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Acid Lipase Deficiency and Lipid Storage in Wolman's Disease and E600-Treated Cells in Culture

Martin George Bialer
Medical University of South Carolina

ACID LIPASE DEFICIENCY AND LIPID STORAGE IN WOLMAN'S DISEASE AND E600-TREATED CELLS IN CULTURE

by

Martin George Bialer

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Department of Pathology

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Approved by:

R. A. Vincent, Jr.
Chairman, Advisory Committee

Lee Silloway

Elsie Taber

John P. Mayro

Emmon Bergdahl

James M. Boggs MD

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ABSTRACT

MARTIN G. BIALER. Acid Lipase Deficiency and Lipid Storage in Wolman's Disease and E600-Treated Cells in Culture. (Under the direction of RUSSELL A. VINCENT, JR., Ph.D).

Fibroblasts obtained from a child with Wolman's disease and maintained in culture demonstrated acid lipase deficiency, reached senescence prematurely and exhibited an abnormal #5 chromosome. When cytogenetic analysis was repeated on frozen and stored cells, the chromosomal defect could no longer be demonstrated, but other anomalies were present. The karyotypes of other Wolman's disease cell cultures and the parents of the proband were normal. The mother's fibroblasts had reduced acid lipase activity, consistent with a carrier-state, but the father's fibroblasts had normal acid lipase activity. It was possible to classify a culture as Wolman's disease, carrier or normal by the ability of medium from the culture to reduce the lipid stored in a Wolman's disease cell culture. The culture of the proband stored more lipid than other Wolman's disease cultures.

Two enzymes present in fetal calf serum possessing paraoxonase activity could be differentiated by their sensitivity to heating at 56°C. The esterase inhibitor E600 was slightly more toxic to Wolman's disease than to normal cells, and was also toxic to E. coli.

Normal cells exhibited lipid storage when treated with 10^{-4}M E600, but a more pronounced time-dependent accumulation of lipid occurred at 10^{-3}M E600. p-Nitrophenol, the major metabolite of E600, had little effect on lipid storage. The amount of lipid stored varried directly with the serum concentration and was unaffected

by heat inactivation of the serum. Histochemical stains and biochemical analyses were performed on cultured fibroblasts. Both Wolman's disease and E600-treated cells showed storage of triglycerides and cholesteryl esters, although one Wolman's disease culture had a normal level of triglyceride. The E600-treated cells also showed a large increase in phospholipid and small increases in free cholesterol and free fatty acid. Wolman's disease cells treated with E600 showed increases comparable to the normal E600-treated cells, but no increase of free fatty acid was seen. Wolman's disease heterozygotes appeared to have normal amounts of lipid. Lipid in Wolman's disease and E600-treated cells fluoresced in ultraviolet light. Cholesteryl esters of untreated normal cells had more stearic (18:0) than oleic (18:1ω9) acid, whereas cholesteryl esters in Wolman's disease, E600-treated cells, and fetal calf serum had three times more oleic than stearic acid, suggesting that serum lipid was taken up, but not degraded, in Wolman's disease and E600-treated cells.

Three Wolman's disease cell cultures exhibited genetic deficiency of acid lipase. Normal fibroblasts had acid lipase activity, but possessed little neutral lipase activity. Acid lipase activity of normal cells was inhibited by E600.

Wolman's disease cells resembled normal cells by scanning electron microscopy. Except in a perinuclear zone which also had not stained with oil red O or neutral red, the cytoplasm of E600-treated cells had numerous bumps which appeared to form ridges along the path of actin stress filaments.

Normal cells treated with free fatty acid or cholesterol, and Wolman's disease cells treated with free fatty acid, exhibited lipid storage in large, peripheral lipid droplets. Lipid was generally stored in smaller granules closer to the nucleus in E600-treated and Wolman's disease cells. E600-treated cells showed increased numbers of lysosomes by neutral red staining and had increased numbers of dense bodies ultrastructurally. The dense bodies of Wolman's disease and E600-treated cells sometimes contained lipid clefts which probably consisted of cholesteryl ester. The dense bodies accumulated colloidal gold, suggesting their identity with secondary lysosomes. Although some differences were observed, E600-treated cells resembled Wolman's disease cells histochemically, biochemically, and ultrastructurally.

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INTRODUCTION

The lysosomal enzyme, acid lipase, was first characterized by Stoffel and Greten (1) in 1967. The enzyme was first reported in human tissue by Patrick and Lake (2) in 1969. In this report, its deficiency in liver and spleen of patients with Wolman's disease was causally linked with this disorder.

In Wolman's disease, triglycerides and cholesteryl esters accumulate in many tissues of the body. The liver is particularly affected, storing so much lipid that it assumes a yellow color. The spleen and gastrointestinal tract are also severely affected. Both adrenals exhibit extensive punctate calcification, a feature that is virtually pathognomonic of the disease. The disease appears to follow an autosomal recessive pattern of inheritance. Infants with Wolman's disease usually present with vomiting or diarrhea and steatorrhea within the first few weeks of life. At this time, hepatosplenomegaly is already present, becoming massive as the disease progresses. The infants show severe malabsorption and fail to thrive. Patients have not benefited from the few attempts at dietary management, and generally die before six months of age. Comprehensive reviews of Wolman's disease have been written by Patrick and Lake (3) and by Sloan and Frederickson (4). It is a severe disorder of lipid storage, an indication of the importance of the acid lipase enzyme to normal function. Its severity may also be related to the high concentration of triglycerides and cholesteryl esters in the body and the continual turnover of these substances. Thus interference with one step in the metabolic pathway may lead to rapid and excessive storage of lipid.

Cholesteryl ester storage disease is another rare disorder of lipid metabolism that appears to be caused by a deficiency of acid lipase (5,6). However, the disease is generally less severe than Wolman's disease. Many patients have shown no symptoms other than hepatosplenomegaly and hypercholesterolemia, although in some families the disease may have a more malignant course leading to death in childhood (7). Although cells from patients with cholesteryl ester storage disease have more residual acid lipase activity than those from patients with Wolman's disease (5,8), the differences have not been deemed to be sufficient to account for the dramatic difference in the courses of the two disorders. However, Goldstein et al. (9) have shown that intact cells from patients with cholesteryl ester storage disease, which have stored approximately three times as much cholesteryl ester as cells derived from normal individuals, show nearly one-third the acid lipase activity of normal cells. The same cells exhibit only 5% of normal activity when assays are carried out on cell-free extracts. Similar studies with intact Wolman's disease cells have not been done. Thus, a small difference in acid lipase activity when only a small amount of activity is present may make a big difference in the amount of lipid stored and the course of the disease. Brown et al. (10) have shown that only 10% of normal acid lipase activity may be necessary for normal function.

The acid lipase enzyme has a pH optimum of approximately 4 to 5 in most assay systems (9,11-15). It is active toward both triglycerides and cholesteryl esters (15), hydrolyzing both lipids at their ester linkages. The enzyme is probably identical to acid cholesteryl esterase (15) and acid esterase (13). It is relatively resistant to E600

(diethyl p-nitrophenyl phosphate, paraoxon), which allows it to be classified as an A-type esterase (16,17) because lipases are considered to be a subtype of esterases (18). It is resistant to diisopropylfluorophosphate, activated by Triton X-100 and taurodeoxycholate and inhibited by cyanide, p-hydroxymercuribenzoate, sodium chloride, 4,4'-diethylaminoethoxyhexestrol, chloroquine and other substances (11,12,14,19,20). Acid lipase is a true lipase because it is more active toward esters of long chain fatty acids than esters of short chain fatty acids (13,17). The enzyme is fairly labile and this has made it difficult to purify (12,13). Cortner et al. (8) have separated three bands of acid lipase activity by cellogel electrophoresis, only one of which was absent in Wolman's disease and cholesteryl ester storage disease. Four discrete bands of acid esterase activity have been separated by isoelectric focusing on polyacrylamide gel (13,21). Wolman's disease fibroblasts were unable to hydrolyze the substrate used to assay acid esterase activity (13,21). It is possible that the isozymes may have different substrate specificities (8).

Acid lipase can be shown to be important to normal function, but neither Wolman's disease nor cholesteryl ester storage disease is very important epidemiologically. There have been approximately 30 cases of Wolman's disease reported since it was first described in 1956 (3,8). Cholesteryl ester storage disease has been observed in about 14 patients since its first description in 1966 (7). However, the study of Wolman's disease has pointed out the clinical importance of acid lipase activity and the study of acid lipase deficient cells has contributed to an understanding of the way lipid is metabolized and stored in the body.

Atherosclerosis is a disease characterized by plaques of lipid material accumulating in the arterial wall. Much of this lipid is cholesteryl ester, and in two animal model systems the cholesteryl ester has been shown to accumulate in lysosomes of aortic smooth muscle cells (22-24). Several reports have suggested that a relative deficiency of acid lipase may be involved in the pathogenesis of atherosclerosis (22-26).

E600 has been used extensively to inhibit esterases in biochemical (27,28) and histological (16,29) studies. Aldridge (27) used the sensitivity of esterases to E600 to distinguish between A and B-type esterases. B-type esterases are inhibited by 10^{-5}M E600 whereas A-type esterases are not inhibited by 10^{-3}M E600. B-type esterases include acetylcholinesterase (27) and liver microsomal esterase (28). A-type esterases include acid lipase (16) and serum paraoxonase (30-32), a serum enzyme that is able to cleave E600. E600 appears to inhibit esterases by transferring a diethylphosphoryl radical to the active site (33). Although E600 has been in use as an esterase inhibitor since 1950 (34), it has rarely been used to treat whole cells. The few studies on whole cells have been toxicological (35,36) or concerned with acetylcholinesterase activity of chick embryo muscle cultures (37,38). No studies of the effect of treating cultured cells with an esterase inhibitor for several days have been published.

In this dissertation, the results of decreased acid lipase activity in genetically deficient Wolman's disease cells and in normal cells rendered dysfunctional for acid lipase by E600 treatment are presented. The dissertation consists of a general introduction, four research sections, and an overall discussion bringing together all the

findings from which certain conclusions are drawn. The first research section presents an unusual case of Wolman's disease and studies on both the parents and the proband. The second research section presents evidence that E600 treatment induces lipid storage in normal cells and provides a histochemical comparison of the lipid stored in Wolman's disease cells with that stored in E600-treated normal and Wolman's disease cells. The third research section presents biochemical studies of Wolman's disease and E600-treated cells, including lipid analyses and enzymatic studies. In the fourth research section, the ultrastructure of Wolman's disease and E600-treated cells is compared and electron microscopic cytochemical studies are presented.

Some results from the first section have been published as an abstract in Laboratory Investigations (39). Some results from the second section have been published as an abstract in the Journal of Cell Biology (40). Data from the second and third sections have been submitted to Experimental Cell Research. Early results from the fourth section were presented at the Southeastern Electron Microscopy Society Meeting (41).

RESEARCH SECTION I

Introduction

Wolman's disease appears to be an inherited disorder with an autosomal recessive mode of transmission. Females are affected as often as males. Of 15 families studied, first cousin marriages were involved in three families, and siblings of the proband were affected in at least four (3). Furthermore, parents of affected children and some of the siblings of affected children have levels of acid lipase activity about half that of normal individuals, falling into the range expected for heterozygotes or carriers of a given trait (3,8,17,42). Carriers for Wolman's disease can thus be detected by assay of acid lipase activity.

Fibroblasts cultured from Wolman's disease patients exhibit acid lipase deficiency (8,19,42,43) and accumulate triglycerides and cholesteryl esters (42). The lipid can be visualized by staining with oil red O (8,42). Kyriakides et al. (42) reported increased lipid storage in fibroblasts cultured from the father of a Wolman's disease patient, but this result has not been repeated.

Cultured fibroblasts have been shown to release lysosomal enzymes into the medium and to take up the enzymes again by a specific receptor-mediated endocytosis (44,45). Cells lacking a specific lysosomal enzyme are able to take up that enzyme if it is introduced into the medium. If medium from normal cells, in which acid lipase can be detected (10), is added to Wolman's disease cells, the acid lipase is endocytosed and transported to lysosomes, where it is able to degrade stored triglycerides and cholesteryl esters. Thus medium from normal cells can be used to correct the Wolman's disease defect in vitro (10,42).

Cytogenetic analysis has only been reported on one Wolman's disease patient (46), and this patient had a normal chromosome complement. Cytogenetic abnormalities are rarely seen in patients with lipid storage disorders. This section presents a case of Wolman's disease in which a chromosomal anomaly was observed, and includes studies done on the parents of this child to provide information about the inheritance of the defect.

Materials and Methods

Cell Culture: Cells were routinely cultured as monolayers in 25 cm^2 polystyrene tissue culture flasks (Falcon or Corning). They were grown in 5 ml of Eagle's Minimal Essential Medium (MEM) with Earle's salts (Flow) or Dulbecco's modified MEM (GIBCO). The medium was supplemented with 8% or 15% fetal calf serum (FCS) (GIBCO) and 1% antibiotic-antimycotic 100X mixture (GIBCO). The same media supplemented with 3% FCS were used for cell maintenance. Medium was filtered sterilized through 0.22 µm cellulose acetate filters (Millipore). Medium was changed weekly. Cells were incubated in a tissue culture incubator (WEDCO) at 37.5°C in a humidified atmosphere of 5 to 8% CO_2. Manipulations requiring sterile conditions were carried out in vertical laminar flow hoods (Baker Co.) For subcultivation, cells were rinsed with 2.5 ml of pH 7.0, Puck's balanced salt solution (PBS) (NaCl, 145.4 mM; Na_2HPO_4, 845.3 µM; NaH_2PO_4, 362.3 µM) and exposed to 1.5 ml of pH 7.1 trypsin solution (DIFCO) (0.25% trypsin with NaCl, 136.9 mM; KCl, 5.4 mM; dextrose, 5.6 mM; $NaHCO_3$, 7.1 mM; EDTA, 537.3 µM) until cells detached. Medium was then added, and the cells were transferred to new culture flasks.

Cell Origin: Fibroblast cultures derived from Wolman's disease patients (GM-1606, GM-2211), normal donors (GM-179, GM-316, GM-37, GM-498, IMR-90), a Wolman's disease heterozygote (GM-2121), and a Chediak-Higashi syndrome

patient (GM-2075) were obtained as monolayers from the Institute for Medical Research (Camden, N.J.). Other cultures (GM-2109, GM-2520, SaJo, GM-3557, GM-3558, JoAl) were established from skin biopsy specimens. GM-2109 (P), a Wolman's disease culture, was established by J.F. Rogers from abdominal skin obtained 2 hr postmortem. The other cultures were established from forearm biopsies: GM-2520 from a 10 year old girl with Bloom's syndrome, JoAl from a 13 year old boy with metachromatic leukodystrophy, SaJo from a 20 year old normal woman, GM-3557 (M) from the 24 year old mother and GM-3558 (F) from the 28 year old father of the child from whom culture GM-2109 (P) was established. The latter three cultures were established with the help of J.F. Rogers. The skin was cleansed and a local anesthetic was applied. A piece of skin was excised with a sterile scalpel and placed in sterile medium with antibiotic. The biopsy specimen was then cut into fragments approximately 0.5 mm in size and each was positioned with 4 or 5 other fragments in one of several 25 cm^2 tissue culture flasks. Two and one-half ml of medium was added to the top surface of each inverted flask and the biopsy fragments were allowed to attach to the bottom for 1 hr at room temperature. Each flask was then carefully turned right side up and the medium was allowed to cover slowly, but not loosen, the fragments. The flasks were incubated at 37.5°C as mentioned above. When outgrowths covered about 75% of the flask, the cells were subcultured. The resultant fibroblast cultures were designated subculture 1. Further subcultures were noted and the passage level of the cells was recorded for all experiments.

Cytogenetic analysis: Chromosomes were examined from fibroblast cell cultures of Wolman's disease (GM-2109 (P), GM-2211, GM-1606) and the

parents of the local proband (GM-3557 (M), GM-3558 (F)). Chromosomes from lymphocytes cultured from the parents were also examined. Fibroblast cultures were treated with colchicine (Lilly) for 3 to 5 hours, swelled in hypotonic solution (.075 M KCl) for 35 minutes and fixed in 3:1 methanol-acetic acid. Lymphocytes were treated with colchicine for 1.5 hours, hypotonic for 25 minutes, and fixed similarly. The cells were spread on slides and burst by air or blaze drying. The slides were stained with 4% Giemsa (Fisher) in pH 6.8 phosphate buffer. For chromosome banding, slides were treated with 2 ml of pancreatin (GIBCO) in 7 ml of Hank's balanced salt solution (GIBCO) and 7 ml of distilled water for 5-6 sec at 37°C.

Chromosomes were counted and examined from at least 20 cells per culture. Karyotypes were prepared on several of the best typical cells and on any atypical cell. The initial karyotypes of GM-2109 (P) were prepared under the direction of J.F. Rogers.

Acid lipase assay: Cells were assayed for acid lipase activity by the fluorometric method of Cortner et al. (8). Generally cells were grown to confluency in a 75 cm^2 flask and trypsinized about one week later. The cells were washed three times in PBS, pelleted and frozen at -70°C until the time of assay. Substrate, 4-methylumbelliferyl oleate (Research Products International) dispersed in lecithin (Sigma) and taurodeoxycholate (Sigma), was prepared beforehand and frozen. On the day of assay the substrate was thawed and one volume was diluted with 7 volumes of 0.2 M acetate buffer, pH 4.0. Eight ml was placed into 25 ml Ehrlenmeyer flasks. Four-methylumbelliferone standards were prepared at concentrations of 0.1 to 2.0 nmol/ml. Then PBS was added to the cell pellets and they were sonicated on ice with a microtip sonicator (Heat Systems - Ultrasonics). Centrifuging at this point could increase the measure of activity, but might also

decrease the reproducibility of the assay. One hundred µl of enzyme source was added to a prewarmed Ehrlenmeyer flask of substrate, mixed, and incubated in a 37°C water bath. At 2, 4, 8 and 16 min, 2 ml of reaction mixture was removed and read on an Aminco-Bowman spectrofluorophotometer at an excitation wavelength of 322 nm and an emission wavelength of 448 nm. Protein concentration in each sample was measured the next day by the method of Lowry et al. (47) or Bradford (48). The specific activity of acid lipase was expressed as nmol of substrate hydrolyzed per min per mg protein.

Correction by conditioned medium: To test the ability of medium from different cell lines to correct the Wolman's disease defect, GM-1606 Wolman's disease cells were seeded onto 22 x 22 mm coverslips in 35 x 10 mm culture dishes at a concentration of 5×10^4 cells per dish. Treatment with conditioned medium was begun 3 days later and lasted 2 weeks. The conditioned medium, with 3% FCS, was pipetted from various cultures, and 1 ml was added to 2 ml of fresh medium, with 8% FCS. Medium was changed every 3 days and conditioned medium was made up fresh each time. After 2 weeks, the cells were fixed for 2 hr in calcium buffered formalin (4% formaldehyde from paraformaldehyde and 2% calcium acetate, pH 7.0-7.2). The coverslips were stained with oil red O and hematoxylin (49) and mounted on slides with glycerol gel. One hundred cells on each coverslip were graded from 0 to 3 in intervals of 0.5 for the extent of sudanophilic granulation. Briefly, grade 0 represents no lipid, grade 3 represents 100% of the cytoplasm exhibiting lipid staining and grade 1.5 represents 50% of the cytoplasm staining. Cells exemplifying the different grades are shown in Figure 1. After grading, the numbers were

summed to give a representation of the amount of lipid present on a given coverslip.

Enzyme addition: Coverslip cultures of GM-1606 were prepared as in the correction by conditioned medium experiment. The cells were treated with varying concentrations of porcine pancreatic lipase (Sigma L-2253), hog liver carboxyesterase (Sigma E-9627) or bovine pancreatic cholesterol esterase (Sigma C-3766) and Sendai virus (10^3 particles per culture dish) to increase permeability of the membrane. After 3 days, the cells were fixed, stained, and graded as in the correction by conditioned medium experiment.

Results

Case Report:

Following a normal delivery, the proband had diarrhea during his first three weeks at home. At 3 months, the child was admitted to the Medical University Hospital with massive hepatosplenomegaly and abdominal distension. No pre-beta or alpha lipoprotein activity was seen by electrophoresis. A liver biopsy showed marked fatty change with massive periportal fibrosis. A flat plate of the abdomen revealed bilateral punctate calcification of the adrenals, virtually pathognomonic of Wolman's disease. The patient died at age 4 months. Autopsy revealed a malnourished black male infant. There was enlargement of the liver, spleen, adrenals and lymph nodes. The liver appeared yellow-orange in color and microscopic examination revealed fat vacuoles in hepatocytes and cholesteryl ester clefts in periportal macrophages. Lipid analysis revealed increased triglycerides, cholesteryl ester, and free cholesterol. The presentation was typical of Wolman's disease, the first case reported in a black child.

Cytogenetic Analysis:

Karyotypes of the GM-2109 (P) Wolman's disease cell culture established from the proband revealed a long B-group chromosome (Fig. 2) which, from banding, appeared to be the #5 chromosome (Fig. 3). The long chromosome was present in 19 of 21 cells examined, and the 2 cells in which it was not seen were very contracted and difficult to analyze. Karyotypes from the GM-1606 (Fig. 4) and GM-2211 (Fig. 5) Wolman's disease cell lines revealed normal female and male karyotypes respectively. Karyotypes from GM-3557 (M) (Fig. 6) and GM-3558 (F) (Fig. 7), cell cultures established from the parents of the proband, revealed no balanced translocation or other abnormality, and the banded karyotypes (Figs. 8,9) did not reveal the presence of a condition (e.g. an inversion) that might dispose the proband to a translocation event.

Cells that had been frozen and stored at the Institute for Medical Research were utilized to repeat cytogenetic analysis on the GM-2109 (P) culture. The cells grew poorly and only 10 metaphase cells were obtained. Of these, 7 were normal, 2 showed a different abnormality of the #5 chromosome (5p+) and 1 cell showed a partial duplication of the #1 chromosome. No cells showed the anomaly seen initially.

Acid lipase assay: The cells of the proband showed a deficiency of acid lipase that was comparable to the 2 other Wolman's disease cell cultures assayed (Table 1).

If this case followed classical Mendelian inheritance, both parents would be expected to be carriers for the disease and the

TABLE 1

ACID LIPASE ACTIVITY OF CELL HOMOGENATES PREPARED FROM CULTURED CELLS OF CLINICALLY NORMAL AND WOLMAN'S DISEASE DONORS.[a]

SAMPLE		ACTIVITY[b]
Apparently normal:		
GM-179		1.51
GM-316		0.93
SaJo		1.98
IMR-90		0.71
GM-2520		2.23
	Mean	1.47 ± 0.65
Wolman's disease:		
GM-1606		0.27
GM-2211		0.46
GM-2109 (proband)		0.29
	Mean	0.34 ± 0.10

[a]Confluent cell cultures were trypsinized, pelleted, and sonicated on ice on a microtip sonicator. Homogenates were assayed for acid lipase by a fluorometric technique.

[b]Nanomoles of 4-methylumbelliferyl oleate hydrolyzed per min per mg protein.

chromosomal anomaly would be unrelated to the disease process. However, when acid lipase was assayed, only the mother (GM-3557 (M)) appeared to be a carrier for Wolman's disease (Table 2). The father (GM-3558 (F)) had a normal level of acid lipase activity. Although this assay is somewhat variable, the mother could be classified as a carrier in 2 of 3 assays though appearing normal in one assay and the father could be classified as normal in 3 of 3.

Correction by conditioned medium: Media from normal cell cultures and a Chediak-Higashi cell culture caused a reduction of lipid storage in Wolman's disease cells (Fig. 10), thus demonstrating the ability to correct the Wolman's disease defect. Media from Wolman's disease cells showed little correction. Medium from a known carrier for Wolman's disease showed some ability to correct the defect but not as much as seen in media from normal cells. Media from the parents of the local proband were also examined. Media from the father's cells corrected the defect as well as normal cells whereas media from the mother's cells did not. All the results correlated well with the level of acid lipase activity of the different cultures, providing further support for classifying the mother, but not the father, as a carrier for Wolman's disease. When the extent of lipid in the treated cultures was plotted against the acid lipase activity shown in assay 1 of Table 2, the points fit a straight line (Fig. 11), statistically significant by linear regression ($p<.05$).

Cells from the parents exhibited little oil red O staining whereas cells from the proband had large numbers of oil red O positive granules (Fig. 12).

TABLE 2

ACID LIPASE ACTIVITY OF CELL HOMOGENATES PREPARED FROM CULTURED CELLS OF CLINICALLY NORMAL, WOLMAN'S DISEASE AND WOLMAN'S DISEASE HETEROZYGOTE DONORS, AND OF THE PARENTS OF THE PROBAND.

SAMPLE	ACTIVITY[a]		
	Assay 1	Assay 2	Assay 3
Apparently normal:			
GM-179	2.49	2.58	1.00
GM-37	2.28	2.59	1.10
GM-498	1.04	2.77	1.06
GM-2075	1.87	5.17	2.50
GM-2520	1.29	1.68	1.96
GM-1492		1.52	
GM-1493		5.43	
JoA1		1.85	1.88
GM-316			1.24
Mean	1.79 ± 0.62	2.95 ± 1.52	1.53 ± 0.58
Wolman's disease:			
GM-1606	0.16	0.17	0.12
GM-2211	0.41		0.29
Mean	0.29 ± 0.18		0.21 ± 0.12
Carrier:			
GM-2121	1.08		1.00
Proband's parents:			
GM-3557 (M)	0.96	2.87	0.57
GM-3558 (F)	2.28	3.13	2.22

[a]Nanomoles of 4-methylumbelliferyl oleate hydrolyzed per min per mg protein.

Figure 1. Cytoplasmic lipid accumulation in cultured cells showing the grades (0-3.0) into which cells were classified according to the extent of oil red O positive staining they exhibited. Stained with oil red O and hematoxylin. X550.

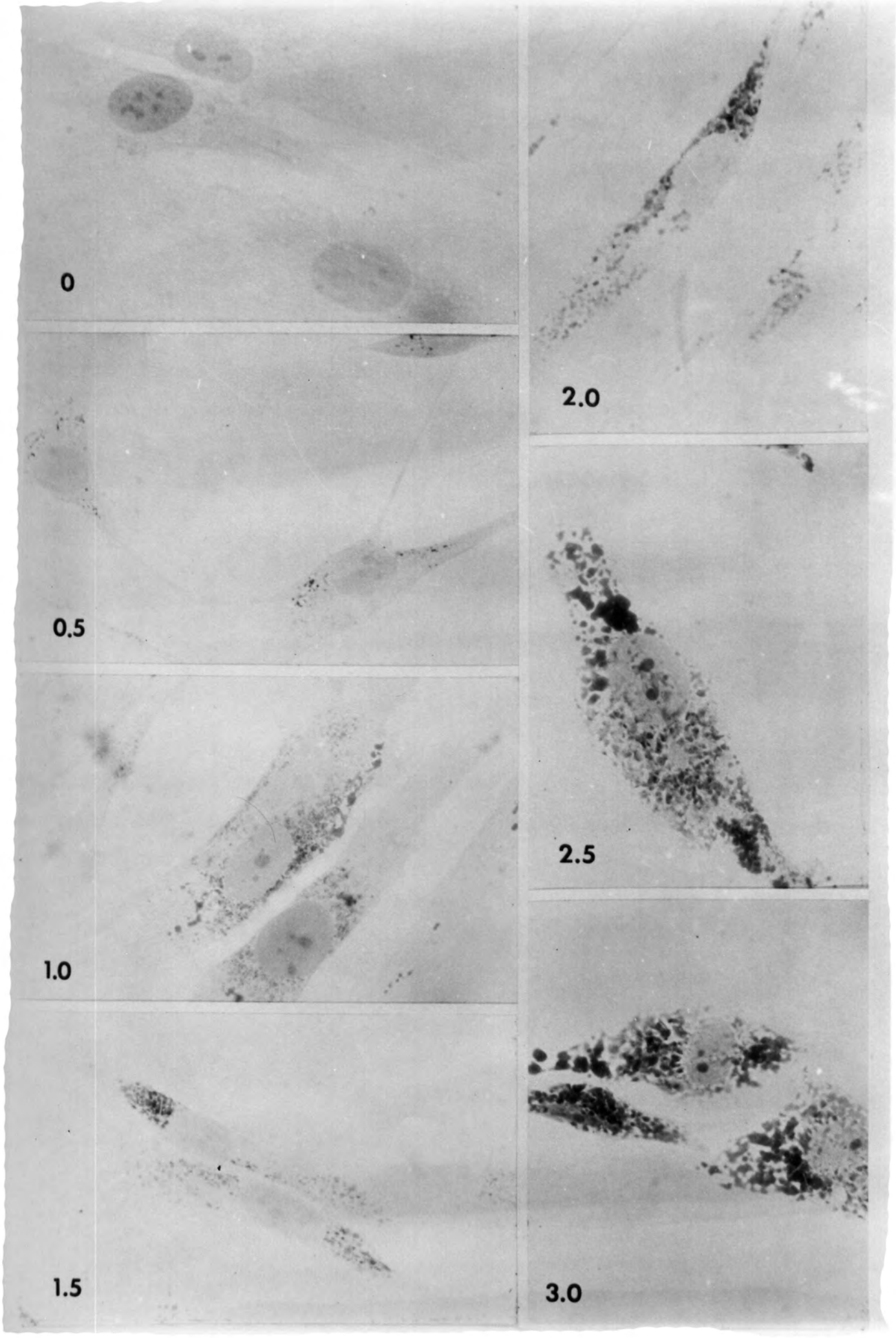
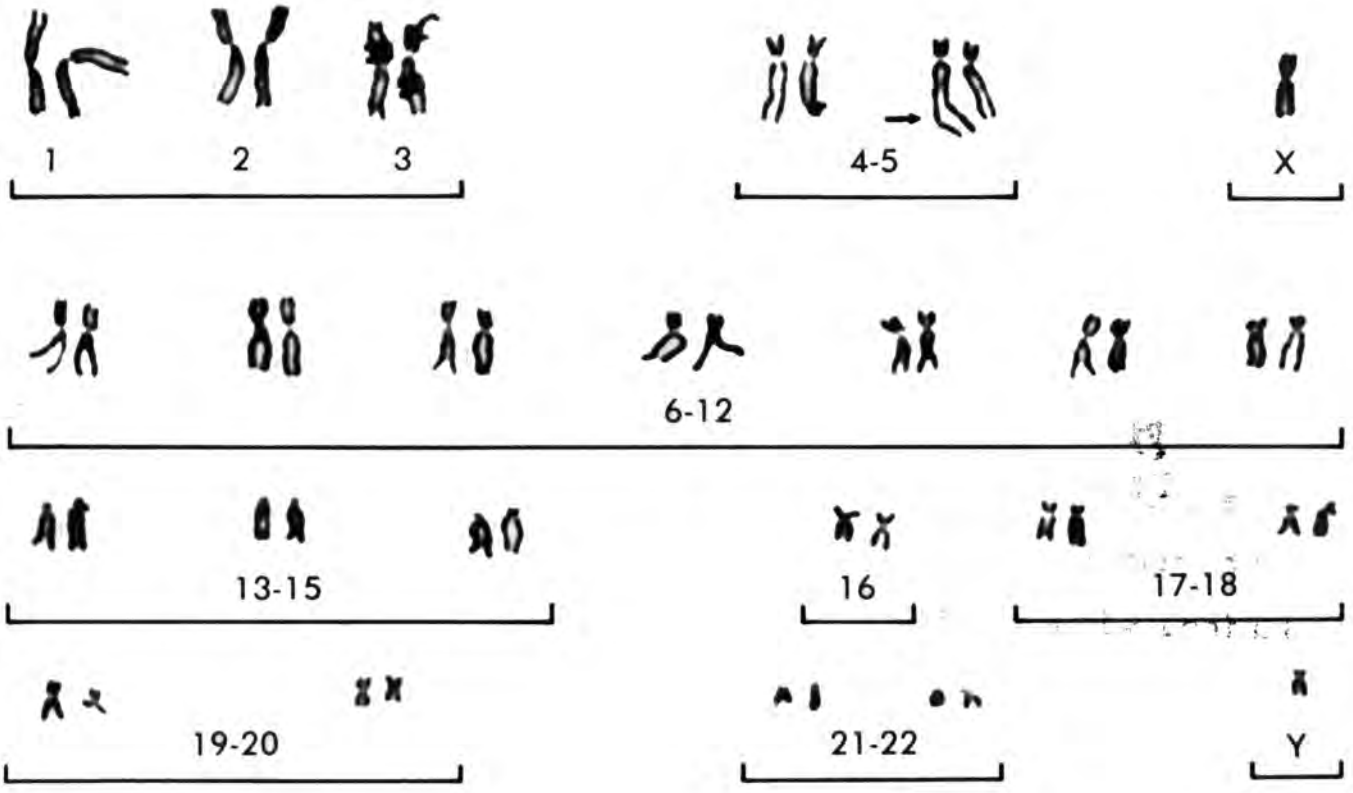

0
0.5
1.0
1.5
2.0
2.5
3.0

Figure 2. Karyotype of the GM-2109 (P) cell culture derived from a child with Wolman's disease. The male karyotype exhibited extra material on the long arm of a B-group chromosome (arrow). Stained with Giemsa.

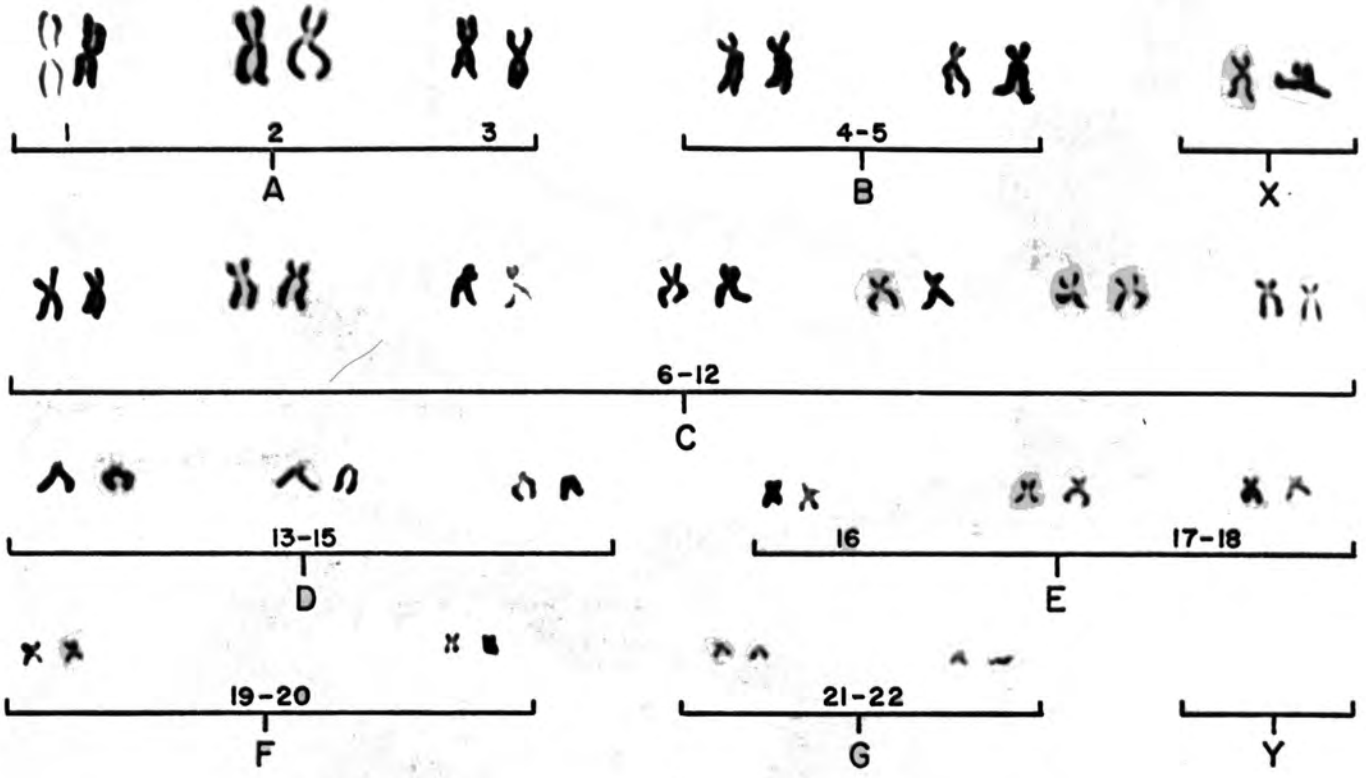
Figure 3. B-group chromosomes of Wolman's disease cell culture GM-2109 (P) stained by a Giemsa banding procedure. The chromosome with the long arm appeared to be the #5 chromosome. The karyotype of the GM-2109 (P) culture was 46,XY,5q+.


1
2
3
4-5
X
6-12
13-15
16
17-18
19-20
21-22
Y


4
5

Figure 4. Karyotype of Wolman's disease cell culture GM-1606. Metaphase spreads from this culture exhibited a normal female karyotype (46,XX). Stained with Giemsa.

Figure 5. Karyotype of Wolman's disease cell culture GM-2211. Metaphase spreads from this culture exhibited a normal male karyotype (46,XY). Stained with Giemsa.


