy. Kamensky et al (1973), in a single passage studied at confluency, reported no abnormalities which distinguished the diseased cells from normal controls.

In the four cell lines derived from patients affected with Duchenne's muscular dystrophy, two abnormalities were noted. One was the presence of

an abnormal number of lipid storage bodies and lysosomes; the second was the presence of abnormal, irregularly-shaped, evenly staining bodies which usually showed the presence of membrane remnants within the single membrane limiting these bodies. No other reports on the ultrastructure of cultured fibroblasts affected with Duchenne's muscular dystrophy have been published.

Of the five fibroblast lines studied which were derived from patients affected with cystic fibrosis, two of the lines were indistinguishable from normals, two others were distinguishable by the presence of abnormal cytoplasmic bodies which were limited by a double membrane and contained material resembling β -glycogen and membrane remnants, and the fifth line was distinguishable by the presence of a lamellar structure wrapped around itself. Bartman et al (1970) studied fibroblasts derived from patients affected with cystic fibrosis and found a difference in the number of cytoplasmic bodies when compared to normals. However, the normals studied at confluency in a single passage also showed large numbers of cytoplasmic inclusions. Tenconi et al (1972) studied fibroblasts from two patients affected with cystic fibrosis but they did not describe the culture conditions or the passage numbers examined. In all the cells, both normal and diseased, they reported that a number of cytoplasmic bodies were present and thus they concluded that the ultrastructural appearance of cultured fibroblasts may not be related to the phenotype of the cells.

It is obvious that great controversy exists in the published reports on the ultrastructure of cultured diseased fibroblasts. The majority of studies must be held to question if normal cells were not studied, or the culture conditions undefined. In addition, in light of the work

published to date on the normal appearance of cultured cells, it must be considered that the use of cells at confluency is questionable, as is also the use of a single passage of the cell line when assessing the presence or absence of abnormalities in a cell line. It can then be concluded that it is possible to distinguish six disease states in cultured postnatal skin fibroblasts using the techniques presented here. These are Pompe's disease, I-cell disease, Fabry's disease, Tay-Sachs disease, metachromatic leukodystrophy and Duchenne's muscular dystrophy. In some cases of cystic fibrosis, it may also be possible to distinguish the disease from normals.

In response to the second question posed in Chapter One, it was necessary to study the ultrastructure of both normal and diseased amniotic fluid fibroblasts. Figure 11 through to Figure 19 give confirmation that the fine structure of normal cultured amniotic fluid fibroblasts is virtually free of the consistent appearance of anomalous cytoplasmic bodies. However, abnormal bodies were very conspicuous in amniotic fluid fibroblasts in three of the genetic diseases studied. One other disease state was indistinguishable from the normal lines. In two amniotic fluid fibroblast lines known to be homozygotic for the allele causing infantile cystinosis, it was not possible using the techniques used in this thesis to distinguish these two lines from normal controls. No other published reports exist on the ultrastructural appearance of amniotic fluid fibroblasts in this condition.

The single amniotic fluid fibroblast line, known to be hemizygotic for the allele causing Fabry's disease, showed the appearance of excessive numbers of bodies resembling lipid storage bodies. These were seen in all the passages studied. No other published reports exist on the ultra-

structural appearance of amniotic fluid fibroblasts in Fabry's disease.

Tay-Sachs disease was studied by electron microscopy for four cultured amniotic fluid fibroblast lines known to be homozygotic for the allele causing the condition. Specific abnormal cytoplasmic bodies were seen in all four lines and in none of the normal controls studied.

In metachromatic leukodystrophy, the single amniotic fluid fibroblast line studied showed an excessive accumulation of single membrane limited bodies which contained a fine granular core. No other data has been reported on the ultrastructural appearance of cultured amniotic fluid fibroblasts with this condition.

It can be concluded that of the four diseases studied in amniotic fluid fibroblasts by electron microscopy three were ultrastructurally detectable. These are Fabry's disease, Tay-Sachs disease, and metachromatic leukodystrophy. In addition, three other disease states have been studied by the ultrastructural examination of cultured amniotic fluid fibroblasts. Hug et al (1973) in the study of Pompe's disease reported that in cultured amniotic fluid fibroblasts there were membrane limited bodies containing material resembling β -glycogen which they considered diagnostic for the disease. However, they did not publish the culturing method, the stage of cell examination nor detailed whether normals had been examined. In the study of I-cell disease, Aula et al (1975) demonstrated the appearance of large numbers of cytoplasmic inclusions in cultured amniotic fluid fibroblasts. The cells were examined six days after passage but no controls were studied. Kohn et al (1975) reported the detection of mucopolipidosis IV prenatally by the ultrastructural examination of cultured amniotic fluid cells. However, no normals were studied, the culturing conditions were undefined, and no studies were

done on other cultured cells prior to the diagnosis being made.

