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Research and Development

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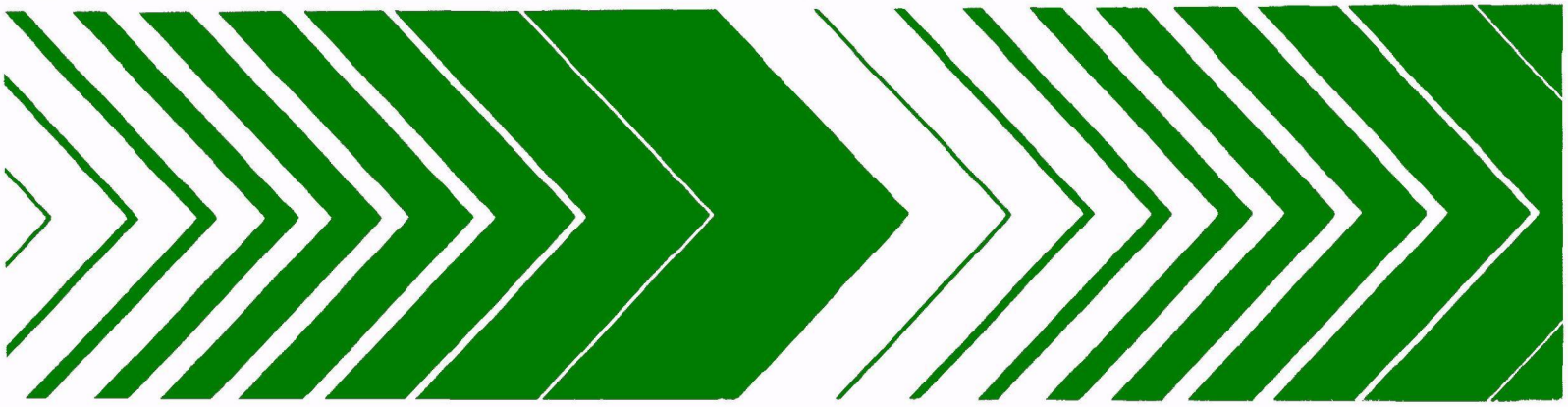
Development of an *in vitro* Model for Screening Organophosphates for Neurotoxicity (Pilot Study)



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DEVELOPMENT OF AN IN VITRO MODEL FOR SCREENING ORGANOPHOSPHATES FOR NEUROTOXICITY (PILOT STUDY)

by

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

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The pilot study was designed to test the feasibility of using murine or human neuroblastoma cells in tissue culture for the assessment of neurotoxicity of organophosphorus insecticides. The following organophosphorus compounds were evaluated: DFP, TOCP, parathion, leptophos, EPN, DEF, and merphos.

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ABSTRACT

An *in vitro* model utilizing neuroblastoma cell lines was developed for the differentiation of organophosphorus compounds which have the potential for induction of delayed degeneration of peripheral nerves and the spinal cord. IMR-32 neuroblastoma cells, derived from a human tumor, showed specific (3H)-norepinephrine uptakes. The greater effect of alkyl over aryl OP compounds suggests a relationship with water solubility, thus equilibrium between adsorption to agar and the surrounding medium or lysosomal contents. The effect of parathion was intermediate, less that that for merphos, DEF and EPN but greater than that for leptophos and TOCP. Electron microscopic observations suggest further studies to define a morphologic lesion peculiar to the clinically delayed neurotoxic OP compounds.

Final Report

EPA Contract 68-02-2953

DEVELOPMENT OF AN IN VITRO MODEL FOR SCREENING ORGANOPHOSPHATES FOR NEUROTOXICITY (PILOT STUDY)

Introduction

One to two weeks after exposure to certain organophosphorus compounds, man and many animals develop degeneration of peripheral nerve and of the long tracts of the spinal cord (1-12). The differentiation of this lesion as neuronal rather than of myelin (1,3) is widely accepted. Whether the focus of injury is within the cell body (13) or in the axon (14,15) has not been settled, but current literature suggests that the concept of a dying-back polyneuropathy, long-postulated to explain toxic, nutritional, and hereditary neuropathies (16-22), does not adequately characterize the sequences of events in organophosphorus-induced delayed neurotoxicity. Rather, it would appear that the initial site of injury is within the axon and that this is then followed by Wallerian degeneration peripheral to the lesion (15,22).

Within the axon, however, the molecular or subcellular target for organophosphorus-induced injury is poorly defined. Aldridge, Johnson, and others have described "neurotoxic esterase", an enzyme activity defined by its capacity to hydrolyze phenyl phenyl-acetate or phenyl valerate and by its inhibition by neurotoxic organophosphorus compounds (4,5,22-25).

Johnson has proposed using inhibition of "neurotoxic esterase" as a screening method for detecting the potential of organophosphorus compounds to induce delayed neurotoxicity and has postulated that inhibition of this enzyme is related to the mechanism by which these agents cause this disorder. While inhibition of

this activity correlates with neurotoxicity potential with rare exceptions, the intracellular location of "neurotoxic esterase", the properties of the enzyme as a protein, its role within the cell, and the relationship between phosphorylation of this enzyme and axonal degeneration, all remain undefined (4). Further, a time-dependent process, termed "aging" is required between phosphorylation and inhibition of this enzyme. This phenomenon is likewise poorly understood and difficult to dissect in whole brain homogenates where, besides "neurotoxic esterase" there are at least 4 additional esterases with similar substrate specificities (4,5).

During the past three years Dr. M.B. Abou-Donia and I have evaluated a series of phenylphosphonothioates for the capacity to induce delayed neurotoxicity in vivo using hens and ducklings (7-12,28). In these studies we have developed a grading system for assassing clinical ataxia and have evolved much-needed standardized treatment protocols in which histopathologic verification of clinical neurotoxicity can be observed as lesions in peripheral nerve and spinal cord. These protocols include daily oral or topical applications of small doses and single large dose studies. In the latter protocols we have been able to test compounds at levels 50 to 100 times the LD_{50} dose through the vigorous prophylactic administration of atropine sulfate to counter the cholinergic manifestations of acute organophosphorus toxicity. Most previous in vivo studies have not included histopathologic study, so that differentiation of the clinical state of acute toxicity from delayed neurotoxicity has been uncertain. It is for this reason that in the development of an in vitro model for differentiating neurotoxic from non-neurotoxic organophosphorus compounds we have chosen to use those which we have characterized in vivo.

In the course of our in vivo studies the need for an in vitro model which could be used for screening became obvious. The neuroblastoma lines we have used as a tool for exploring the pathogenesis of Parkinson's disease (26,27) have now become

a model system for organophosphorus-induced delayed neurotoxicity. The advantages of neuroblastoma cells in tissue culture as an in vitro system are many. These cells differentiate in tissue culture, form neurites, demonstrate electrical activity, and synthesize and store specific neurotransmitters (28-32). Thus these cell lines provide neurobiologists with the heretofore unavailable opportunity to study neuronal metabolism and pathology divorced from blood-tissue barriers and the interposition of myelin and glial cells. Further, using cloned lines from either human or murine sources, statements can be made in reference to one type of neuron as compared with another (e.g., adrenergic vs cholinergic), a distinction not readily achieved in vivo. In addition, cloned neuroblastoma lines in tissue culture present a pure population of neurons in an optimal setting for morphological observations (phase and electron microscopy, autoradiography) and are available in sufficient quantities for chemical studies.

This pilot study was designed to test the feasability of using murine or human neuroblastoma cells in tissue culture for the assessment of neurotoxicity of organophosphorus insecticides. By testing a variety of agents known to result in delayed neurotoxicity in chickens and, in some cases, in man, it was hoped that morphologic and/or biochemical end points could be defined which distinguished these insecticides from those not associated with delayed neurotoxicity. The tissue culture lines utilized were the N115 clone of the C1300 murine neuroblastoma and the human IMR-32 neuroblastoma line. The following organophosphorus compounds were evaluated: DFP, TOCP, parathion, leptophos, EPN, DEF, and merphos.

Materials and Methods

Tissue culture lines: The N115 adrenergic clone of the murine C1300 neuroblastoma, developed in the Nirenberg laboratory at NIH was obtained from Dr. David Duch, Burroughs Wellcome Co. and was maintained in serial passage in Dulbecco's modification of Eagle's MEM medium with 20% fetal calf serum, penicillin, (250 i.u/ml), streptomycin (100 mg/ml) and glutamine. IMR-32 adrenergic neuroblastoma was started from frozen stock in the laboratory of Dr. Darell Bigner at Duke. It, too, was grown in Dulbecco's medium.

Organophosphorus compounds: DFP (diisopropyl phosphofluoridate) was obtained from Aldrich Chemical Co., Milwaukee, WI, EPN from E.I. duPont deNemours and Co., Inc., Wilmington, Del., leptophos from Velsicol Chemical Co., Chicago, Ill., DEF and merphos from Chemagro Corp., Kansas City, Mo., TOCP from Eastman Kodak Co., Rochester, N.Y., parathion from Pfaltz and Bauer.