1
2
3
A
4-5
B
X
6-12
C
13-15
D
16
17-18
E
19-20
F
21-22
G
Y

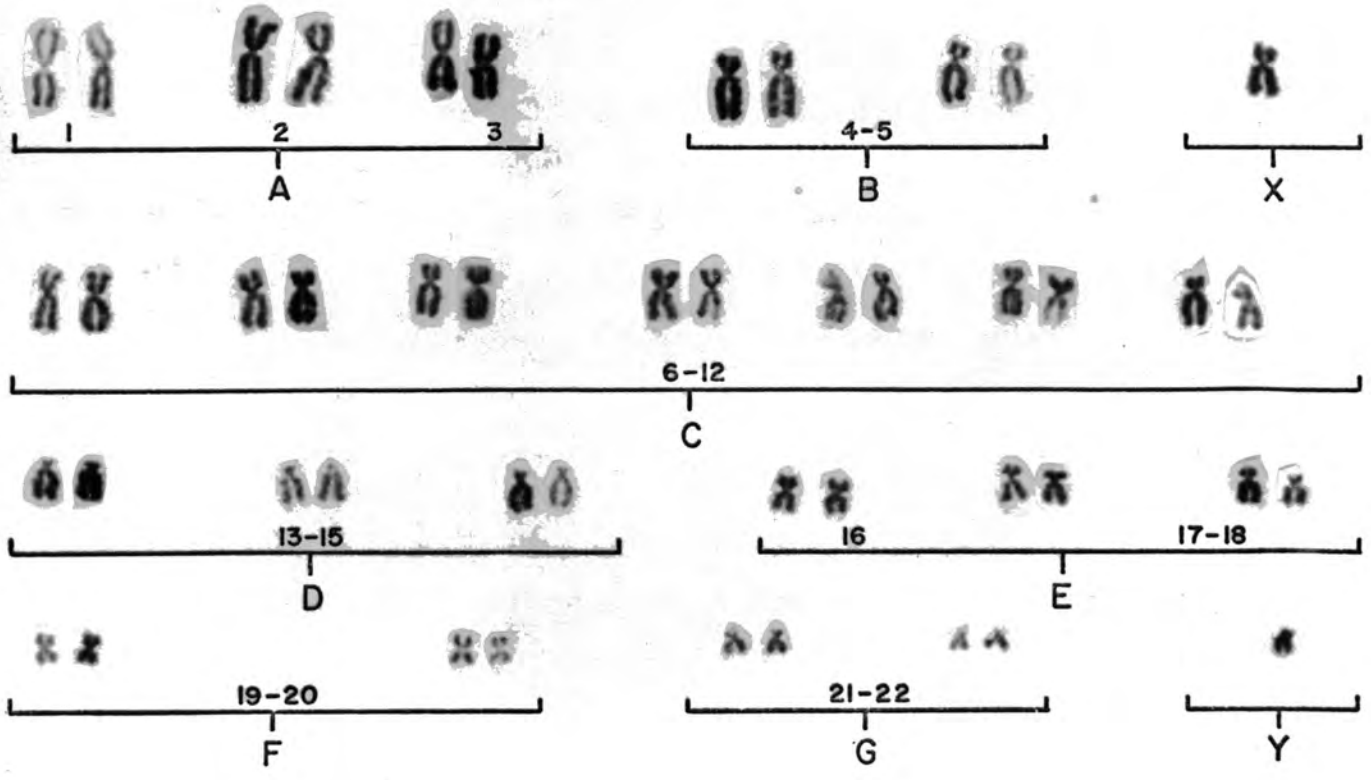
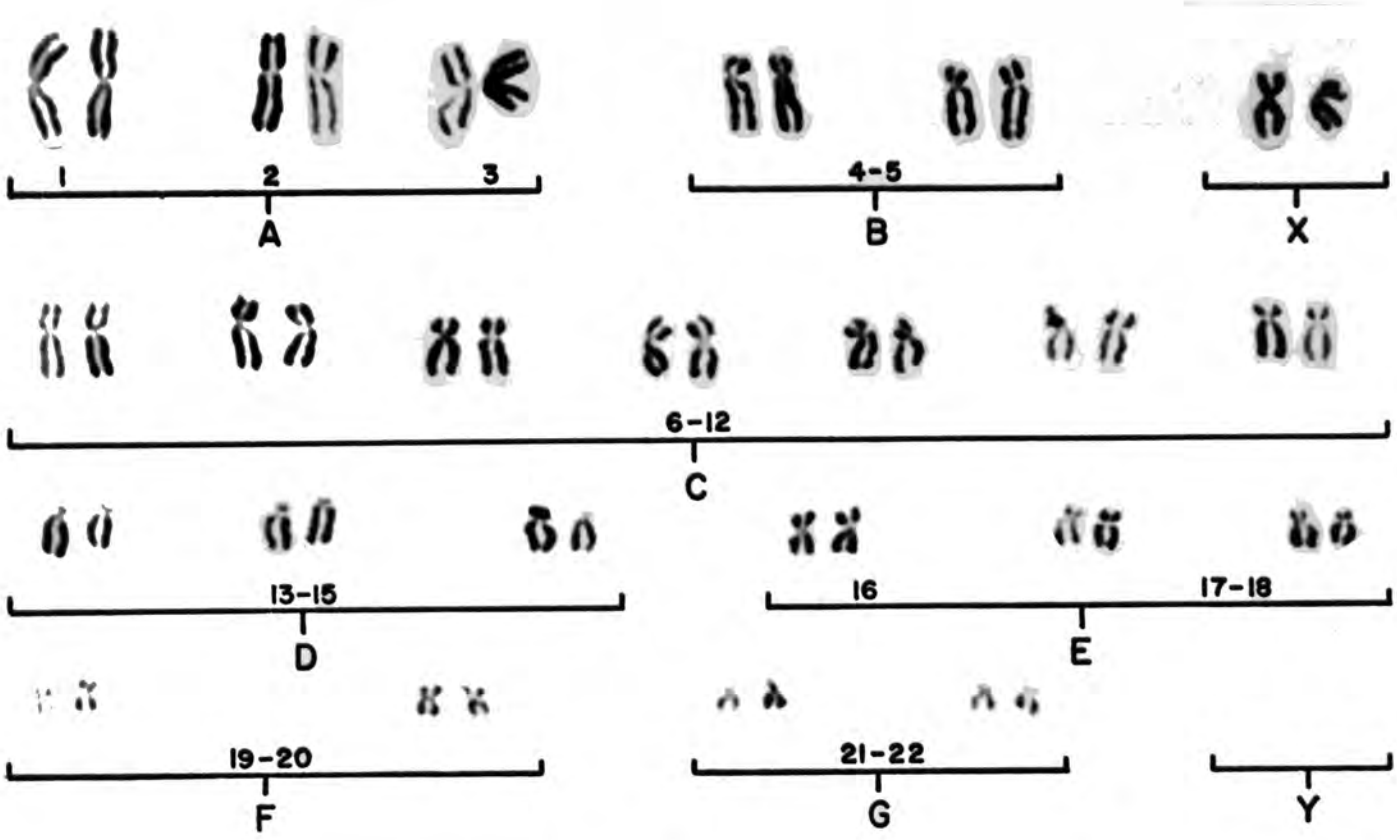

1
2
3
A
4-5
B
X
6-12
C
13-15
D
16
17-18
E
19-20
F
21-22
G
Y

Figure 6. Karyotype of the GM-3557 cell culture, derived from the mother of the proband (GM-2109). Metaphase spreads from this culture exhibited a normal female karyotype (46,XX). Stained with Giemsa.

Figure 7. Karyotype of the GM-3558 cell culture, derived from the father of the proband (GM-2109). Metaphase spreads from this culture exhibited a normal male karyotype (46,XY). Stained with Giemsa.


1
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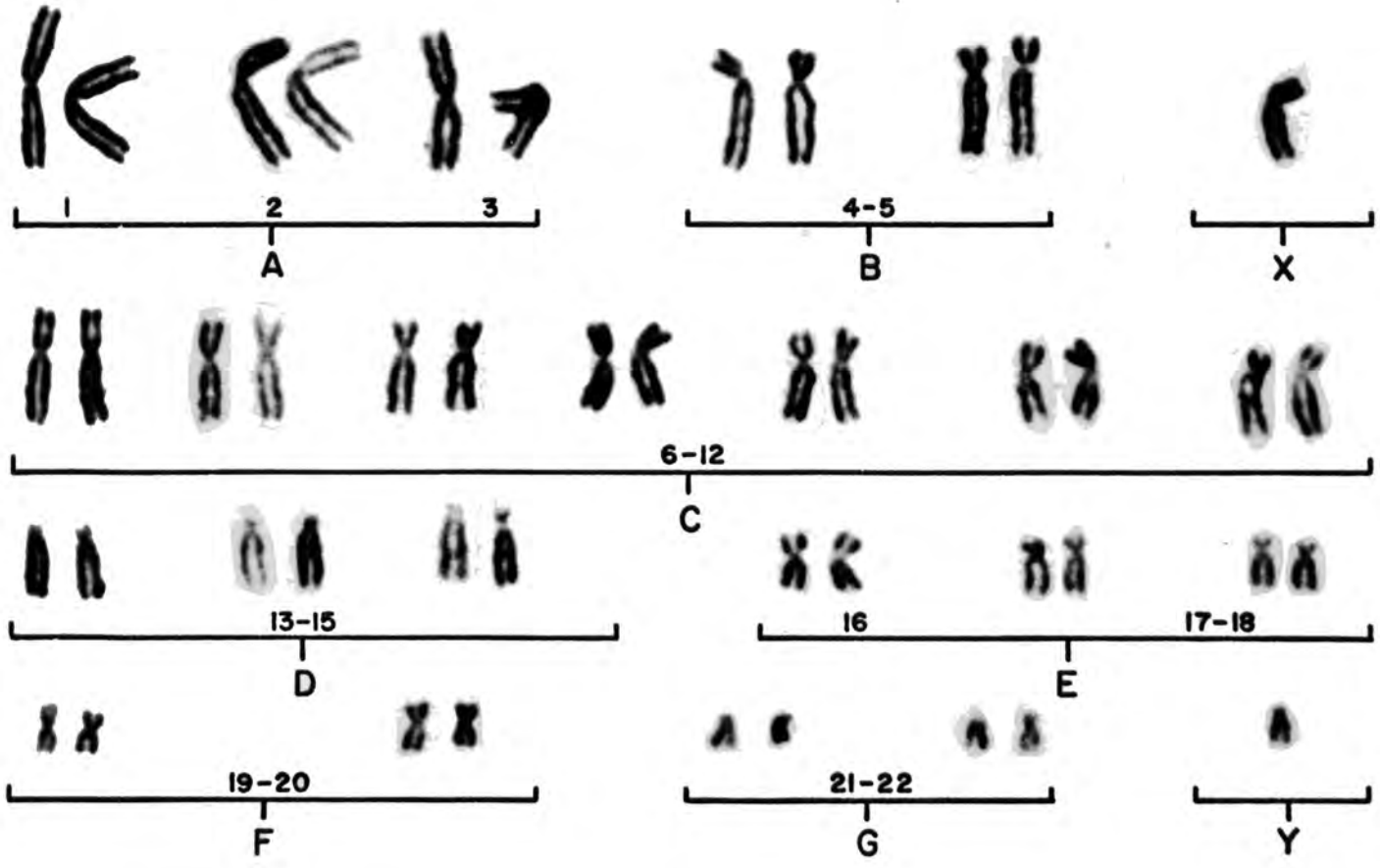
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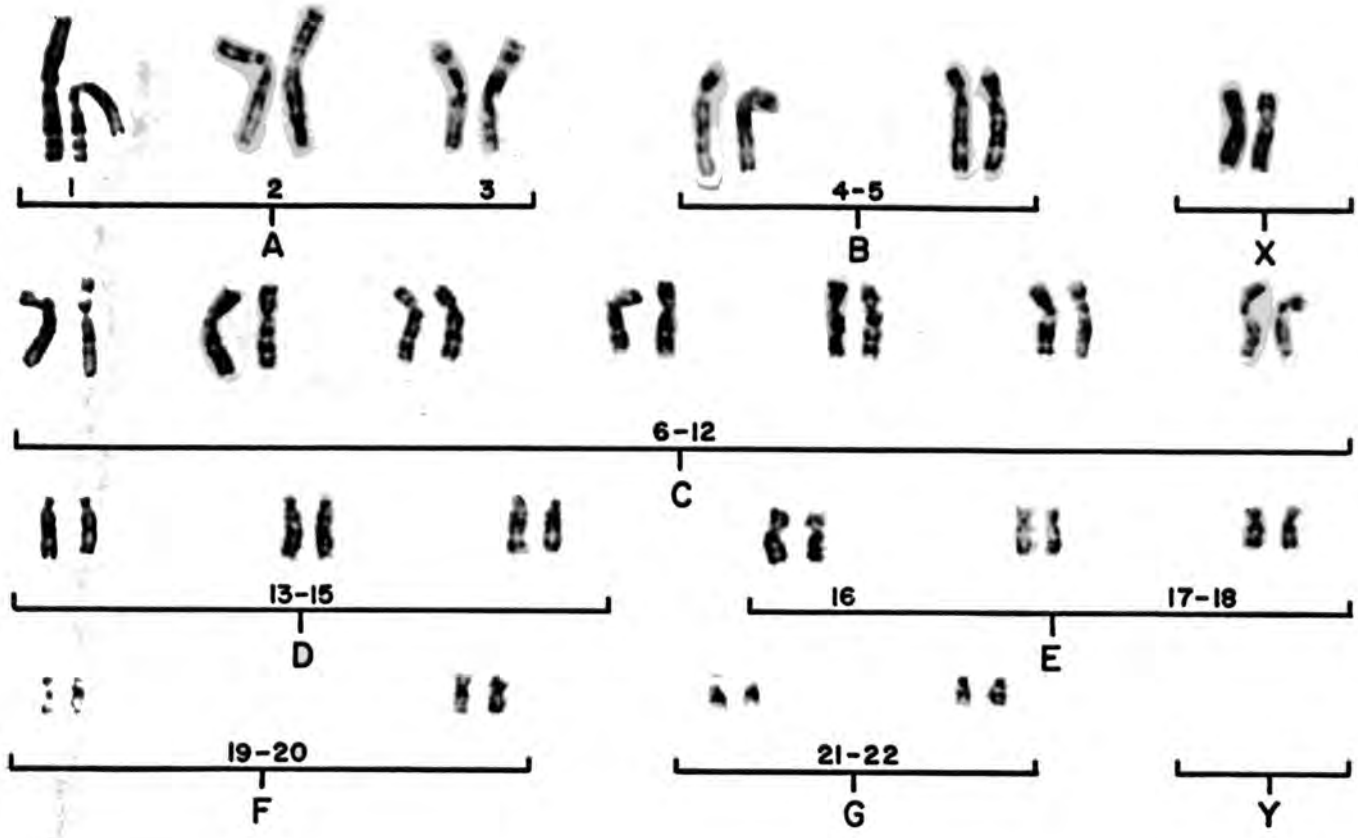
Y

Figure 8. Giemsa-banded karyotype of the GM-3557 cell culture, derived from the mother of the proband (GM-2109). The karyotype appears normal.

Figure 9. Giemsa-banded karyotype of the GM-3558 cell culture, derived from the father of the proband (GM-2109). The karyotype appears normal.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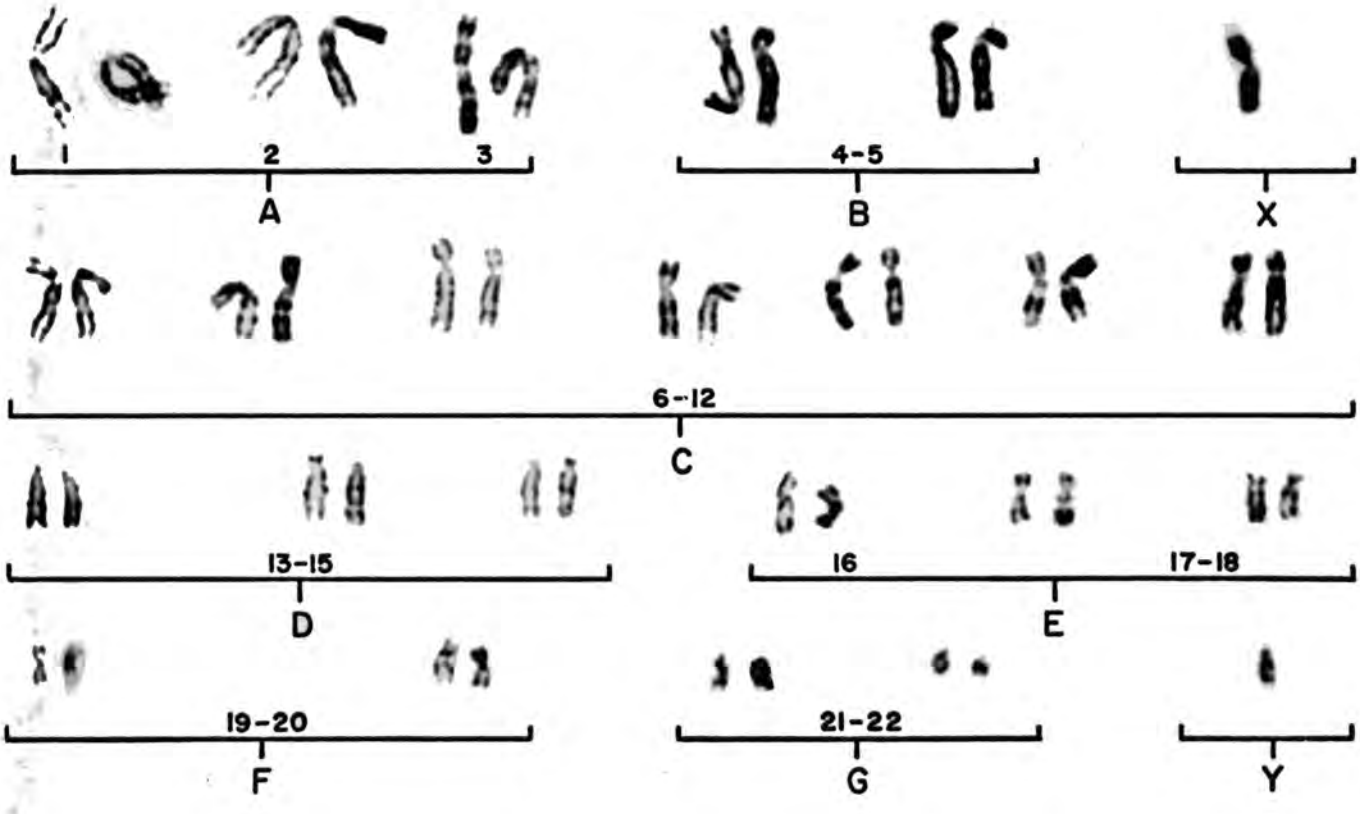
Y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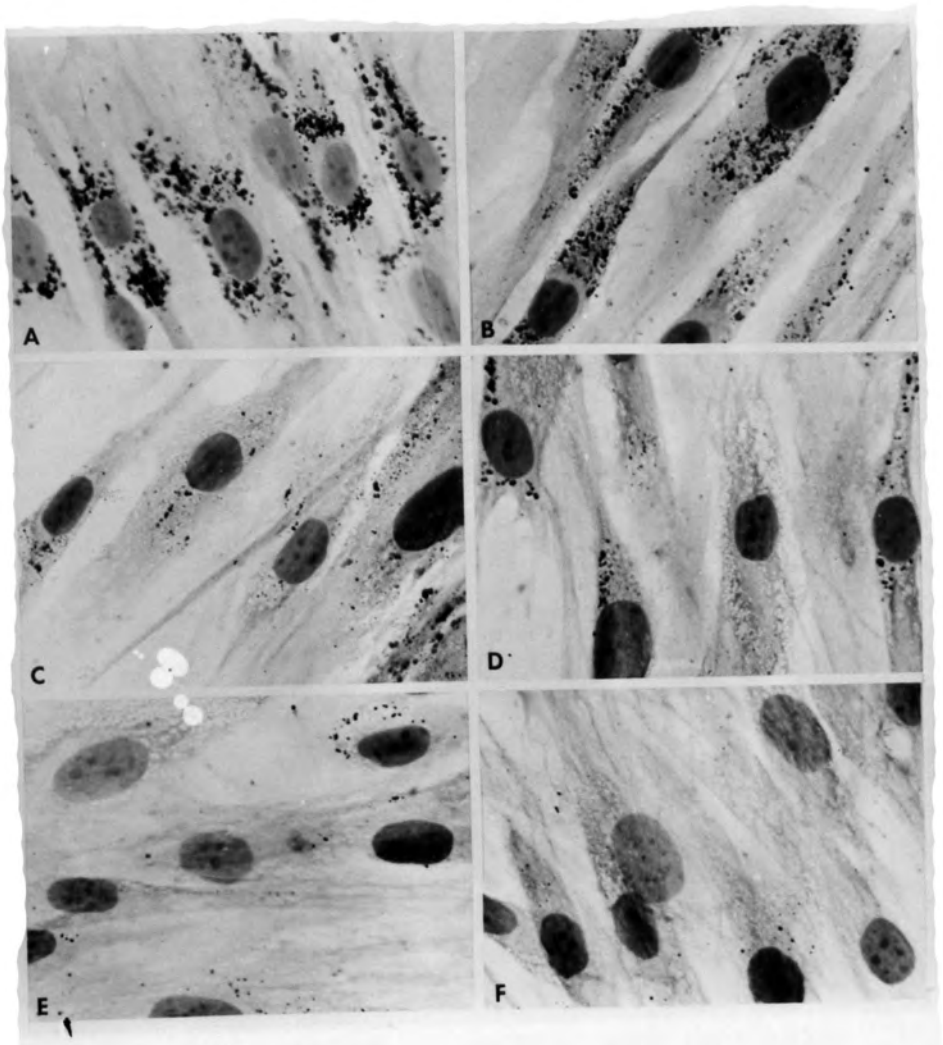
Y

Figure 10. Correction by conditioned medium. Cells from the GM-1606 Wolman's disease culture, which normally stores lipid, were grown for 2 weeks in the presence of conditioned media from other cell cultures. (A) Fresh medium; (B) GM-1606, Wolman's disease; (C) GM-2121, Wolman's disease heterozygote; (D) GM-179, normal; (E) GM-3557, mother of proband; (F) GM-3558, father of proband. Stained with oil red O and hematoxylin. X350.

Figure 11. Correlation of the extent of correction of lipid storage in GM-1606, Wolman's disease cells treated with conditioned media from other cell cultures with the acid lipase activity of those cell cultures (Assay 1 of Table 2). One hundred cells per point were graded for the extent of oil red O positive granulation according to the grades shown in Fig. 1 and the numbers were summed to give the ordinate value. The line was statistically significant by linear regression ($p<.05$).


A
B
C
D
E
F

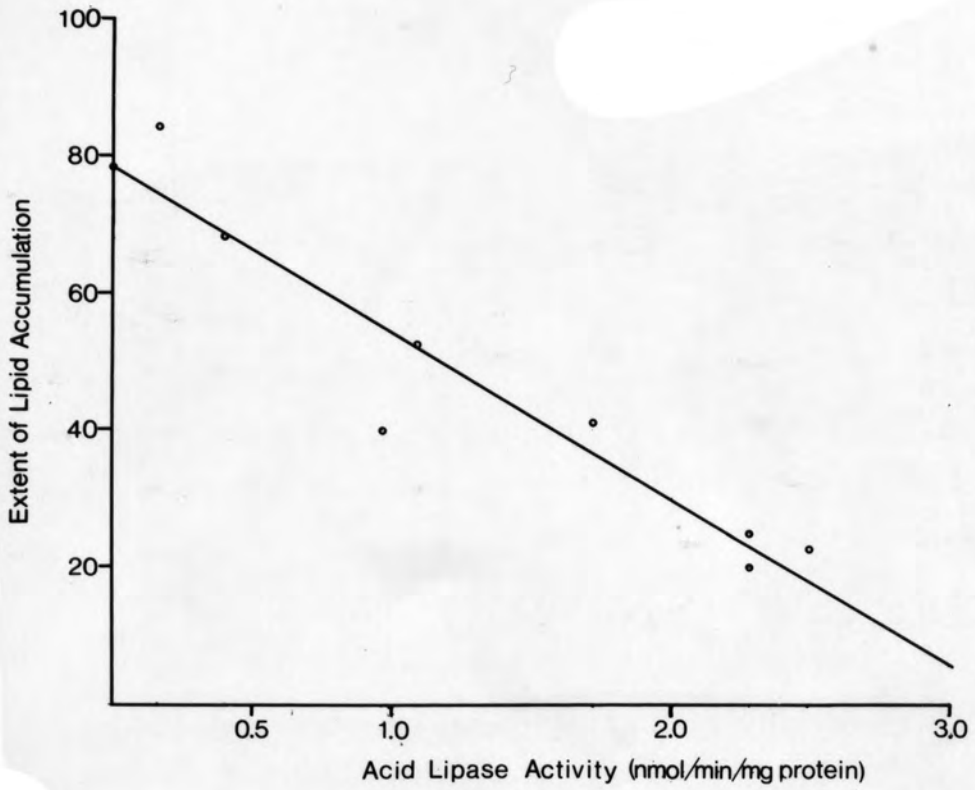
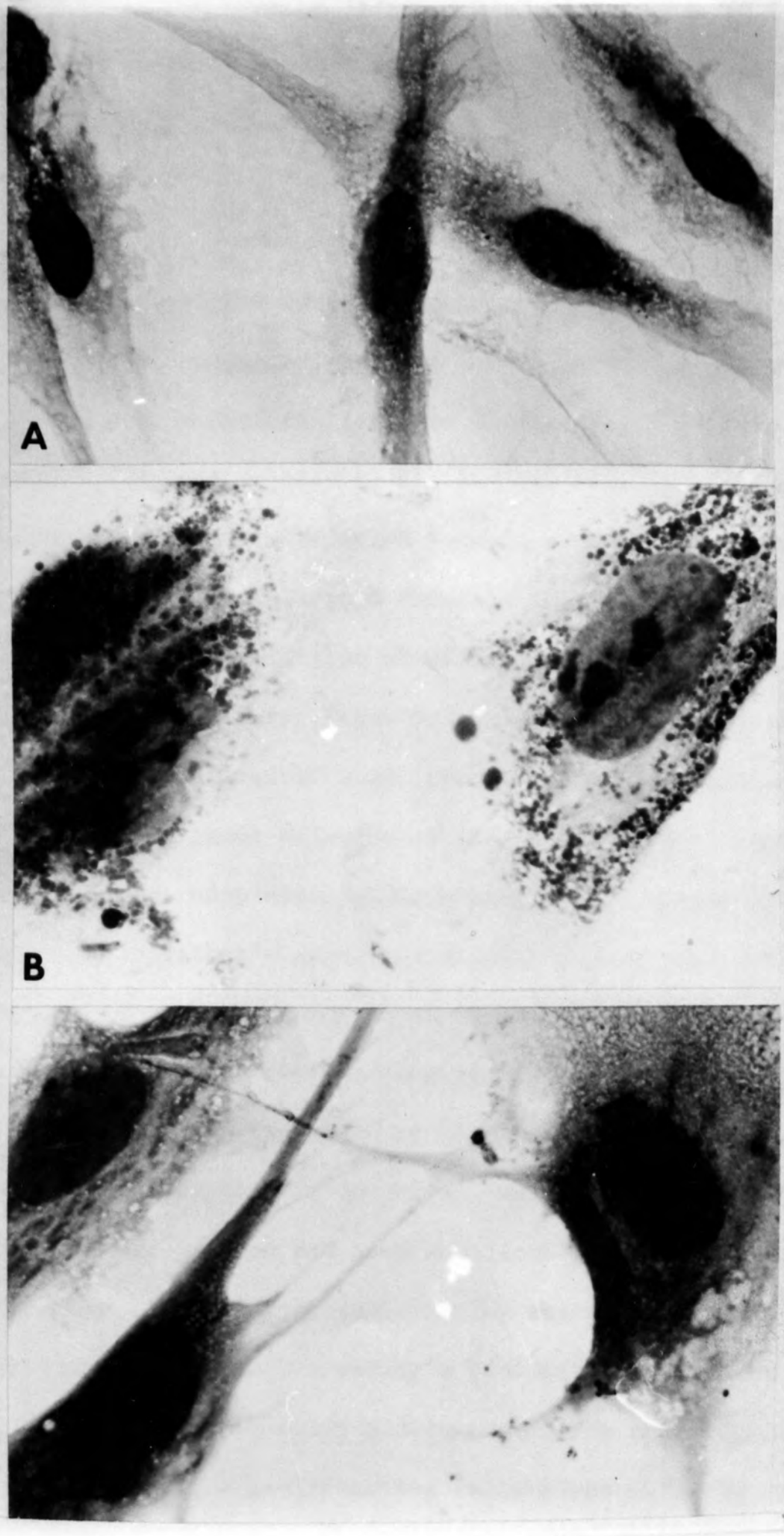

100
80
60
40
20
Extent of Lipid Accumulation
0.5
1.0
2.0
3.0
Acid Lipase Activity (nmol/min/mg protein)

Figure 12. Cytoplasmic lipid accumulation in cells cultured from a patient with Wolman's disease and his parents. (A) GM-3558, father of the proband; (B) GM-2109, Wolman's disease proband; (C) GM-3557, mother of the proband. Significant lipid accumulation was only seen in cells from the proband. Stained with oil red O and hematoxylin. X700.


A
B

Enzyme addition: Wolman's disease cells treated with lipase (0.2, 0.02, 0.002 mg/ml), esterase (0.2, 0.02, 0.002 mg/ml), and cholesterol esterase (0.4, 0.04, 0.004 mg/ml) showed no difference in lipid storage from untreated cells.

Discussion

The initial cytogenetic findings, obtained when the cells were actively proliferating, suggested that the karyotype of the proband contained a translocation onto the long arm of chromosome #5 (46,XY,5q+). Cytogenetic and biochemical studies of the parents were undertaken in order to elucidate the possible relation between a chromosomal anomaly and the inheritance of a lipid storage disease. Results of acid lipase assays and conditioned media addition experiments indicated that, though the mother appeared to be a carrier for Wolman's disease, the father appeared to have a normal level of acid lipase. This focused attention on the father, because without deletion of his normal acid lipase gene the child would not have been expected to exhibit the symptoms of Wolman's disease. The father's karyotype did not show a balanced translocation nor was there evidence of an inversion or other rearrangement that could have disposed the proband to a translocation event. The results could be explained by a translocation during meiosis of the germ cells of the father or during an early mitotic division of the zygote. Cells from the proband had been submitted to the Institute for Medical Research for ultracold storage. During the course of the studies, we encountered difficulties in recovering a proliferative culture from storage, presumably due to the early senescence of the culture. After approximately 10 attempts, a proliferating culture was obtained and the

cytogenetic analyses were repeated. On this occasion, no cells showed the 5q+ anomaly and, although two cells showed other rearrangements involving chromosome #5, seven cells appeared normal. The cells still stored lipid. These results raise the possibility that the initial cytogenetic finding did not truly reflect the karyotype of the proband.

Although the karyotypes of cultured human fibroblasts are usually stable and diploid (50,51), it is possible for clones with marker chromosomes to emerge in culture (52,53). Even though the aberrent chromosome was present in at least 90% of the metaphases examined, it remains possible that in a poorly proliferating culture the population could become enriched by selection for clonally derived cells with increased proliferative capacity and an associated cytogenetic anomaly. If the proband had a normal karyotype, the father should have been a carrier for Wolman's disease. His apparently normal level of acid lipase activity may have been a false negative due to individual variation in acid lipase activity and variation in the assay. If this is true, further cases of Wolman's disease may appear in this family. If the proband were not the son of the presumed father, the true father could still be a carrier or his karyotype could contain a balanced translocation. The possibility that this case of Wolman's disease followed an X-linked recessive mode of inheritance cannot be ruled out. However, few diseases normally inherited autosomally can be inherited as X-lined recessives (54,55). The fact that only one gene product appeared to be affected favors the hypothesis that the karyotype of the proband was normal because most chromosome aberrations result in multiple developmental anomalies.

In favor of the linkage of the acid lipase defect to the cytogenetic anomaly were the normal level of acid lipase in the father and

the presence of the cytogenetic anomaly in the cells of the proband before ultracold storage and the manifestation of senescence. The GM-2109 (P) culture reached senescence after only 18 population doublings (Research Section II), and senescent cultures characteristically exhibit increased aneuploidy and tetraploidy (56). The effects of senescence, lipid storage, and freezing on the emergence of karyotypically distinct cells are unclear at this time. It is possible that an initial translocation which deleted the acid lipase gene was susceptible to further genetic rearrangement and was lost. In this case, although the cells might appear to have a normal chromosome complement, a small deletion might still be present. A deletion from the q terminal end of chromosome #5 would be consistent with the initial cytogenetic findings. If the acid lipase gene were located in this region, it might exhibit linkage with diphtheria toxin sensitivity, a marker which has been localized to the 5q15→5qter region (57). If Wolman's disease was caused by the translocation in this case, future children in this family would not be expected to inherit Wolman's disease.

No final conclusions about the inheritance of this case of Wolman's disease can be drawn. If the couple should have another child with Wolman's disease, it would appear likely that the chromosomal anomaly was an artifact of tissue culture. If the acid lipase gene is mapped to chromosome #5 it would appear likely that the proband had a chromosomal aberration and the other conclusions discussed above would follow. Mapping experiments on acid lipase are currently being conducted by other laboratories (P. Coates, personal communication).

The enzymes (lipase, esterase, cholesterol esterase) added to the Wolman's disease culture should have been able to degrade some of the stored lipid. The fact that they did not suggests that the enzymes

did not reach the lysosomes where the lipid was stored, or that their pH optima differed greatly from the lysosomal pH, resulting in minimal enzyme activity. It is also possible that the enzymes were unable to enter the cell. The activities of the enzymes were not checked prior to their use, so the possibility that a loss of activity had occurred cannot be ruled out.

It is difficult to evaluate the effect lipid storage may have had on cell aging or karyotypic instability in the GM-2109 (P) culture, but it is possible that lysosomal neutral lipid storage may have more profound effects on cultured cells than is currently believed.

RESEARCH SECTION II

Introduction

The esterase inhibitor, E600 (diethyl p-nitrophenyl phosphate), is an extremely toxic substance. It has been employed as a pesticide under the trade name paraoxon. Parathion, the sulfur analogue of E600, is still in wide use as a pesticide. In the insect's body, the parathion is oxidized to E600, which blocks acetylcholinesterase activity and ultimately kills the insect (36). Although E600 is much more toxic to whole animals than either parathion or the hydrolysis product of E600, p-nitrophenol, the toxic relationships may be reversed in cultured cells. Both parathion and p-nitrophenol are more toxic to HeLa cells and fibroblasts than E600 (36), though this result was not seen in chick embryo muscle cells (38).

As mentioned previously, E600 has been used to differentiate between B-type (E600-sensitive) esterases and A-type (E600-resistant) esterases (27). Although E600 appears to inactivate most esterases by phosphorylating the enzymes at their active site, an A-type esterase present in mammalian sera which binds normally can remain active, presumably by releasing the phosphoryl group, thus catalyzing the hydrolysis of E600 to p-nitrophenol (30). This esterase or "paraoxonase" activity of mammalian sera has been much studied (30-32,58) and can be reduced by the use of inhibitors such as EDTA and aluminon (31) or heat inactivation (32).

This section presents evidence that treating fibroblast cultures with E600 induces lipid storage in the cells. Different parameters of E600-induced lipid storage, including dose and time dependence, origin

of the lipid, and histochemical characterization of the lipid are shown. Other histochemical data will be presented to compare lipid storage in different Wolman's disease cell lines, to compare the effect of adding various lipids to normal and Wolman's disease cell cultures, and to determine the effect of E600 treatment on neutral red granules. The effect of E600 on bacterial cultures is also shown.

Materials and Methods

E600 Treatment: Stock solutions of E600 (Sigma Chemical Co.) were prepared in 100% ethanol at 200X the desired final concentration. Treated cells received 0.5% of the stock solution. Controls were treated with 0.5% ethanol, a concentration which is reportedly not harmful to cultured cells (35) and appeared to have no effect on lipid storage. In the growth curve experiment, the E600 was carried in 1% ethanol. Cell cultures were usually exposed to E600 1 to 4 days after seeding. Stock E600 was added to fresh medium shortly before each change of medium.

Paraoxonase Inactivation: Fetal calf serum (FCS, GIBCO) was pipetted into clean sterile plastic test tubes and heated at 56°C or 70°C in a water bath or subjected to gamma radiation in a gammator (Isomedix, Inc.) for specified periods.

MEM without phenol red (GIBCO) but containing 10^{-3}M E600 was prepared with 3% FCS, 8% FCS, and 15% FCS for each inactivation time. Controls lacking serum or E600 were prepared. Tubes with 5 ml of the different media were incubated at 37°C for 4 days and the p-nitrophenol formed by the hydrolysis of E600 was assayed according to the method of Aldridge (30). p-Nitrophenol was extracted with 5 ml of n-butanol-toluene (1:1). From the upper layer, 2.5 ml was drawn off and 1.5 ml of 20% ammonium hydroxide in 100% ethanol was added to it. The volume was adjusted to 5 ml with approximately 1 ml of n-butanol-toluene. The absorbance of

the samples was read on a Gilford spectrophotometer at a wavelength of 420 nm. p-Nitrophenol standards were prepared in medium and treated similarly. The concentration of p-nitrophenol in each sample was calculated from the linear regression of the standard curve.

Growth Curve: Normal fibroblasts (GM-179 or SaJo) and Wolman's disease fibroblasts (GM-2109 (P) or GM-1606) were seeded into 35 x 10 mm culture dishes at a concentration of 5×10^4 cells per dish. No less than 24 hr later, treatment with different concentrations of E600 was begun. At this time 3 culture dishes for each culture were trypsinized and counted in a hemocytometer. At days 2, 4, 8, 12 and 16, 3 culture dishes each for untreated normal, treated normal, untreated Wolman's disease, and treated Wolman's disease cultures were trypsinized and counted in a hemocytometer. A trypan blue viability stain (59) was performed on every culture counted. The counts for the 3 culture dishes were averaged, giving one value $\pm$ standard deviation that represented the number of cells in a given culture on a given day.

Relative Toxicity: Normal fibroblasts (GM-179) and Wolman's disease fibroblasts (GM-1606) were seeded into 35 x 10 mm culture dishes at a concentration of 5×10^4 cells per dish. After 24 hr the cells were treated with varying concentrations of E600 (4×10^{-3}M, 2×10^{-3}M, 10^{-3}M, 5×10^{-4}M, 10^{-4}M, none) in MEM supplemented with 15% FCS. At 2 days and 6 days, 3 culture dishes per E600 concentration were trypsinized and counted, and the counts were averaged. The percentage of nonviable cells was determined by trypan blue staining and used to calculate the number of viable cells. The percent inhibition was calculated for the different E600 concentrations and the ID_{50} determined (35).

Dose and Time Dependence: Normal fibroblasts (GM-37, GM-316) and Wolman's disease fibroblasts (GM-1606) were seeded onto 22 x 22 mm sterile coverslips in 35 x 10 mm culture dishes at a density of 5×10^4 cells per dish. The cells were treated with varying concentrations of E600 (10^{-2}M, 3×10^{-3}M, 10^{-3}M, 3×10^{-4}M, 10^{-4}M, 10^{-5}M, 10^{-6}M, none) in MEM plus 8% FCS heat inactivated 1 hr at 56°C. The cells were fixed after specified periods (0.5, 1, 2, 4, 8, 12 and 16 days) in an aqueous solution of 4% formaldehyde and 2% calcium acetate for 1 to 2 hr. The coverslips were stained with oil red O, counterstained with hematoxylin, and scored for the extent of oil red O positive staining as described in Research Section I (Fig. 1).

Histochemistry: Normal fibroblasts (SaJo, GM-179, GM-37) and Wolman's disease fibroblasts (GM-2109 (P), GM-1606, GM-2211) were seeded and fixed as in the dose and time dependence experiments described above. Fixation in the Gomori esterase procedure was for 10 min. Untreated cells and cells treated with 10^{-3}M E600 for 4 to 9 days were examined. The staining techniques employed are shown in Table 3. Most stain reactions were carried out in accordance with standard protocols for lipids (49) and complex carbohydrates (60). The permanganate-aldehyde fuchsin followed the method of Landing et al. (61) and Gomori's α-naphthyl acetate method for esterases was performed according to the procedure given by Hunt (18). Unstained, unfixed cells were examined for autofluorescence on a Zeiss Photomicroscope II with an HBO 200 mercury source, a BG3 excitation filter, and 41 and 53 barrier filters. Formaldehyde-fixed cells were examined by phase contrast microscopy.