The third question posed in the introduction can now be answered. In Tay-Sachs disease, Fabry's disease, metachromatic leukodystrophy and infantile cystinosis, the appearance of the cultured postnatal skin fibroblast was similar to that of cultured amniotic fluid fibroblasts affected with the same disease. In Tay-Sachs disease, characteristic cytoplasmic bodies were seen in both the cultured skin fibroblasts and the cultured amniotic fluid fibroblasts. These bodies were not observed in any other cell lines of either type. The appearance of this anomaly indicates that the mutant autosomal recessive alleles responsible for the condition of Tay-Sachs disease are morphologically detectable in the prenatal period. Tay-Sachs disease has been successfully diagnosed biochemically prenatally on several different occasions (Becker and Seegmiller, 1975). Thus, it may now be possible to use ultrastructural studies in confirming these biochemical methods, thereby providing two methods of detecting the disease prenatally.

In Fabry's disease, abnormalities were detected in both the cultured postnatal skin fibroblasts and the cultured amniotic fluid fibroblasts. The abnormality noted was similar in both cell types, that is, there was an excessive accumulation of material resembling lipid storage bodies. The appearance of this abnormality in both cell types indicate that the mutant X-linked allele responsible for the condition is morphologically detectable in the amniotic fluid fibroblasts. Prenatal diagnosis of Fabry's disease has been successfully accomplished in several instances by biochemical assay (Becker and Seegmiller, 1975). Thus, it may now be possible to use electron microscopy as a second method to detect the disease prenatally.

Metachromatic leukodystrophy was also studied in both cultured amniotic fluid cells and cultured postnatal skin fibroblasts. In both cell types similar abnormalities were observed, with the occurrence of excessive numbers of membrane limited, evenly staining bodies which had a fine granular core being observed. The presence of the abnormality in both cell types indicates that the mutant autosomal recessive alleles responsible for the disease are morphologically detectable in the amniotic fluid fibroblasts. Prenatal diagnosis of metachromatic leukodystrophy has been successfully performed on several occasions using biochemical methods (Becker and Seegmiller, 1975). Thus, it may be possible now to use electron microscopy as a confirmatory technique in the prenatal diagnosis of metachromatic leukodystrophy.

In infantile cystinosis, amniotic fluid fibroblasts and cultured postnatal skin fibroblasts showed no detectable abnormality by ultrastructural examination of the cells. This does not necessarily exclude the use of electron microscopy in the study of infantile cystinosis, but it does indicate that the techniques used in the work presented here are not capable of detecting any abnormality. Prenatal diagnosis of the disease is possible by biochemical assay (McKusick, 1975).

The results described in Chapter Four demonstrate that the ultrastructural examination of cultured human fibroblasts has major implications in at least two specific directions. Firstly, the use of electron microscopy has immediate clinical application for prenatal diagnosis; and secondly, it has a long term potential in the study and interpretation of mechanisms of pathogenesis of genetic disorders in the prenatal period.

For ultrastructural phenomena in cultured fibroblasts to be of value in prenatal diagnosis it is essential that a similar ultrastructural

appearance be present in amniotic fluid fibroblasts but not manifest in fibroblasts from heterozygotes for the disease locus in question. In Tay-Sachs disease, Fabry's disease, and metachromatic leukodystrophy, it was demonstrated here that the amniotic fluid fibroblasts showed similar inclusion bodies to the skin fibroblasts. These bodies were absent from the heterozygotic skin fibroblasts available for study. In addition, diagnostic lesions have been described in amniotic fluid fibroblasts of Pompe's disease (Hug et al, 1973) and I-cell disease (Aula et al, 1975). In the homozygotic cell lines for Pompe's disease and I-cell disease this report confirms the appearance of abnormalities. Also, the heterozygotic I-cell disease fibroblasts available were indistinguishable from normal controls. The above results indicate that fetuses affected with a specific disease should be distinguishable from carriers for the condition under study, as well as the normals. If the concordance between the abnormalities in cultured postnatal skin fibroblasts and cultured amniotic fluid fibroblasts is applicable for Duchenne's muscular dystrophy, a condition for which no definitive biochemical tests exist, a specific diagnosis in high risk situations for this disorder might be possible. Only by a more extensive examination of skin fibroblasts from patients with genetic disorders, in particular those for which there is no biochemical understanding, will the full potential of this approach for prenatal diagnosis be established. It can be concluded from this study that electron microscopy of cultured amniotic fluid cells provides another means to supplement, and in some diseases, complement the methods that are currently being used in the detection of disease prenatally. It should not be thought that these abnormalities can be considered to be specific for any disease state. These lesions do, however, indicate that