Exposure of neuroblastoma cells to organophosphorus compounds: In experiments with DFP, ampoules were chilled on dry ice and opened in a fume hood, immediately diluting in isopropanol in order to reduce hydrolysis. Isopropanol solutions were then added to medium without fetal calf serum, mixed and rapidly added to the tissue culture flasks. Control cells were exposed to medium to which an equal volume of isopropanol had been added. Exposure was for 3 hours, after which the cells were evaluated for the capacity for (^{3}H)-norepinephrine uptake or washed and covered with tissue culture medium containing fetal calf serum in the multiple-exposure experiments. In the latter, on successive days neuroblastoma cells in Falcon flasks were washed with medium deficient in fetal calf serum prior to exposure to DFP for 3 hour periods at 37^{o}C.

Exposure to the remaining organophosphorus compounds required that methodology be developed for the uniform exposure of tissue culture cells to agents poorly soluble in aqueous media. Each compound was dissolved in ether,

then layered over a rapidly stirring sterile suspension of agar particles (0.25%). Stirring continued until the ether had evaporated and the organophosphorus compound had been adsorbed by the agar. Then aliquots were pipetted into medium to yield a final concentration of organophosphorus compound of 1 mg/ml. The uniform suspension was then pipetted into flasks containing neuroblastoma cells, with incubation for 3 hours at 37°C. Control cultures were treated with medium containing an equal amount of agar. At the end of the exposure the cells were examined by phase contrast microscopy, then separate flasks were exposed to (^{3}H)-norepinephrine or prepared for electron microscopy as described below.

Electron microscopy: Cells were fixed in situ with a) glutaraldehyde (4% in 0.1M sodium cacodylate buffer pH 7.4), b) 4% aqueous $KMnO_4$, or c) 4% aqueous $KMnO_4$ after 30 minutes exposure to 10 μM 5-hydroxydopamine (3,4,5-trihydroxy-phenylethylamine). Glutaraldehyde-fixed cells were post-fixed in 1% OsO_4. All preparations were then dehydrated and embedded in Epon. After solidification the plastic flasks were broken away from the sheet of epon and sites chosen for thin sections. Grids were examined and photographed on a Hitachi HS 11 transmission electron microscope.

(^{3}H) norepinephrine uptake: (^{3}H) norepinephrine (25-30 Ci/m mole) was added to complete medium to a final concentration of 10^{-7}M and pipetted to the bottom of a vertical Falcon flask. Flasks were then laid flat to allow contact of medium with neuroblastoma cells for 10 min at 37°C in a 5% CO_2 atmosphere. At the end of this period the flasks were placed vertically in ice and (^{3}H) norepinephrine medium withdrawn. The cells were then dissociated with trypsin, washed with centrifugation using phosphate-buffered saline, then the (^{3}H)-norepinephrine extracted with 0.1M perchloric acid for scintillation counting. The perchloric acid pellets were then digested with 1.0 M NaOH for protein determinations.

Protein concentrations: Protein content was quantified by the Pulley and Grieve modification of the Lowry method in which protein is precipitated in cold 10% TCA, then dissolved in 1N NaOH after which buffered sodium dodecylsulfate is added to prevent precipitation upon addition of the Folin-Phenol Reagent (33).

Scintillation counting: Aliquots of perchloric acid extracts of neuroblastoma cells exposed to (^{3}H)-norepinephrine were added to 10 ml. volumes of aquasol II (New England Nuclear Corp., Boston, MA) and counted in a Beckman LS-150 scintillation spectrometer.

Results

Initial studies with the N115 adrenergic clone of the murine C1300 neuroblastoma showed a 21% reduction in (^{3}H)-norepinephrine uptake after 3 hours exposure to DFP at 1 mg/ml in the absence of fetal calf serum. Two observations gave us reason to doubt the adrenergic differentiation of these cells, which would diminish the usefulness of (^{3}H)-norepinephrine uptake as a sensitive index of neurite injury: norepinephrine could not be detected in perchloric acid extracts of these cells, and (^{3}H)-norepinephrine uptake was only slightly inhibited by 10^{-5}M cocaine.

Accordingly, we focused our efforts on another cell line, the human IMR-32 neuroblastoma. In medium containing 1mM ascorbate and 2 uM iproniazid these cells demonstrated active (^{3}H)-norepinephrine uptake, linear for at least 10 minutes, which was inhibited $\geq$ 90% by 10^{-5}M cocaine. Perchloric acid extracts of the IMR-32 cultures however, have not been demonstrated to contain norepinephrine. Our working hypothesis is that these cells are dopaminergic rather than noradrenergic; this will be established through the demonstration of dopamine and tyrosine hydroxylase and the concommitant absence of norepinephrine and dopamine β-hydroxylase.

In order to study the effects of DFP on the IMR-32 cells we had to deal with two properties of this organophosphorus compound, its rapid hydrolysis in aqueous solutions and its reactivity with nucleophiles in serum proteins. Accordingly, DFP dilutions were made in isopropanol, and exposure to neuroblastoma cells was effected in medium without fetal calf serum as soon after addition of DFP to medium as was possible. Under these conditions exposure to DFP at 1 mg/ml for 3 hours resulted in a reduction of (^{3}H)-norepinephrine uptake from (mean $\pm$ SEM) 35,245 $\pm$ 1,087 cpm/mg protein to 19,680 $\pm$ 1,507 cpm/mg protein (44.2% reduction, $p < 0.005$). Reducing the concentration of DFP to 0.5 or 0.25 mg/ml and daily exposure up to 8 days at 0.25 mg/ml yielded minor to no differences in (^{3}H)-norepinephrine uptake.

Exposure of neuroblastoma cells to the remaining organophosphorus compounds required that water-insoluble compounds be dispersed in a form that would allow for uniform exposure to the cells in tissue culture. Adsorption to agar particles gave excellent agreement among triplicate assays for (^{3}H)-norepinephrine uptake, and electron microscopy disclosed evidence for phagocytosis of the agar particles by the neuroblastoma cells, as will be discussed below.

In Table I are presented the results of experiments in which IMR-32 cells were exposed to medium containing agar particles, with and without adsorbed organophosphorus compounds. In each instance the final concentration was 1 mg/ml. The greatest inhibition of (^{3}H)-norepinephrine uptake was observed with the alkyl organophosphorus compounds, merphos, DEF, and parathion, while lesser degrees of inhibition were found with the aryl compounds TOCP, EPN, and leptophos. In all instances the reductions were statistically significant.

Only a limited amount of electron microscopy was completed in this study. Cells were examined from each exposure group reported in Table I. In these studies we were able to ascertain that adsorped organophosphorus compounds at a final concentration were not cytotoxic, that neurofibrils, neurotubules, and dense core vesicles typical of catecholamine neurons could be observed, that the agar particles were taken into the neuroblastoma cells by phagocytosis, and that on two occasions, after exposure to leptophos and DEF, tightly packed bundles of microfilaments were observed within the neuroblastoma cells.

Representative control cells are illustrated in Figs. 1-4. The neuroblastoma cultures are a replicating population of cells with continuous cell division (Fig. 2) and cell death. Dead cells lose their adsorption to plastic and would thus be washed off prior to (^{3}H)-norepinephrine exposure; relating (^{3}H)-norepinephrine uptake to protein concentration, therefore, served as a reliable method for correcting for the flask to flask variation in cell density and cell death. The close

apposition between cells and neurite processes from other cells was readily observed (Figs. 3,4). Vacuoles with debris suggesting agar phagocytosis were observed (Fig. 1-4) and are more clearly seen in later figures.

Neuroblastoma cells which had been exposed to parathion are seen in Figs. 5 and 6. The large vacuoles containing agar are most clearly seen in Fig. 5. In Fig. 6 the two adjacent parathion exposed cells are seen to be well-preserved without changes of cell injury. Again the agar particles can be seen in phagocytic vacuoles.

Similarly after exposure to TOCP there was no increase in the proportion of dead cells, and those remaining showed no evidence for cell injury. Continuation of replication side-by-side with cell death can be seen in Fig. 8 in which cells had been exposed to EPN/agar for 3 hours. Among the EPN-exposed cells dense core vesicles typical of catecholamine-storage vacuoles, were observed in cell bodies and neuritic processes along with neurotubules (Fig. 9).

After leptophos exposure, dividing cells and dead cells were also observed (Fig. 10). Dense core granules could be seen in neurites (Fig. 11) and cell bodies (Fig. 14). Cytoplasmic extensions or neurites were noted to contain closely-packed bundles of microfilaments (actin) (Figs. 12,13) which were not observed in control or parathion-exposed cells but were observed after DEF exposure (Fig. 15).

The marked reductions in (^{3}H)-norepinephrine uptake effected by DEF and merphos were not accompanied by excess cell death (Figs. 15-17). Neurosecretory granules (dense core vesicles) were readily observed in cell bodies (Fig. 16).

DISCUSSION

This study has shown that hydrophobic organophosphorus compounds can be exposed to neuroblastoma cells in tissue culture through adsorption to agar particles. By electron microscopy numerous phagocytic vacuoles could be observed, and in many, debris probably representing agar was readily seen. Close agreement was seen between triplicate flasks with regard to the capacity for (^{3}H)-norepinephrine uptake, suggesting that cell exposure was relatively uniform.

IMR-32 neuroblastoma cells, derived from a human tumor, showed specific (^{3}H)-norepinephrine uptake (>90% inhibition by 10^{-5}M cocaine). The uptake of (^{3}H)-norepinephrine was reduced after a 3 hour exposure to all organophosphorus compounds tested. The greater effect of alkyl over aryl organophosphorus compounds suggests a relationship with solubility in water, thus the equilibrium between adsorption to agar and the surrounding medium or lysosomal contents.