Comparison of Wolman's Disease Cell Cultures: Wolman's disease fibroblasts (GM-2109 (P), GM-1606, GM-2211) were grown on coverslips and fixed as described in the dose and time dependence experiments above. One coverslip of

GM-2211 was trypsinized and allowed to re-attach for 7 hr before fixation. The coverslips were stained with oil red 0, counterstained with hematoxylin, and scored for the extent of oil red 0 positive staining as described in Research Section I (Fig. 1).

Lipid Addition: Normal (GM-498) and Wolman's disease (GM-1606) cells were seeded on coverslips in culture dishes as described in the dose and time dependence experiment. The cells were treated with varying concentrations of cholesterol (Sigma), cholesteryl ester (cholesteryl palmitate, Sigma), and free fatty acid (cis-vaccenic acid, Sigma). After dissolving 4 mg of lipid in 0.5 ml of ethanol, which required heating for cholesteryl palmitate, the solutions were added to 8 ml of FCS with mixing and serial dilutions were made. Dulbecco's MEM supplemented with 8% FCS was prepared with the different lipid-fortified fetal calf sera. The cells were treated for 3 days and then fixed, stained, and scored for the extent of oil red 0 staining as described in Research Section I (Fig. 1).

Effect of Serum Concentration and Inactivation: Normal cells (GM-316) were seeded on coverslips in culture dishes as described in the dose and time dependence experiments above. Replicate cultures were grown in media with 10^{-3}M E600 and either fresh FCS or FCS that had been heat-inactivated 1 hr at 56°C. The following FCS concentrations were employed: 1%, 5%, 15%, and 30%. The cells were fixed after 8 days of treatment with calcium-buffered formalin as described in the dose and time dependence experiments. The coverslips were stained with oil red 0, counterstained with hematoxylin, and scored for the extent of oil red 0 positive staining as described in Research Section I (Fig. 1).

Comparison of E600 and p-Nitrophenol: Normal cells (GM-179) were seeded on coverslips in culture dishes as described in the dose and time dependence experiments above. Cultures were grown in MEM supplemented with 15% heat-inactivated (1 hr at 56°C) FCS and varying concentrations of E600 (10^{-4}M, 3 x 10^{-4}M, 10^{-3}M) or p-nitrophenol (10^{-4}M, 3 x 10^{-4}M, 10^{-3}M, 3 x 10^{-3}M). After 2 days treatment, the cells were fixed with calcium-buffered formalin as described in the dose and time dependence experiments. The coverslips were stained with oil red O, counterstained with hematoxylin, and scored for the extent of oil red O staining as described in Research Section I (Fig. 1). To estimate toxicity, 5 Chartpak squares, whose approximate area was 2.58 mm^2 each, were placed on each coverslip, the number of cells per square were counted and used to calculate the number of cells per coverslip.

Neutral Red Staining: Normal cells (GM-179, GM-37), Wolman's disease cells (GM-1606), Wolman's disease heterozygote cells (GM-2121, GM-3557 (M)), and Bloom's syndrome cells (GM-2548) were seeded on coverslips in culture dishes as described in the dose and time dependence experiments. Untreated cells and cells treated with 10^{-4}M E600 for 9 days were stained 30 min in 0.005% neutral red (filtered before use) and scored for the number of positive-staining granules as described for oil red O stained granules in Research Section I (Fig. 1).

Effect on Bacterial Growth: Each of 2 tubes containing 10 ml of trypticase soy broth was inoculated with 0.3 ml of Escherichia coli cultured WP_2, a strain which requires tryptophan. One tube was treated with 10^{-3}M E600, the control tube with 0.5% ethanol. The tubes were incubated with aeration for approximately 2 hr. The optical density was read on a

Spectronic 20 (Bausch & Lomb) at intervals. When the optical density of the control reached 0.42, dilutions of both tubes were prepared in Puck's balanced salt solution (PBS). Triplicate pour plates in brain heart infusion agar were prepared at the 10^{-6}, 10^{-7}, and 10^{-8} dilutions of the control, and at the 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions of the E600-treated tube. The plates were incubated at 37°C for 2 days and counted.

Results

Paraoxonase Inactivation: FCS showed significant paraoxonase activity which increased linearly with the concentration of FCS (Fig. 13). Heating the FCS at 56°C reduced paraoxonase activity (Fig. 13). There appeared to be a significant percentage of paraoxonase activity that was resistant to heating at 56°C (Fig. 14). However, heating at 70°C for 10 min abolished much of this activity (Fig. 13). Gamma irradiation had no effect on FCS paraoxonase activity (Fig. 15).

Growth Curve: Growth was not inhibited by either 10^{-5}M or 10^{-4}M E600. At 10^{-3}M E600 both normal and Wolman's disease cultures showed growth inhibition (Figs. 16,17). Wolman's disease cells appeared to be somewhat more susceptible to E600 than normal cells. The trypan blue viabilities were comparable, except on day 12 and 16 of Fig. 16, in which the E600-treated GM-2109 (P) cells showed less viability than the untreated cells.

Relative Toxicity: There was little difference between the normal cells and Wolman's disease cells in sensitivity to E600. After 2 days the normal cells appeared to be slightly more susceptible than the Wolman's disease cells (Fig. 18). The ID_{50} for GM-179 was calculated to be 0.7 mM E600 and the ID_{50} for GM-1606 was 0.95 mM. However, by 6 days the situation was reversed, as the Wolman's disease cells exhibited slightly

more inhibition that the normal cells (Fig. 19). The ID_{50} for GM-179 now measured 0.4 mM and the ID50 for GM-1606 was 0.24 mM E600.

Dose and Time Dependence: A conspicuous increase in lipid storage in cells exposed to E600 was noted in both normal cultures, GM-37 (Fig. 20) and GM-316 (Fig. 21). After seeding, 10 to 24% of the untreated normal cells exhibited low levels of lipid storage. This proportion increased to as much as 80% with increased time in culture. However, the extent of lipid storage never approached the level seen in cells treated with 10^{-4}M E600 or untreated Wolman's disease cells. Normal cells treated with 10^{-6}M E600 showed an extent of lipid storage similar to that of time-matched controls. Although culture GM-37 appeared to show some lipid storage after treatment with 10^{-5}M E600, the GM-316 culture did not clearly express lipid storage until treated with 10^{-4}M E600. At this concentration very little time-dependent increase in lipid accumulation was observed. Normal cells treated with 3×10^{-4}M E600 showed a slight increase in time-dependent lipid storage, whereas cells treated with 10^{-3}M E600 showed a pronounced time-dependent build-up of lipid. At 3×10^{-3}M, some lipid storage was observed, but the cells died within 4 days. All cells were killed by 10^{-2}M E600 within 12 hr.

Untreated Wolman's disease cells showed a more marked lipid accumulation than normal cells with time in culture after seeding (Fig. 22). The extent of lipid already present in the cells made it difficult to detect increased lipid storage in treatment with 10^{-4}M and 3×10^{-4}M E600. However, at 10^{-3}M E600, an increase in lipid accumulation was evident.

When cells that had been treated with 10^{-3}M E600 for 12 hr to 4 days were removed from E600 and grown in normal media, the levels of stored lipid dropped, approaching those of untreated cells (Figs. 20,21).

Generally, the longer cells had been treated, the longer it took to reverse the lipid storage. GM-37 cells treated for 12 hr required more than 1.5 but less than 3.5 days to return to controls levels. GM-37 cells treated 1 day took more than 3 but less than 7 days, whereas cells treated for 4 days had not reached control levels 9 days after removal of E600. The GM-316 culture appeared to reverse lipid storage more rapidly than the GM-37 culture. GM-316 cells treated 12 hr returned to control levels 12 hr later and those treated for 1 day returned to control levels within 3 days.

Histochemistry: Refractile granules were observed by phase contrast microscopy in the cytoplasm of all fixed, unstained Wolman's disease cell cultures as well as in normal cultures treated with 10^{-3}M E600 (Fig. 23). Crystalline inclusions were also visible in all but the untreated controls. Similar inclusions were observed in unfixed cells.

Like untreated Wolman's disease cells, normal cells treated with 10^{-3}M E600 showed the presence of neutral lipid, as evidenced by positive staining with oil red O, sudan black B, and nile blue sulfate (Table 3). The oil red O positive lipid was pyridine extractable. Both E600-treated normal cells and untreated Wolman's disease cells gave intense staining for cholesterol, both by the perchloric acid naphthoquinone and Schultz reactions. The Wolman's disease cells did not stain by Schnabel's procedure for differentiating cholesterol and cholesteryl esters by which only free cholesterol stains. The E600-treated cells, both normal and Wolman's disease, showed a small number of positive granules. This indicates that both Wolman's disease and E600-treated cells stored a large amount of cholesteryl ester, and that the E600-treated cells also stored a small

TABLE 3

HISTOCHEMISTRY OF NORMAL AND WD CELLS, UNTREATED AND TREATED WITH 10^{-3}M E600

Staining Technique	Substance Stained	Stain Intensity of Cytoplasmic Granules			
		Normal cells	WD cells	E600-treated normal cells	E600-treated WD cells
Sudan black B	neutral lipid, phospholipid	0	++++	++++	
Lillie's oil red O	neutral lipid	0	++++	++++	++++
Pyridine extraction, oil red O	non-lipid, oil red O + material		0	0	0
Cain's Nile blue sulfate	neutral lipid (red) acidic lipid (blue)	0	+++[a]	++++[a]	++++[a]
Morton's bromine-silver	unsaturated lipid	+	+	+	+
Performic acid-Schiff	unsaturated lipid	0	0	0	
Copper phthalocyanin	phospholipid	+[b]	+[b,c]	++[b,d]	++[b,d]
Holczinger's copper-rubeanic acid	free fatty acid	+[e]	+[e]	++[e]	++[e]
Schultz method	cholesterol, cholesteryl esters	0	+++[f]	+++[f]	+++[f,g]
Schnabel's differentiation of Schultz method	cholesterol, not cholesteryl esters	0	0	+	+
Adam's perchloric acid-naphthoquinone	cholesterol, cholesteryl esters	0	+++	++++	++++
Autofluorescence		+	++++	++++	
Periodic acid-Schiff (PAS)	glycolipid, glycogen, mucosubstance (cytoplasmic matrix/ granules)	+ to ++/ 0 to +, +++[h]	+ to ++/ 0 to +, ++[i]	++/0 to +	+++/0 to +
PAS-Diastase		same as above	same as above	same as above	same as above
Permanganate-aldehyde fuchsin	probably lipofuscin	0	0, ++[j]	0	0
Gomori's esterase pH 7.4	non-specific esterase	0 to +	0 to +	0 to +	
Gomori's esterase pH 5.0	non-specific esterase	0 to +	0 to +	0 to +	
Aldehyde fuchsin-alcian blue	sulfated (purple), carboxyl (blue) mucosubstance	0	0		0
Alcian blue, pH 2.5-PAS	neutral (magenta), acidic (blue-purple) mucosubstance	+[k]	+, ++[i,k]	+[k]	+[k]
Alcian blue, ph 2.5	acidic mucosubstance	0	0	0	0
Alcian blue, pH 1.0	sulfated mucosubstance	0	0	0	0

[a]Cells stained pink indicating presence of neutral lipid. E600-treated cells showed pink cytoplasmic staining and pink-staining granules, whereas Wolman's disease cells only showed the latter.

[b]Nuclei and nucleoli stain.

[c]GM-2211 showed some small dark staining granules in cytoplasm.

[d]Darker staining in perinuclear location, possibly representing Golgi zone.

[e]Nucleoli stain.

[f]Wolman's disease cells showed small to medium-sized, blue-green granules. E600-treated cells showed gray-green granules, some of which were quite large.

[g]Exocytosis of lipid granules was visible.

[h]GM-37 showed strong staining around large cytoplasmic granules.

[i]GM-2211 showed many small PAS-positive granules.

[j]GM-2109 showed small PMAF-positive granules throughout cytoplasm.

[k]Cytoplasmic matrix stained with PAS as above, nuclei stained with alcian blue.

but definite amount of unesterified cholesterol. Untreated Wolman's disease cells and E600-treated normal cells were both highly autofluorescent (Fig. 24).

Holczinger's fatty acid stain was weakly positive for normal and Wolman's disease cells and somewhat more strongly positive for E600-treated cells. In both the nile blue sulfate and fatty acid stains, the reactivity in the E600-treated cells appeared to include staining that was not associated with granules. The copper phthalocyanin procedure for phospholipid stained a perinuclear region of E600-treated cells that may have represented the Golgi zone. The GM-2109 (P) Wolman's disease culture, but not Wolman's disease culture GM-1606, was positive for the permanganate-aldehyde fuchsin stain. This stain is thought to be reactive to lipofuscin-like material (62). Neither untreated nor E600-treated normal cells showed any reactivity to permanganate-aldehyde fuchsin. The presence of lipofuscin-like material may be related to the early senescence of the GM-2109 (P) culture. No difference in reactivity was observed among controls, Wolman's disease cells, and E600-treated cells for unsaturated lipid, glycolipid, or mucosubstance. The stain reactivity of the Wolman's disease cells treated with 10^{-3}M E600 was comparable to that of the similarly treated normal cells, although somewhat more intense in the former.

Comparison of Wolman's Disease Cell Cultures: There was considerable variation in the amount of oil red O positive lipid stored by the 3 Wolman's disease cultures (Fig. 25). Culture GM-1606 showed little to no lipid accumulation when first obtained, but after approximately 3 months in culture significant lipid accumulation was observed. Culture GM-2211 and GM-2109 (P) both exhibited lipid accumulation from the time they were

obtained. Culture GM-2109 (P) stored the most lipid, grew the slowest, and reached senescence after only approximately 18 population doublings.

No significant differences were seen in the amount of oil red O positive lipid stored by the GM-2211 Wolman's disease culture before and after trypsinization (Fig. 26).

Lipid Addition: Increased lipid storage was seen in normal cells treated with 4.0 mg% cholesterol, but no lipid storage was seen at lower concentrations or in Wolman's disease cells (Fig. 27). Treatment with free fatty acid resulted in increased lipid storage in both normal and Wolman's disease cells at concentrations of 0.04 mg% and 0.4 mg% (Fig. 27). More lipid was stored at the higher concentrations. The highest concentration, 4.0 mg%, was toxic to the cells. In both treatment with cholesterol and free fatty acid, the observed lipid storage differed from that seen in Wolman's disease or E600-treated cells. The lipid droplets were larger and located more peripherally (Fig. 28). No change in lipid storage was seen after treatment with cholesteryl ester.

Effect of Serum Concentration and Inactivation: The amount of lipid stored in normal cultures treated with 10^{-3}M E600 varied directly with the concentration of serum in the medium (Fig. 29). At higher serum concentrations, the cells grown in heat-inactivated serum appeared to store more lipid than those grown in fresh serum.

Comparison of E600 and p-Nitrophenol: p-Nitrophenol, the major metabolite of E600 (35,36), appeared to have little effect on the induction of lipid storage (Fig. 30). When the concentration of 10^{-3}M p-nitrophenol was reached, lipid accumulation became noticeable, but at this concentration less than half of the cells stored lipid, whereas 90% of cells treated with 10^{-4}M E600 showed lipid accumulation. p-Nitrophenol appeared to be slightly more toxic to the cells than E600 (not shown).

TABLE 4

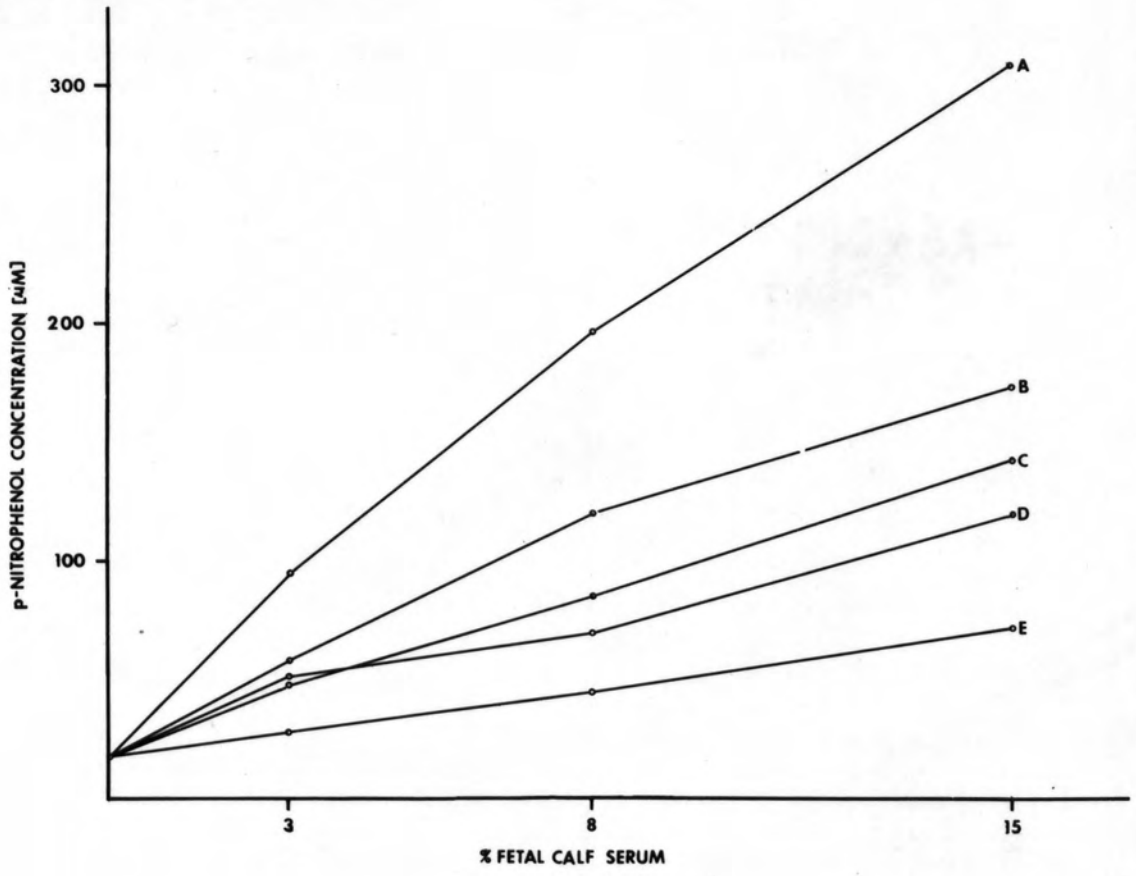
MEAN COLONIES OF E. COLI PER PLATE, UNTREATED AND TREATED WITH 10^{-3}M E600.[a]

DILUTION		CONTROL	E600-TREATED
10^{-8}		7.7	1.7
10^{-7}		65.0	23.3
10^{-6}		537.7	217.0
	Mean	6.52×10^{8}	2.06×10^{8}

[a] E. coli stain WP_2 treated with 10^{-3}M E600 or 0.5% ethanol for controls were grown in trypticase soy broth for approximately 2 hr with aeration, and 10^{-8}, 10^{-7}, and 10^{-6} dilutions were pour plated in brain heart infusion agar, incubated at 37°C and counted 2 days later.

Figure 13. Paraoxonase activity of fetal calf serum heat-inactivated for varying amounts of time. (A) 0 hr, 56°C; (B) 0.5 hr, 56°C; (C) 1 hr, 56°C; (D) 2 hr, 56°C; (E) 10 min, 70°C. Increased heating time resulted in a decrease in paraoxonase activity.

Figure 14. Paraoxonase activity of fetal calf serum heated at 56°C. Significant activity was resistant to heating at 56°C indicating the presence of two enzymes with paraoxonase activity.


300
200
100
p-NITROPHENOL CONCENTRATION [uM]
A
B
C
D
E
3
8
15
% FETAL CALF SERUM

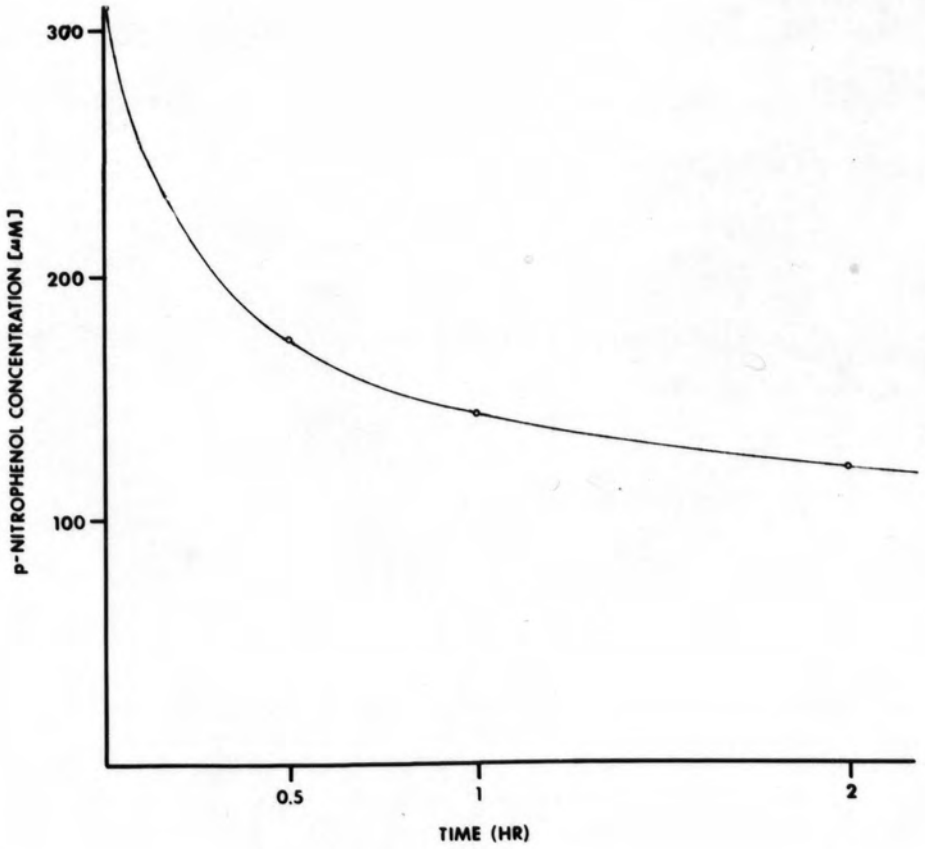

300
200
100
p-NITROPHENOL CONCENTRATION [uM]
0.5
1
2
TIME (HR)

Figure 15. Paraoxonase activity of fetal calf serum exposed to gamma irradiation or heating. (A) 0 kr; (B) 25 kr; (C) 50 kr; (D) 100 kr; (E) 200 kr; (F) heated at 56°C for 2 hr; (G) heated at 70°C for 10 min. Gamma irradiation, unlike heating, did not affect paraoxonase activity.

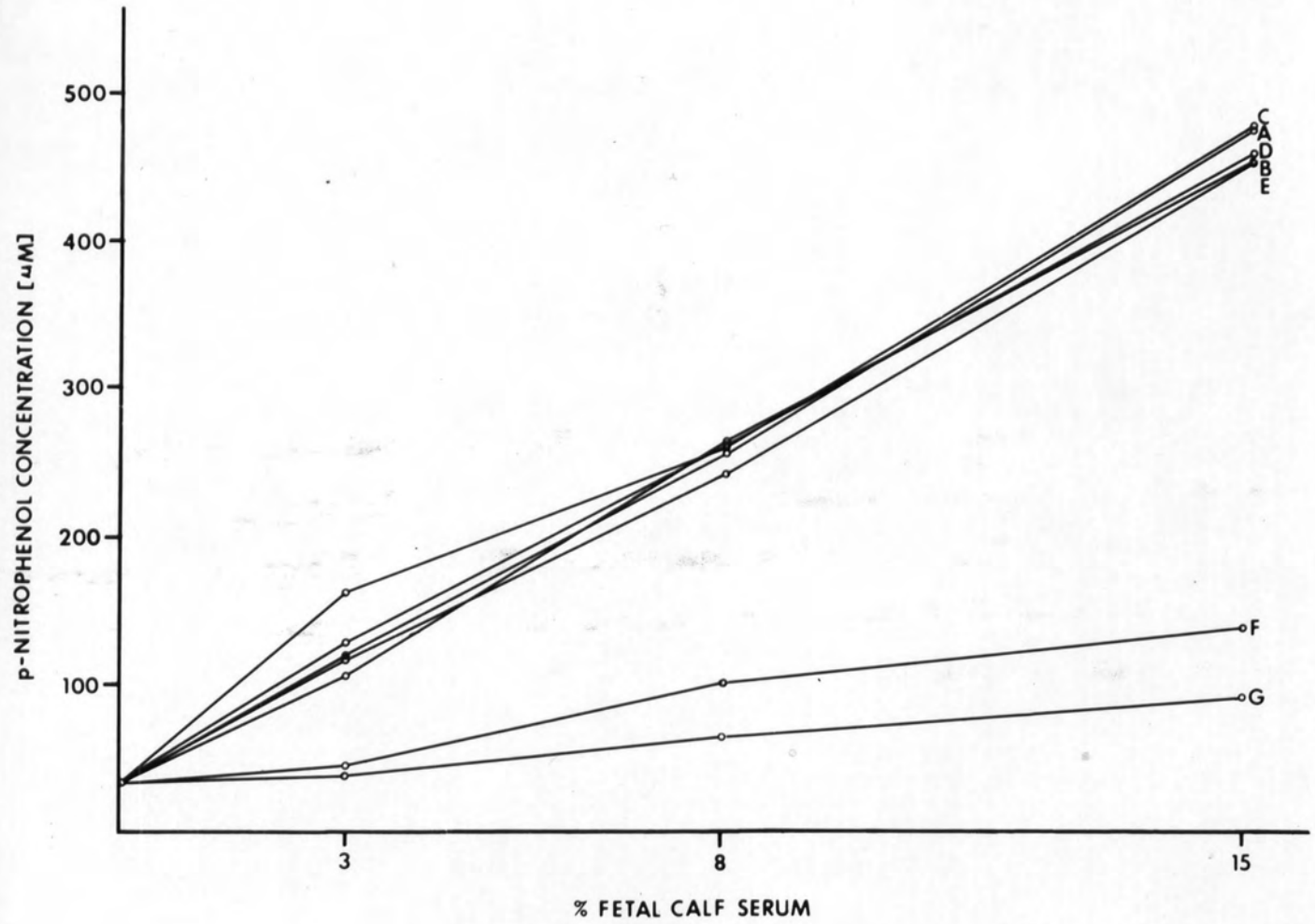
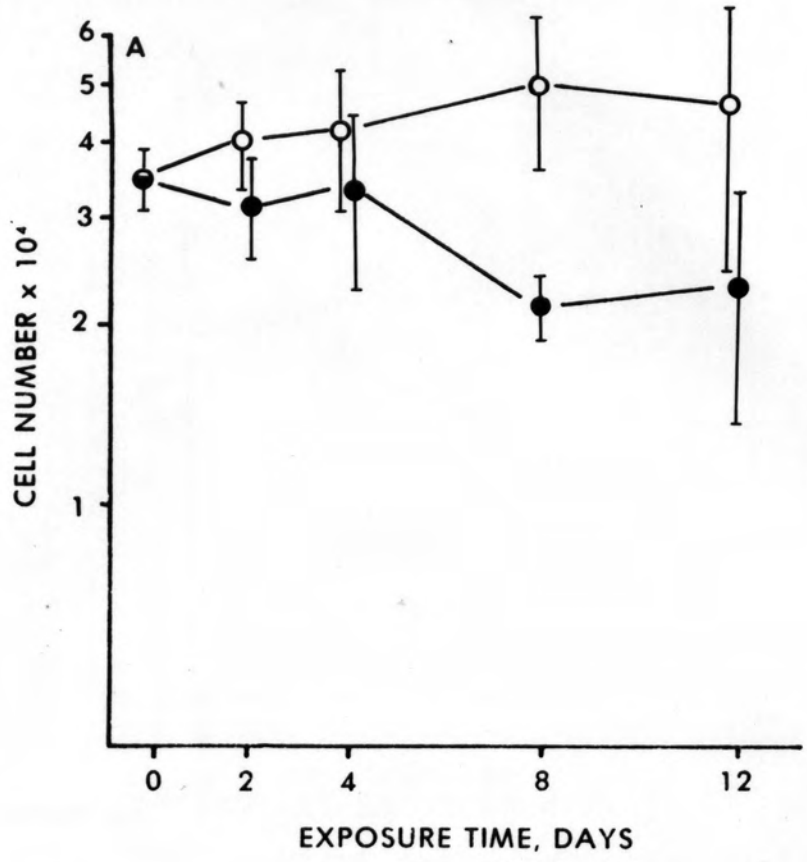

500
400
300
200
100
p-NITROPHENOL CONCENTRATION [μM]
C
A
D
B
E
F
G
3
8
15
% FETAL CALF SERUM

Figure 16. Growth curve of (A) SaJo, normal, and (B) GM-2109 (P), Wolman's disease cells, untreated (○ □) and treated with 10^{-3}M E600 (● ■). E600-treatment was more toxic to Wolman's disease than to normal cells.


A
6
5
4
3
2
1
CELL NUMBER x 10⁴
0
2
4
8
12
EXPOSURE TIME, DAYS

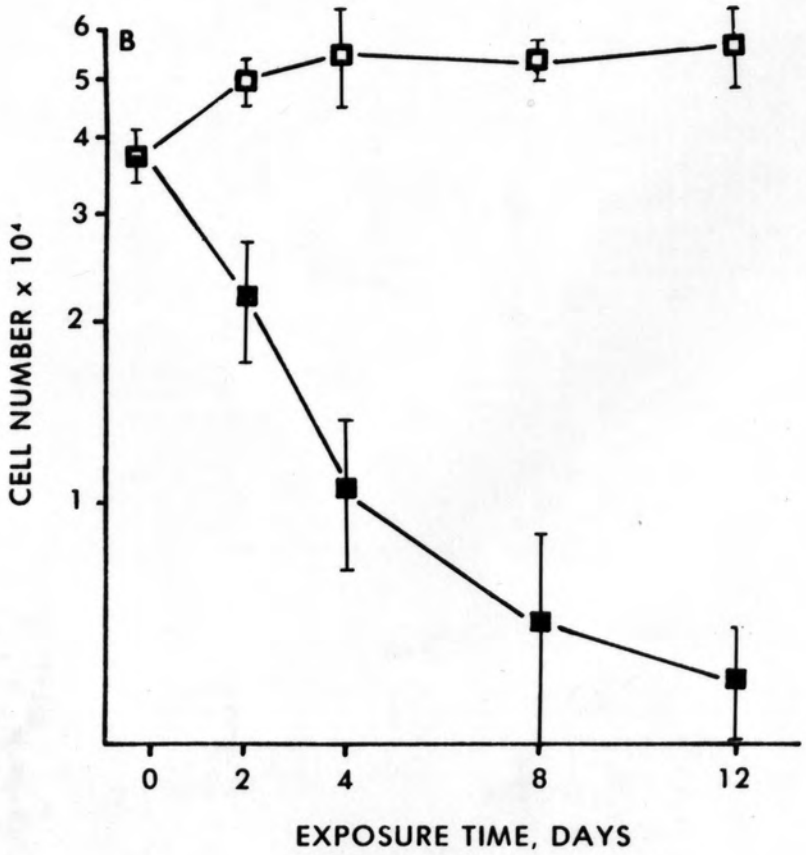
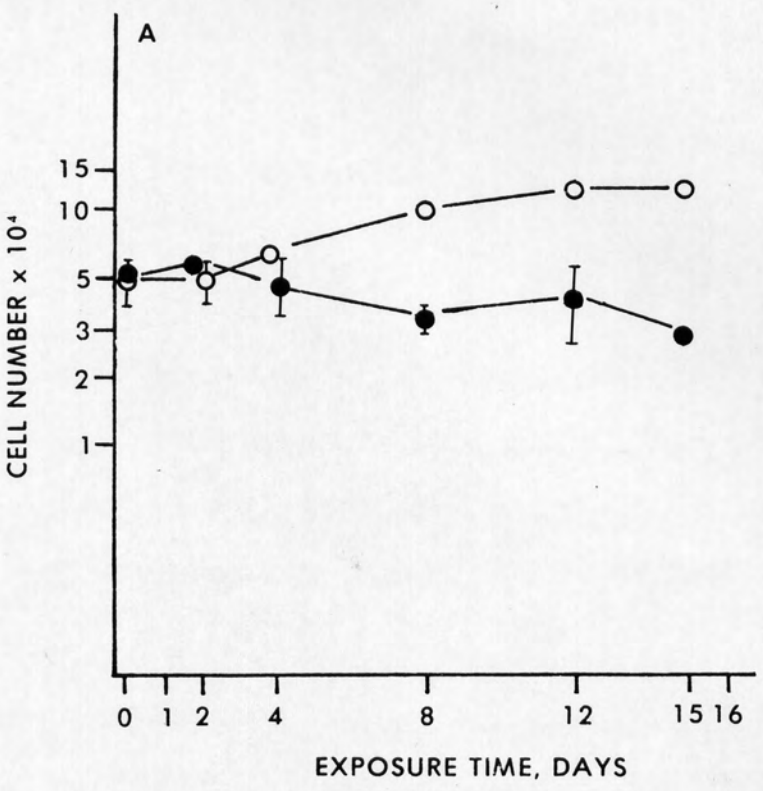

B
6
5
4
3
2
1
CELL NUMBER x 10⁴
0
2
4
8
12
EXPOSURE TIME, DAYS

Figure 17. Growth curve of (A) GM-179, normal, and (B) GM-1606, Wolman's disease cells, untreated (○ □) and treated with 10^{-3}M E600 (● ■). E600-treatment was slightly more toxic to Wolman's disease than to normal cells. In points without error bars, the error bars fell within the outlines of the point.


A
CELL NUMBER x 10⁴
15
10
5
3
2
1
0 1 2 4 8 12 15 16
EXPOSURE TIME, DAYS

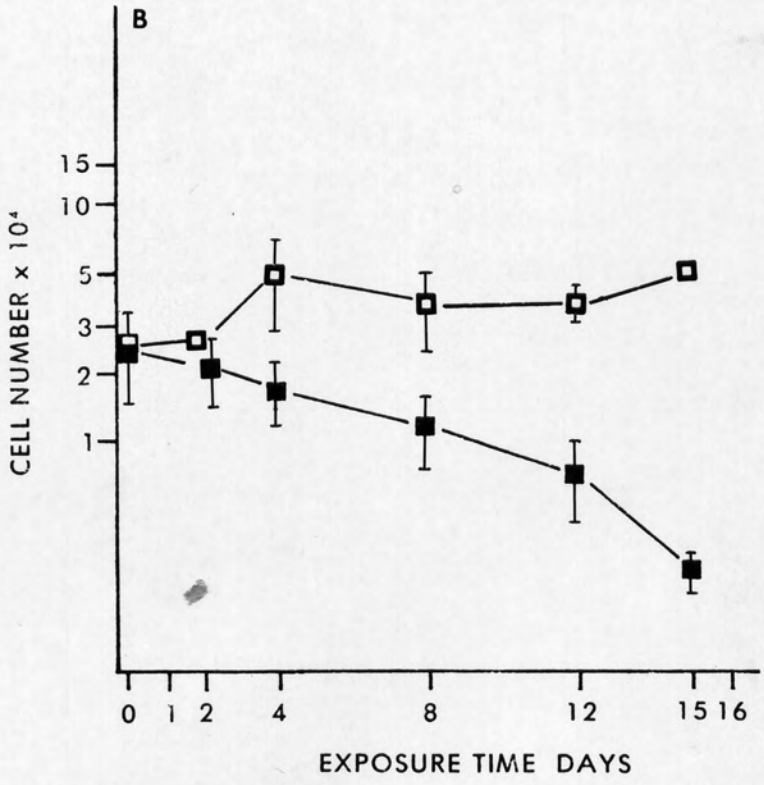
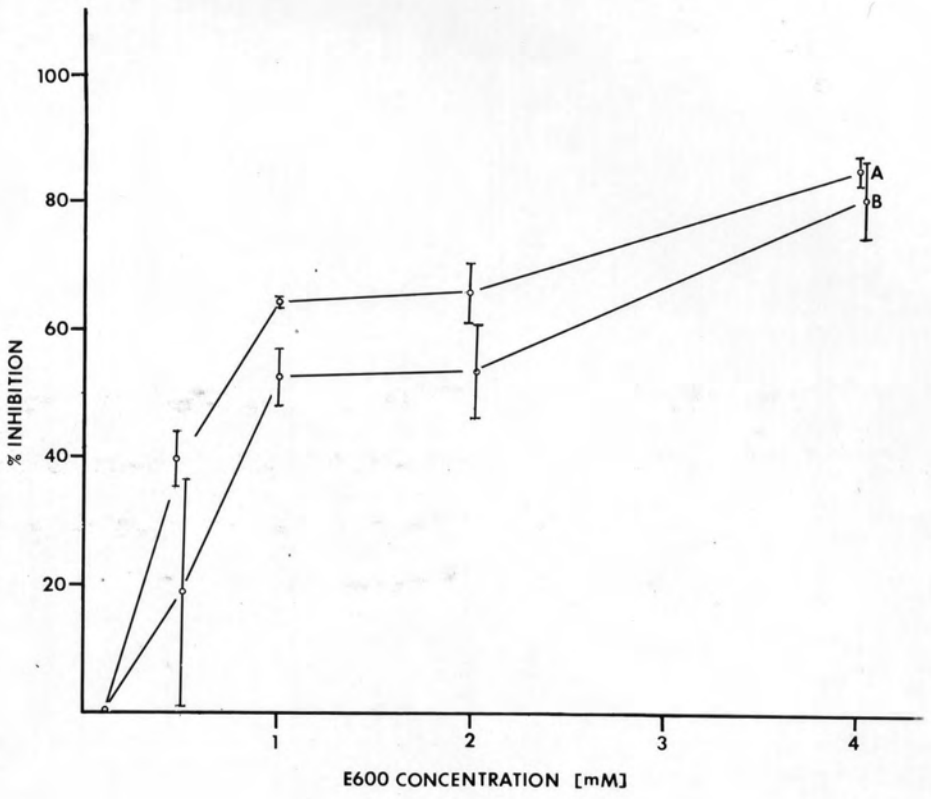

B
CELL NUMBER x 10⁴
15
10
5
3
2
1
0 1 2 4 8 12 15 16
EXPOSURE TIME DAYS

Figure 18. Toxicity of E600 to (A) GM-179, normal, and (B) GM-1606, Wolman's disease cells treated for 2 days with varying concentrations of E600. E600 appeared to be slightly more toxic to normal cells than to Wolman's disease cells.