affected genotypes in certain disease states alter the morphology of the cell to produce abnormalities which make it possible to distinguish diseased cells from the normal controls. It cannot be assumed that each genetic disease studied in cultured human fibroblasts will be characterized by a specific morphological aberration. This possible lack of specificity should in no way negate the potential use for prenatal diagnosis by electron microscopy since the presence of abnormal inclusions or alterations in the cellular morphology would still be indicative of a pathological condition in the cell.

The second, and perhaps more important, use of the electron microscopic study of cultured human fibroblasts is associated with the potential use of this system to study mechanisms of disease. The ability to change the environment in which the cells grow permit two types of manipulation. The first is the deprivation of specific components from the cellular environment in an attempt to induce alteration in the morphology of the cells. This might be used in determination of enzyme function by putting undue stress on heterozygotes for particular conditions, thus causing malfunctions in metabolic capacities and thereby observe the effect that these malfunctions have on the structure of the cell. The second type of manipulation that can be carried out on cultured cells would be the opposite, that is, loading the cells with excess amounts of metabolites with the intent of altering the functioning of the cell and thereby its morphology. This approach could be used in the detection of heterozygotes by loading the metabolic path under study and determining the effects of the cell's inability to metabolise at the normal rate. This loading of cells can also be used in the investigation of the possibility of utilizing enzyme replacement therapy in the treatment of

genetic disorders. The effects of the addition of a specific isolated enzyme to a cell population can be morphologically studied to determine how rapidly the enzyme is uptaken, how the enzyme is uptaken in the cell, and where the enzyme acts in the cells can be studied by electron microscopy which would provide information on the suitability of enzyme replacement therapy.

The study of genetic diseases can also utilize electron microscopy as a method of determining the deficient enzyme in conditions where it is not known. For example, if the morphological abnormality in Duchenne's muscular dystrophy demonstrated in this thesis can be confirmed by independent sources, it may be possible to fractionate the affected cells, isolate the abnormal body, determine its biochemical composition and make efforts to determine which biochemical pathway would produce the abnormal product if the pathway was disturbed.

Two developments must occur before it will be possible to introduce electron microscopy for prenatal diagnosis at the clinical level. Firstly, there is an urgent need for definitive studies on normal cell lines. To date, only cursory work has been done on the ultrastructure of normal, cultured cell lines - both amniotic fluid fibroblasts and postnatal skin fibroblasts. In addition, more extensive work must be done in regard to comparisons between postnatal fibroblast lines and amniotic fluid fibroblast lines. The cell lines studied in this work represent the more extensive survey of normal fibroblast lines published. It is obvious that far more work needs to be done before conclusive statements can be made without reservation. Secondly, more complete and thorough studies of both specific disease states and their corresponding carrier states must be undertaken to ensure that it is possible to distinguish the affected

homozygote from the unaffected carrier.

CHAPTER SIX

SUMMARY

1. It was demonstrated that it was possible to minimize the number of unique cytoplasmic bodies which appeared in normal cell lines by careful control of the culturing conditions under which cells were grown, and by the ultrastructural examination of the cells in the non-confluent stage.
2. It was demonstrated that it was possible to detect genetic disorders by the ultrastructural examination of cultured postnatal skin fibroblasts. Abnormalities were detected in Pompe's disease, Fabry's disease, Tay-Sachs disease, I-cell disease, metachromatic leukodystrophy, and Duchenne's muscular dystrophy.
3. It was demonstrated that it was possible to detect genetic disorders by the ultrastructural examination of cultured amniotic fluid fibroblasts. Abnormalities were detected in Fabry's disease, Tay-Sachs disease, and metachromatic leukodystrophy.
4. It was concluded that it is now potentially possible to utilize electron microscopy in the prenatal diagnosis of genetic disease.

CHAPTER SEVEN

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