The effect of parathion, which has not been demonstrated to cause delayed neurotoxicity in vivo, was intermediate, less than that for merphos, DEF, and EPN, but greater than that for leptophos and TOCP.

Two alternative explanations can be proposed for these results. One is that there is no relationship between reduced (^{3}H)-norepinephrine uptake and the capacity of a given organophosphorus compound to cause delayed neurotoxicity. The other is that parathion might be capable of causing delayed neurotoxicity but cannot be demonstrated to do so in vivo because of the severe cholinergic poisoning caused by this compound.

Electron microscopic observations suggest that further study may define a morphologic lesion peculiar to the clinically delayed neurotoxic organophosphorus compounds. Cells exposed to leptophos and DEF were found to contain tightly packed bundles of microfilaments (actin). This finding was not present in control or parathion-exposed cells, yet should be viewed with caution until a more complete electron microscopic study can be completed.

Publication: Graham, D.G., Lee, J.S., and Abou-Donia, M.B., "The use of human neuroblastoma cells in tissue culture in the study of organophosphorus-induced delayed neurotoxicity," To be presented at the annual meeting of the Society of Toxicology, Washington, D.C., March 10, 1980.

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Principal Investigator

December 12, 1979

TABLE I

The Effect of Agar-Adsorbed Organophosphorus Compounds on (^{3}H) Norepinephrine Uptake by IMR-32 Neuroblastoma

Compound	(^{3}H) norepinephrine uptake (cpm/mg protein ± SEM)	% Inhibition	P
1. Control	76,271 ± 1,565	-	-
Leptophos	63,629 ± 3,890	16.6	0.05
EPN	47,548 ± 1,286	37.7	0.001
2. Control	203,627 ± 24,081	-	-
DEF	110,625 ± 4,580	45.7	0.01
Merphos	76,357 ± 9,415	62.5	0.01
3. Control	120,555 ± 1,566	-	-
TOCP	84,942 ± 4,391	29.5	0.005
Parathion	75,360 ± 1,896	37.5	0.001

LEGENDS TO FIGURES

Fig. 1. Control cells. The IMR-32 human neuroblastoma line grows well in tissue culture. This figure shows the cytoplasmic vacuolization which is seen when these cells are exposed to medium containing agar particles (left side of figure). On the right are portions of 3 dead cells in among well-preserved cells, illustrating that in tissue culture there is continuous cell turnover and cell death. X12,500.

Fig. 2. Control cells. Along with occasional dead cells, evidence that these cells are replicating is regularly observed as here where a cell is found in mitosis (telophase). Vacuoles resulting from exposure to agar particles are seen in the lower right corner. X21,000.

Fig. 3. Control cells. By electron microscopy one can see what is readily apparent by phase contrast microscopy, that these cells send out cytoplasmic processes which come into close apposition with each other. One in the bottom center contains neurotubule-like structures. Two in the upper portion of the figure contain vacuoles in which the debris may represent agar particles. X42,500.

Fig. 4. Control cells. The ribosome-rich cell below contains cytoplasmic vacuoles. Processes from neighboring neuroblastoma cells come into close contact with the cell body and each other. The larger process above contains neurotubule-like structures and a less well preserved dense core vacuole. X42,500.

Fig. 5. Parathion-exposed cells. The vacuoles in the cytoplasm of these cells contain lacy ingested agar particles. X12,500.

Fig. 6. Parathion-exposed cells. Two neuroblastoma cells with intact nuclear and cytoplasmic structures are seen here. In the cell on the right, neurotubule-like structures run roughly parallel to the plasma membrane. The cell on the left contains agar particles in cytoplasmic vacuoles. X42,500.

Fig. 7. TOCP-exposed cells. As in control cultures scattered dead cells are observed among cells with no evidence of cell injury.

Fig. 8. EPN-exposed cells. A dead cell (upper center) is seen among well-preserved neuroblastoma cells, one of which is dividing (left center). An agar particle, to which EPN was adsorbed in this experiment, can be seen in the lower right corner. X12,500.

Fig. 9. EPN-exposed cells. One of the cytoplasmic processes (neurites) in the upper portion of this figure contains the dense core vacuoles typical of catechola mine-storage vesicles. Additional dense core vesicles are seen in the lower right along with neurotubule-like structures, ribosomes and mitochondria. X42,500.

Fig. 10. Leptophos-exposed cells. Cytoplasmic vacuoles reflecting phagocytosis of leptophos - containing agar particles can be seen in most of the cells. The cell on the left with islands of chromatin is dividing, while on the right a cell is seen with the pyknotic nucleus characteristic of cell death. X12,500.

Fig. 11. Leptophos-exposed cells. The cell in the lower right contains numerous cytoplasmic vacuoles. A cytoplasmic process (left center) contains many dense core vesicles. X21,000.

Fig. 12. Leptophos-exposed cells. The cell in the lower half contains numerous cytoplasmic vacuoles. In the upper right the neuroblastoma cell contains a densely packed bundle of microfilaments. X42,500.

Fig. 13. Leptophos-exposed cells. The cell on the right contains cytoplasmic vacuoles plus filaments and neurotubule-like structures. A portion of a cell containing bundles of microfilaments is seen in left center. X42,500.

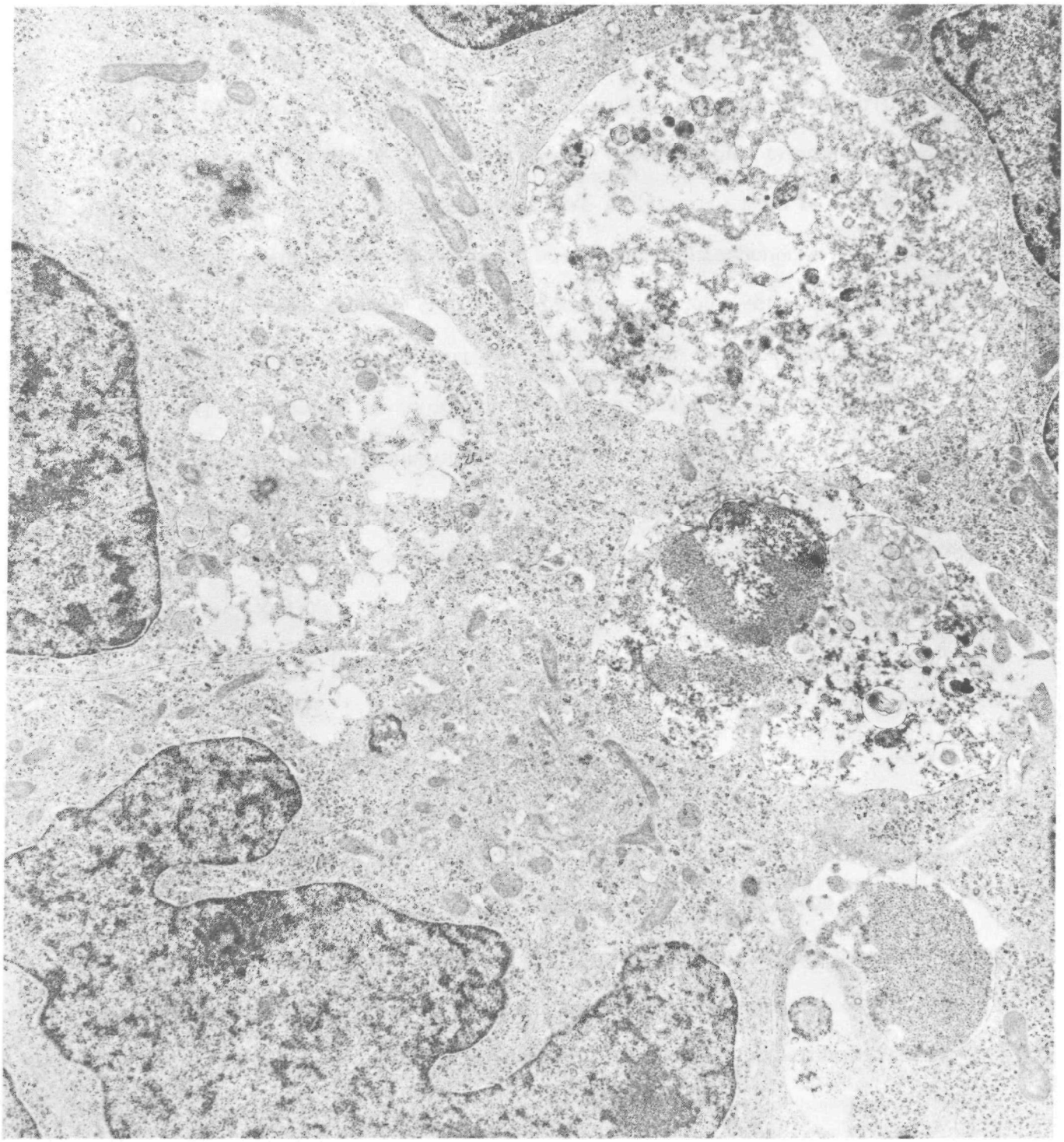
Fig. 14. Leptophos-exposed cells. High magnification detail of dense core granules reveals the morphology of catecholamine-containing vesicles. X70,000

Fig. 15. DEF-exposed cells. A dividing cell is observed in the lower left corner. The cells contain cytoplasmic vacuoles. Two cells contain bundles of tightly packed neurofilaments. X12,500.