Figure 19. Toxicity of E600 to (A) GM-179, normal, and (B) GM-1606, Wolman's disease cells treated for 6 days with varying concentrations of E600. E600 appeared to be slightly more toxic to Wolman's disease than to normal cells. E600 was more toxic after 6 days than after 2 days (Fig. 18).


100
80
60
40
20
% INHIBITION
A
B
1
2
3
4
E600 CONCENTRATION [mM]

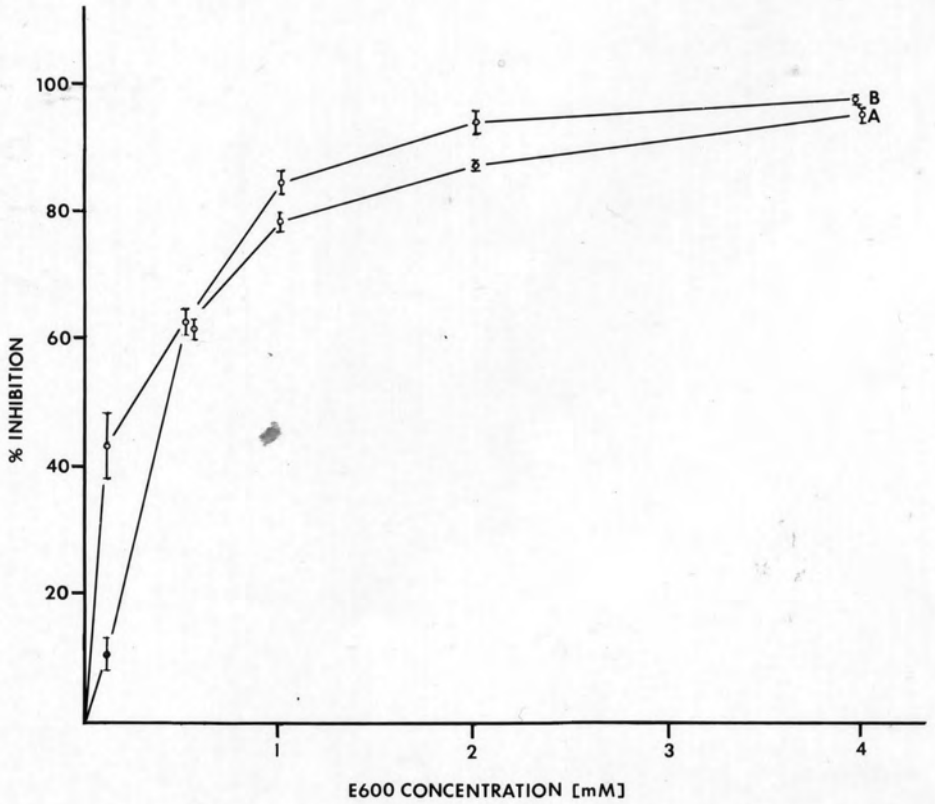

100
80
60
40
20
% INHIBITION
B
A
1
2
3
4
E600 CONCENTRATION [mM]

Figure 20. Dose and time dependence of E600-induced lipid accumulation in GM-37, normal cells and its reversibility. One hundred cells per box were graded for the extent of oil red O positive granulation according to the grades shown in Fig. 1 (A) control, exposed to 0.5% ethanol; (B) 10^{-6}M E600; (C) 10^{-5}M E600; (D) 10^{-4}M E600; (E) 3 x 10^{-4}M E600; (F) 10^{-3}M E600; (G) 10^{-3}M E600, 12 hr, untreated thereafter; (H) 10^{-3}M E600, 24 hr, untreated thereafter, (I) 10^{-3}M E600, 48 hr, untreated thereafter; (J) 10^{-3}M E600, 4 days, untreated thereafter. GM-37 cells showed time-dependent increases in lipid storage which were minimal at 10^{-4}M E600 and pronounced at 10^{-3}M E600. (G-J) cells were treated with 10^{-3}M E600 for the specified period, rinsed with Puck's balanced salt solution and incubated in medium containing 0.5% ethanol until fixation. After removal of the E600, the cellular lipid accumulation returned to control levels (A).

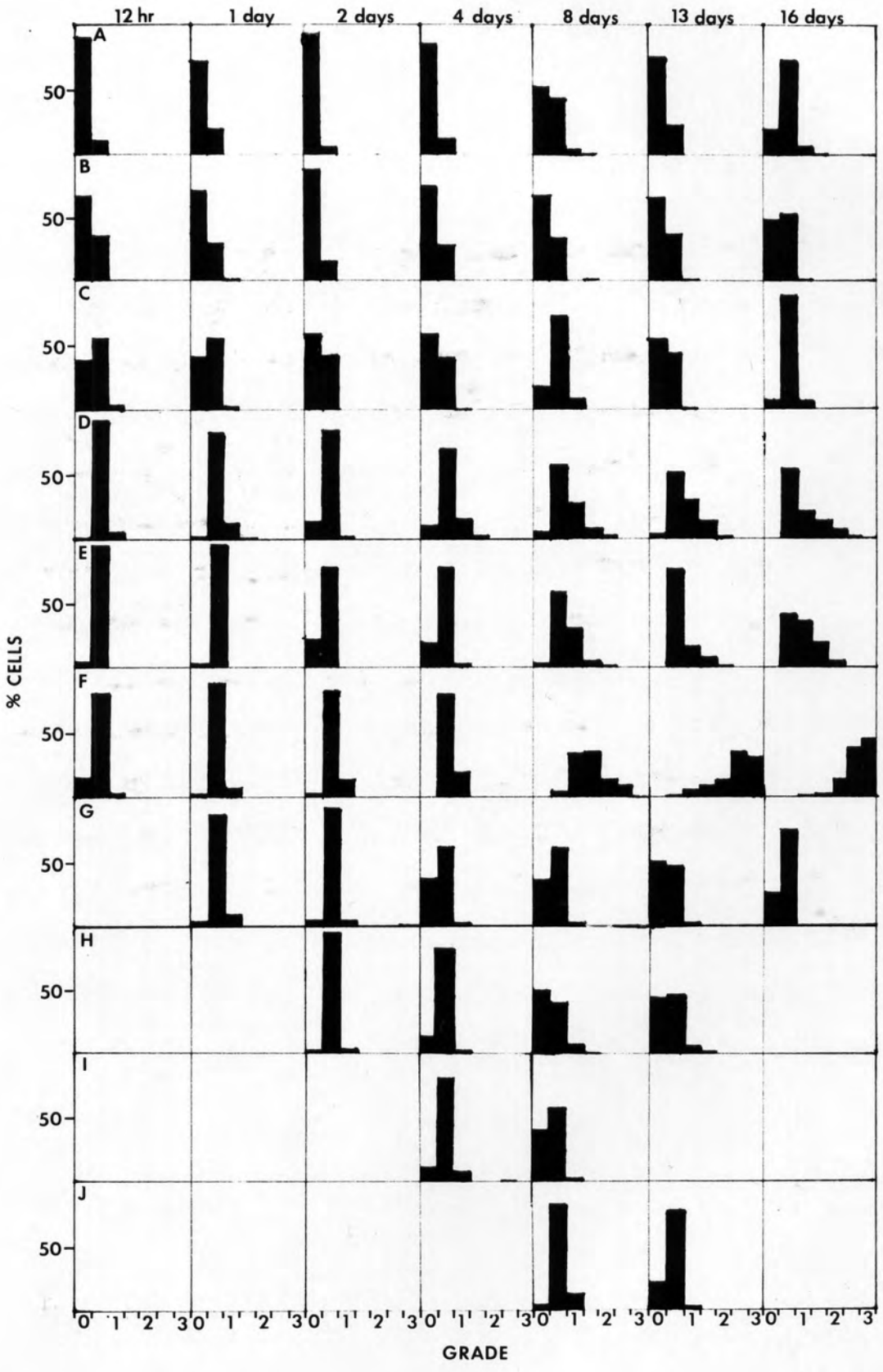

12 hr
1 day
2 days
4 days
8 days
13 days
16 days
A
B
C
D
E
F
G
H
I
J
50
% CELLS
0 1 2 3
GRADE

Figure 21. Dose and time dependence of E600-induced lipid accumulation in GM-316, normal cells and its reversibility. One hundred cells per box were graded for the extent of oil red O positive granulation according to the grades shown in Fig. 1. (A) control, exposed to 0.5% ethanol; (B) 10^{-6}M E600; (C) 10^{-5}M E600; (D) 10^{-4}M E600; (E) 3 x 10^{-4}M E600; (F) 10^{-3}M E600; (G) 3 x 10^{-3}M E600; (H) 10^{-3}M E600, 12 hr, untreated thereafter. (I) 10^{-3}M E600, 48 hr, untreated thereafter. GM-316 cells showed time-dependent increases in lipid storage which were minimal at 10^{-4}M E600 and more pronounced at 3 x 10^{-4}M and 10^{-3}M E600. Toxicity was observed at 3 x 10^{-3}M E600. (H,I) Cells were treated with 10^{-3}M E600 for the specified period, rinsed with Puck's balanced salt solution and incubated in medium containing 0.5% ethanol until fixation. After removal of the E600, the cellular lipid accumulation returned to control levels (A).

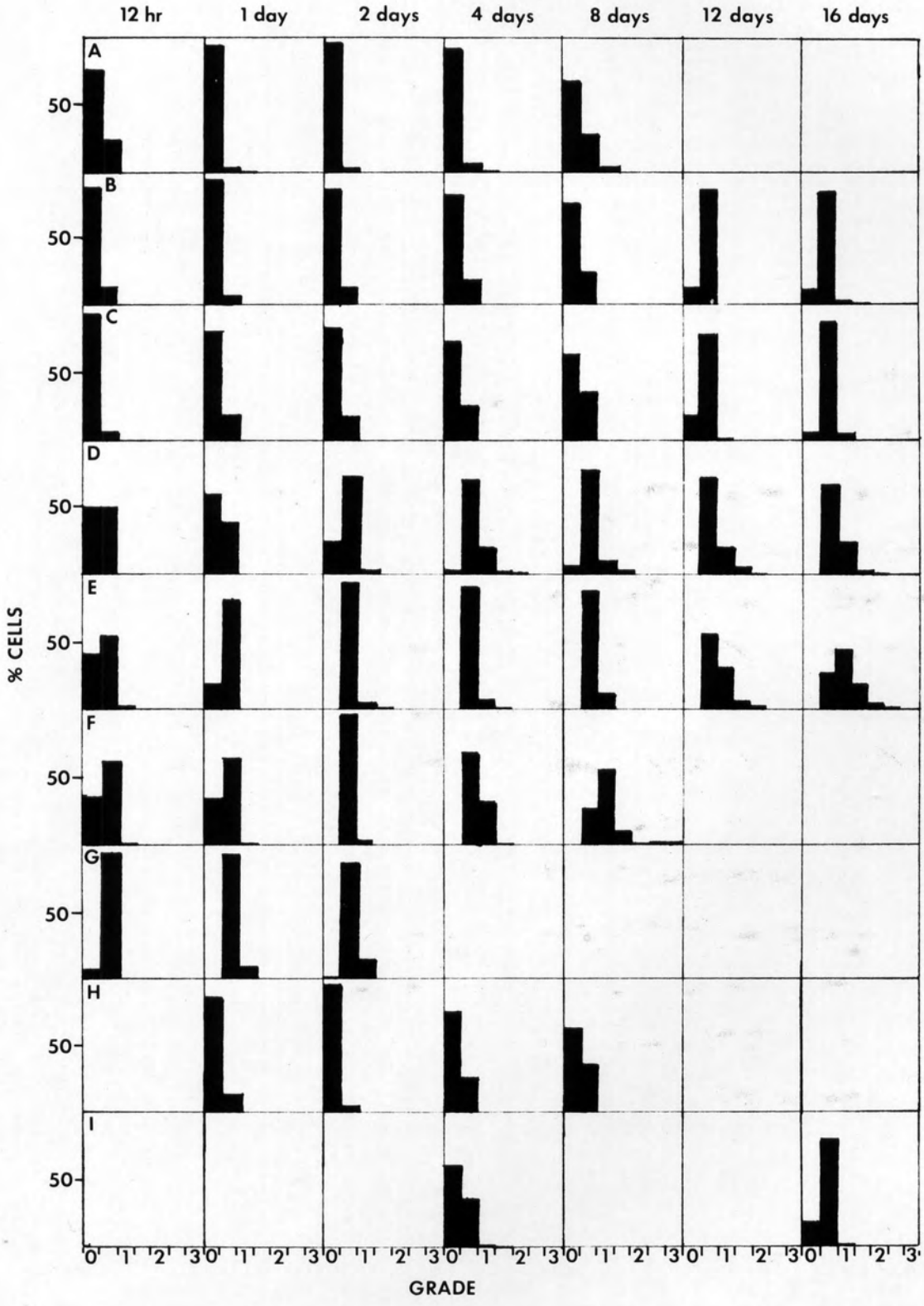

12 hr
1 day
2 days
4 days
8 days
12 days
16 days
A
B
C
D
E
F
G
H
I
50
% CELLS
0 1 2 3
GRADE

Figure 22. Dose and time dependence of E600-induced lipid accumulation in GM-1606, Wolman's disease cells. One hundred cells per box were graded for the extent of oil red O positive granulation according to the grades shown in Fig. 1. (A) control, exposed to 0.5% ethanol; (B) 10^{-6}M E600; (C) 10^{-5}M E600; (D) 10^{-4}M E600; (E) 3×10^{-4}M E600; (F) 10^{-3}M E600; (G) 3×10^{-3}M E600. Untreated cells showed pronounced lipid accumulation with increased time in culture. Further increases were seen in cells treated with 10^{-3}M and 3×10^{-3}M E600, though the latter concentration was toxic to the cells.

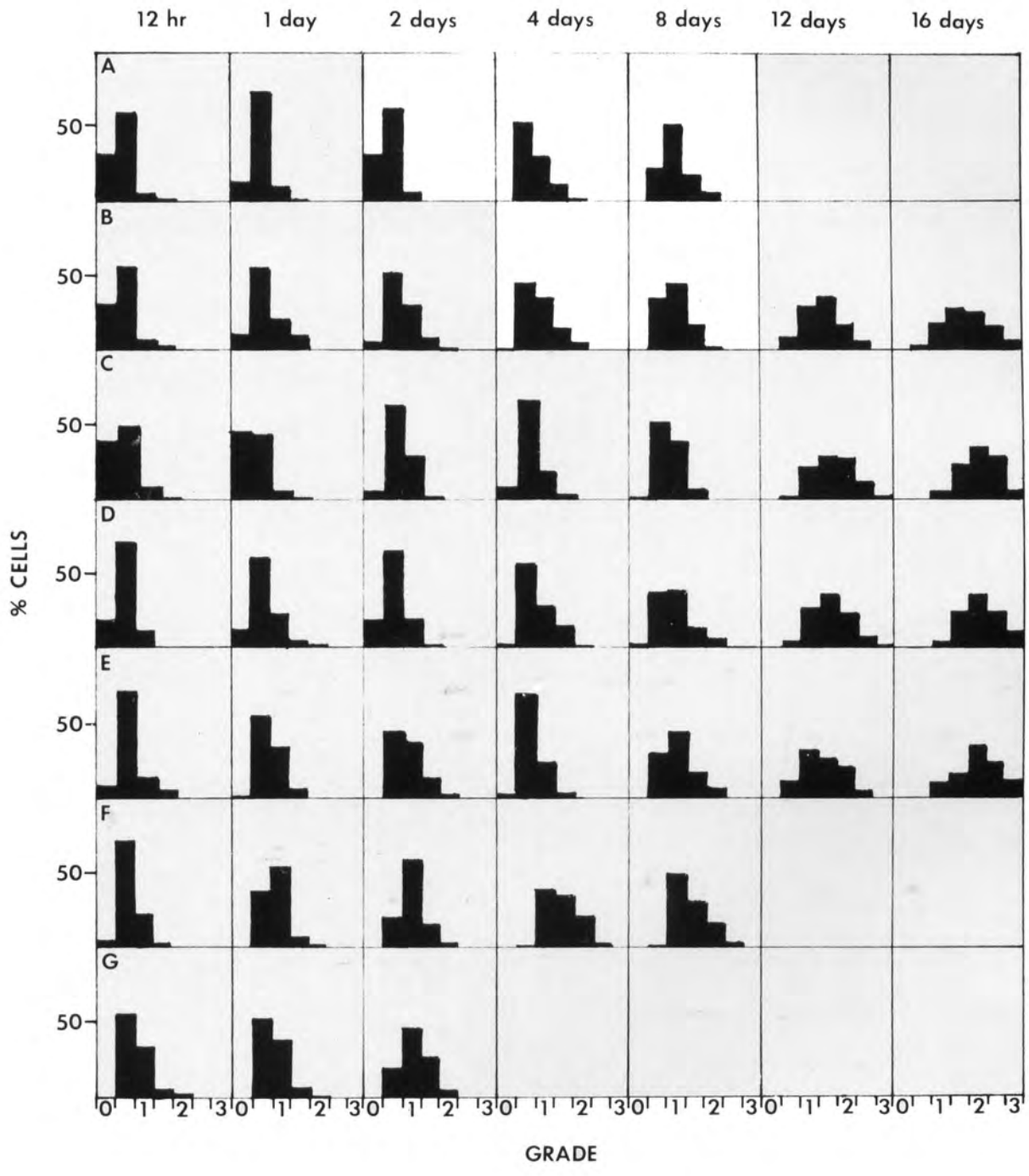
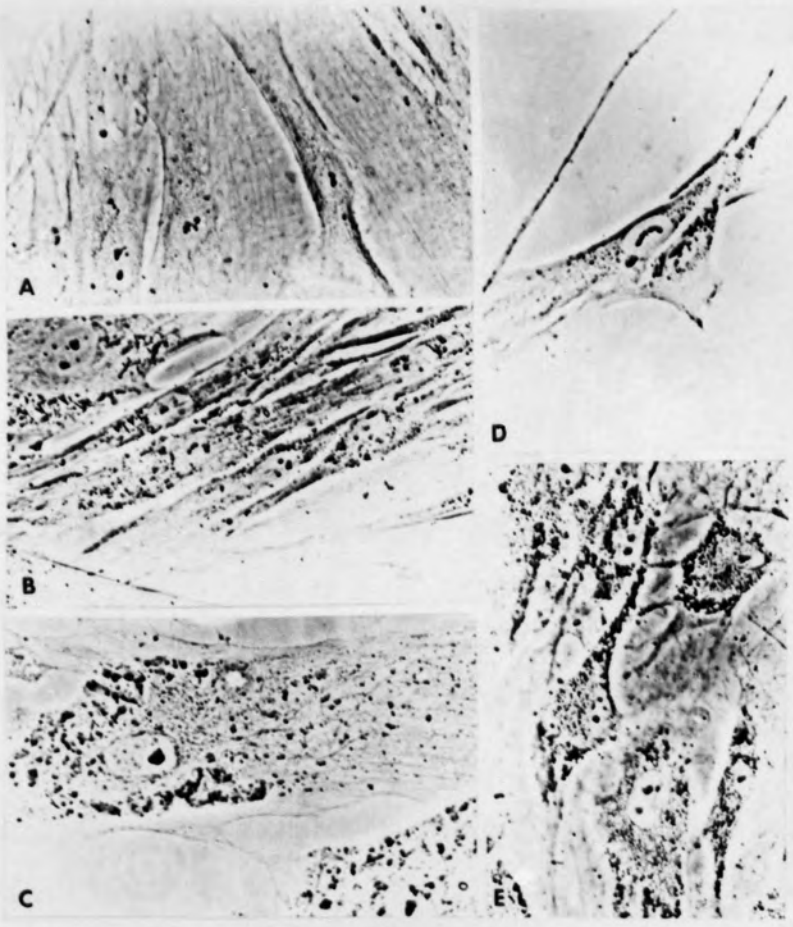

12 hr
1 day
2 days
4 days
8 days
12 days
16 days
A
B
C
D
E
F
G
50
% CELLS
0 1 2 3
GRADE

Figure 23. Lipid droplets and crystals in the cytoplasm of Wolman's disease and E600-treated normal cells. (A) GM-179, normal, untreated; (B) GM-1606, Wolman's disease; (C) GM-2211, Wolman's disease; (D) GM-2109 (P), Wolman's disease; (E) GM-179, normal, treated with 10^{-3}M E600. Cells were fixed in calcium buffered formalin and observed by phase contrast microscopy. X260.

Figure 24. Autofluorescent lipid in the cytoplasm of Wolman's disease and E600-treated normal cells. (A) GM-37, normal, untreated; (B) GM-37, normal, 10^{-3}M E600 for 7 days (C) GM-2211, Wolman's disease, untreated. X200.


A
B
C
D
E

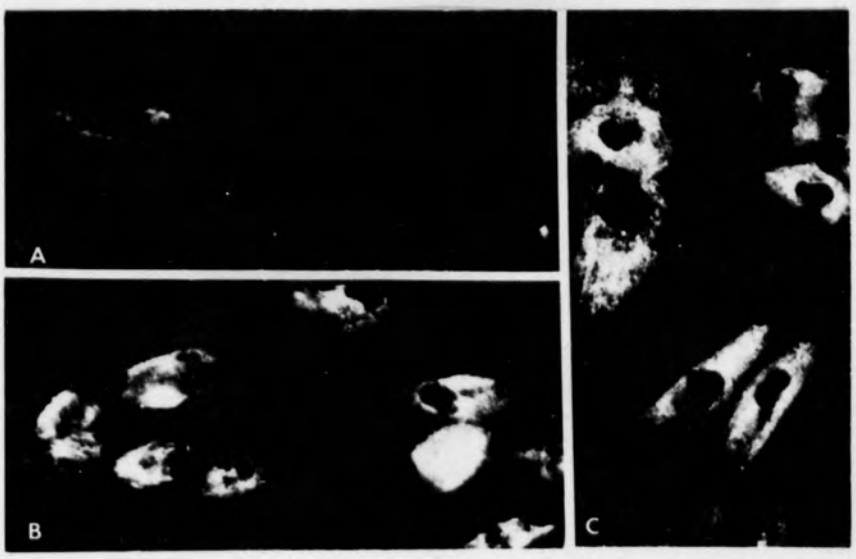
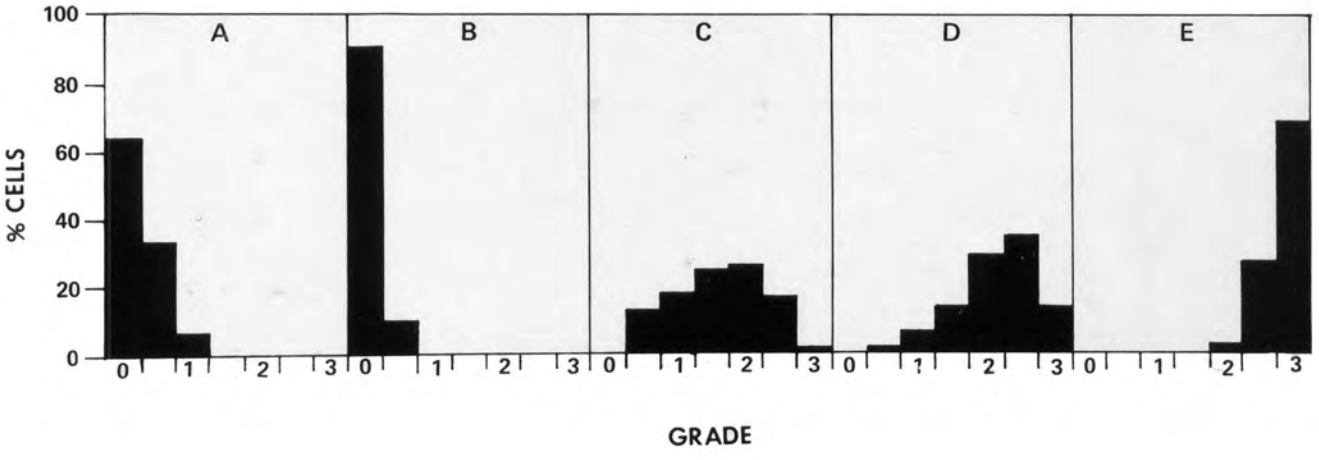

A
B
C

Figure 25. Extent of lipid storage in different Wolman's disease cell cultures and a normal control. One hundred cells per box were graded for the extent of oil red O positive granulation according to the grades shown in Fig. 1. (A) GM-179, normal; (B) GM-1606, Wolman's disease, 2 months in culture; (C) GM-1606, Wolman's disease, 3 months in culture; (D) GM-2211, Wolman's disease, 2 months in culture; (E) GM-2109 (P), Wolman's disease, 8 months in culture. Culture GM-1606 did not accumulate more lipid than normal cultures until in culture for 3 months.

Figure 26. Lipid storage in GM-2211, Wolman's disease cells (A) before and (B) after trypsinization. One hundred cells per box were graded for the extent of oil red O positive granulation according to the grades shown in Fig. 1. Trypsinization appeared to have little effect on lipid storage.


100
80
60
40
20
0
% CELLS
A
B
C
D
E
0
1
2
3
GRADE

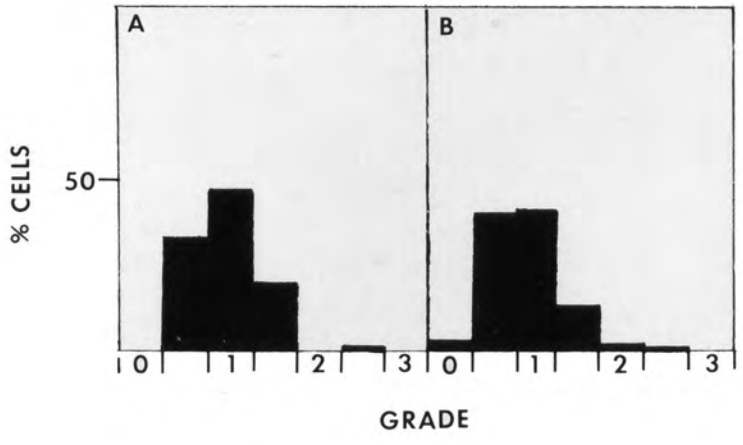

50
% CELLS
A
B
0
1
2
3
GRADE

Figure 27. Lipid storage in GM-498, normal (A,B,C) and GM-1606, Wolman's disease (D,E,F) cells treated with varying concentrations of cholesterol (A,D), cis-vaccenic acid (B,E), and cholesteryl palmitate (C,F). One hundred cells per box were graded for the extent of oil red O positive granulation according to the grades shown in Fig. 1. Cis-vaccenic acid at 4.0 mg% was toxic to the cells. (The O concentration values are the means of the grades of 300 cells for each culture.)

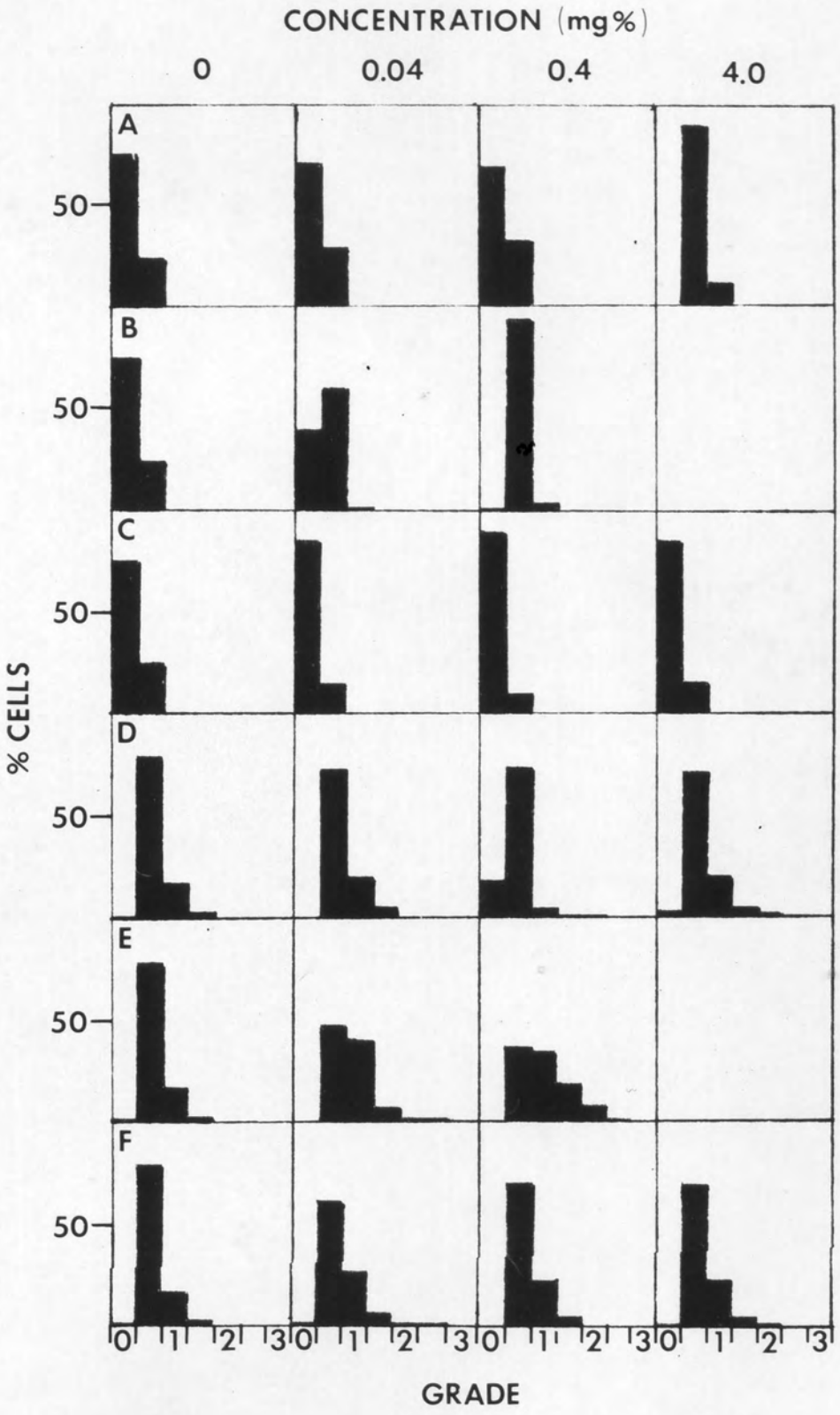

CONCENTRATION (mg%)
0
0.04
0.4
4.0
A
B
C
D
E
F
50
% CELLS
0
1
2
3
GRADE

Figure 28. Lipid storage in GM-498, normal and GM-1606, Wolman's disease cells treated with varying concentrations of cholesterol, free fatty acid, and cholesteryl ester. (A) untreated; (B) 4.0 mg% cholesterol; (C) 0.4 mg% cis-vaccenic acid; (D) 4.0 mg% cholesteryl palmitate. Normal cells treated with cholesterol or free fatty acid and Wolman's disease cells treated with free fatty acid exhibited large peripheral lipid droplets. Stained with oil red O and hematoxylin. X500.

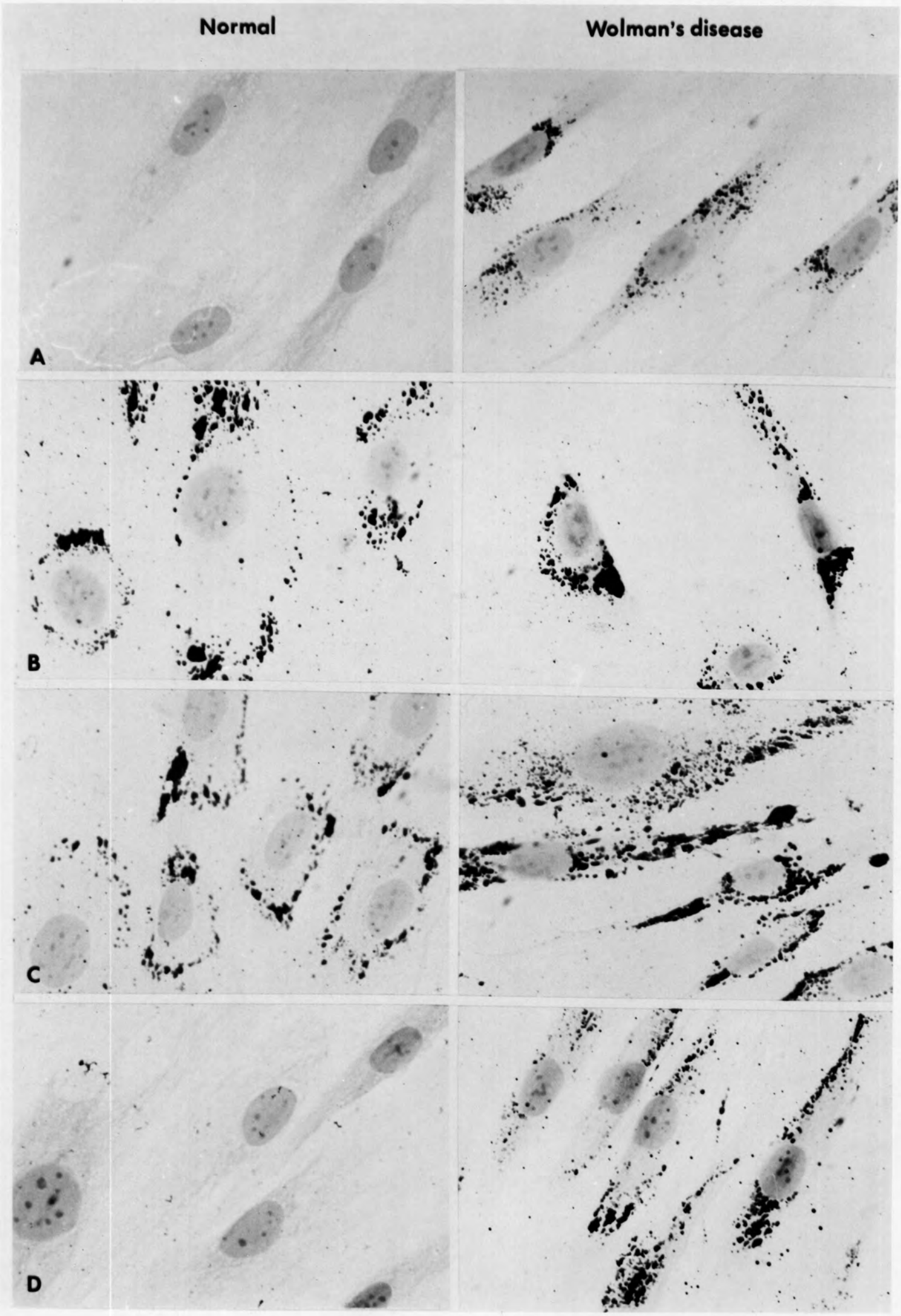
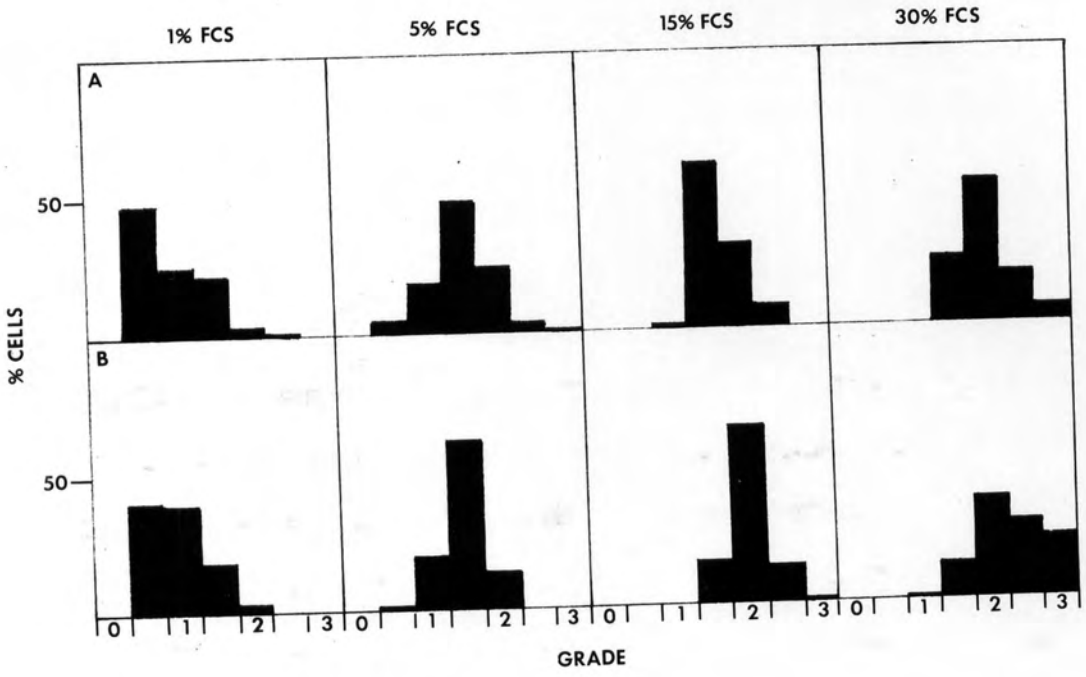

Normal
Wolman's disease
A
B
C
D

Figure 29. Lipid storage in GM-316, normal cells treated with 10^{-3}M E600 in the presence of varying concentrations of fetal calf serum. One hundred cells per box were graded for the extent of oil red O positive granulation according to the grades shown in Fig. 1. (A) fresh fetal calf serum; (B) fetal calf serum heat-inactivated 1 hr at 56°C. E600-induced lipid accumulation varied directly with the serum concentration.

Figure 30. Lipid storage in cells of normal culture GM-179 exposed to identical concentrations of (A) E600 and (B) p-nitrophenol. One hundred cells per box were graded for the extent of oil red O positive granulation according to the grades shown in Fig. 1. E600, though less toxic than p-nitrophenol, produced significantly more lipid accumulation. Results for the untreated control were from the same coverslip.


1% FCS
5% FCS
15% FCS
30% FCS
A
B
50
50
% CELLS
0
1
2
3
GRADE

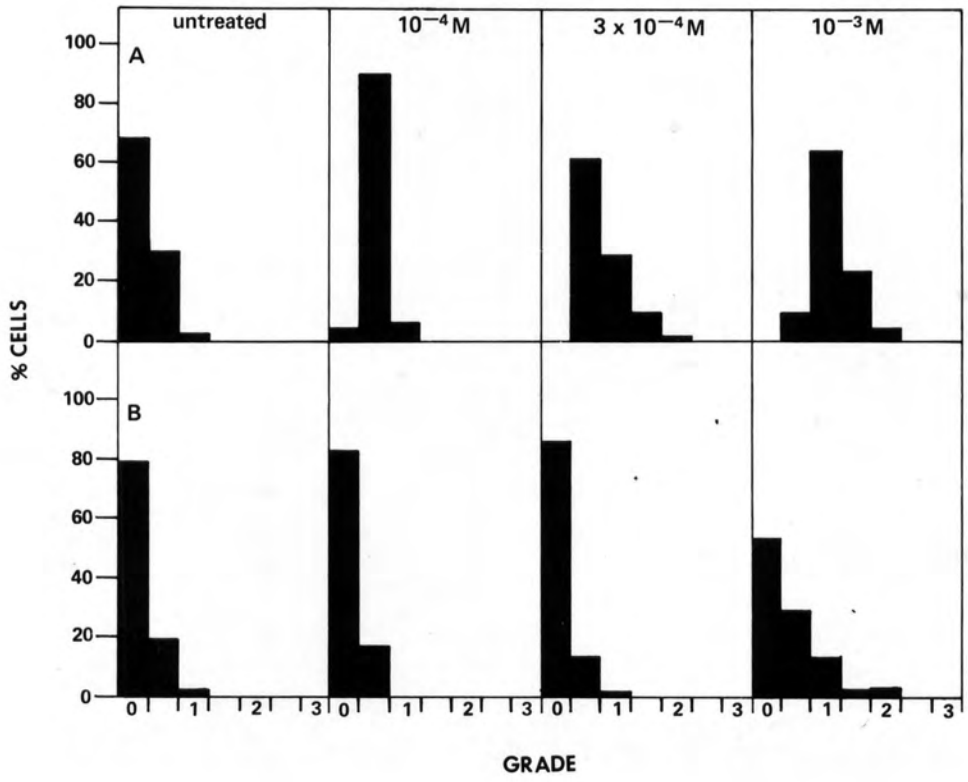

untreated
10⁻⁴M
3 x 10⁻⁴M
10⁻³M
A
B
100
80
60
40
20
0
% CELLS
0
1
2
3
GRADE

Figure 31. Neutral red staining of various cell cultures, untreated and treated with 10^{-4}M E600. One hundred cells per box were graded for the extent of neutral red granulation according to grades corresponding to those shown for oil red O staining in Fig. 1. (A) GM-37, normal; (B) GM-179, normal; (C) GM-2548, Bloom's syndrome; (D) GM-3557, mother of proband; (E) GM-1606, Wolman's disease. E600-treatment resulted in increased neutral red granulation.

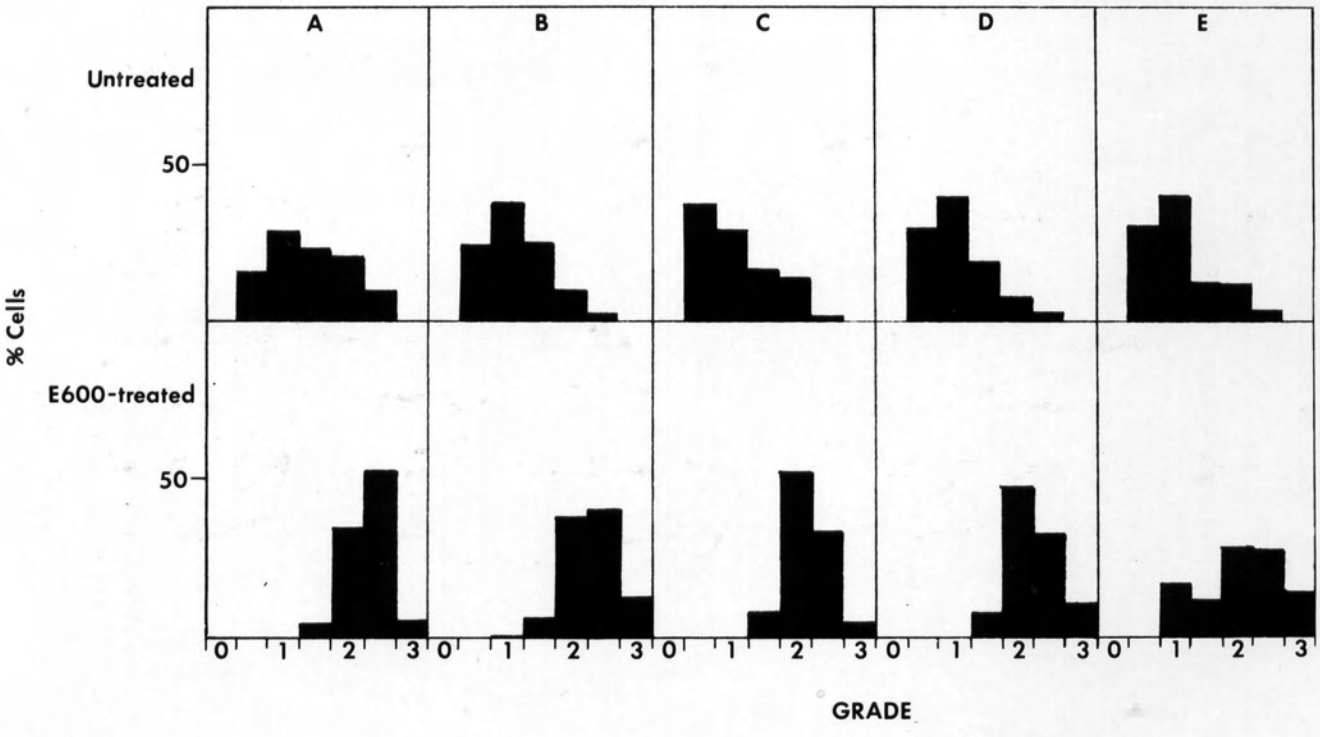

A
B
C
D
E
Untreated
50
E600-treated
50
% Cells
0
1
2
3
GRADE

Figure 32. Neutral red staining of various cell cultures, untreated and treated with 10^{-4}M E600. (A) GM-37, normal; (B) GM-179, normal; (C) GM-1606, Wolman's disease. Clear granules are also visible in the E600-treated cells. A clear perinuclear area is visible in the E600-treated cells. X425.

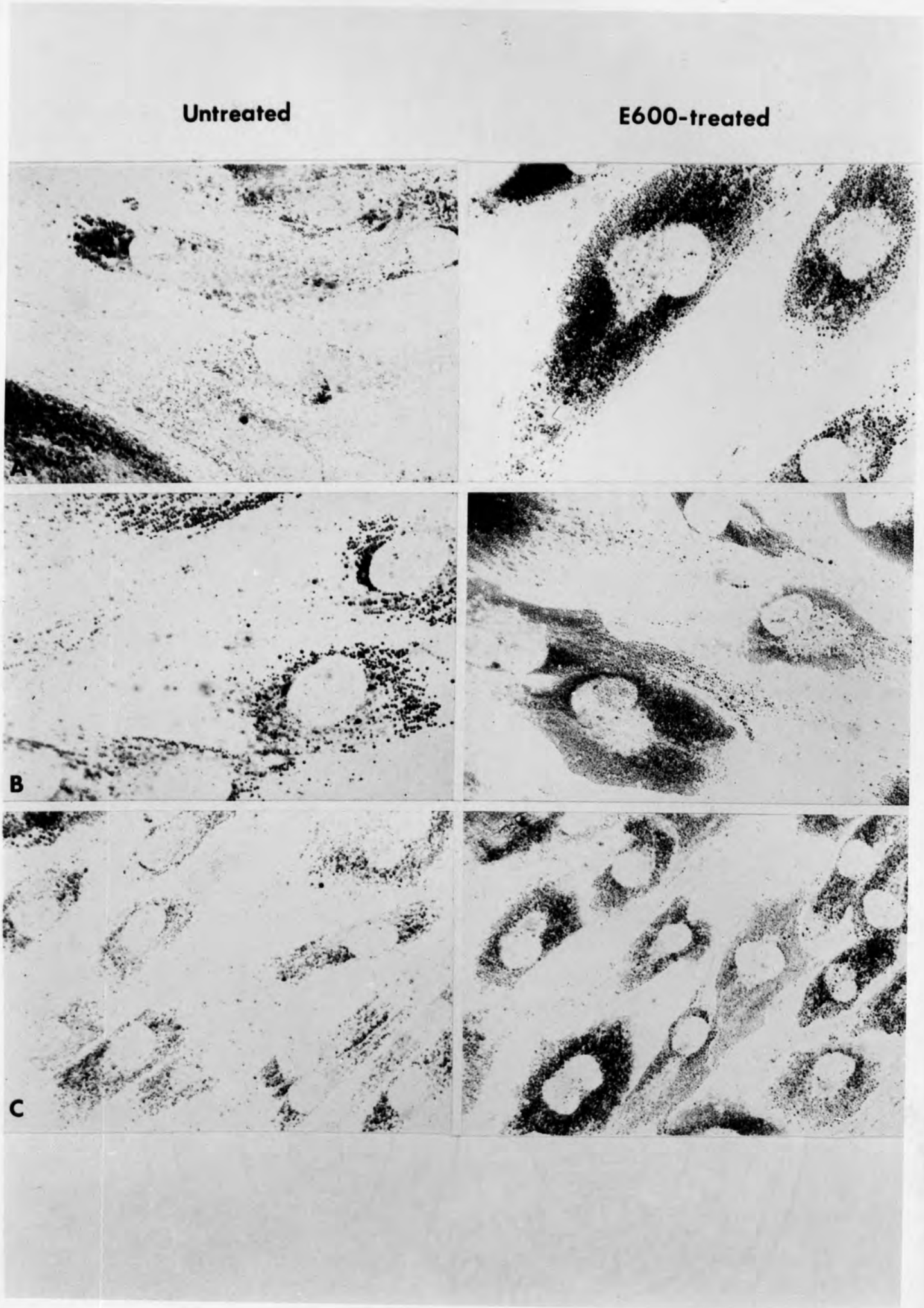
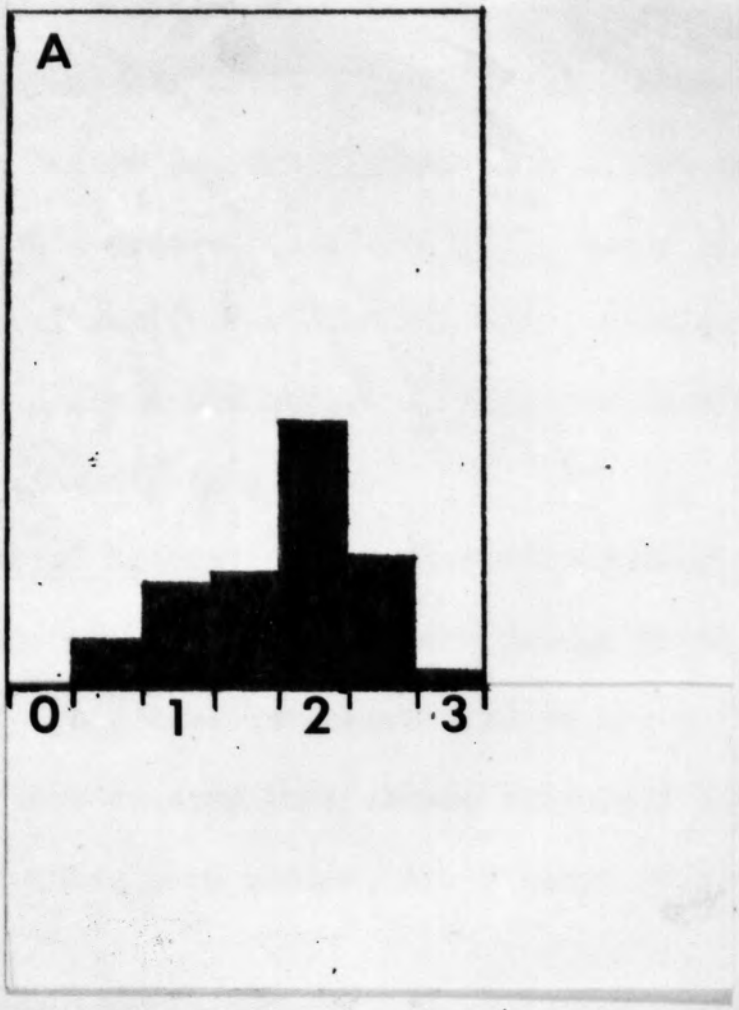
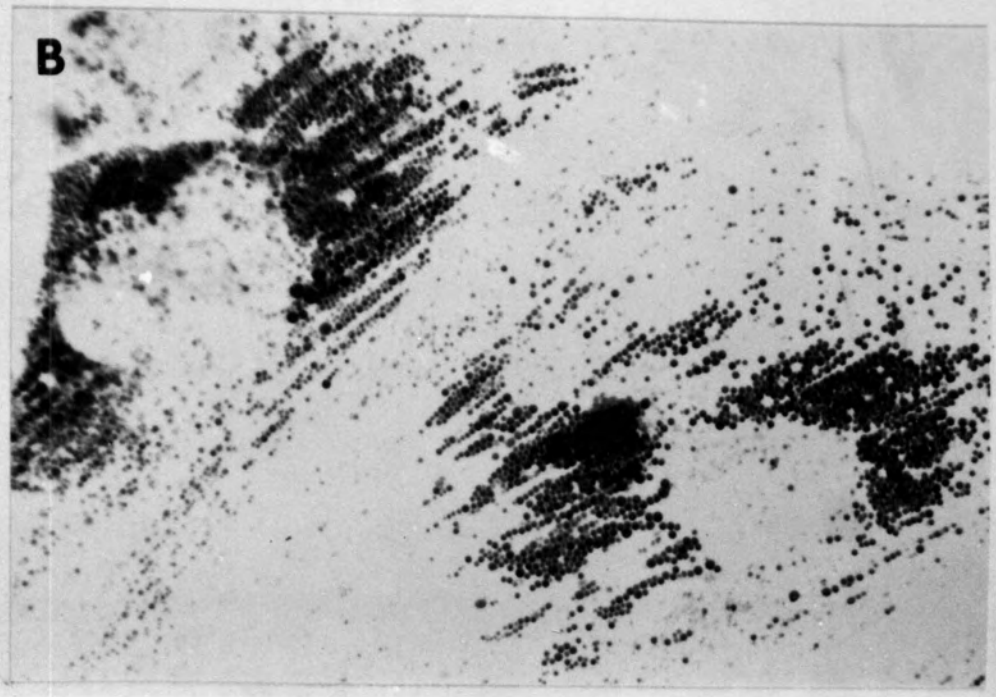

Untreated
E600-treated
A
B
C

Figure 33. Neutral red staining of a Wolman's disease heterozygote cell culture, GM-2121. (A) One hundred cells were graded for the extent of neutral red granulation according to grades corresponding to those shown for oil red O staining in Fig. 1. (B) Linear arrangement of neutral red granules. X570. The GM-2121 cell culture had more neutral red granules than other untreated cell cultures (Figs. 31,32). Clear granules are also visible.


A
0
1
2
3


B

Neutral Red Staining: Wolman's disease cells did not show more neutral red granules than normal cells (Figs. 31,32). E600-treated cells showed an increased number of neutral red granules. E600-treated cells, both normal and Wolman's disease, also exhibited clear granules that did not stain with neutral red (Fig. 32). The GM-2121 culture showed more neutral red granules than the other untreated cultures, and the granules appeared to be arranged linearly (Fig. 33).

Effect on Bacterial Growth: The number of colonies per plate are shown in Table 4. When the results from each concentration were averaged together, the untreated E. coli tube was calculated to number 6.52×10^8 organisms per ml whereas the E600-treated tube showed only 2.06×10^8 organisms per ml. At the time the tubes were plated, their respective optical densities were 0.42 and 0.26.

Discussion

The shape of the curve for paraoxonase inactivation (Fig. 14) suggests that FCS contains two enzymes with paraoxonase activity which can be separated on the basis of their sensitivity to heating (31,32). The paraoxonase activity of FCS can be reduced to less than half by heating at 56°C for 1 hr. Because further heating produced only a slight reduction of paraoxonase activity but could have had effects on other factors necessary to cell growth, FCS was inactivated for 1 hr at 56°C in experiments in which cells were treated with E600. Heat inactivation did not result in decreased lipid storage (Fig. 29). In fact, a slight increase in lipid storage was seen, presumably due to the maintenance of a higher E600 concentration in medium with heat-inactivated serum.

No evidence for E600 toxicity was seen at 10^{-5}M or 10^{-4}M E600 in the growth curve, although the relative toxicity experiment showed some inhibition

at 10^{-4}M E600 by day 6. At 10^{-3}M E600 both the growth curve and the relative toxicity experiment showed some toxicity to cells. Increased time of exposure resulted in increased toxicity in both experiments. Typically the extent of cell toxicity is proportional to the test concentration and the duration of incubation (63). Wolman's disease cells appeared to be slightly more susceptible to E600-induced killing than normal cells (Figs. 16,17,19). Wolman's disease cells, because they are deficient in acid lipase, might be expected to rely more on other enzymes that are sensitive to E600. However, the lack of any significant toxicity at 10^{-5}M and 10^{-4}M E600, concentrations that should completely inhibit most esterase enzymes (27), makes this hypothesis less likely. It is possible that cytoplasmic enzymes are exposed to a lower concentration of E600 than the lysosomal enzymes, because lysosomes fuse with phagocytic vacuoles which may carry E600 into the cells. Perhaps the lipid storage already present in Wolman's disease cells caused them to reach toxic levels of lipid slightly before the E600-treated normal cells. Respiration may be affected by organophosphates like E600 (38,64). Because the Wolman's disease cells are already under stress due to their enzyme deficiency, they may show a decreased ability to resist other stressful stimuli.

E600 appeared to be quite toxic to bacteria. The tube grown without E600 showed 3.2 times as many viable organisms as the tube with 10^{-3}M E600. The optical density, which shows the live and dead organisms, showed only 1.6 times as many cells in the untreated tube. This discrepancy could be explained if the E600-treated tube contained 2.06×10^{8} viable organisms and 1.98×10^{8} dead organisms. Because the tubes were incubated for only 2 hr it is unlikely that the E600 had degraded sufficiently to cause an increase in optical density. It is unclear whether the growth

inhibition is due to bacterial dependence upon lipase or esterase activity for growth or an effect of E600 on bacterial respiration.

The wide variation seen in the amount of lipid stored by the 3 Wolman's disease cultures (Fig. 25) may indicate different levels of residual acid lipase activity, but it may also reflect the different growth rates of the 3 cultures. When the GM-1606 Wolman's disease culture was first obtained, it proliferated rapidly and exhibited little lipid accumulation for about 2 months. After 3 months, in cells of a similar passage level, the growth rate had slowed and lipid accumulation was noticeable and has continued to be since. Kyriakides et al. (42) also observed a Wolman's disease fibroblast culture that did not store lipid until it had been in culture 3 months. The GM-2109 (P) culture was the slowest growing Wolman's disease culture and it accumulated the most lipid. After only 18 population doublings, the cells exhibited the typical characteristics of a senescent culture including lower growth rate, low density, and large cells (Fig. 23), which Kontermann and Bayreuther have called type II fibrocytes (65). It is not known whether or not the presence of large amounts of stored lipid may shorten the lifespan of a fibroblast cell culture. In actively growing cultures there are fewer non-proliferative cells, and the short generation times and the rapid formation of new cytoplasmic material in the proliferative cells may have the effect of diluting the stored lipid. The rapidly proliferating cells have a high metabolic activity as well, and may be able to utilize more lipid for energy or membrane biosynthesis. Thus lipid storage may not be manifested as clearly in the rapidly proliferating cells. Regular subcultivation and other culture conditions may be important in determining the degree of expressivity of this mutation in vitro.

The lipid stored in cells treated with free fatty acid was probably triglyceride and phospholipid (66,67). Storage may have increased more in the Wolman's disease cells than in the normal cells. The reverse appeared to be true in cells treated with cholesterol. The Wolman's disease cells may require more cholesterol for membrane synthesis because they cannot obtain cholesterol from cholesteryl ester. The normal cells, therefore, may be experiencing a greater effective concentration of cholesterol because they have less use for the cholesterol and are also still metabolizing cholesteryl esters. This may result in cytoplasmic esterification of the cholesterol. The morphological difference between these cells and E600-treated cells may result from the difference between cytoplasmic and lysosomal storage of lipid. Cholesteryl ester is normally taken up as a component of low density lipoprotein (LDL). The lack of lipid storage in cells treated with cholesteryl ester was most likely due to the form of the lipid. If isolated LDL had been added, the result would probably have been quite different (9).

Some of the crystals seen in Wolman's disease and E600-treated normal cells were most likely cholesteryl ester, but some may also represent triglyceride that had been crystallized by formaldehyde fixation (68). Cholesteryl ester crystals have also been visualized by electron microscopy in cultured Wolman's disease cells (Research Section IV) and tissue from Wolman's disease patients (16, 69, 70). The autofluorescent granules seen in the Wolman's disease and E600-treated normal cells were probably lipid. It is not clearly established whether or not neutral lipids or free fatty acids are fluorescent (71-73). Various oxidized derivatives of neutral lipids are believed to be fluorescent (74-76)

and fluorescence is a fundamental characteristic of lipofuscin and ceroid. Oxygenated steryl esters (77) and ceroid (78) have been reported to be present in tissues from Wolman's disease patients, but the reported lipids have not been shown to be autofluorescent. Partin & Schubert (79) reported autofluorescent foam cells in an intestinal biopsy from a patient with cholesteryl ester storage disease. The autofluorescence we saw in Wolman's disease and E600-treated cells may be due to oxidized and peroxidized lipids or to the low grade fluorescence from large accumulations of cholesterol, fatty acids, and triglyceride (71).

E600-treated cells were quite similar to Wolman's disease cells histochemically. Both showed large amounts of neutral lipid and cholesteryl esters. Both did not differ from controls in staining for mucosubstance, glycogen, or unstaturated lipid. However, the E600-treated cells showed slight increases in free fatty acid, free cholesterol, and perhaps phospholipid which were not observed in Wolman's disease cells. The E600-treated cells may resemble Wolman's disease cells because they have deficient acid lipase (Research Section III), but if so, they probably have reduced activity of other enzymes as well.

The lipid stored in E600-treated cells was derived from serum, an expected finding for cultured cells (9,66,67,80), and much of it appeared to be cholesteryl ester. Goldstein et al. (9,80) have shown that cholesteryl esters are taken up as a component of LDL at a specific LDL receptor and are degraded in the lysosomes. The free cholesterol generated leaves the lysosome and exerts several regulatory actions, including the suppression of the synthesis of LDL receptor molecules and the resultant reduction of the amount of LDL taken up. In acid lipase deficient cells (Wolman's disease and cholesteryl

ester storage disease), the cleavage of cholesteryl esters is reduced, so no free cholesterol is formed resulting in no inhibition of LDL receptor synthesis. Under these conditions, the cell continues to take up large amounts of LDL-cholesteryl esters. The storage of cholesteryl esters in E600-treated cells may have a similar mechanism.

Treating cells with 10^{-6}M and 10^{-5}M E600 appeared to have little effect on them, but at 10^{-4}M E600 definite lipid accumulation was observed. This concentration of E600 had little toxicity. p-Nitrophenol, at the same concentration, had no apparent effect. Cells treated with 10^{-4}M and 3×10^{-4}M E600 showed little progressive build-up of lipid with time, suggesting that at these concentrations enzyme inhibition and lipid storage were balanced by E600 degradation, the metabolism of stored lipid, and the synthesis of new enzyme. At 10^{-3}M E600 this balance may have been upset, for a more progressive time-dependent accumulation of lipid was observed. Although E600 is somewhat toxic to the cells at this concentration, this is probably not related to the lipid storage. p-Nitrophenol, at the same concentration, is slightly more toxic, but has much less effect on lipid storage. Although p-nitrophenol has been reported to be significantly more toxic than E600 (35,36), if the authors had measured the concentration in molarity rather than parts per million, the results would have been different. There would have been no difference in toxicity to fibroblasts, although p-nitrophenol would still have been more toxic to HeLa cells.

The reversal of lipid accumulation that occurred when E600 was removed from the medium was probably due in part to the synthesis of new enzyme (37) and in part to the slow return of activity to inhibited enzyme (81). The return of activity may be caused by hydrolysis of the phosphate group

transferred to a functional site of the enzyme by E600 during inactivation (81). Two ethyl groups are attached to the transferred phosphate group in E600-induced enzyme inactivation and the return of activity is quite slow, but with dimethyl p-nitrophenyl phosphate, which has 2 methyl groups attached to the phosphate, the rate of hydrolysis is more rapid (81).

Neutral red granules are believed to be lysosomes (82,83). The GM-1606 Wolman's disease culture which was at an early passage level, showed the same number of neutral red granules as the normal cells. However, the GM-2211 culture, which stored more lipid, appeared to have increased neutral red granules in an earlier assay. It may be that the age and condition of the culture, cell density, FCS concentration and variation between FCS lots may be more important in determining the number of neutral red granules in a culture than the presence of a lysosomal enzyme deficiency. However, the fact that E600 treatment consistently resulted in a significant increase in the number of neutral red granules suggests that the presence of a large amount of stored lipid in the lysosomes may stimulate cells to increase the formation of lysosomes. Typically, lysosomal storage diseases show increases by biochemical assay of some lysosomal enzymes other than the deficient one (17,84,85). Increased neutral red granules may be a morphological reflection of this, representing an attempt by the cells to clear the stored material and, if the material interfered with normal degradation, to maintain sufficient operational lysosomes. The granules not stained by neutral red that were observed in the E600-treated cells are structures that are not at acid pH and may represent non-lysosomal lipid storage, lysosomal bodies that have lost their acid pH, perhaps due to the buffering action of storage products, or vacuoles unrelated to lipid storage. The linear arrangement of neutral

red granules apparent in the GM-2121 culture and hinted at in other cultures suggests that lysosomes may be organized by microfilaments, microtubules, or other structural components of the cell.

RESEARCH SECTION III

Introduction

The similarity of the histochemistry of E600-treated cells and Wolman's disease cells shown in Research Section II suggests that E600 (diethyl p-nitrophenyl phosphate) may be inhibiting acid lipase. E600 has been shown to inhibit porcine pancreatic lipase by transferring a diethylphosphoryl group to a serine group at an essential site of the enzyme (33). The micellar concentration of E600 that inhibits pancreatic lipase is similar to the concentration that we have shown to induce lipid storage (86).

The histochemical results from Research Section II provided a preliminary characterization of the lipid stored in E600-treated cells and Wolman's disease cells. Although the lipid stored in tissues of Wolman's disease patients has been analyzed thoroughly (2,46,69,77,78,87-91), analysis of fibroblast cultures has been limited (42).

Biochemical techniques such as thin layer chromatography and gas-liquid chromatography are useful in quantitation and more specific characterization of the stored lipid. This section presents the biochemistry of E600-treated and Wolman's disease cells, including attempts to assay the lipase activity present, the effect of E600 treatment of cells on acid lipase activity, and biochemical characterization of the lipids stored.

Materials and Methods

Acid lipase assay: To determine the lipase activity of normal and Wolman's disease fibroblast cultures, cells grown to confluency

in one 75 cm^2 and one 150 cm^2 flask were pooled and prepared as described in Research Section I. Substrate was prepared as described in 0.2 M acetate buffer, pH 4.0, but an equal amount was prepared in 0.1 M phosphate buffer, pH 7.0. The assay was performed as described in Research Section I, but the same enzyme source was used and 40 µl of specified concentrations of E600 were added to the assay flasks. In some experiments the enzyme was pretreated with the appropriate concentration of E600 for 30 min at room temperature. Protein was not determined because the same enzyme source was used, and thus all assay flasks received equal protein.

To determine the long term effects of E600 on acid lipase activity, 75 cm^2 flasks of normal cells were treated with specific concentrations of E600 and prepared and assayed as described in Research Section I.

Thin Layer Chromatography and Lipid Analysis: Three 150 cm^2 flasks of the following cultures, whose origin is listed in Research Section I, were grown to confluency: GM-498, GM-2520, GM-2075, GM-2121, GM-3557 (M), GM-3558 (F), and JoAl. Six flasks of GM-179, GM-37, and GM-1606 were grown. Only two 150 cm^2 flasks of GM-2211, which grew slowly, could be obtained. Three flasks were also grown of normal mouse and beige mouse fibroblasts, derived from primary abdominal skin cultures of 6 week old female normal black (C57 BL/65, +/+) and beige (C57 BL/65, bg/bg) mice. The beige mouse bears the trait of the Chediak-Higashi syndrome. The cells were grown in medium with 8% FCS supplemented with 100 units of mycostatin per ml. Mycostatin did not appear to affect lipid

storage. Three flasks of GM-179, GM-37, GM-1606 were treated with 10^{-3}M E600 whereas the other three received 0.5% ethanol. Heat-inactivated serum was used for these flasks. After 9 days of treatment, the cells were washed twice in Puck's balanced salt solution (PBS), trypsinized, and each culture was pooled in a 50 ml centrifuge tube which was placed on ice. The cells were counted on a hemocytometer and the cell number in each sample was computed. The samples were centrifuged at 1000-1100 rpm for 10 min on an International Centrifuge, Model CM. The supernatant was drawn off and the pellets were frozen until extraction. The samples were thawed in 5 ml of PBS, sonicated for 15 sec on ice on a Heat Systems-Ultrasonics microtip sonicator at 50 watts and assayed for protein by the method of Bradford (48). A tube containing 5 ml of fetal calf serum (FCS) was prepared along with the other samples after the protein was determined.

Lipids were extracted (92) in 10 ml of 1:1 chloroform-methanol (Fisher) and the lower chloroform layer was recovered following centrifugation. The upper layer was extracted with another 5 ml of chloroform and this second extraction was pooled with the first. The solvent was evaporated with a stream of air and the lipid resuspended in 4.5 ml of chloroform.

The total lipid in each sample was assayed by a technique based on the ability of lipid to reduce dichromate (93) with pooled Paramecium lipid (1.17 µg/ml) as a standard. One hundred or 200 µl of sample was added to an acid-washed tube and solvent was evaporated. To each tube, 2 ml of acid dichromate was added (0.75 g of $K_2Cr_2O_7$ was dissolved with heating in 5 ml of deionized water, cooled and made

up to 500 ml with 98% H_2SO_4). Tubes containing lipids were heated to 100°C in an oven or water bath for 20 min. After cooling, 4.5 ml of deionized water was added to each tube with mixing. A blank was prepared by reducing the dichomate with 0.1 ml of 10% Na_2SO_3 (Baker, anhydrous). The color reaction was read on a Gilford spectrophotometer at a wavelength of 440 nm. The results of 3 assays were averaged to determine the total lipid present in the samples.

Up to 500 µg of lipid was applied to silica gel plates which had been heated at 120°C for 20 min (Supelcosil 12A, Supelco Inc., Bellefonte, Pa.), and the neutral lipid classes were separated by thin layer chromatography (94) in a solvent system of petroleum ether-diethyl ether-acetic acid (80:20:0.5, Fisher). Standards of cholesterol (Sigma), cis-vaccenic acid (Sigma), tripalmitin (Eastman), and cholesteryl palmitate (Sigma) were co-chromatographed to identify lipid classes. After development, the plates were air-dried. Some were sprayed with 50% sulfuric acid (Fisher) and charred in an oven at 100°C for 10 min to visualized the lipid spots. Other plates were examined under long wave ultraviolet light and the spots were circled and scraped into tubes. Separate dichromate assays were conducted for cholesterol, free fatty acid, cholesteryl ester, triglyceride and phospholipid with cholesterol, cis-vaccenic acid and cholesteryl palmitate as standards for the former three and cottonseed oil (Fisher) as a standard for the latter two lipid classes. The relative percentages of each lipid class

were computed for each sample and the already determined values for total lipid were used to calculate the quantity of each lipid class as the quantity of lipid per cell and lipid per mg protein.

Gas-liquid chromatography and phospholipid analysis: Two 150 cm^2 flasks of GM-179 and GM-1606 cells were grown to confluency. One of each was treated with 10^{-4}M E600 for 8 days. The cells were prepared as in the lipid analysis experiment. Briefly, cells were washed twice in PBS, trypsinized, pelleted, and counted. They were sonicated and protein was assayed. A sample of 5 ml of FCS was also assayed for protein.

Lipid was extracted from the 5 samples with chloroform-methanol (1:1) and two subsequent chloroform extractions. The neutral lipid classes were separated on Supelcosil 12A silica gel plates and dried. The plates were sprayed with 2',7'-dichlorofluorescein (Eastman) and observed by long-wave ultraviolet light. The spots were circled and scraped into acid-washed tubes.

Fatty acid methyl esters were prepared from the free fatty acid, triglyceride, and cholesteryl ester fractions. Two ml of 1% methanolic sulfuric acid and 4 drops of petroleum ether were added to each tube. The tubes were vortexed lightly and heated at 60°C for 18 hr. The methyl esters and other lipids were extracted with 2 ml of petroleum ether after the addition of 1 ml of water. The petroleum ether (upper) layer was pipetted into a clean tube. The original tube was extracted with another 2 ml of petroleum ether

and the upper layer was pooled with the first extract. The solvent was evaporated and the samples were dissolved in 50 µl of petroleum ether and spotted on Supelcosil 12A silica gel plates. The plates were developed in petroleum ether-diethyl ether-acetic acid (80:20:0.5) and dried. After spraying the plates with 2',7'-dichlorofluorescein, the methyl ester spots were circled and scraped into clean tubes. The methyl esters were extracted with 4 ml of chloroform-methanol (1:1) and one drop of water. The tubes were vortexed, centrifuged to bring down the silica gel, and the extract was poured into a clean tube. This was repeated twice, pooling the extracts from each sample. The solvent was evaporated under a stream of air. Fatty acid methyl esters were analyzed by gas chromotography on a Hewlett-Packard flame ionization 5711A gas chromatograph fitted with a 50 meter x 0.5 mm glass wall-coated open-tubular column coated with Apolar-5CP (Applied Science Corp.). The conditions were: oven temperature 180°C, injector 250°C, detector 300°C, helium pressure 60 psig. Fatty acid methyl ester species were identified by comparison with the retention times of appropriate fatty acid methyl ester standards. The product of the retention times and peak heights for each sample were used to calculate the relative percentage of each fatty acid in the sample.

The phospholipids separated earlier were extracted from the silica gel with 4 ml of chloroform-methanol (1:1) and one drop of water. The tubes were vortexed and centrifuged to bring down the silica gel. The extract was poured into a clean tube and the procedure was repeated, pooling the two extracts. The samples were

dried, redissolved in 50 µl of chloroform and each sample was spotted on the right-hand corner of a Supelcosil 42A silica gel plate. Two dimensional thin-layer chromatography was performed in solvent systems consisting of chloroform-methanol-water (65:35:4) for the first dimension and, in chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5) for the second dimension. Some plates were sprayed with 50% sulfuric acid and charred to visualize spots. Other plates were developed in iodine (Fisher) to visualize spots. After the iodine had faded, they were sprayed with ninhydrin (0.2% in butanol, Pierce) to detect phospholipids with amine groups. The spots were circled and scraped into clean tubes. An attempt was made to assay the samples for phosphorous (95) using $Na_2HPO_4 \cdot 7H_2O$ (Fisher) as a standard.

Results

A normal fibroblast culture homogenate showed greater lipase activity at pH 4 than at pH 7 (Table 5). The activity at both pH's exhibited little inhibition at 10^{-5}M E600 but significant inhibition at 10^{-3}M E600. However, at 10^{-3}M E600, the optical density at the first point (2 min) dropped from 20 to 6, making the interpretation of the results more difficult. The lipase activity of the untreated Wolman's disease culture homogenate, though less than normal, was greater at pH 4 than at pH 7 and also exhibited little inhibition at 10^{-5}M E600 but significant inhibition at 10^{-3}M E600 at both pH's. Pretreating the enzyme source with E600 for 30 min did not appear to change the inhibition greatly.

TABLE 5

ACID LIPASE ACTIVITY OF NORMAL AND WOLMAN'S DISEASE CELL HOMOGENATES TREATED WITH VARYING CONCENTRATIONS OF E600.[a]

Cell strain	pH	E600 concentration (M):	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
GM-37 (Normal)	4	Activity[b]	2.21	2.12	1.94	1.59	0.01
		%	100	96	88	72	0
	7	Activity	0.97	1.00	0.83	0.66	0.08
		%	100	103	86	68	8
GM-1606 (Wolman's disease)	4	Activity	0.64	0.81	0.63	0.27	0.04
		%	100	127	98	42	6
	7	Activity	0.36	0.48	0.56	0.35	0.06
		%	100	133	156	97	17
GM-37[c] (Normal)	4	Activity	1.44	1.91	1.24	0.80	0.0
		%	100	133	86	56	0

[a]Confluent cell cultures were trypsinized, pelleted, and sonicated on ice with a microtip sonicator. Homogenates were assayed for acid lipase by a fluorometric technique with the appropriate concentration of E600 in the assay mixture.

[b]Nanomoles of 4-methylumbelliferyl oleate hydrolyzed per min per mg protein.

[c]Cells were exposed to the specified concentration of E600 for 30 min at 24°C prior to the assay.

Whole normal cells treated with E600 exhibited reduced acid lipase activity (Table 6). The reduction in acid lipase activity paralleled increases in the concentration of E600 except at the highest concentration, 10^{-3}M. Normal cells treated with 3 x 10^{-4}M E600 exhibited levels of acid lipase activity comparable to those seen in Wolman's disease cells. When cells that had been treated with 10^{-3}M E600 for 24 hr were removed from E600 and grown in normal medium, there was no indication of the return of acid lipase activity.

Separation of the major lipid classes by thin layer chromatography (Fig. 34) revealed a large increase in cholesteryl ester in both Wolman's disease and E600-treated cells. An increase in triglyceride was also seen. The FCS lipid included much cholesteryl ester, but little triglyceride. The proportion of total lipid that was free fatty acid decreased in E600-treated cells. However, because the total lipid per mg protein of the E600-treated cells was much more than that of normal cells (Table 7), the free fatty acid content of E600-treated normal cells actually showed a significant increase ($p<.02$) when the chromatographically separated lipids were quantitated by dichromate assay and expressed per mg protein (Table 8). The E600-treated normal cells also showed significant increases ($p<.01$) in cholesteryl ester, triglyceride, free cholesterol, and phospholipid. The untreated Wolman's disease cells showed increased cholesteryl ester and a slight increase in free fatty acid. The GM-1606 culture, but not the GM-2211 culture, showed increased triglyceride. The E600-treated Wolman's disease cells showed increases comparable to the E600-treated normal cells, except that no further increase in free fatty acid was observed. GM-3557 (M), GM-3558 (F), the parents of the local

TABLE 6

ACID LIPASE ACTIVITY OF HOMOGENATES OF E600-TREATED NORMAL CELLS.[a]

Exposure time	E600 concentration (M):	0	10^{-6}	10^{-5}	10^{-4}	3×10^{-4}	10^{-3}	10^{-3}[b]
24 hr.	Activity[c]	0.71	0.33	0.13	0.07	0.07	0.15	0.05
	%	100	46	18	10	10	21	7
11 days	Activity	4.06	4.21	0.80	0.51	0.19	1.00	0.72
	%	100	104	20	13	5	25	18

[a]Cell cultures were exposed to the appropriate concentration of E600 for the specified period and then trypsinized, pelleted and sonicated on ice with a microtip sonicator. Homogenates were assayed for acid lipase by a fluorometric technique.