Fig. 16. DEF-exposed cells. Cells have debris-containing cytoplasmic vacuoles, dense core granules, and neurotubule-like structures. X42,500.

Fig. 17. Merphos-exposed cells. A mixture of vacuole-containing living cells and dead cells is seen. X12,500.

Figure 1



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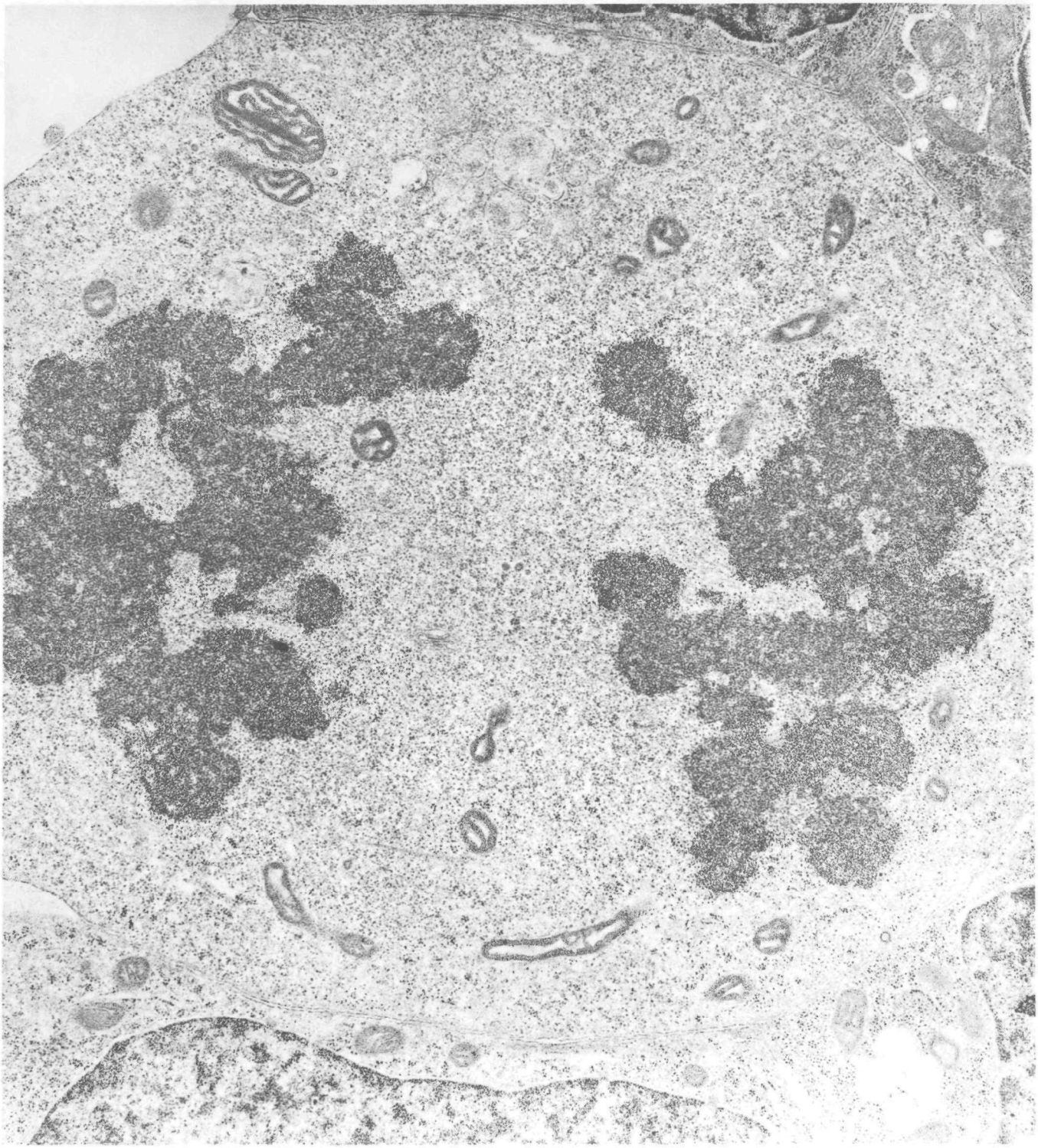

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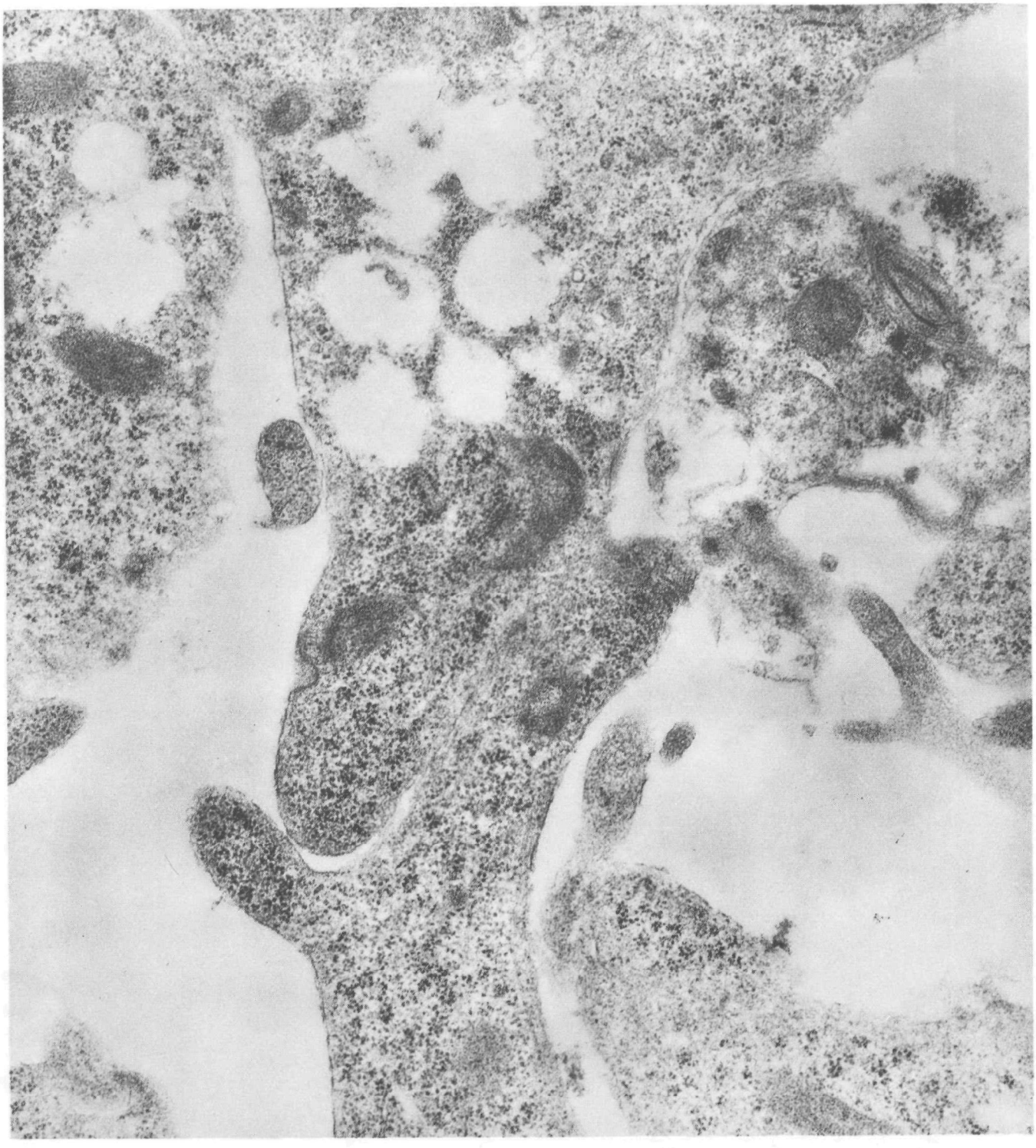