[b]Cells were treated for 24 hr and 10 days respectively with 10^{-3}M E600. They were rinsed with PBS and incubated in control medium for 24 hr.

[c]Nanomoles of 4-methylumbelliferyl oleate hydrolyzed per min per mg protein.

TABLE 7

LIPID AND PROTEIN CONCENTRATIONS OF VARIOUS FIBROBLAST CELL CULTURES.[a]

Cell strain	E600 treatment	Cell # ($\times 10^6$)	Protein/Cell (pg)	Total lipid ± S.D. (µg)	Lipid/Cell (pg)	µg Lipid / mg Protein
E600-treated cultures:						
GM-179 (Normal)	-	5.9	659	720 ± 98	121	184
	+	2.3	655	1167 ± 316	519	792
GM-37 (Normal)	-	6.8	822	1081 ± 63	159	193
	+	5.8	506	1813 ± 83	311	615
GM-1606 (Wolman's disease)	-	28.4	205	2422 ± 309	85	416
	+	5.2	203	1168 ± 134	223	1096
Untreated cultures:						
GM-498 (Normal)	-	12.7	364	1153 ± 13	91	250
GM-2211 (Wolman's disease carrier)	-	0.6	1255	189 ± 94	343	273
GM-2121 (Wolman's disease)	-	25.8	289	1994 ± 545	77	268
GM-3557 (Proband's mother)	-	22.3	333	1706 ± 346	76	230
GM-3558 (Proband's father)	-	46.6	248	2812 ± 507	60	243
GM-2520 (Bloom's syndrome)	-	6.2	555	524 ± 118	85	153
JoA1 (Metachromatic leukodystrophy)	-	9.4	709	1481 ± 161	158	222
GM-2075 (Chediak-Higashi syndrome)	-	13.8	396	1120 ± 32	81	205
Black mouse (Normal)	-	42.6	285	2583 ± 630	61	213
Beige mouse (Chediak-Higashi syndrome)	-	37.6	277	2108 ± 255	56	203
Fetal calf serum	-	-	(21 mg/ml)	1336 ± 166	(267 µg/ml)	13

[a]Confluent cell cultures, some treated with 10^{-3}M E600 for 9 days, were trypsinized, counted on a hemocytometer, pelleted, and sonicated on ice with a microtip sonicator. After assaying for protein, the lipid was extracted in chloroform-methanol (1:1) and assayed three times. The calculations were corrected for the lipid removed during the lipid and protein assay procedures.

TABLE 8

MAJOR LIPID CLASSES OF VARIOUS FIBROBLAST CELL CULTURES (µg lipid/mg protein).[a]

Cell strain[b]	E600 treatment	Phospholipid	Free Cholesterol	Free fatty acid	Triglyceride	Cholesteryl ester
E600-treated cultures:						
GM-179	-	125	39	11	10	0
	+	373	57	23	138	200
GM-37	-	124	39	10	13	7
	+	327	51	16	83	138
GM-1606	-	165	47	24	42	139
	+	402	85	23	207	379
Untreated cultures:						
GM-498	-	155	37	15	27	17
GM-2211	-	110	34	26	8	96
GM-2121	-	170	45	17	22	14
GM-3557 (M)	-	143	38	19	19	11
GM-3558 (F)	-	165	45	12	16	6
GM-2520	-	103	35	4	2	9
JoAl	-	143	45	19	10	5
GM-2075	-	145	29	10	15	6
Black mouse	-	138	33	8	7	27
Beige mouse	-	146	41	9	7	0
Fetal calf serum	-	5	1	1	2	5

[a] Lipid classes were separated by thin layer chromatography and assayed by an acid dichromate technique. Values were calculated from relative percentages utilizing data from Table 7.

[b] Diagnosis of cell strains shown in Table 7.

[c] May include some sulfatide storage.

proband, and GM-2121, a known Wolman's disease heterozygote, appeared to have normal amounts of lipid. When the concentrations of each lipid class per cell were calculated (Table 9) the results were similar, but the differences were not as significant, probably due to the added variable of cell size being introduced. The amount of protein per cell did not appear to change significantly with E600 treatment (Table 7). The metachromatic leukodystrophy culture, JoAl, did not appear to be storing large amounts of sulfatide by phase contrast microscopy. Sulfatide probably was not separated from phospholipid by the thin layer chromatographic technique employed, so that any sulfatide stored in the JoAl cells was included in the value shown for the phospholipid class (Tables 8,9), which did not appear to be unusually high.

Both Wolman's disease and E600-treated cells showed fatty acid patterns similar to that of fetal calf serum, unlike the untreated normal cells (Table 10). The normal cells showed more stearic acid (18:0) than oleic acid (18:1ω9) in all three lipid classes analyzed, whereas Wolman's disease cells, E600-treated cells, and fetal calf serum all had more oleate than stearate. This was particularly noticeable in the cholesteryl ester fraction, where all but the normal cells showed more than three times as much oleate as stearate. The cholesteryl esters of normal cells also appeared to contain more short chain fatty acids. E600-treated normal and E600-treated Wolman's disease cells appeared to contain more 16:1ω7 than the untreated cells.

The phospholipid plates showed good separation. There appeared to be increased sphingomyelin and lysophosphatidyl ethanolamine in the E600-treated cells. The results from the phospholipid analysis were poor but appeared to indicate some increases in sphingomyelin, phosphatidyl ethanolamine and, perhaps, phosphatidyl choline in the E600-treated cells.

TABLE 9

MAJOR LIPID CLASSES OF VARIOUS FIBROBLAST CELL CULTURES (pg lipid/cell).[a]

Cell strain[b]	E600 treatment	Phospholipid	Free Cholesterol	Free fatty acid	Triglyceride	Cholesteryl ester
E600-treated cultures:						
GM-179	-	82	25	7	7	0
	+	244	37	15	90	131
GM-37	-	102	32	8	11	6
	+	165	26	8	42	70
GM-1606	-	34	10	5	9	28
	+	82	17	5	42	77
Untreated cultures:						
GM-498	-	56	13	5	9	6
GM-2211	-	138	42	33	10	120
GM-2121	-	49	13	5	6	4
GM-3557 (M)	-	48	13	6	6	4
GM-3558 (F)	-	41	11	3	4	1
GM-2520	-	57	19	2	1	5
JoA1	-	102	32	14	7	4
GM-2075	-	57	12	4	6	2
Black mouse	-	39	10	2	2	8
Beige mouse	-	40	11	2	2	0
Fetal calf serum	-	98 µg/ml	23 µg/ml	13 µg/ml	32 µg/ml	100 µg/ml

[a]Lipid classes were separated by thin layer chromatography and assayed by an acid dichromate technique. Values were calculated from relative percentages utilizing data from Table 7.

[b]Diagnosis of cell strains shown in Table 7.

[c]May include some sulfatide storage.

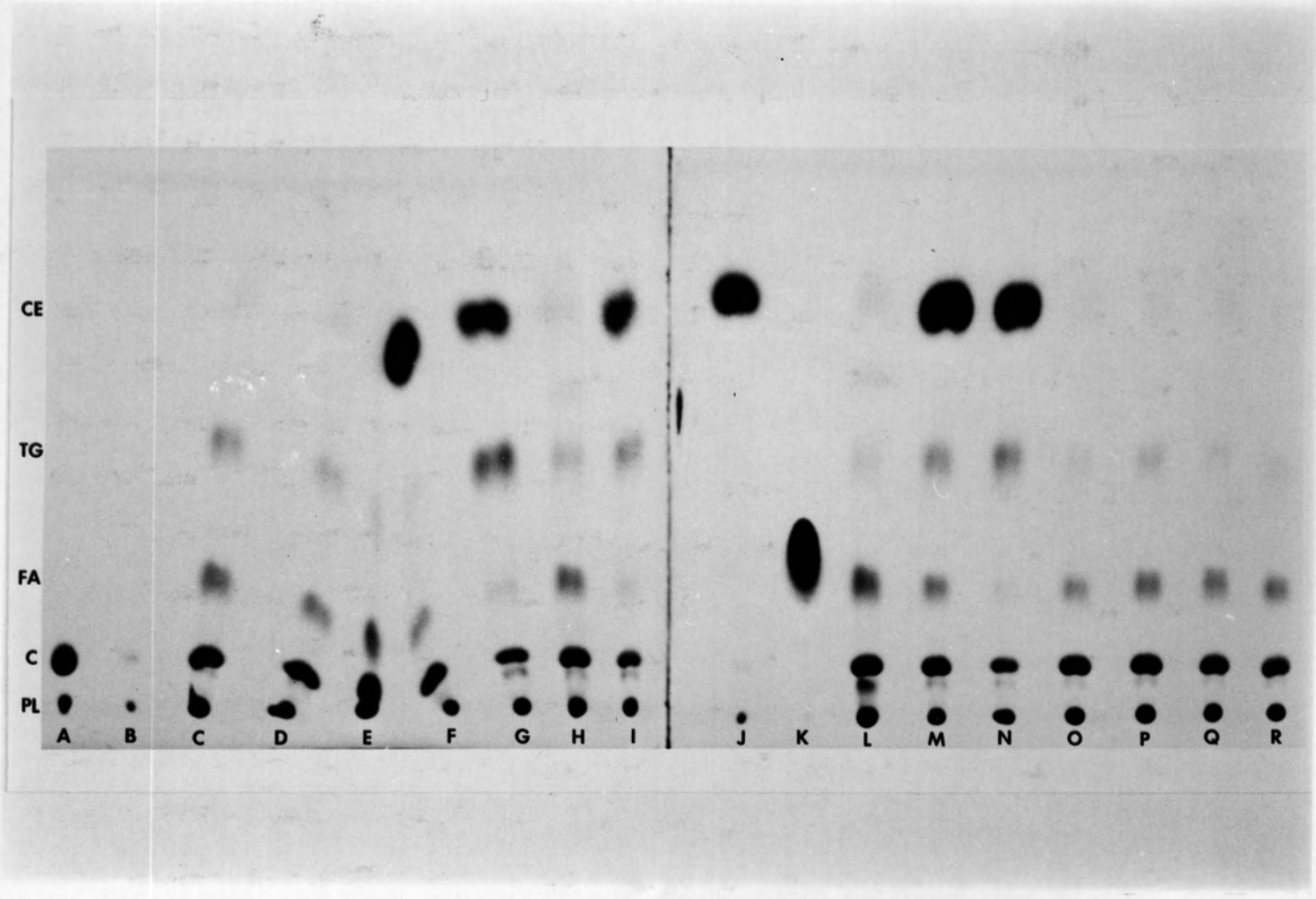
TABLE 10

DISTRIBUTION OF FATTY ACIDS IN DIFFERENT LIPID CLASSES OF NORMAL, WOLMAN'S DISEASE, AND E600-TREATED CELL CULTURES.[a]

Lipid class	Sample	Distribution of fatty acids (%)								
		<16:0	16:0	16:1ω9	16:1ω7	18:0	18:1ω9	18:1ω7	18:2ω6	other
Free fatty acid	GM-179	1.9	22.7	1.8	2.3	29.7	27.2	7.0	4.2	3.3
	GM-179 + E600	3.9	19.9	3.2	6.0	22.5	32.7	11.8	-	-
	GM-1606	2.0	19.4	3.9	2.4	21.4	34.5	11.1	1.9	3.4
	GM-1606 + E600	0.8	18.1	3.5	7.7	16.8	34.7	11.6	-	6.8
	Fetal calf serum	1.6	27.1	2.5	2.1	21.7	28.6	9.8	6.6	-
Triglyceride	GM-179	6.4	30.6	5.9	-	26.7	23.2	-	10.6	-
	GM-179 + E600	1.9	21.6	3.8	5.6	13.5	38.4	15.2	-	-
	GM-1606	-	24.8	-	-	22.9	34.6	17.7	-	-
	GM-1606 + E600	1.1	22.8	3.8	8.6	11.7	37.0	15.2	-	-
	Fetal calf serum	9.6	50.0	-	-	-	-	-	-	40.5
Cholesteryl ester	GM-179	7.2	32.7	4.9	7.6	23.5	19.3	-	-	4.7
	GM-179 + E600	-	22.7	2.8	4.0	9.7	32.9	9.5	13.3	5.2
	GM-1606	1.8	26.6	2.4	5.9	7.1	35.1	8.6	5.7	7.2
	GM-1606 + E600	2.1	27.2	2.7	7.6	7.2	31.1	9.6	5.7	6.7
	Fetal calf serum	2.6	32.6	2.6	5.3	7.2	23.0	9.4	9.4	7.9

[a] Confluent cell cultures were trypsinized, pelleted, and sonicated on ice with a microtip sonicator. Lipid was extracted with chloroform-methanol (1:1) and the major lipid classes were separated by thin layer chromatography. Fatty acid methyl esters were prepared and purified by thin layer chromatography. The retention times and peak heights were recorded on a gas chromatograph and used to calculate the relative percentages.

Figure 34, Separation of major lipid classes of various cell cultures by thin layer chromatography. (A) Free cholesterol control; (B) Triglyceride control; (C) Black mouse, normal; (D) Beige mouse, Chediak-Higashi syndrome; (E) GM-2075, Chediak-Higashi syndrome; (F) Fetal calf serum; (G) GM-179, normal, treated with 10^{-3}M E600; (H) GM-37, normal, untreated; (I) GM-37, normal, treated with 10^{-3}M E600; (J) Cholesteryl ester control; (K) Free fatty acid control; (L) GM-498, normal; (M) GM-1606, Wolman's disease, untreated; (N) GM-1606, Wolman's disease, treated with 10^{-3}M E600; (O) GM-3558, father of proband; (P) GM-3557, mother of proband; (Q) GM-2121, Wolman's disease heterozygote; (R) JoAl, metachromatic leukodystrophy. E600 treatment lasted 9 days. Lipid was extracted from pelleted cells and separated on silica gel plates. The plates were dried, sprayed with sulfuric acid, and heated to visualize the lipids. From 350 to 500 µg of lipid were used for each culture, so plates show relative percentages of each lipid class. Triglyceride control did not char after sulfuric acid spray and heating because it contained only saturated fatty acids. PL-phospholipid; C-cholesterol; FA-fatty acid, TG-triglyceride; CE-cholesteryl ester.


CE
TG
FA
C
PL
A
B
C
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E
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Discussion

Fibroblasts differ from liver cells in having more lipase activity at acid pH than at neutral pH (9,14,96). The observed lipase activity was resistant to 10^{-5}M E600, indicating that it probably represented acid lipase at both pH's (16,17). Acid lipase usually shows a more narrow pH range for activity (9,14). The wide range seen may be due to the buffer system or substrate, or it may reflect the relatively low activity of the material examined and the consequent decreased accuracy of the assay. The conclusion that even the lipase activity at pH 7 was due to acid lipase is supported by the findings of other investigators that nearly all the lipase activity of fibroblasts is due to acid lipase (9,14).

E600-induced inhibition of acid lipase was generally consistent with the histochemical dose and time dependence studies in Research Section II (Figs. 20-22). Although 10^{-6}M E600 had no detectable effect on lipid storage or acid lipase activity, at 10^{-5}M E600 there was an 80% inhibition of acid lipase with little to no effect on lipid storage. Brown et al. (10) showed that as little as 10% of normal acid lipase activity may be sufficient for normal function in fibroblast cultures. An 88% inhibition of acid lipase and definite lipid accumulation were seen in cells treated with 10^{-4}M E600. Treating cells with 3×10^{-4}M E600 caused a 95% inhibition of acid lipase with slightly more lipid accumulation than at 10^{-4}M. Although 10^{-3}M E600 induced a more progressive time-dependent accumulation of lipid, acid lipase activity did

not appear to be maximally inhibited at 10^{-3}M E600. In two assays, acid lipase activity decreased as a function of E600 concentration until 3 x 10^{-4}M, but showed less inhibition at 10^{-3}M than 3 x 10^{-4}M. Cell cultures treated with 10^{-3}M E600 for 3 hr, 1 day, and 9 days each exhibited approximately 75-80% inhibition of acid lipase activity. It is not clear why this occurs. Although E600 did not totally inhibit acid lipase, assays for acid lipase in which esters of 4-methylumbelliferone are utilized, generally show appreciable residual activity even in acid lipase deficient cultures (7).

The analysis of lipid in Wolman's disease and E600-treated cells supported the histochemical findings in showing increased storage of cholesteryl esters in the cells. The results also showed increased triglyceride, which cannot be specifically demonstrated histochemically. Slight increases in free fatty acid and cholesterol in E600-treated cells were observed both histochemically and by lipid analysis. The latter finding was unexpected, because the deficiency or inhibition of an enzyme in a metabolic pathway usually leads to a decreased level of the products of the inhibited reaction. Degradation of the stored lipid by normal or artifactual processes may account for this finding. Free fatty acid has been reported to be increased in histological (89,97) and biochemical (46,97) studies of tissues from Wolman's disease patients. Increased free fatty acid was also seen in biochemical analysis of the liver of the donor of Wolman's disease culture GM-2109 (P) (Dr. H. Jonsson, personal communication). The increase in phospholipid found in E600-treated cells was not observed histochemically,

although there was increased phospholipid staining in a perinuclear region of the cells which, it was thought, might represent the Golgi region. Numerous amphiphilic compounds have been shown to induce phospholipidosis (98-100). However, E600 lacks the positively charged, nitrogen containing, hydrophilic side chain common to these compounds. The analysis of the phospholipid classes gave no definite result, but it appeared that sphingomyelin, phosphatidyl ethanolamine, lysophosphatidyl ethanolamine and phosphatidyl choline might all be elevated. No significant increase in any lipid class was seen in known or suspected Wolman's disease heterozygotes. This supports the histochemical findings shown in Fig. 12. Although cells from one carrier were reported to store lipid (42), most carriers probably do not.

The observed lipid accumulation in cells treated with E600 appears to result, at least in part, from the specific inhibition of acid lipase, explaining the similarities of E600-treated cells to Wolman's disease cells. Although acid lipase is fairly resistant to E600 (13,16,17,101), fibroblasts appear to have little other lipase activity (9,14) so that no cellular effect may be manifested until acid lipase is inhibited. However, the storage of additional triglycerides and cholesteryl esters in Wolman's disease cells treated with 10^{-4}M E600 indicates either the inhibition of residual acid lipase activity or the inhibition of additional enzymes involved in lipid metabolism. Gartner et al. (8) were able to separate, by cellogel electrophoresis,

three isozymes with the ability to cleave 4-MUO under acid conditions, only one of which was deficient in Wolman's disease. It is possible that E600 is also blocking the other isozymes which may have different substrate specificities (8). The increased levels of phospholipids in E600-treated cells suggest that one or more enzymes involved in phospholipid degradation may be inhibited by E600 treatment as well.

The gas chromatography results support the conclusion that the cholesteryl ester stored in Wolman's disease and E600-treated cells is derived from the cholesteryl ester component of low density lipoprotein in the serum and has not been metabolized in the cell. However, normal cells degrade the lipid and, when the cholesterol is transported from the lysosome to the cytosol, it is re-esterified. The normal cells showed a different pattern of fatty acids in the cholesteryl ester fraction, suggesting that more stearic acid (18:0) is available for re-esterifation than oleic acid (18:1ω9) or that the cholesteryl ester synthetase preferentially esterifies the saturated fatty acid. There was little triglyceride present in fetal calf serum. The triglyceride present in the cells was probably derived from the serum free fatty acid (66,67). The E600-treated cells may have preferred oleic acid (18:1ω9) to stearic acid (18:0) in the synthesis of triglyceride. The gas chromatography of cultured Wolman's disease fibroblasts gave results similar to those of other analyzed Wolman's disease tissues (46,77,78,91) except for an increase in stearic acid in the triglyceride fraction.

RESEARCH SECTION IV

The ultrastructural appearance of the storage products of lysosomal storage diseases is characteristic of the disease and in some cases can be used for diagnosis (102). Although fibroblast cultures do show morphology typical for a specific disease (102,103), Ornoy et al. (103) have suggested that amniotic fluid cells are more reliable for diagnostic purposes. In Wolman's disease, the ultrastructure of liver (16,69,104), adrenal (69), lymph node (46), duodenum (104), lymphocytes (101), and for the peripheral and central nervous system (70) have been reported. Numerous lipid droplets and clefts are structures typically observed. Some investigators have reported that lipid can be either membrane-limited or free in the cytoplasm (69,70) whereas others have reported that the lipid is membrane-bound (3,16,101). The lipid has been observed in lysosomes, associated with acid phosphatase activity (3,16,69). In cholesteryl ester storage disease, Partin and Schubert not only demonstrated lipid droplets and cholesteryl ester clefts in a detailed study of small intestinal biopsies (79), but they also demonstrated the presence of membrane-bound crystals in fibroblasts cultured from the patient (105). Drug-induced lipidoses resemble, ultrastructurally, inherited lipid storage diseases (98,100) particularly those involving a similar storage product.

This section presents ultrastructural and cytochemical studies on Wolman's disease and E600-treated fibroblasts and

attempts to correlate the information with the histochemical and biochemical data.

Materials and Methods

Normal (GM-179) and Wolman's disease (GM-1606) cells were seeded in 35 x 10 mm culture dishes at a concentration of 5×10^4 cells per dish. Half the normal cells were treated 11 days with 10^{-4}M E600 (diethyl p-nitrophenyl phosphate) and embedded *in situ* by the method of Douglas *et al.* (106). The cells were rinsed 3 times in Puck's balanced salt solution (PBS) (0.9% NaCl for acid phosphatase cells) and fixed 30 min in 2.5% glutaraldehyde (EMS, Fort Washington, Pa.) in 0.1 M sodium cacodylate (Fisher) buffer. The cells were rinsed in 0.2 M cacodylate buffer and post-fixed 30 min in 1% osmium tetroxide (EMS) in 0.1 M cacodylate buffer. After dehydration in graded alcohols, the cells were infiltrated in Epon-ethanol (1:1) and then 4 changes of Epon (EMS). The next morning, after 2 additional changes of Epon, the culture dishes were polymerized at 55°C for 12 hr. The polymerized cells were removed from the culture dish with pliers and cured for 30 hr at 60°C. The dishes were stained with toluidine blue and suitable areas were marked. These sections were cut out with a coping saw and placed in a Beem Capsule in which they were flat-embedded with Epon. Thin sections were examined on an AEI (EM-68) or Hitachi (HS-8F-1) electron microscope. The GM-1606 monolayers partially detached during glutaralydehyde fixation, so less cells were available for examination.

Morphology: After thin sections were cut and placed on grids, the sections were stained in saturated uranyl acetate (Fisher) in 50% ethanol, rinsed in distilled water and stained in lead citrate (from lead nitrate, Fisher, and sodium citrate, EMS).

Colloidal Gold: Approximately 55 ml of colloidal gold (Harleco) was boiled down to 6 ml with stirring. This was diluted in medium (1:2) and used to treat the cells for either 2 or 24 hr before fixation.

Acid Phosphatase: After glutaraldehyde fixation, staining solution was prepared by dissolving 63 mg of β-glycerophosphate (Sigma) in 5 ml of 0.2 M Tris maleate buffer, pH 5.0 (Sigma) and 10 ml of distilled water. The pH was readjusted to 5.0 with hydrochloric acid. To this solution was added 10 ml of 0.2% lead nitrate, drop by drop with stirring. Control solution was prepared similarly, but lacked β-glycerophosphate. The cells were treated for 1 hr at 37°C.

Peroxidase: After glutaraldehyde fixation, staining solution was prepared by dissolving 3 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 10 ml of 0.05 M Tris buffer pH 7.2-7.6 (Fisher) and approximately 0.02 ml of 3% hydrogen peroxide (Parke-Davis). Control solution was prepared without DAB. The cells were treated for 1 hr at room temperature.

Scanning Electron Microscopy: Normal cells (GM-37) and Wolman's disease cells (GM-1606) were seeded on coverslips in culture dishes as described in Research Section I. One dish of each was treated 8 days with 10^{-4} M E600. The cells were fixed

30 min in 2.5% glutaraldehyde in 0.2 M cacodylate buffer, rinsed in buffer, post-fixed one hr in 1% osmium tetroxide in 0.1 M cacodylate buffer, and dehydrated in graded alcohols. Critical point drying was performed in a Technics CPA-II critical point drying apparatus and, after attaching pieces of coverslip with cells to specimen mounts with silver paint (Dotite Electroconductives), the samples were coated with gold for 2.5 min on a Hummer II vacuum evaporator (Technics). Cells were examined on a Jeol JSM-25 scanning electron microscope and Polaroid pictures were taken.

Results

Transmission electron microscopy (TEM): Normal fibroblast cells fixed *in situ* and examined by TEM revealed features typical of an active cell (Figs. 35,36). Abundant rough endoplasmic reticulum and long thin mitochondria were present. Round dense bodies probably representing lysosomes were seen scattered throughout the cytoplasm and osmiophilic cytoplasmic lipid droplets were occasionally encountered. Large clear vacuoles were also observed. Thin filaments which were probably microtubules and microfilaments, were numerous. The nucleus usually appeared rounded and two or more nucleoli were visible. The cells appeared to have minimal acid phosphatase or peroxidase activity. After 2 hr little colloidal gold had entered the cells, although some was visible at the cell surface. After 24 hr however, more colloidal gold had entered the cells and could generally be seen in dense bodies, large clear vacuoles, or occasionally

in small endocytic vacuoles of intermediate density (Figs. 37,38).

Wolman's disease fibroblasts showed morphology similar to normal cells though some dilation of rough endoplasmic reticulum was observed. Crystalloid clefts probably representing cholesteryl ester were visible within dense bodies that were often deformed by the shape of the crystals (Figs. 39-41). Large clear vacuoles were numerous and appeared more angular than in normal cells. Again no significant reaction was seen for acid phosphatase or peroxidase and little colloidal gold had entered the cell after 2 hr. After 24 hr the colloidal gold appeared to have had some negative effects upon the cells. The mitochondria were swollen and had areas of clearing. Colloidal gold was usually seen in dense bodies, some of which contained lipid clefts, or in the large clear vacuoles (Figs. 42,43).

Normal cells treated with 10^{-4}M E600 exhibited numerous lipid clefts. They generally appeared to be present in dense bodies which, as in the Wolman's disease cells, often were deformed by the shape of the crystalloid material (Figs. 44-46). The cells showed increased numbers of dense bodies (Fig. 44). Large clear vacuoles were numerous. An increase was also observed in multilamellar structures (Fig. 45), similar to the lamellar bodies typically seen in drug-induced phospholipidosis (98). Cells treated with colloidal gold for 24 hr exhibited colloidal gold particles in endocytic vesicles and dense bodies, some of which contained lipid clefts (Figs. 47,48). Colloidal gold particles were also seen in the large clear vacuoles. Aside from an increase in the number of dense bodies, the E600-treated cells resembled Wolman's disease cells. Osmiophilic cytoplasmic lipid droplets

Figure 35, Ultrastructure of a GM-179, normal fibroblast fixed *in situ*. Abundant rough endoplasmic reticulum is visible. Mitochondria are long and thin. The few dense bodies are clustered near the nucleus or scattered throughout the cytoplasm. N-nucleus; Nu-nucleolus; DB-dense body; ER-rough endoplasmic reticulum; M-mitochondrion. Stained with uranyl acetate and lead citrate. X5500.

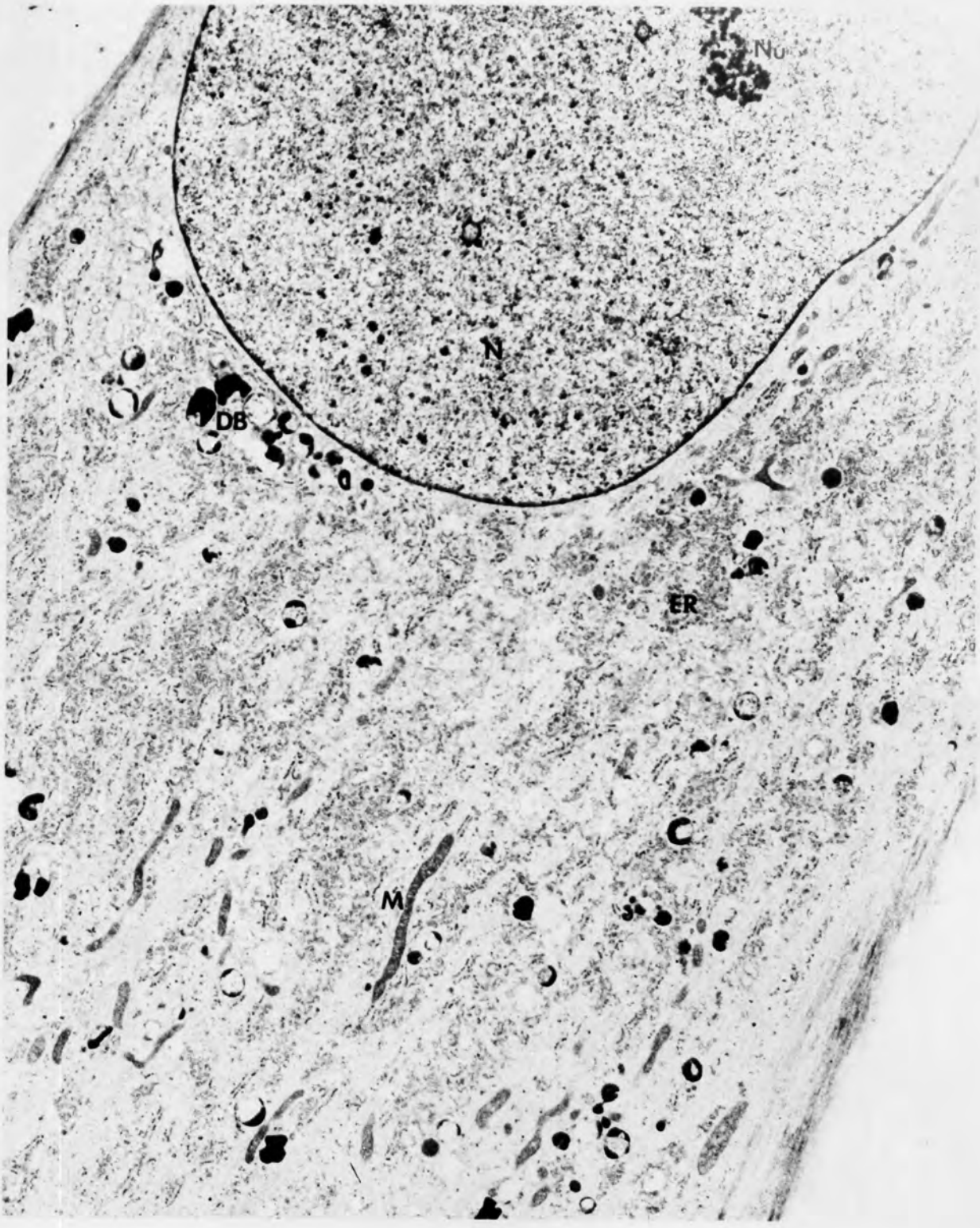

Nu
N
DB
ER
C
M

Figure 36. A GM-179, normal fibroblast viewed at a higher power. Microtubules and numerous microfilaments are present in the cytoplasm. Dense bodies and less dense vacuoles are also present. N-nucleus; DB-dense body; ER-rough endoplasmic reticulum; M-mitochondrion; Mfb-microfilament bundle. Stained with uranyl acetate and lead citrate. X15,800.

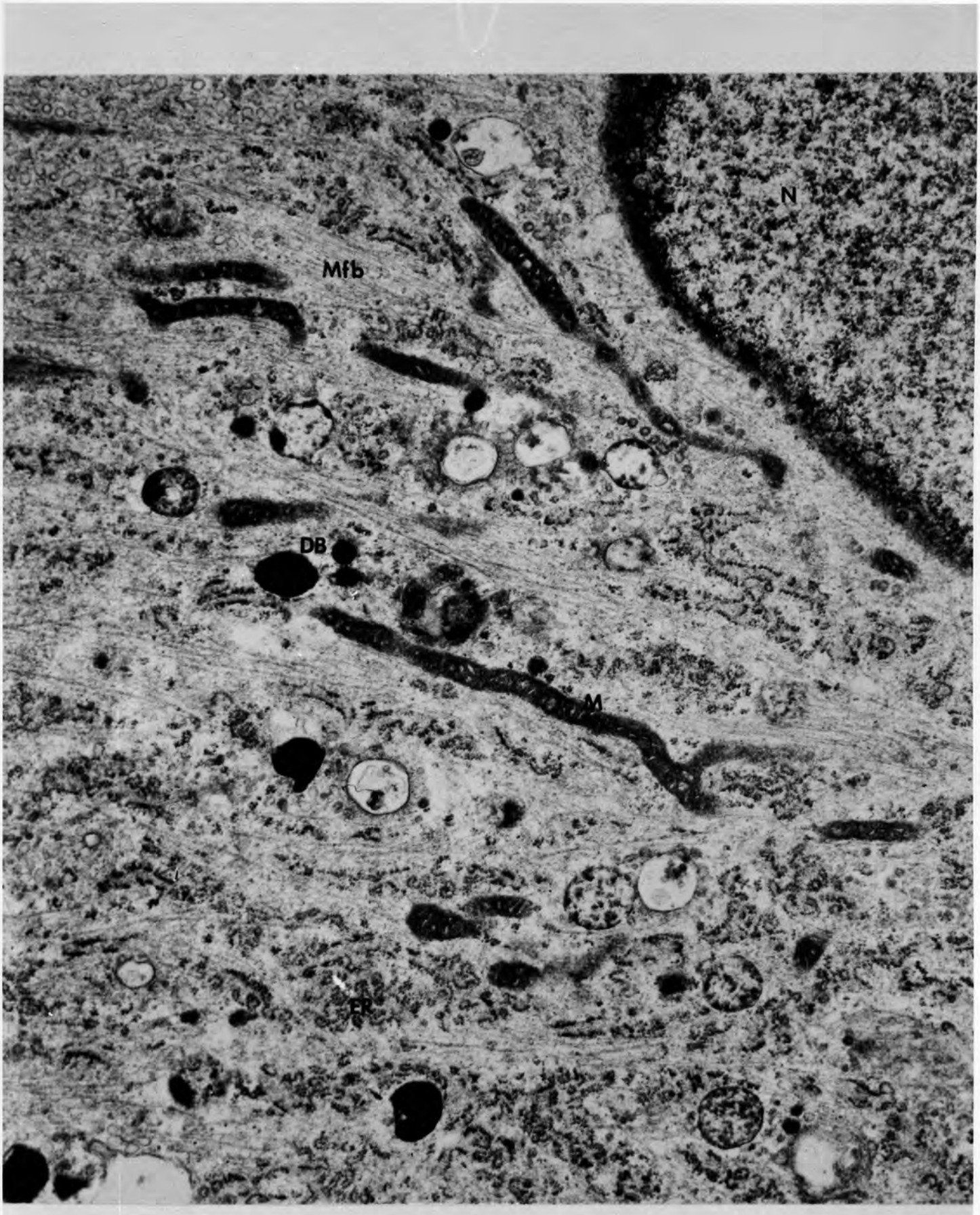

N
Mfb
DB
M
ER

Figure 37. Colloidal gold particles within dense bodies of a GM-179, normal fibroblast. One colloidal gold particle appears to be within a small endocytic vesicle (arrow). L-probable cytoplasmic lipid droplet. Treated with colloidal gold for 24 hr. X17,000.

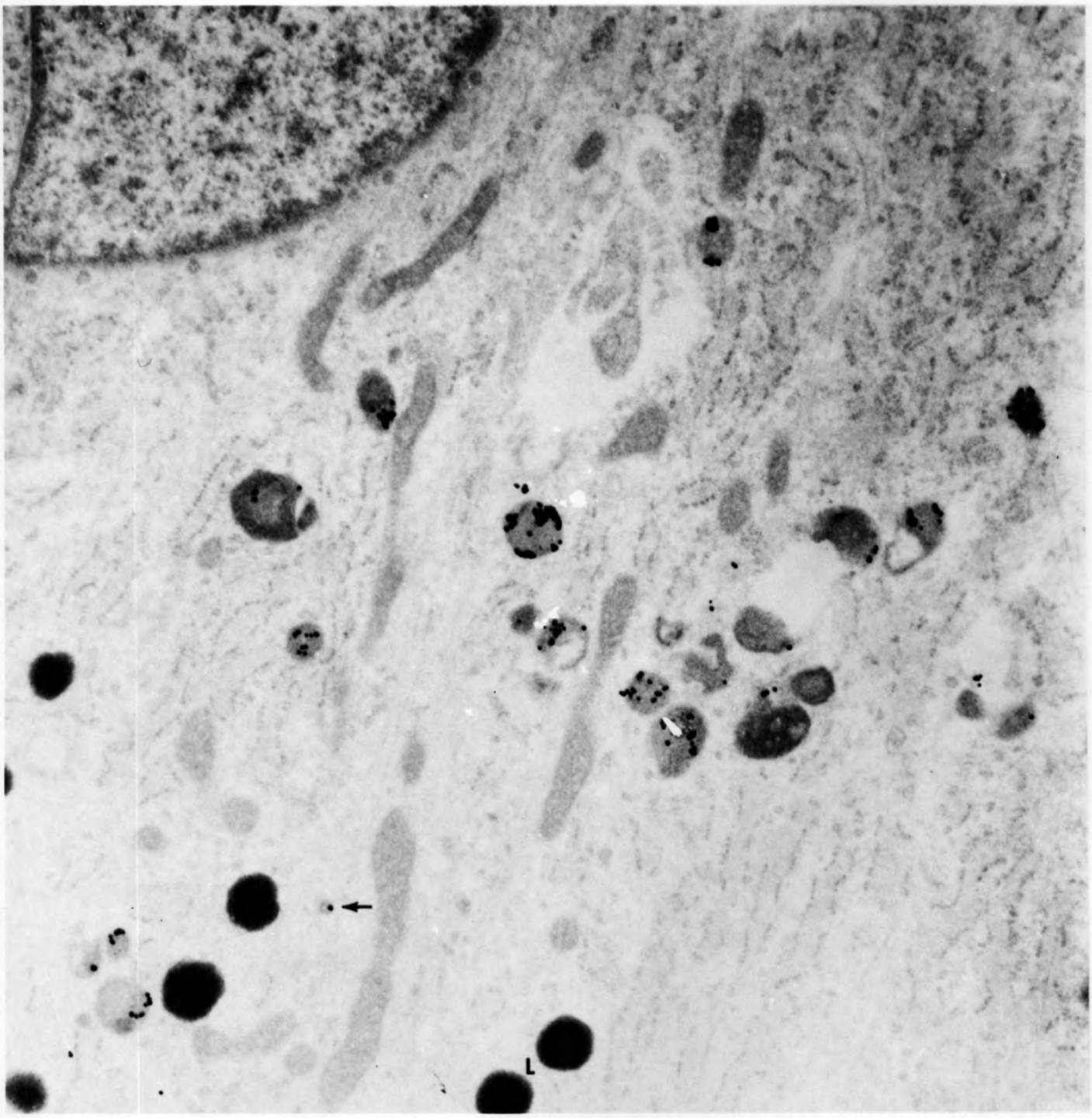


Figure 38. Colloidal gold particles in a GM-179, normal fibroblast accumulate in dense bodies and large clear vacuoles (arrows). L-probable cytoplasmic lipid droplet. Treated with colloidal gold for 24 hr. X16,800.