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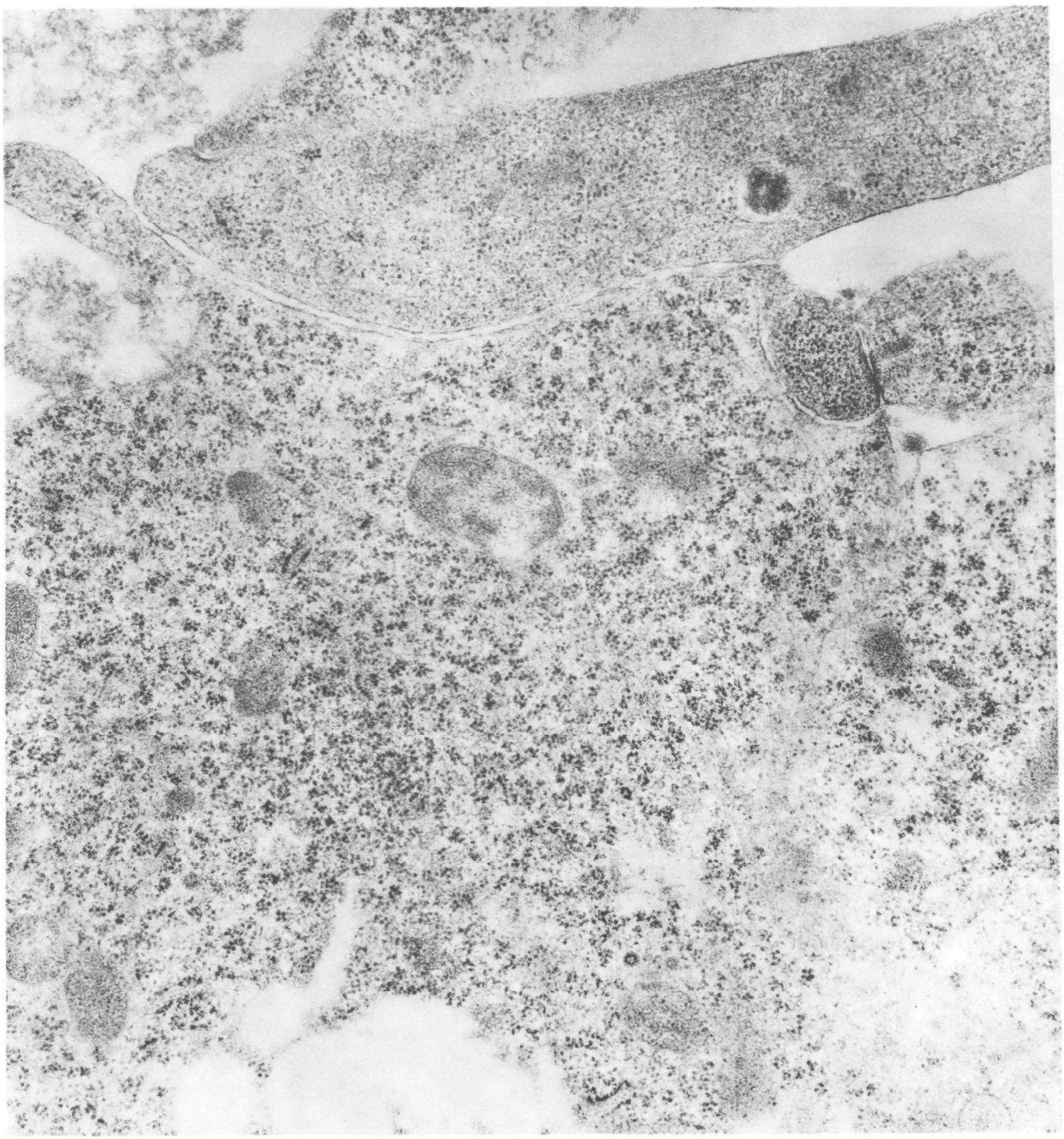

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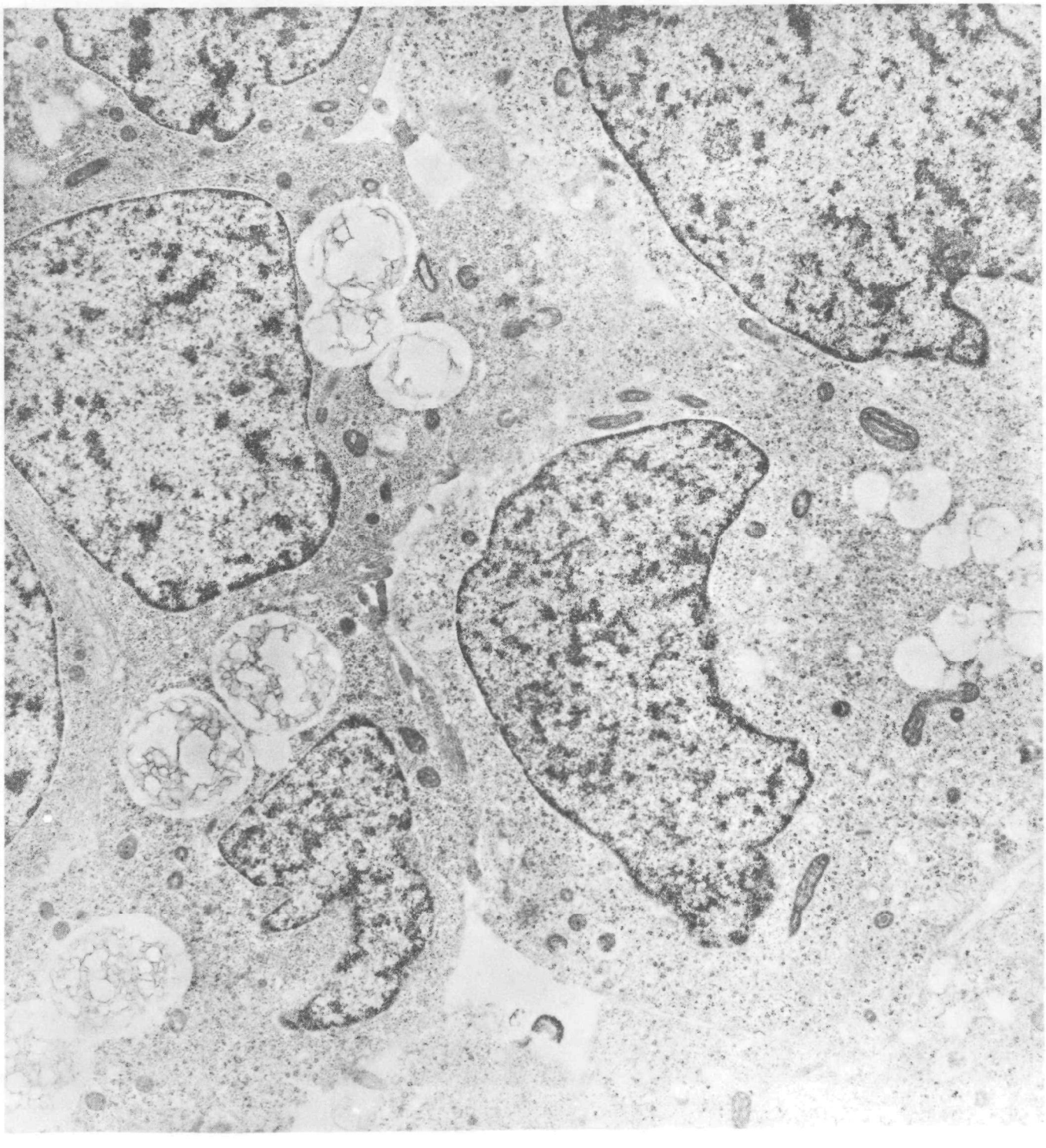

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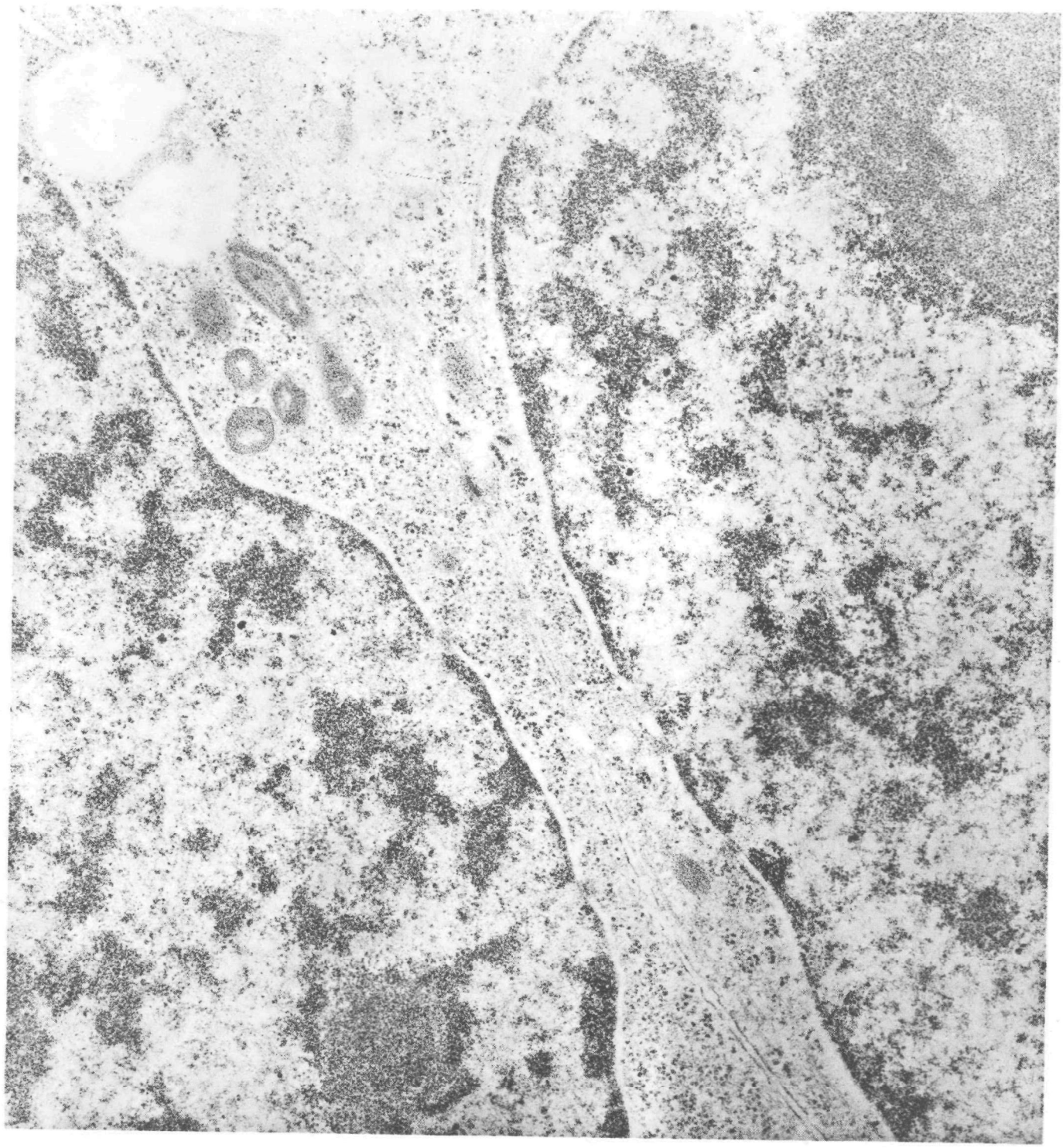

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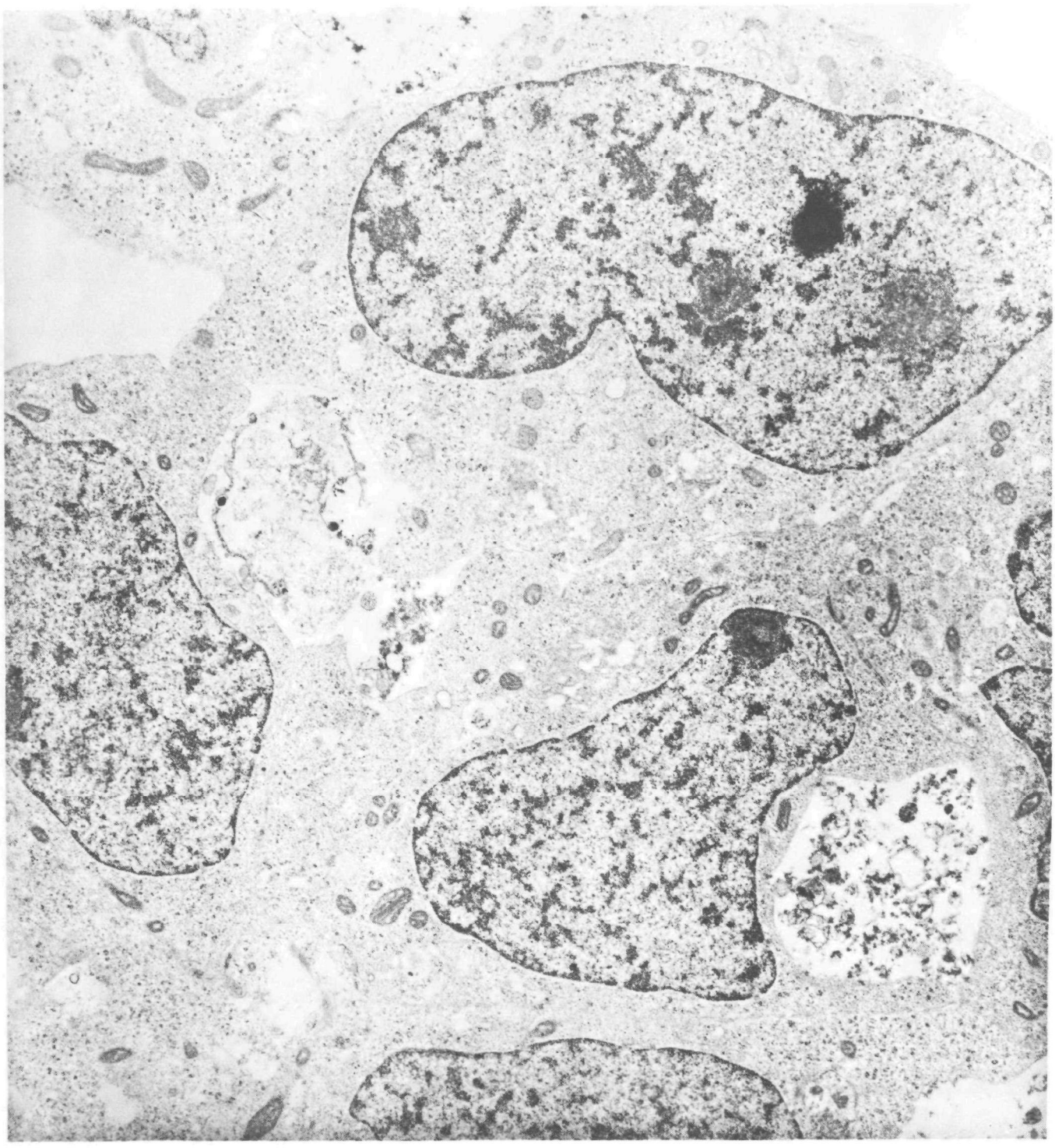

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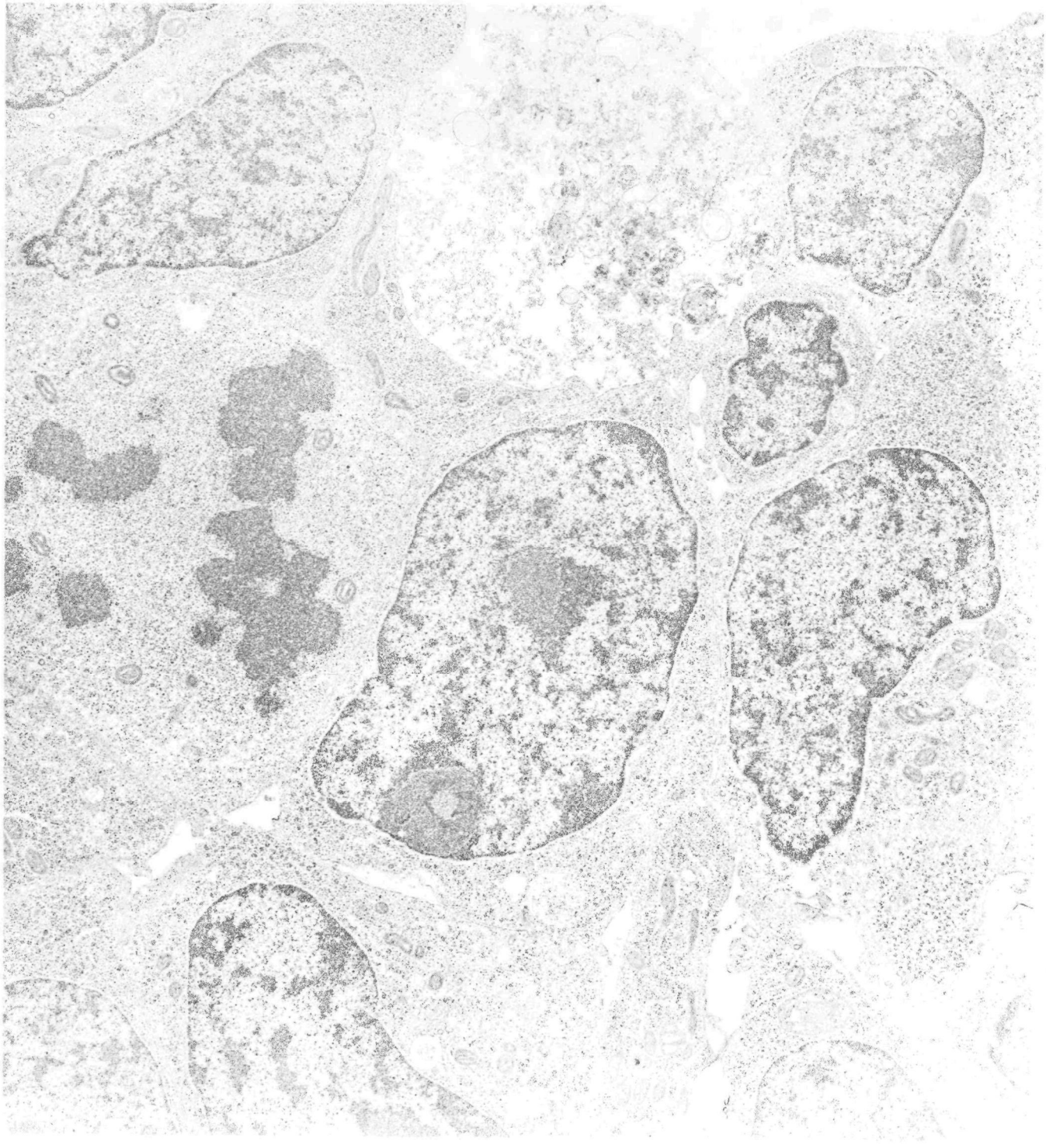