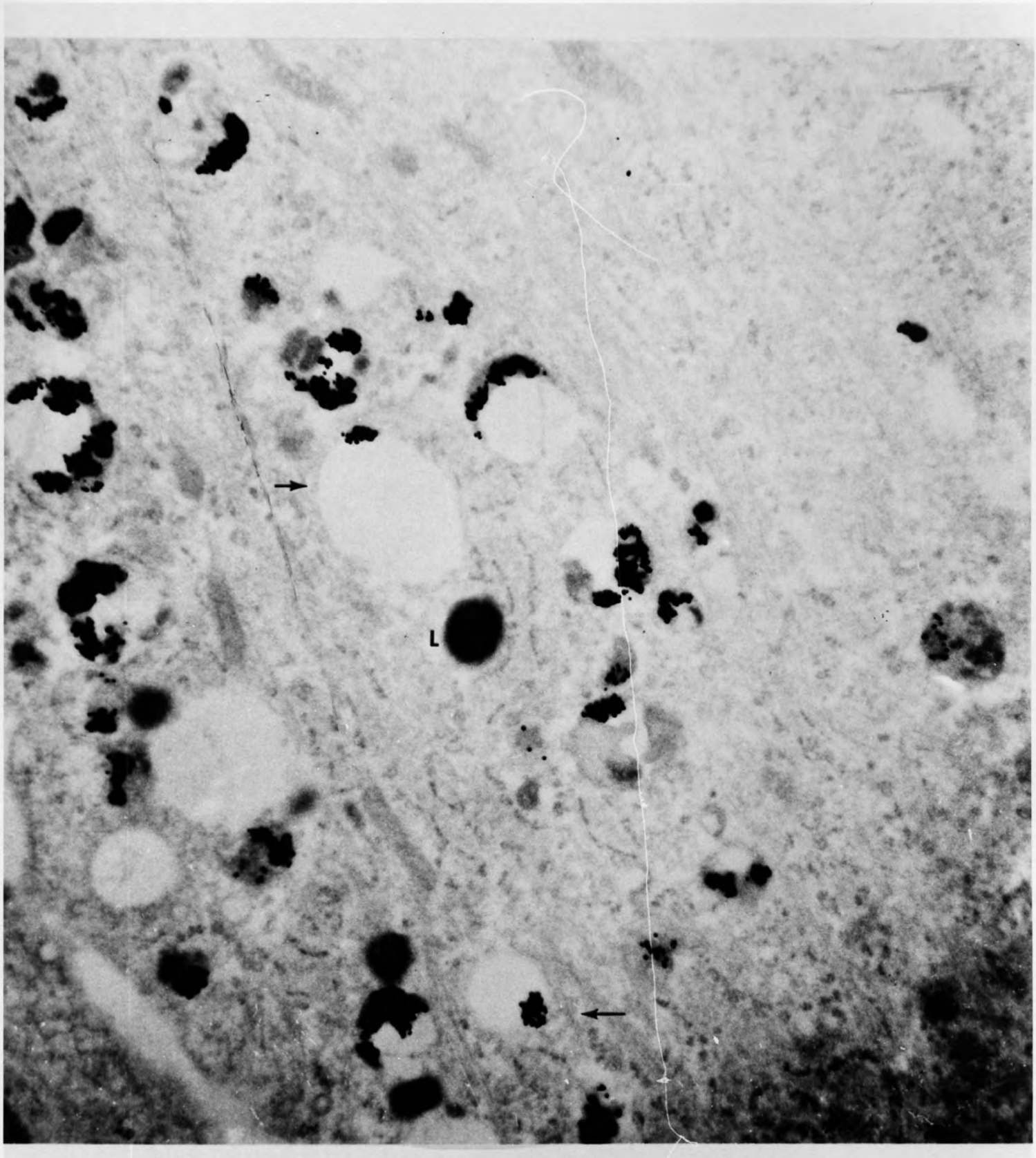

L

Figure 39. Ultrastructure of a GM-1606, Wolman's disease fibroblast fixed *in situ*. The cytoplasm contains many long crystalloid clefts which probably represent cholesteryl ester dissolved during processing. The crystalloid was often present in dense structures resembling lysosomes. Few normal dense bodies are seen. Large clear vacuoles are present, but appear more angular than in normal cells (arrows). N-nucleus; ER-rough endoplasmic reticulum; M-mitochondrion. Stained with uranyl acetate and lead citrate. X5600.

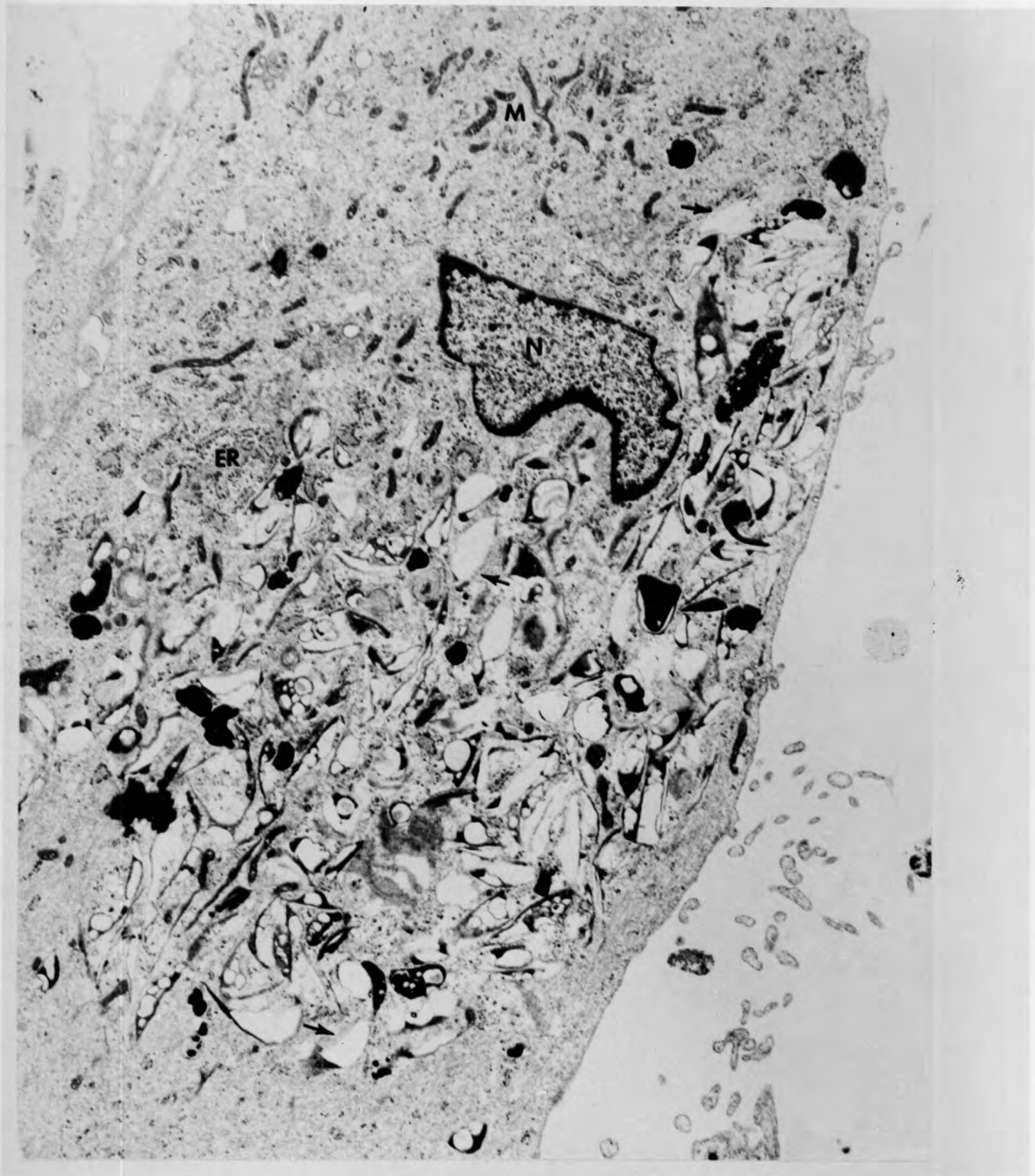

M
N
ER

Figure 40. A GM-1606, Wolman's disease fibroblast viewed at a higher power. Numerous clefts are visible within dense bodies, which are often deformed by the crystalloid (small arrows). Membrane is visible surrounding some crystalloid-containing structures (large arrows). The rough endoplasmic reticulum is dilated. N-nucleus; Nu-nucleolus; ER-rough endoplasmic reticulum. Stained with uranyl acetate and lead citrate. X15,500.

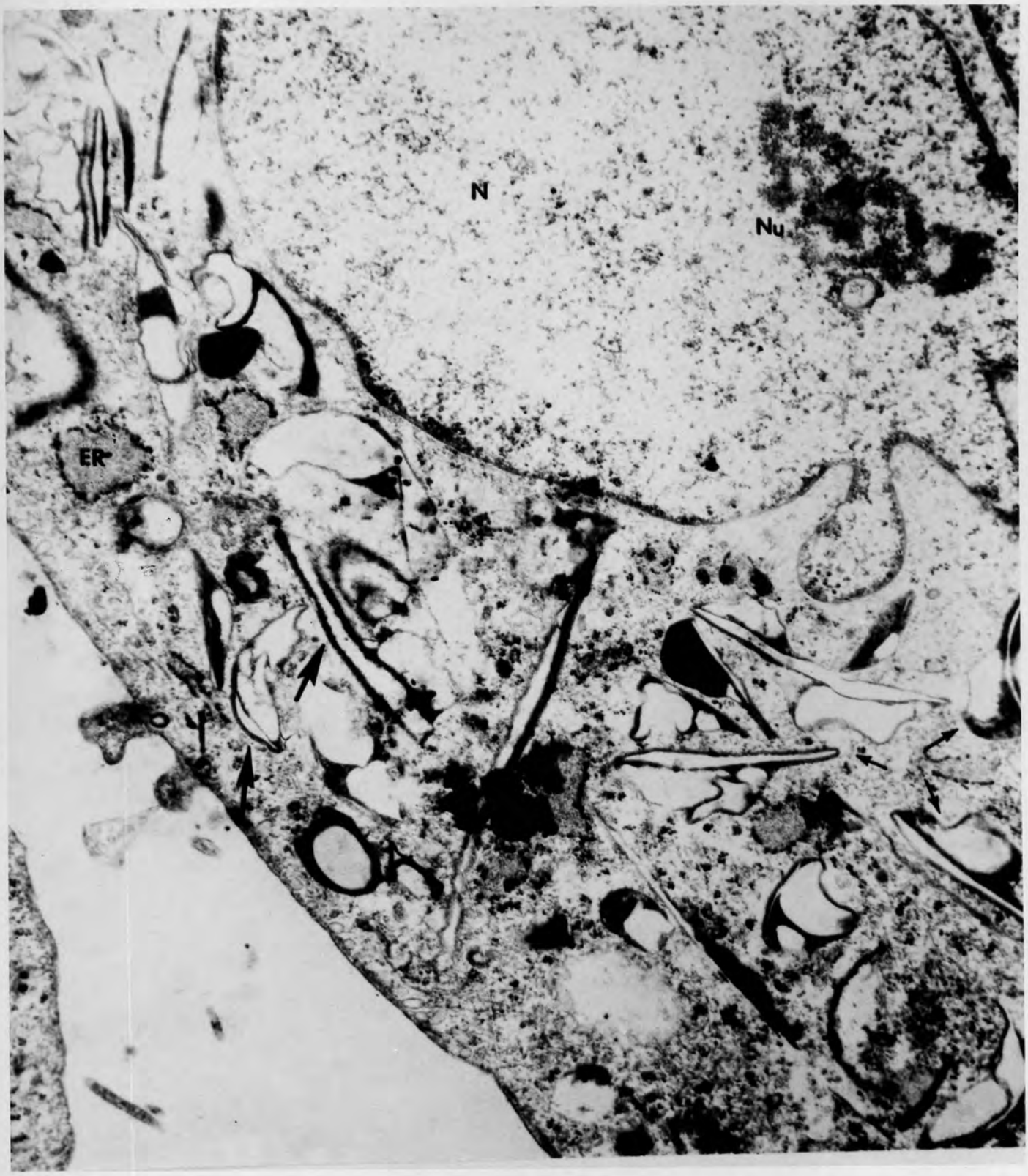

N
Nu
ER

Figure 41. Unusual structures in a GM-1606, Wolman's disease fibroblast. In addition to the more typical crystalloid-containing dense bodies (small arrow), some cells contained large multivesicular bodies. Some of the multivesicular bodies also appeared to contain crystalloid (large arrows). Stained with uranyl acetate and lead citrate. X32,300.

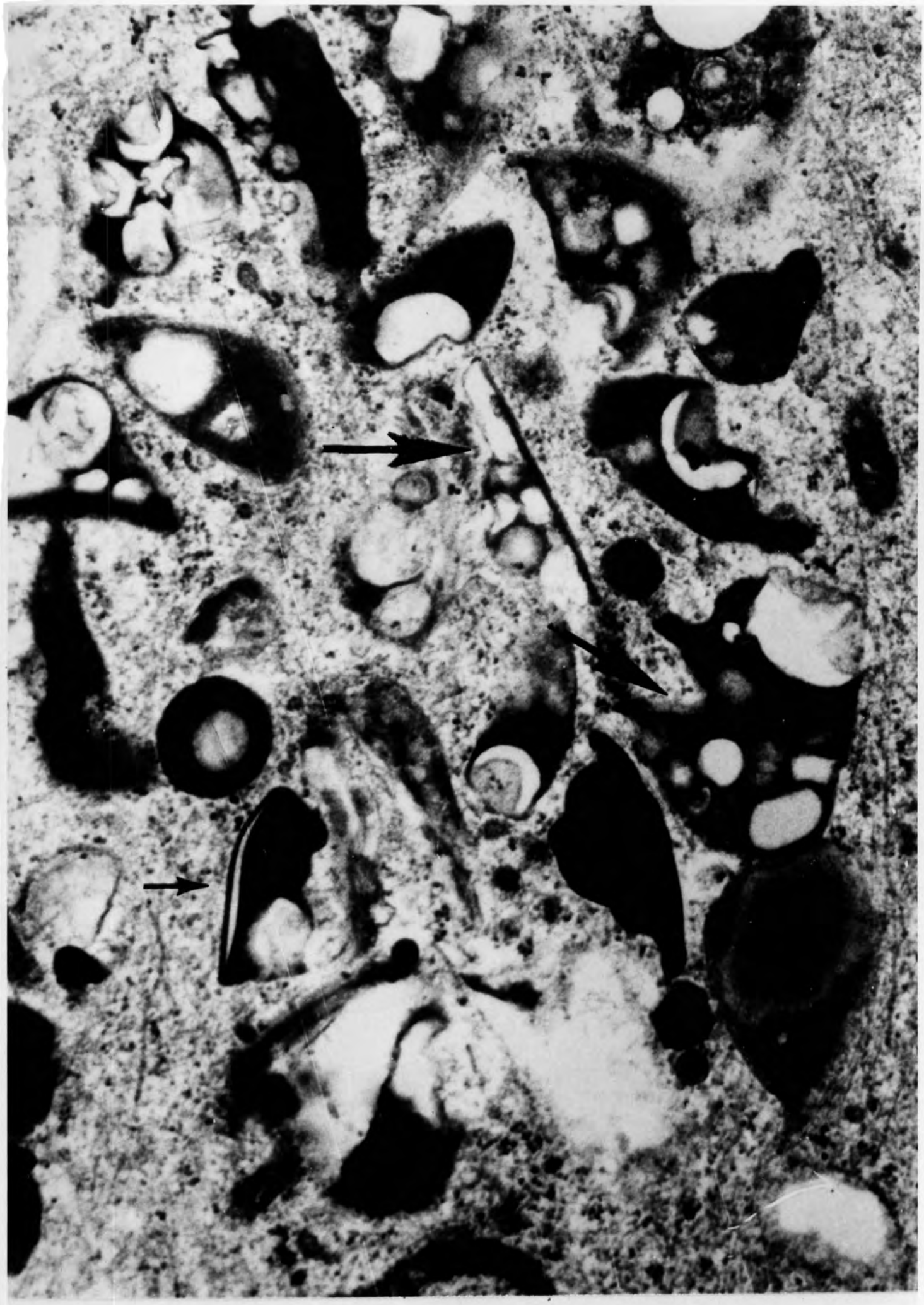


Figure 42. Colloidal gold particles in GM-1606, Wolman's disease fibroblasts showing localization to dense bodies and large clear vacuoles (large arrows). Some crystalloid-containing structures also accumulate colloidal gold particles (small arrows). Cytoplasmic lipid droplets did not appear to be associated with colloidal gold particles. Treated with colloidal gold for 24 hr. X15,000. Inset X32,000.

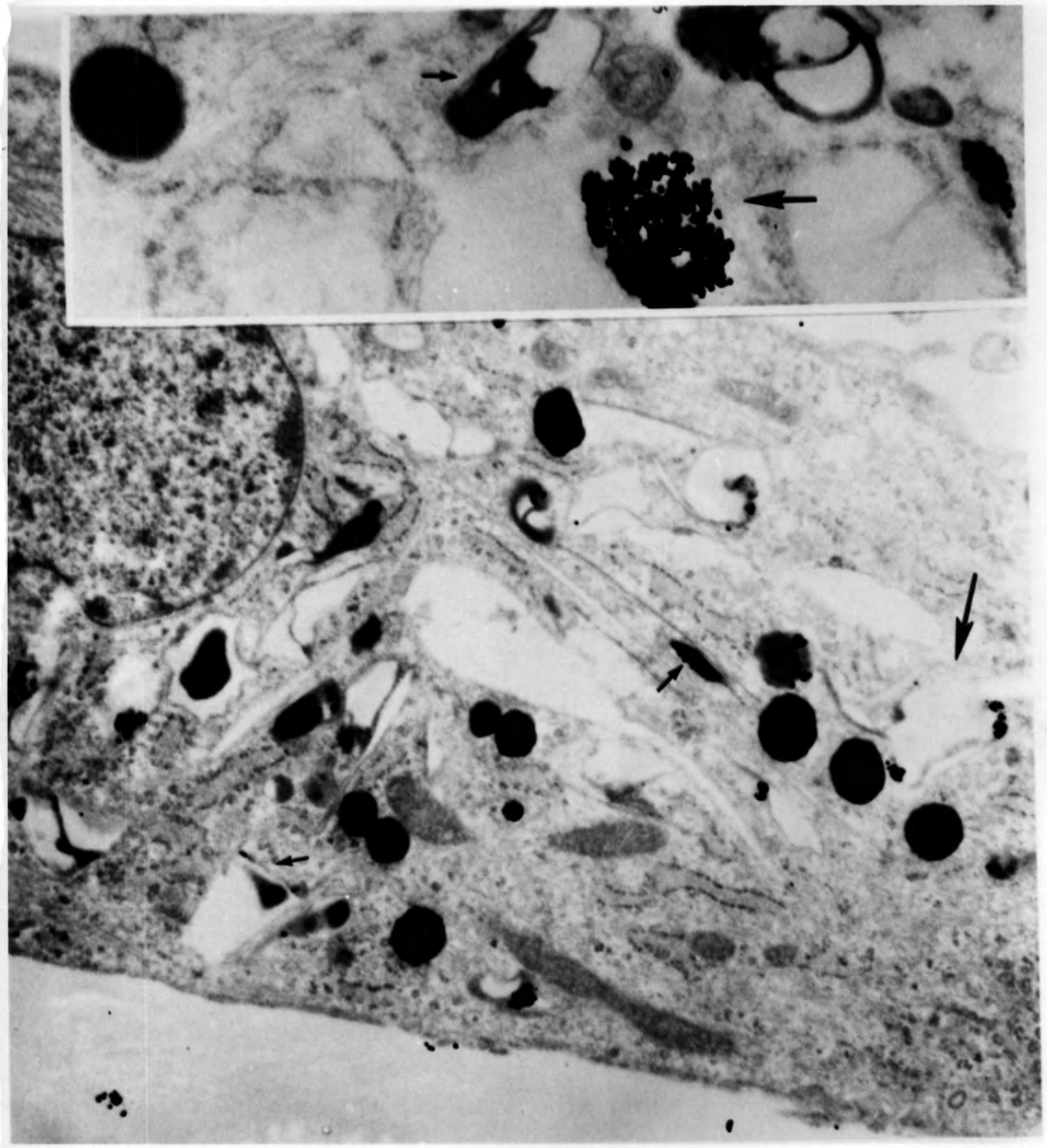


Figure 43. A GM-1606, Wolman's disease fibroblast exhibiting colloidal gold accumulation within dense bodies and large clear vacuoles (arrows) which appear angular and may contain crystalloid material. Colloidal gold particles are also seen at the cell surface, but are not associated with cytoplasmic lipid droplets. Treated with colloidal gold for 24 hr. X14,700.

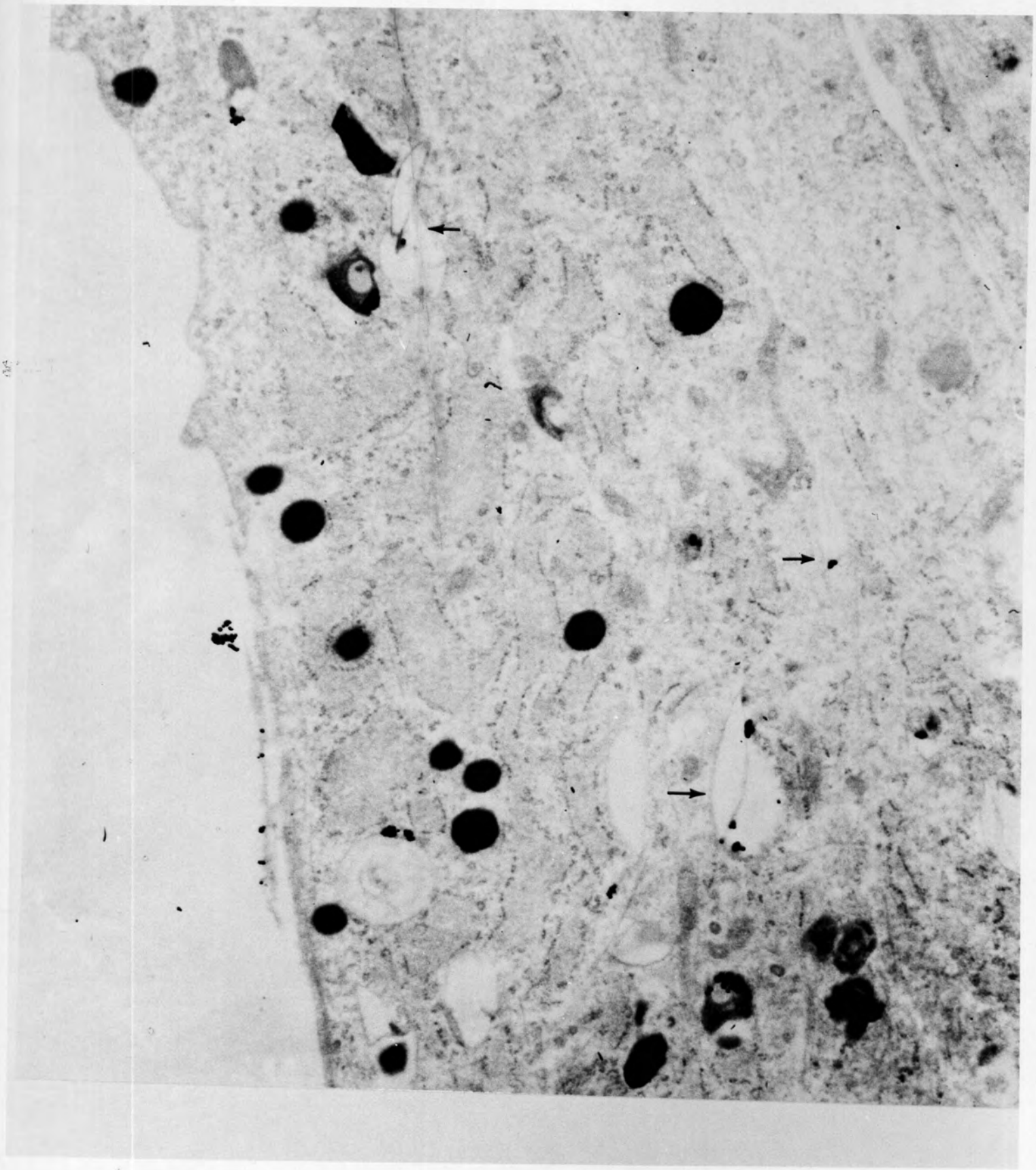


Figure 44. Ultrastructure of a GM-179, normal fibroblast treated with 10^{-4}M E600 for 11 days and fixed *in situ*. The cytoplasm is nearly filled with large clear vacuoles and dense bodies which often contain semilunar or angular electron lucent areas that probably represent cholesteryl ester dissolved during processing. A small perinuclear zone lacking dense bodies is visible (arrow) which contains mitochondria, rough endoplasmic reticulum, and microfilament bundles. N-nucleus; M-mitochondrion. Stained with uranyl acetate and lead citrate. X5400.

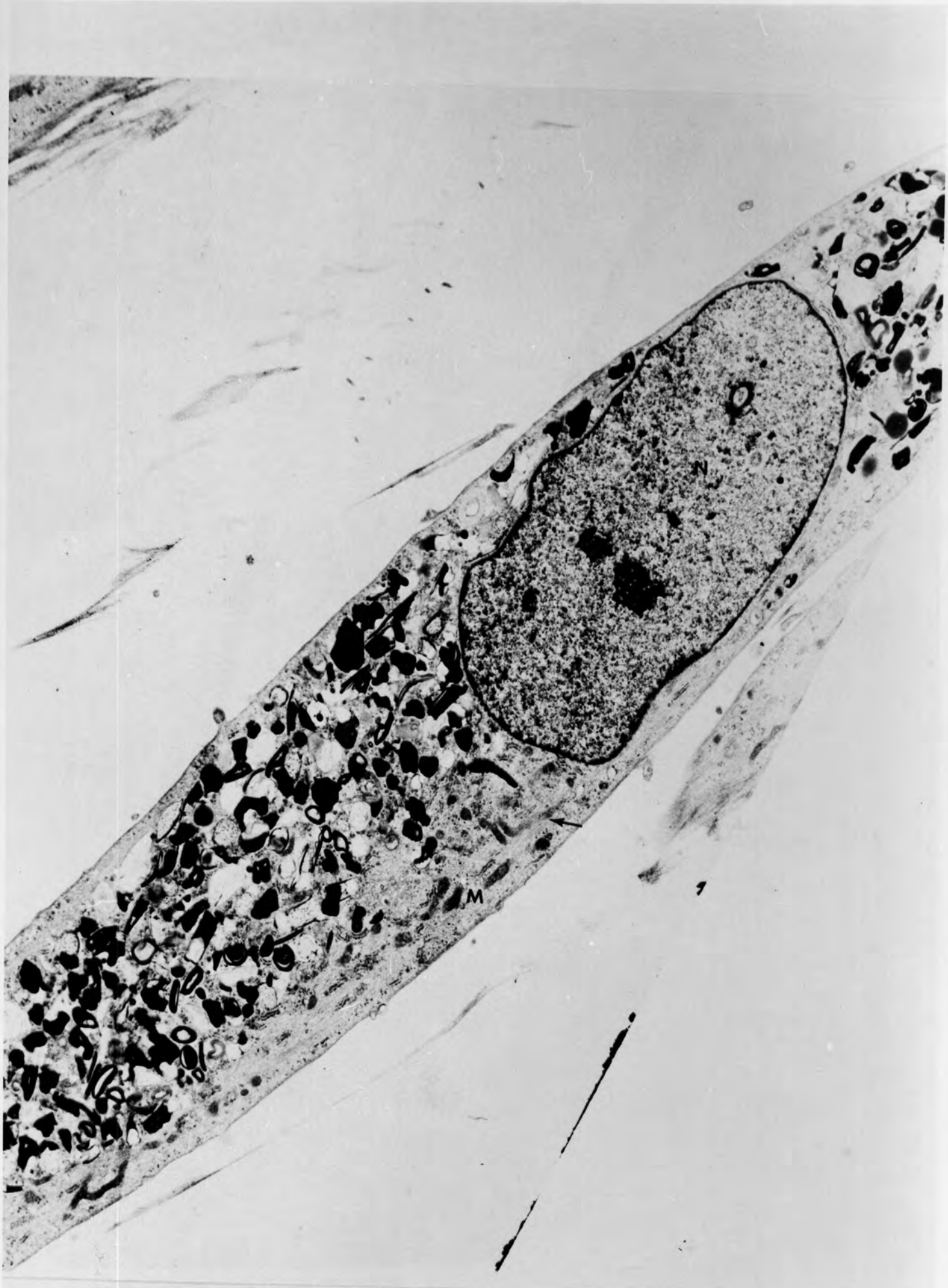

N
M

Figure 45. An E600-treated, normal (GM-179) fibroblast viewed at a higher power. Numerous dense bodies contain angular and semilunar clefts, some of which deform the shape of the dense bodies (small arrows). Some bodies appear to contain concentrically arranged membranous materials (large arrows). N-nucleus; ER-rough endoplasmic reticulum; M-mitochondrion; V-large clear vacuole; Mfb-microfilament bundle. Stained with uranyl acetate and lead citrate. X16,500.

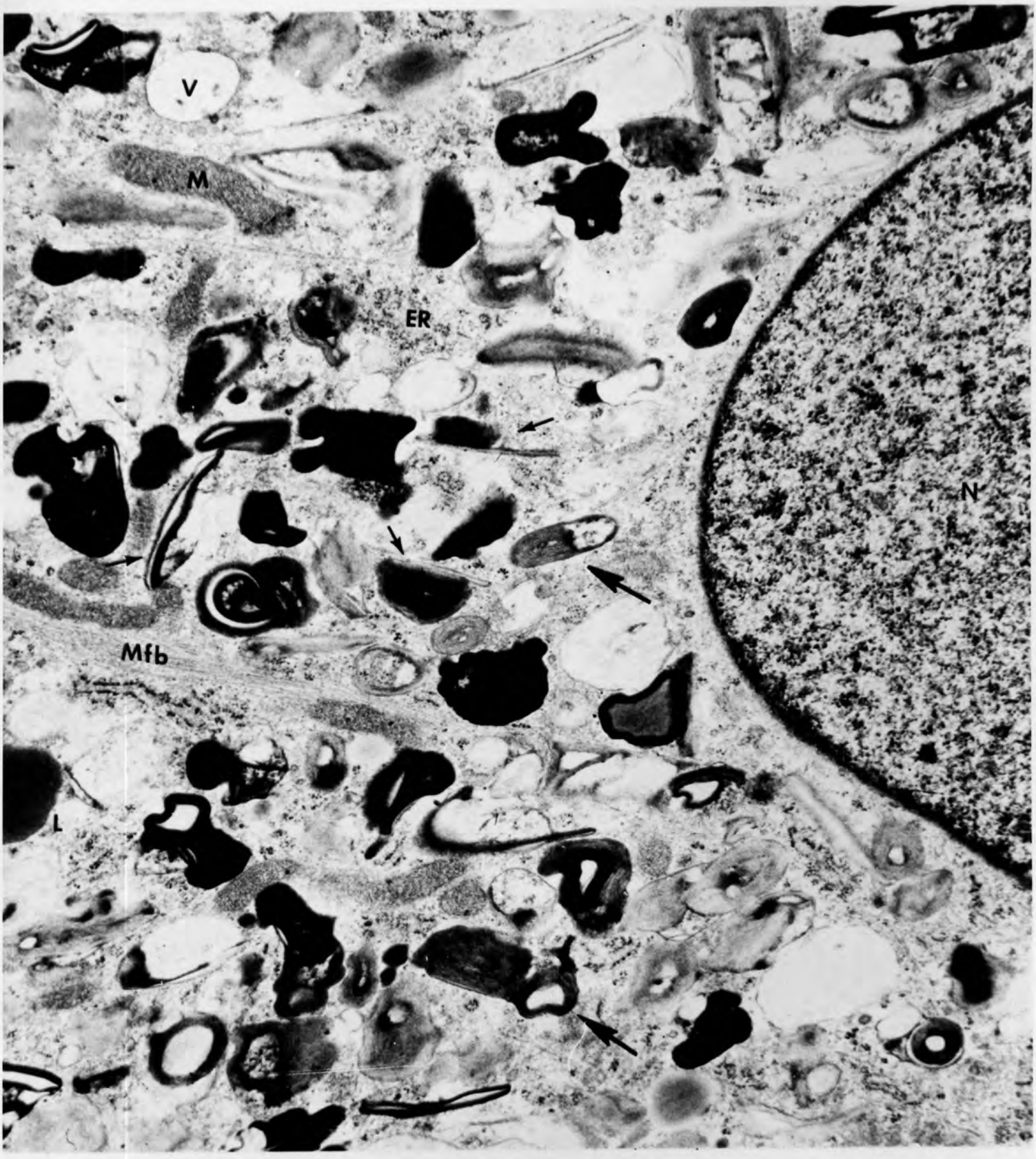
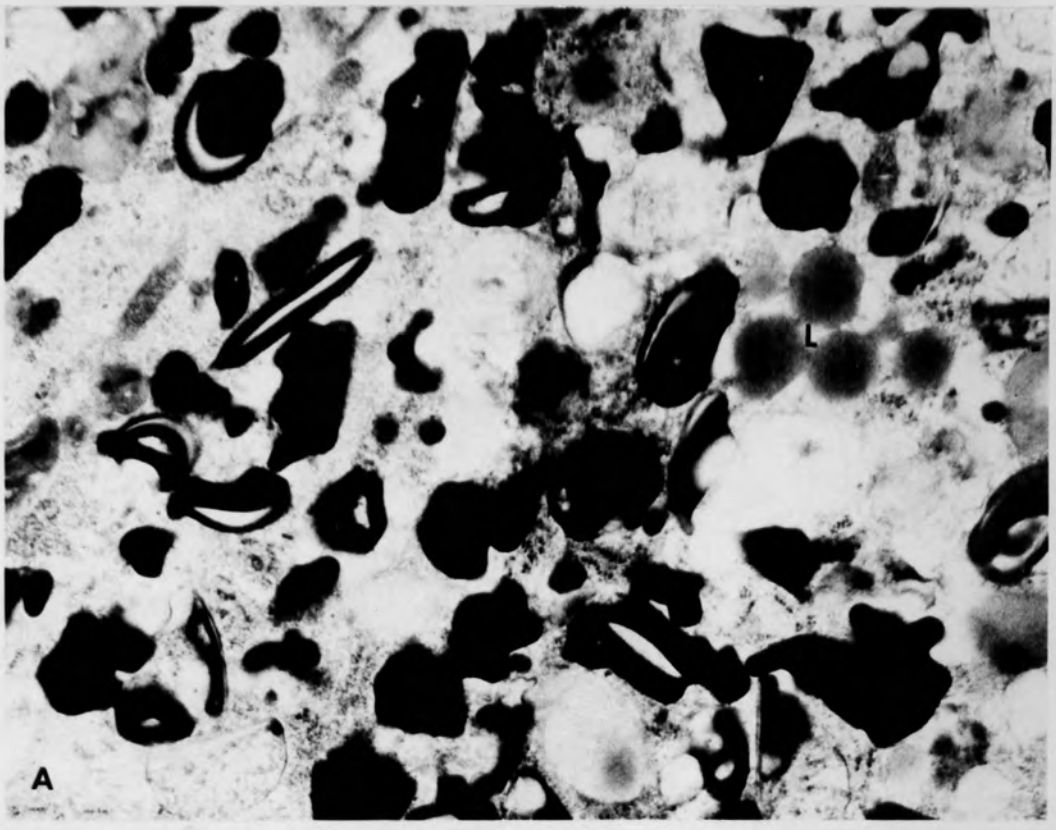

V
M
ER
N
Mfb
L

Figure 46. A. Dense bodies, clear vacuoles, and cytoplasmic lipid droplets filling the cytoplasm of an E600-treated, GM-179, normal fibroblast. Angular and semilunar clefts are visible within dense bodies. L-probable cytoplasmic lipid droplet. X17,500. B. Dense body appears to be fusing with a lipid cleft which is already within a dense matrix. X15,500.


L
A

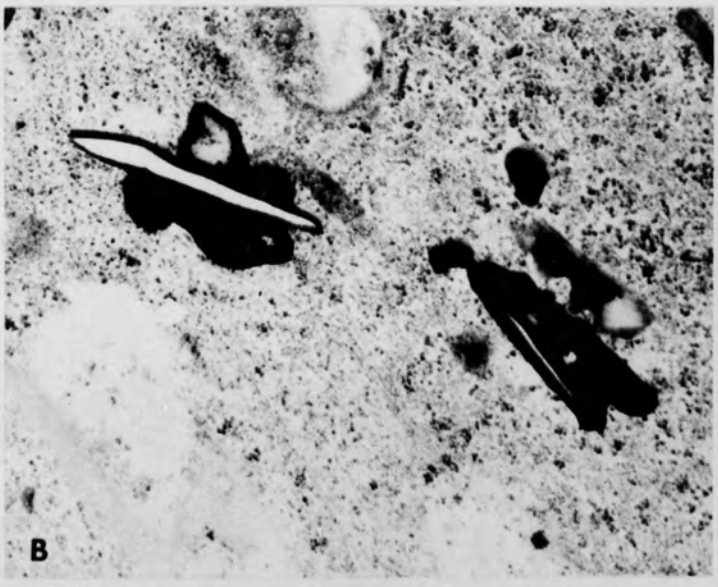

B

Figure 47. Colloidal gold particles in dense bodies and large clear vacuoles (large arrows) of an E600-treated, GM-179, normal fibroblast. Some crystalloid-containing structures accumulate colloidal gold particles (small arrows). Treated with colloidal gold for 24 hr. X16,600.