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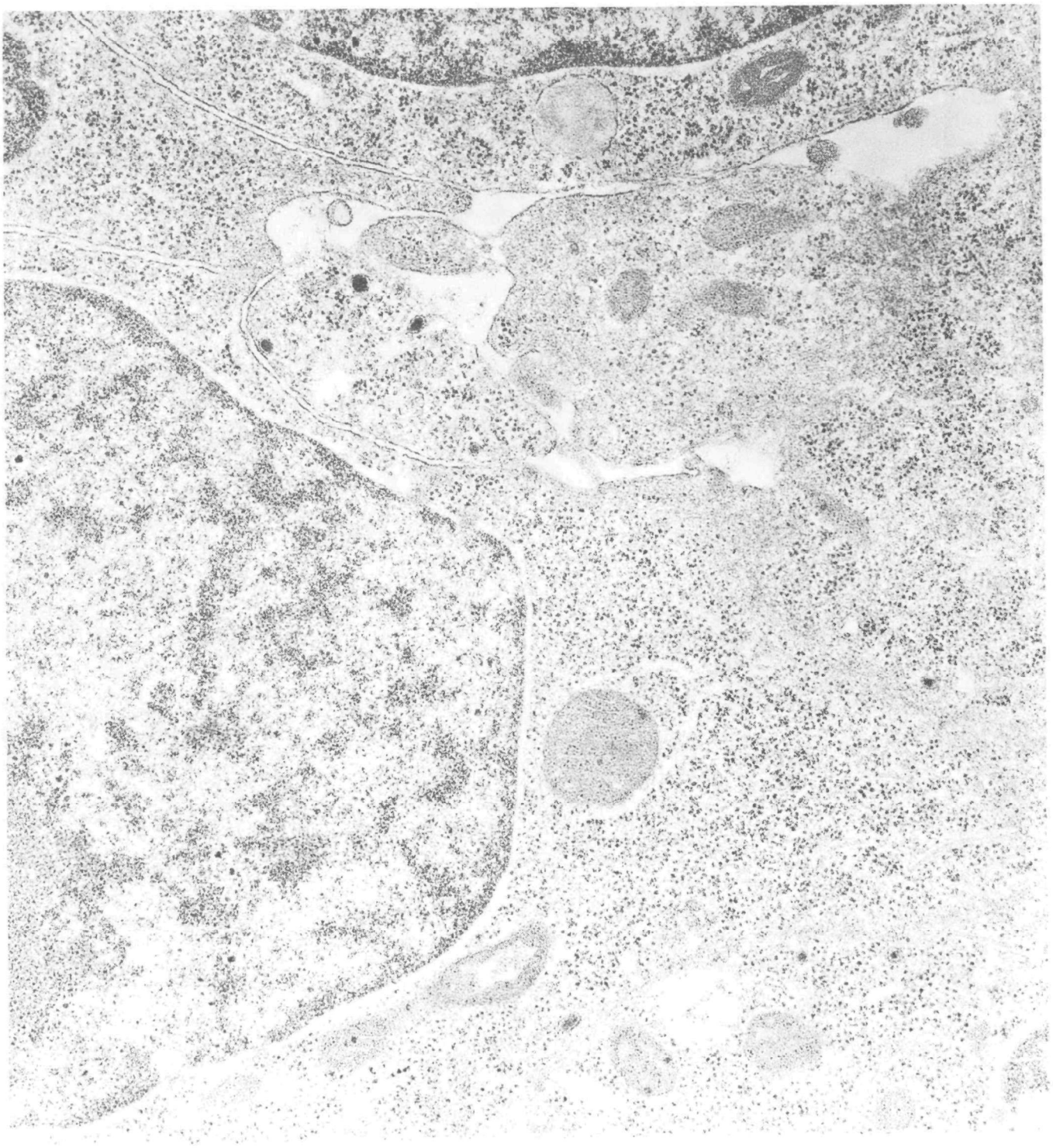

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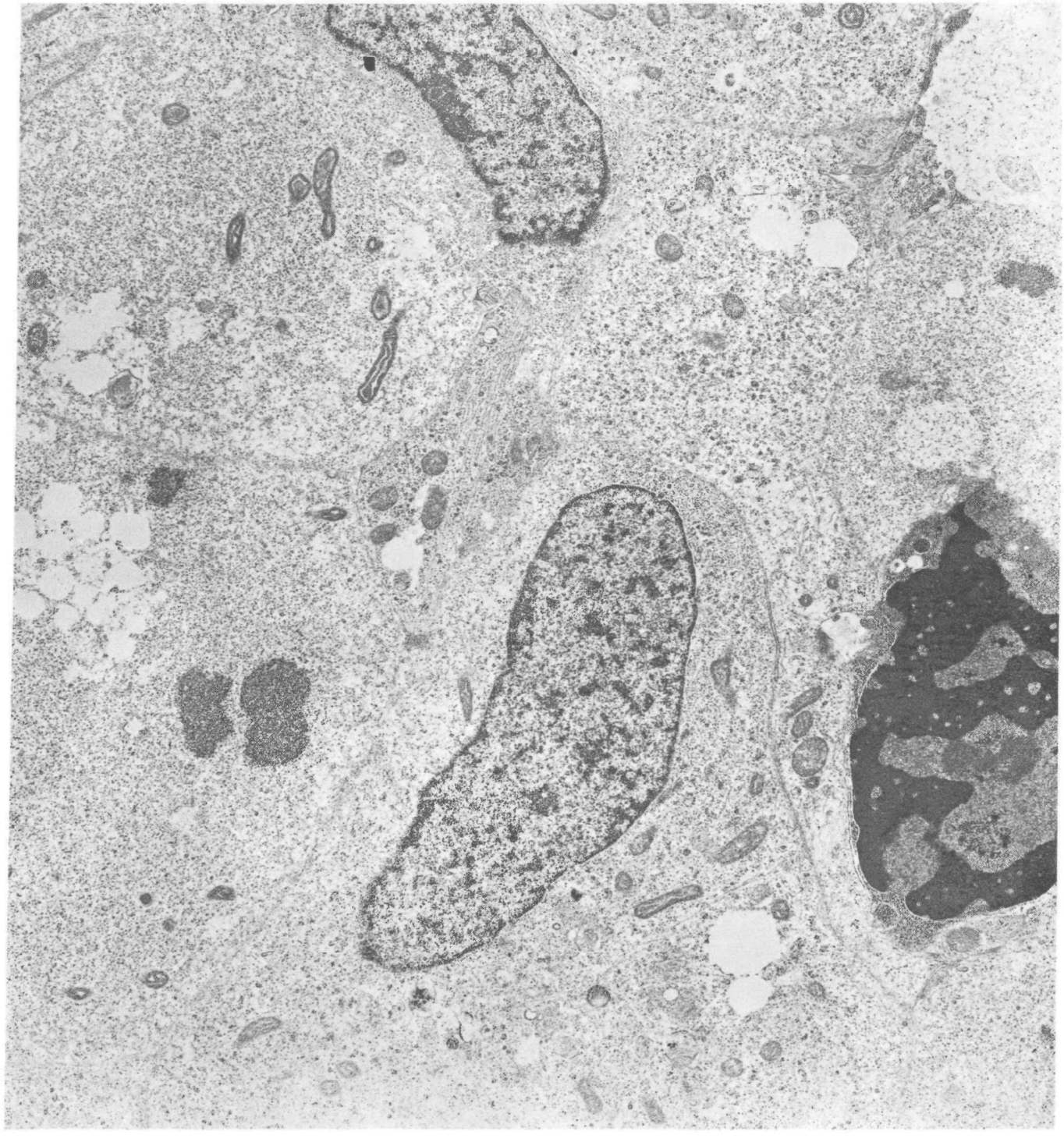

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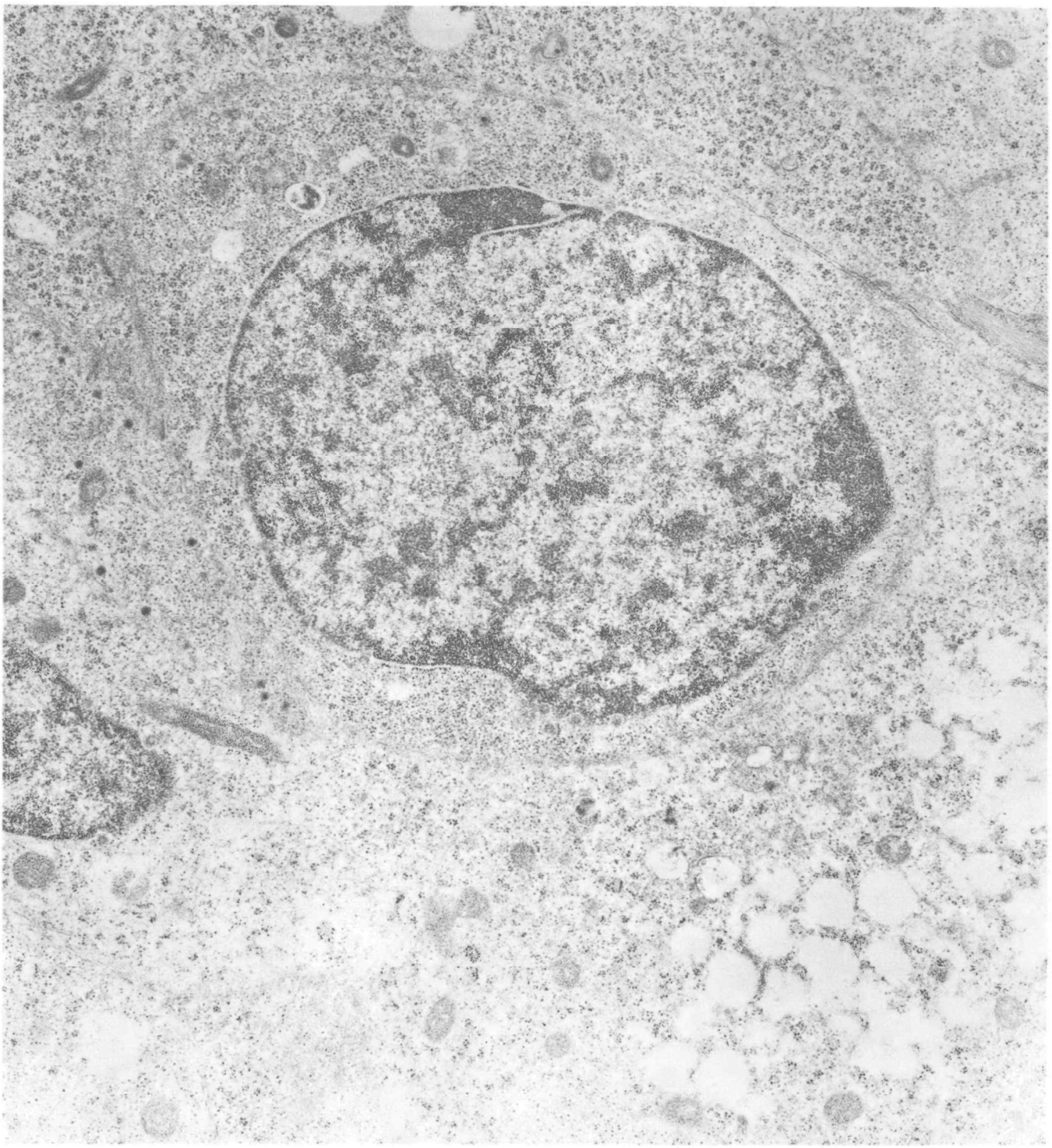