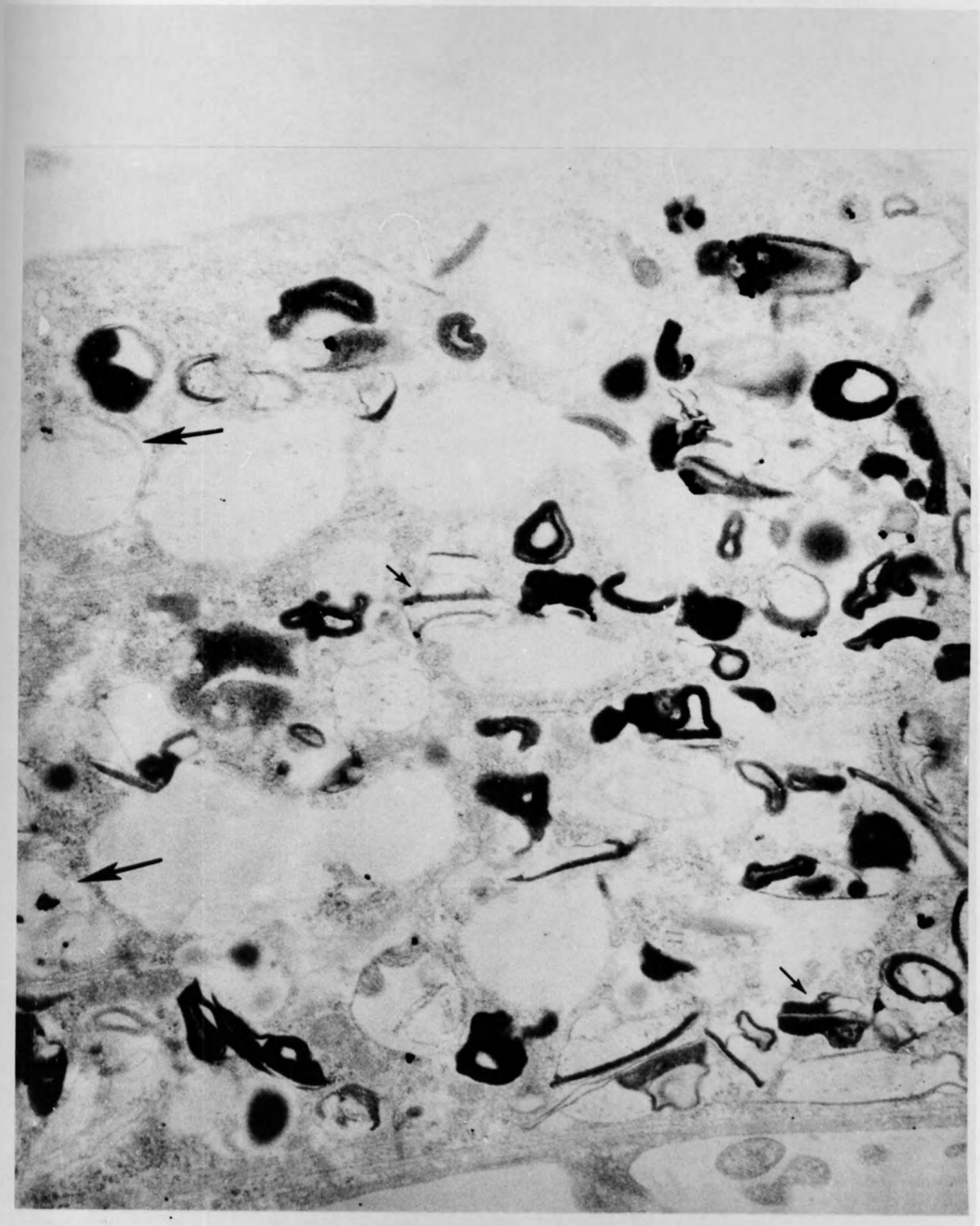


Figure 48. Accumulation of colloidal gold particles in dense bodies of an E600-treated, GM-179, normal fibroblast. Colloidal gold is sometimes associated with crystalloid-containing structures. One colloidal gold particle is associated with a cytoplasmic lipid droplet (large arrow), but this droplet may be fusing with a secondary lysosome next to it which contains colloidal gold. Treated with colloidal gold for 24 hr. X15,500.

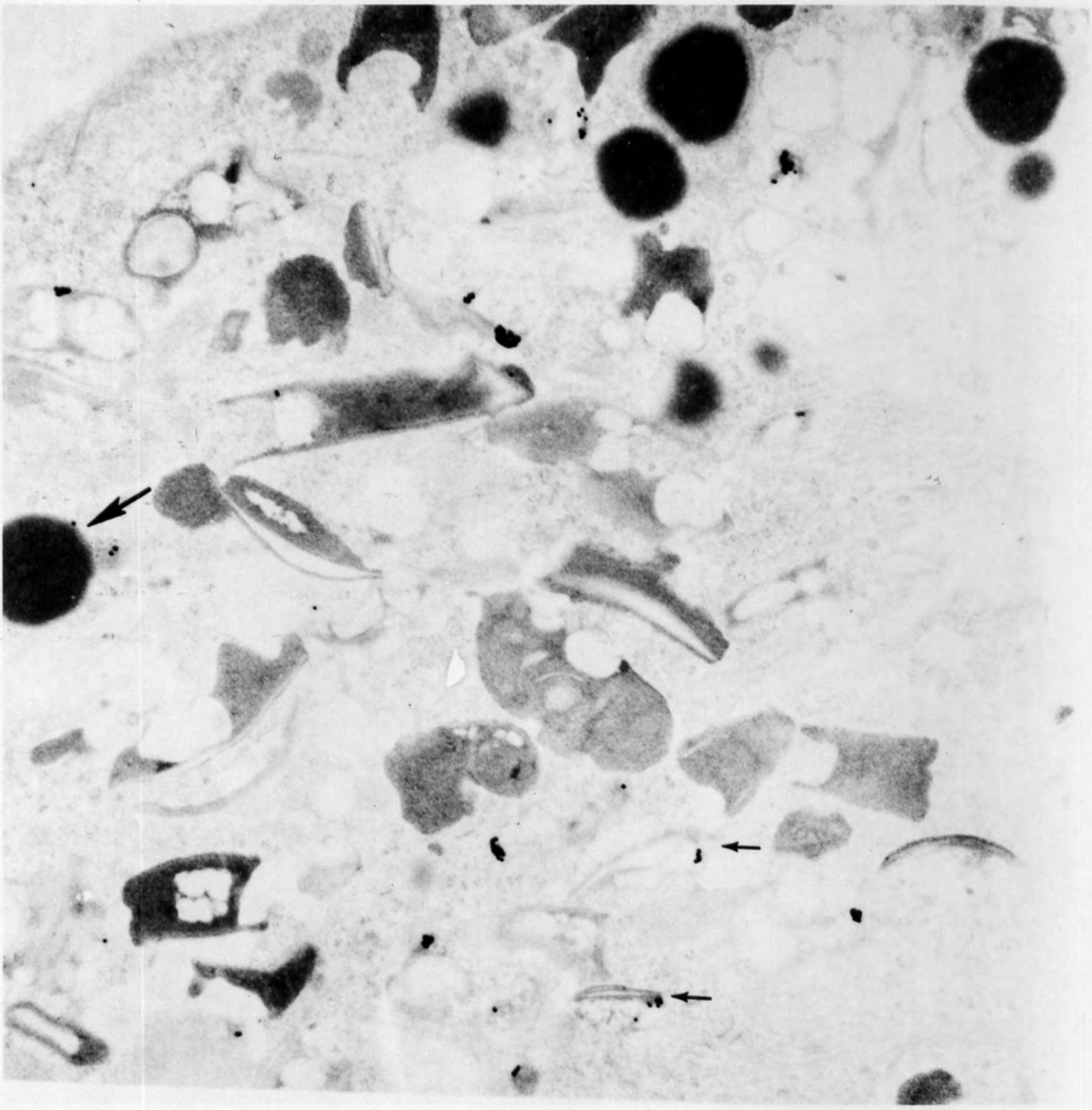


Figure 49. Normal, GM-37 (A,C) and Wolman's disease, GM-1606 (B,D) fibroblasts, untreated (A,B) and treated with 10^{-4}M E600 (C,D) viewed by scanning electron microscopy. Cells show nuclei, nucleoli, and microfilaments running lengthwise through the cells. The E600-treated cells show bumps throughout the cytoplasm except for a perinuclear clear zone (arrows). The bumps appear to form ridges along microfilaments. X1400, 45° angle.

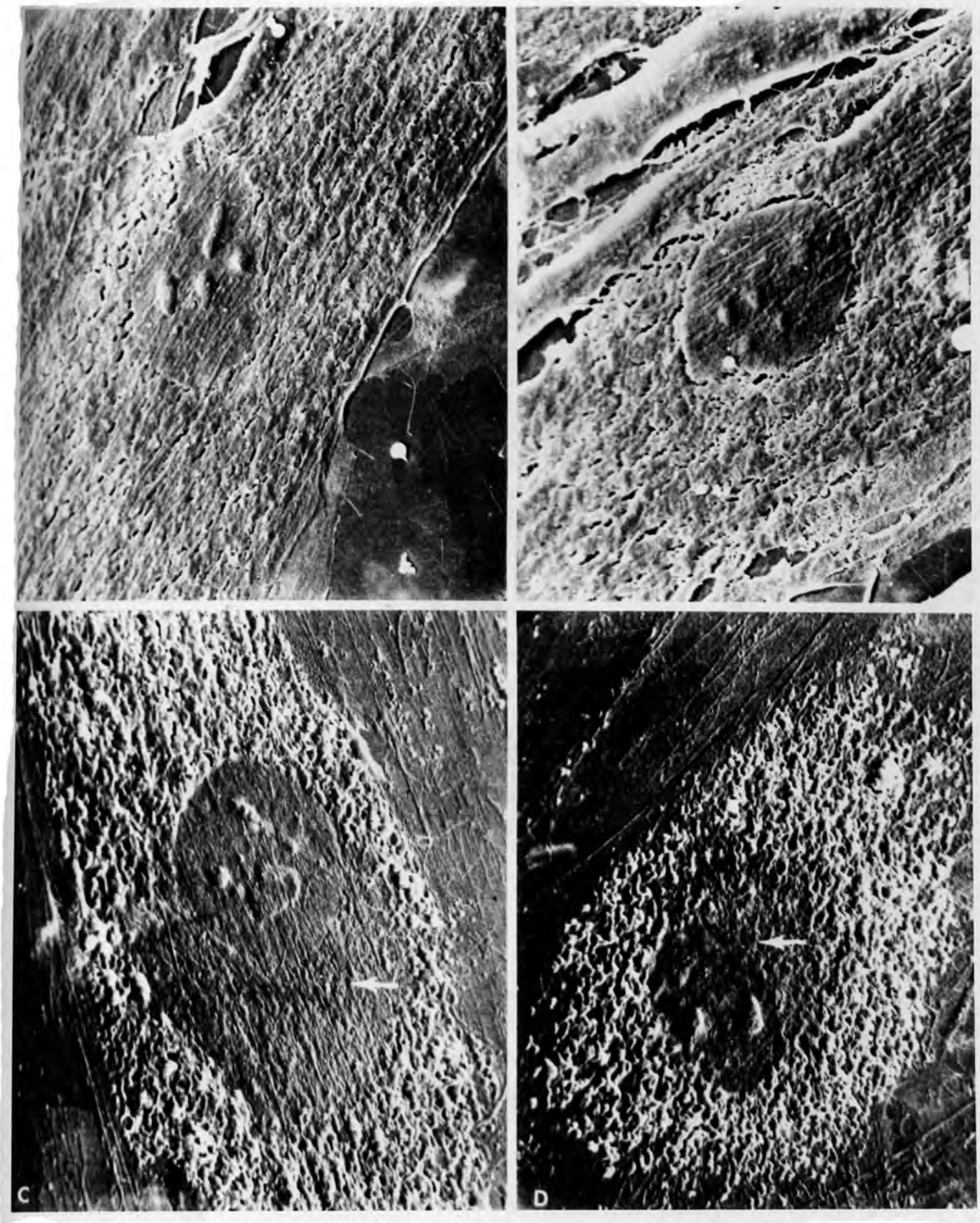
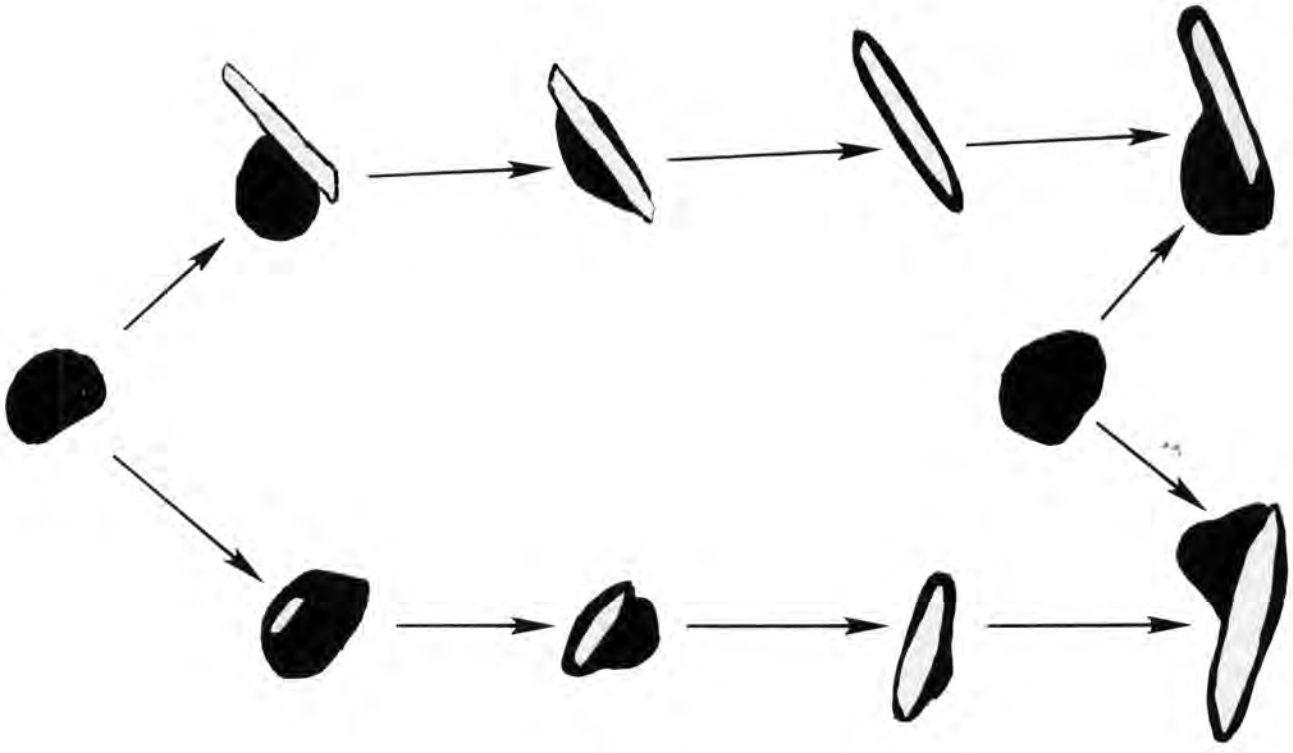

C
D

Figure 50. Possible mechanisms to explain the presence of crystalloid material within secondary lysosomes. A. A lysosome fuses with a lipid cleft lying free in the cytoplasm. The lysosome engulfs the cleft, which thereby alters its shape. Eventually another lysosome may fuse with this structure. B. A lipid cleft begins forming within the lysosome which is unable to degrade it. As more lipid is taken up, the cleft expands, stretching the lysosome. As in A, eventually another lysosome may fuse with this structure.



did not appear to be increased in either Wolman's disease or E600-treated cells and did not generally contain colloidal gold particles. The amount of colloidal gold uptake varied from cell to cell but was not appreciably different in either quantity or location in the normal, E600-treated, or Wolman's disease cells.

Scanning electron microscopy (SEM): Normal cells were indistinguishable from Wolman's disease cells by SEM (Fig. 49 A,B). The nuclei appeared as smooth ovoid areas with 2 to 4 raised nucleoli. Thin strands could be observed running parallel to the longitudinal axis of the cell. Aside from these filaments, the cytoplasm appeared smooth. Pits were present in the cytoplasm and may have reflected the presence of vacuoles covered by fragile areas of cytoplasm or they may have represented artifacts of critical point drying.

Normal cells and Wolman's disease cells treated with 10^{-4}M E600 showed a distinct change in morphology (Fig. 49 C,D). The cytoplasm became bumpy and the bumps sometimes appeared to be linear, running along the filaments. A perinuclear zone was observed which showed no bumps. This was comparable to a clear perinuclear area seen by neutral red (Fig. 32) and oil red O staining. The bumps thus appeared to represent the lysosomal lipid storage granules.

Discussion

Although lipid clefts have been noted in both light and electron microscopic studies of Wolman's disease, only Lake and Patrick (16) reported that the crystals were membrane-limited, but they showed no illustrations. Partin and Schubert (105) reported

that fibroblasts from a patient with cholesteryl ester storage disease contained "semilunar or angular electron lucent images immersed in an electron dense material contained in membrane bounded structures probably lysosomes." This is similar to what we observed in Wolman's disease and E600-treated cells. The deformation of the structures by crystalloid material has not been previously noted. The presence of the clefts and the concomitant alteration in morphology did not appear to interfere with the activity of the structures in fusing with endocytic vesicles containing colloidal gold.

Acid hydrolases are manufactured in the cell on the ribosomes of the rough endoplasmic reticulum and are transported via the endoplasmic reticulum to the Golgi. In the Golgi the hydrolases are packaged and, when they bud off the Golgi membrane, they are known as primary lysosomes. After pinocytotic vesicles and phagocytic vacuoles are formed at the cell surface, they are transported through the cytoplasm and fuse with a primary lysosome which thereafter is called a secondary lysosome (phagolysosome). The secondary lysosome digests the endocytosed material and continues to fuse with new endocytic vesicles. Eventually the lysosome accumulates indigestible material and becomes less active. It is now called a residual body. Colloidal gold particles are large and inert and they cannot pass through the plasma membrane, but they are taken up by endocytosis. When the colloidal gold-carrying endocytic vesicle fuses with a lysosome, the colloidal gold remains in the secondary lysosome for it cannot be digested. Thus secondary lysosomes accumulate colloidal gold (107). The crystalloid-containing dense bodies, which were generally larger and

denser than endocytic vacuoles, contained colloidal gold and were thus considered to be secondary lysosomes. Because Wolman's disease and E600-treated cells stored vastly more cholesteryl ester than normal cells (Research Section III), it is likely that the crystalloid clefts consisted mainly of cholesteryl ester.

Two mechanisms can be hypothesized to result in the presence of crystalloid within secondary lysosomes. If cholesteryl ester clefts were found free in the cytoplasm, perhaps in a structure like the clear vacuole, one or more lysosomes might fuse with the crystalloid and engulf it (Fig. 50A). Such a mechanism is suggested by Fig. 46. The accepted mechanism of cholesteryl ester uptake via the low density lipoprotein pathway suggests that the clefts may form in the lysosomes due to the aggregation of storage material (Fig. 50B).

The large clear vacuoles were seen in all cell types, but appeared to be more numerous and to be more angular in Wolman's disease and E600-treated cells. The angularity may have been due to the presence of cholesteryl ester crystals. The vacuoles may represent non-osmiophilic lipid droplets, which Partin and Schubert noted in macrophages in cholesteryl ester storage disease (79) or macropinocytotic vesicles (108). The vacuoles were often seen in association with lysosomes and sometimes the lysosomal membrane appeared to extend around the vacuole as if the lysosome were attempting to engulf the clear vacuole. The presence of colloidal gold in some of the clear vacuoles, generally at the periphery of the vacuole, suggests that some of these vacuoles did fuse with lysosomes. Alternately, the clear vacuole may form within a lysosome and cause expansion. Other lysosomes may then fuse with it. These

hypotheses are analogous to those discussed for cholesteryl ester crystals and depicted in Fig. 50.

No morphological evidence of damage was noted in cells treated with E600. The dilation of rough endoplasmic reticulum seen in Wolman's disease cells may have been due to the glutaraldehyde fixation procedure. The damage appeared to be slightly more severe in the Wolman's disease cells incubated in colloidal gold for 24 hr.

Increased numbers of dense bodies, many containing crystalloid clefts, were obvious in E600-treated cells. This supports the conclusion shown in Research Section II that E600-treated cells, because they show increased numbers of neutral red granules, have an increased number of lysosomes. Although direct stimulation cannot be ruled out, it seems likely that this is caused indirectly and represents an attempt by the cells to clear the lipid accumulations induced by E600 treatment.

The longitudinal filaments observed by SEM have the same morphology as actin stress fibers in human (109) and mouse (110) fibroblasts. Actin filaments are thought to be involved in the spreading of fibroblasts (111) and the maintenance of cell shape as well as such diverse and important processes as growth, adhesion to the substratum, and cellular motility (109). The apparent allignment of the E600-induced lysosomes along actin filaments supports the observation of linear neutral red granules that were particularly noticeable in the GM-2121 culture (Fig. 32). Although fixation and drying might cause organelles to line up against resisting structures like bundles of actin filaments, the apparent association of lysosomes and actin filaments in the unfixed neutral red stained cells makes it likely that this is not an

artifact of fixation. Actin has been shown in fibroblasts surrounding phagolysosomes containing latex beads (112). An association between lysosomes and microfilaments has been reported in polymorphonuclear leukocytes (113), by both TEM and SEM. It is tempting to speculate that the contractile protein actin may direct or at least modulate the intracellular movements of lysosomes.

The clear perinuclear area visible by SEM and neutral red staining of E600-treated cells was stained by the copper phthalocyanin procedure for phospholipids (Research Section II). By TEM, little Golgi was observed in this area. Lipid-containing lysosomes, which filled most of the cytoplasm, may have displaced other cell organelles to this area. This area did seem to show a slight increase in the concentration of rough endoplasmic reticulum and mitochondria by TEM (Fig. 44). Perhaps the concentration of membrane in this area increased sufficiently to be visualized by the phospholipid staining procedure.

DISCUSSION

Fibroblasts derived from a child with Wolman's disease exhibited a long #5 chromosome, but this finding was called into question when cells that had been stored frozen did not show the anomaly. If the initial finding was representative of the karyotype of the proband, a translocation probably occurred in the father's germ cells or in an early mitotic division of the zygote, resulting in the deletion of the father's normal acid lipase gene and suggesting that the acid lipase gene locus may be on chromosome #5. If the initial karyotypic finding was an artifact of tissue culture, either individual variation in the father's acid lipase level and variation within the assay resulted in his not appearing to be a carrier, or the presumed father was not the true father of the child and the true father was a carrier for acid lipase deficiency. The different Wolman's disease cell cultures stored different amounts of lipid which may have depended on the rate of proliferation of the cell cultures.

Normal cells treated with the esterase inhibitor E600 exhibited time dependent lipid storage that appeared maximal at 10^{-3}M E600. The stored lipid appeared to be derived from serum lipid. As in Wolman's disease, triglycerides and cholesteryl esters accumulated. In addition, however, phospholipid storage and slightly increased free cholesterol and free fatty acid storage were observed. Wolman's disease cells, which are deficient in acid lipase, after treatment with E600, showed comparable increases in all lipid classes but free fatty acid, implying that E600 inhibited some enzyme activity present in Wolman's disease cells. The phospholipid storage suggested inhibition

of an additional enzyme. E600-treated cells exhibited reduced acid lipase activity, showing that acid lipase deficiency was probably partially responsible for the lipid storage.

Ultrastructurally, Wolman's disease and E600-treated normal cells showed lipid clefts within dense bodies that were sometimes associated with colloidal gold particles. The E600-treated cells exhibited increased numbers of dense bodies and had increased neutral red granules by light microscopy.

When viewed by scanning electron microscope, Wolman's disease cells resembled normal cells, but E600-treated cells showed bumps that appeared to represent structures arranged lineraly along actin filaments. A similar arrangement was suggested by neutral red staining.

Although E600 was found to induce lipid storage in all tests, in the early experiments presented in much of Research Section II, the optimum concentration appeared to be 10^{-3}M E600, whereas in later experiments, such as the gas chromatography and electron microscopy, the optimum concentration appeared to be 10^{-4}M E600. In the later experiments 10^{-3}M E600 was more toxic to the cells. There are two likely explanations for this finding. E600-treated cells showed less toxicity when grown on glass than when grown on polystyrene. In all the histochemical experiments the cells were grown on glass coverslips. For the lipid biochemistry and electron microscopy however, the cells were grown in polystyrene flasks or culture dishes respectively. It is worthy of note that the relative toxicity and growth curve experiments, early experiments in which the cells were grown in polystyrene culture dishes, showed significant toxicity

at 10^{-3}M E600. Perhaps the E600 binds to the plastic, presenting an increased local concentration where the cells are present. However, there still appeared to be an increased effect of 10^{-4}M E600 on cells grown on glass coverslips in late experiments. Different lots of FCS were utilized in the early and late experiments. It is possible that the different lots varied in paraoxonase activity. If the FCS used in the later experiments had less paraoxonase activity, the treated cells would experience an effective increase in the concentration of E600.

The increase in phospholipid observed in E600-treated cells is difficult to interpret. Histochemically an increase in phospholipid staining was visible in a perinuclear location, but ultrastructurally this area appeared essentially normal. If the increase were due to inhibition of a lysosomal phospholipase, the lipid would be expected to be stored in the lysosome and to be discernible by histochemical staining. If the storage were cytoplasmic or represented an increase in the amount of membrane, it would be difficult to detect histochemically. The major increase in membranes in E600-treated cells appeared to be due to a vast increase in the number of lysosomes. This may at least account for a part of the phospholipid increase. An increased number of lamellar bodies may also have accounted for some phospholipid storage. Because phospholipid degradation requires cleavage of glyceryl fatty acid esters and phosphate esters, it is quite possible that E600-induced inhibition of a phospholipolytic enzyme is also involved.

The evidence appears good that, as in Wolman's disease, the storage of triglycerides and cholesteryl esters in E600-treated

cells occurs in lysosomes. Firstly, E600 inhibited acid lipase, a lysosomal enzyme. Acid lipase deficiency results in lysosomal lipid storage in Wolman's disease (16,101). Secondly, the cholesteryl esters in both Wolman's disease and E600-treated cells had the same fatty acid pattern as cholesteryl esters in FCS, suggesting that they had not been degraded. Since cholesteryl esters are normally taken up at receptor sites and transported to the lysosomes for degradation by acid lipase (9), the lack of degradation suggests that the deficiency of acid lipase resulted in the storage of the cholesteryl esters in the lysosomes. A third line of evidence that the storage of lipid in E600-treated cells is lysosomal is indicated by the morphology of the oil red O granules. In Wolman's disease and E600-treated cells, the granules were small and close to the nucleus. In contrast, cells treated with large amounts of free fatty acid or cholesterol stored the lipid in large droplets at the periphery of the cells. Because little lipid was seen in areas where the lysosomes were most abundant, lipid transported to these areas may have been degraded. A fourth line of evidence that the storage was lysosomal is the presence in E600-treated cells of a clear perinuclear zone surrounded by numerous granules that stained with oil red O and neutral red, appeared as bumps by SEM, and as dense bodies containing lipid clefts by TEM. The similar appearance of the clear zone suggests that these structures are identical and, because they stained with both oil red O and neutral red, that they represent lipid-containing lysosomes. As a fifth line of evidence, the electron-dense structures that sometimes contained lipid

clefts by TEM could be shown to be associated with colloidal gold particles, indicating that they were probably secondary lysosomes.

In several animal model systems of atherosclerosis, cholesteryl esters have been shown to be stored in lysosomes of cells of the aortic wall (22,114,115). An accompanying increase was observed in lysosomal size (22,115), number (22,115) and activity (114). Increased lysosomal number and activity have also been reported in aortic smooth muscle cells of hypertensive rats (116). Aortic smooth muscle cells have acid lipase activity (15,23) and evidence indicates that this activity may be reduced in atherosclerosis (26,117,118) and hypertension (25). It has been proposed that a relative deficiency of acid cholesterol esterase (22-24) or acid esterase (25,26,117,118), which appear to be identical to acid lipase (13,15), may play a role in the pathogenesis of atherosclerotic disease. Autopsy findings from patients with acid lipase deficiency diseases give support to this hypothesis. Arterial accumulation of fat is a typical finding in Wolman's disease (8). Advanced atherosclerosis has been seen in two of the three autopsies performed on patients with cholesteryl ester storage disease (5,7).

Although aortic smooth muscle cells and fibroblasts in culture store little lipid, when treated with certain chemicals they can be induced to store cholesteryl esters and reproduce features of atherosclerotic cells. Cells treated with low density lipoprotein (LDL) modified to carry a strong positive charge by a covalent linkage to N,N-dimethyl-1,3-propanediamine (DMPA) stored massive amounts of cholesteryl esters (119-121). The lipid droplets did not appear

to be membrane limited in either fibroblasts (120) or smooth muscle cells (121). DMPA-LDL appears to be a useful method of inducing non-lysosomal cholesteryl ester storage. Fibroblasts treated with 25-hydroxycholesterol also appeared to store cholesteryl esters in the cytoplasm (9,80,122). Cytoplasmic esterification is normally stimulated by an increase in cytoplasmic cholesterol which suppresses the cholesterol synthetic pathway (9,80,122). This may result from the hydrolysis of cholesteryl esters in the lysosomes. Cytoplasmic hydrolysis of endogenously synthesized cholesteryl esters is not dependent on lysosomal acid lipase and occurs equally well in normal, cholesteryl ester storage disease, and chloroquine-treated cells (9).

Chloroquine treatment appears to induce lysosomal cholesteryl ester storage in fibroblast (9,80,120,123) and aortic smooth muscle (124) cultures, and its use has been proposed in a model system for studying the role of the lysosomal system in atherosclerosis (124). Chloroquine-induced cholesteryl ester storage probably results from the inhibition of acid lipase (20,80) in keeping with the hypothesized role for acid lipase in the etiology of atherosclerosis. However, chloroquine inhibits other lysosomal hydrolases involved in the degradation of mucopolysaccharides (125) and proteins (126). These results are thought to stem from increases in the lysosomal pH (127). Chloroquine may have other complex actions on cells, including inhibition of receptor-mediated endocytosis of lysosomal enzymes (45). Because E600-induced accumulation of cholesteryl esters appears to be caused by specific inhibition of acid lipase whereas chloroquine-induced

accumulation is thought to result from a non-specific alteration of the intralysosomal environment, E600 may be more suitable for inducing lysosomal cholesteryl ester storage as a means of studying atherosclerosis *in vitro*.

Chloroquine-induced cholesteryl ester accumulation has been reported to be maximal after 48 hr (124) and the cholesteryl ester accumulation has not exceeded 102.0 µg/mg protein (123). E600-induced lipid accumulation thus results in greater cholesteryl ester accumulations than chloroquine (Table 8) and the lipid continues to accumulate with time (Figs. 20-22), a situation that may be more analogous to the development of atherosclerosis. In addition, E600-treated cells exhibit increased numbers of lysosomes (Figs. 32,44) as has been observed in atherosclerosis (22,115). This has not been shown in chloroquine-treated cells. The stored lipid, in chloroquine-treated cells, is still bound to the protein component of LDL (9,80). In Wolman's disease, the protein appears to be degraded normally (10). Whether or not the chloresteryl ester stored in atherosclerosis or in E600-treated cells is still bound to protein is not presently known.

There is much room for further research on Wolman's disease and E600-treated cells and on their relation to atherosclerosis. The major difference between the lipids stored in Wolman's disease and E600-treated cells was the large increase in phospholipid storage after E600-treatment. Although a preliminary separation of the phospholipid classes was carried out, further phospholipid analysis might prove rewarding. Cells could be grown in the presence of

E600 and inorganic phosphate with radiolabeled phosphorus. After extracting the lipids and separating the phospholipids by two dimensional thin layer chromatography, the phospholipid spots could be scraped from the plates, extracted from the silica gel, and the radioactivity measured on a liquid scintillation counter. This experiment would give a more accurate numerical representation of the phospholipid classes stored in E600-treated cells.

Chloroquine has been shown to inhibit LDL degradation for seven hr after its removal (80). It would be interesting to determine how long after removal of E600 the treated cells begin to hydrolyze the stored cholesteryl esters. Replicate culture dishes of cells, after growing for 24 hr in lipoprotein deficient serum with or without E600, could be switched to a medium containing cholesteryl ester-LDL in which the cholesterol has been radiolabeled. At this time and every hr afterward, lipid could be extracted from duplicate dishes and separated by thin layer chromatography. The spots could be scraped, the lipid extracted, and the extent of radioactivity measured on a liquid scintillation counter. The total amount of free radiolabeled cholesterol would represent the cholesteryl esterolytic activity (9). The results of this experiment should show clearly at what time activity returns to E600-treated cells as well as demonstrating again that the E600 initially causes inhibition.

Goldstein et al. (9) demonstrated that when substrate built up sufficiently in cholesteryl ester storage disease cells, the monolayers showed 1/3 the activity of normal cells although extracts

from the cells showed only 5% of the normal activity. It is possible that the small difference present in acid lipase activity of Wolman's disease and cholesteryl ester storage disease cells may be magnified when increased substrate is presented to the enzyme in intact cells, resulting in the extreme difference in symptoms between the two diseases. This could be tested by growing replicate culture dishes of cells (normal, cholesteryl ester storage disease, and Wolman's disease) for 48 hr in lipoprotein deficient serum and then switching them to a growth medium containing cholesteryl ester-LDL in which the cholesterol has been radiolabeled. At this time and every hr afterward, duplicate culture dishes would be extracted and prepared as outlined in the experiment above. Not only might this experiment distinguish between cholesteryl ester storage disease and Wolman's disease cells, but, if the Wolman's disease cells do show some residual acid lipase activity, it might explain why E600-treated Wolman's disease cells, in which residual acid lipase activity could be inhibited, stored additional triglycerides and cholesteryl esters. The effect of E600 could be tested in this system to determine if the residual acid lipase activity is truly inhibited.

Cytoplasmic cholesteryl ester synthesis and hydrolysis have been shown to be normal in cholesteryl ester storage disease and chloroquine-treated cells (9). Because E600 inhibits esterase enzymes whereas chloroquine inhibits lysosomal degradation, E600 may well have an effect upon the cytoplasmic cholesteryl ester hydrolase and perhaps even the synthetase (fatty acyl-CoA:cholesteryl acyltransferase). The cytoplasmic cholesteryl ester hydrolase

may not be as resistant to E600 as acid lipase, so different E600 concentrations should be evaluated. It would also be interesting to compare cholesteryl ester storage disease and Wolman's disease cells, to determine if a difference in this system may account for the more serious nature of the latter disease. Normal, cholesteryl ester storage disease and Wolman's disease cells, after incubation in lipoprotein deficient serum, would be incubated in replicate culture dishes with LDL and radiolabeled oleate with or without E600 for 20 hr. This would label endogenously synthesized cholesteryl esters. The cells would be switched to lipoprotein deficient serum with or without E600. At this time and at intervals up to 24 hr, the cholesteryl oleate would be extracted from replicate dishes, separated by thin layer chromatography, and the amount of radioactivity in the cholesteryl ester spots would be measured on a liquid scintillation counter. The decrease in cholesteryl ester radioactivity serves as a measure of cytoplasmic cholesteryl esterase activity and the initial level measures synthetase activity.

Because an increase in lysosome number was seen in E600-treated cells, it would be interesting to see if other events that have been reported in animal model systems of atherosclerosis also occur in E600-treated cells. The activity of other lysosomal hydrolases in E600-treated cells should be tested to determine if similar increases to those seen in atherosclerotic cells (114) occur. In addition, studies of lysosomal phospholipases would be worthwhile. No increase in lysosomal size was noted by TEM in E600-treated cells, though authors have reported this in atherosclerosis model systems (22,115).

Reduced lysosomal density due to lipid accumulation was reported in aortic cells in rabbit experimental atheroma (114). Because of the massive lipid storage, and the apparent involvement of lysosomes in E600-treated cells, a similar result might be expected. This could be tested by differential centrifugation to purify lysosomes and centrifugation in sucrose gradients to determine the density of the lysosomes.

In order to determine the importance of acid lipase in the etiology of atherosclerosis and how closely E600-treated cells resembled atherosclerotic cells it would be important to determine if the cholesteryl ester remains bound to the protein component of LDL as in chloroquine-treated cells (9,80) or if the protein is degraded normally as in cholesteryl ester storage disease (9,10) and Wolman's disease (10). If both protein and cholesteryl ester degradation were inhibited, a general overloading of lysosomes with relative decreases in proteolytic and cholesteryl esterolytic hydrolase activities would be indicated, whereas if only cholesteryl ester degration were inhibited, a relative deficiency of acid lipase would be indicated. In an animal rendered atherosclerotic by cholesterol feeding, the aorta could be removed and perfused with ^{125}I-labeled LDL or cholesteryl ester-LDL with radiolabeled cholesterol. The ability of the tissue to degrade the LDL and cholesteryl esters could be compared to that of normal animals. For E600-treated cells in culture, after incubation of replicate culture dishes in lipoprotein deficient serum, the labeled LDL and E600 could be added. At this time and every hr after this, duplicate cultures would be extracted with trichloroacetic acid for ^{125}I-labeled

acid soluble material (9,10,80) or with chloroform-methanol for radiolabeled free cholesterol (9,10) and the radioactivity measured on a liquid scintillation counter.

The results reported here suggest that E600 may be useful for loading cultured cells with cholesteryl esters in order to study the clearing of cholesteryl esters accomplished by different pharmacological agents. Stein et al. (123,124) showed that medium enriched in high density apolipoprotein and sphingomyelin led to increased depletion of cellular cholesterol and cholesteryl ester in cells pretreated with chloroquine. E600 pretreatment could prove useful in similar experiments. A drug could also be tested for its ability to block LDL uptake by treating cells in the presence of E600. If the drug were effective, there would be a reduction in E600-induced lipid accumulation. E600 treatment could potentially be employed on any cell type which can be maintained in vitro.

In addition, E600 might prove useful in assessing the role of acid lipase in the lysosomal digestion of more complex substances and microorganisms, although in phagocytic cells E600 would probably inhibit other esterase enzymes as well as acid lipase.

A great deal of study remains to be done on acid lipase. The enzyme has not been purified nor has the gene been mapped. Studies in this area may have important therapeutic consequences. Enzyme replacement therapy appears to be helpful in Fabry's disease and Gaucher's disease (128), two lipid storage diseases in which symptoms are not due to lipid accumulations in the central nervous system. To obtain sufficient acid lipase for enzyme replacement therapy would probably

require genetic engineering utilizing recombinant DNA techniques. Once large amounts of purified acid lipase can be obtained, not only might enzyme replacement therapy be of use in treating patients with Wolman's disease and cholesteryl ester storage disease, but more importantly it might also permit the reduction of atherosclerotic plaques, providing a safe and simple therapy for what is today the leading cause of death in the United States.

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