Figure 12

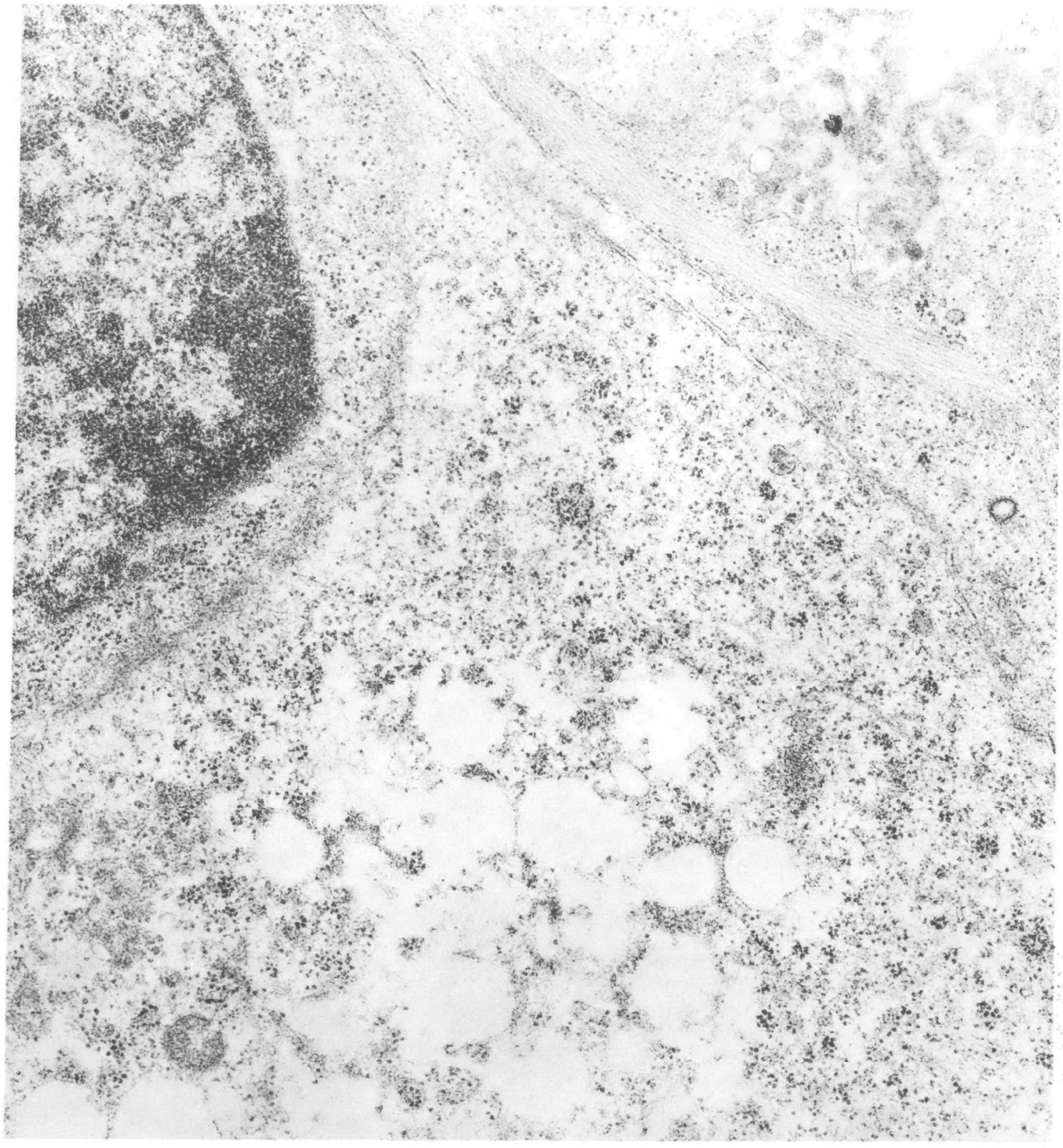
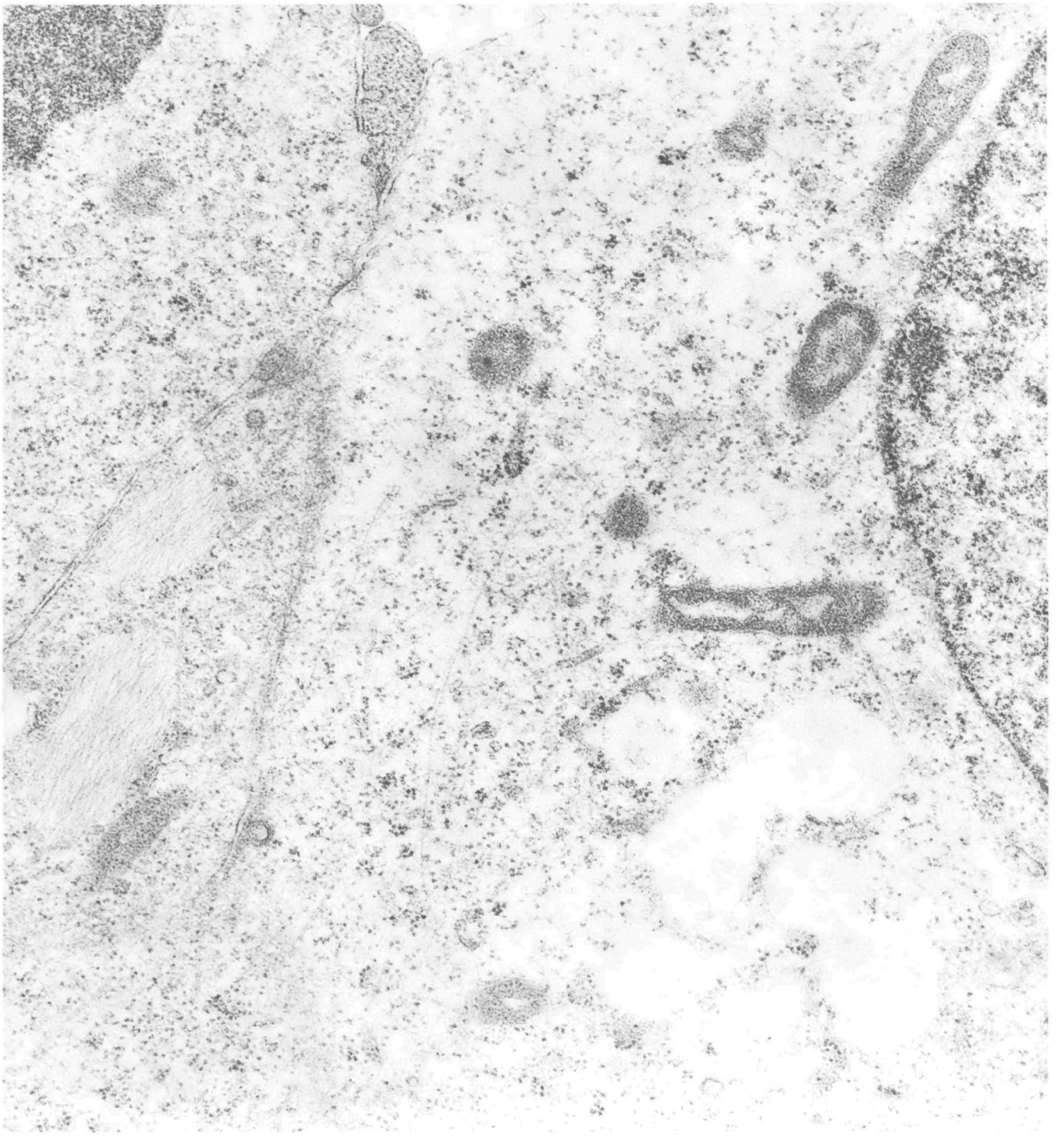


Figure 13



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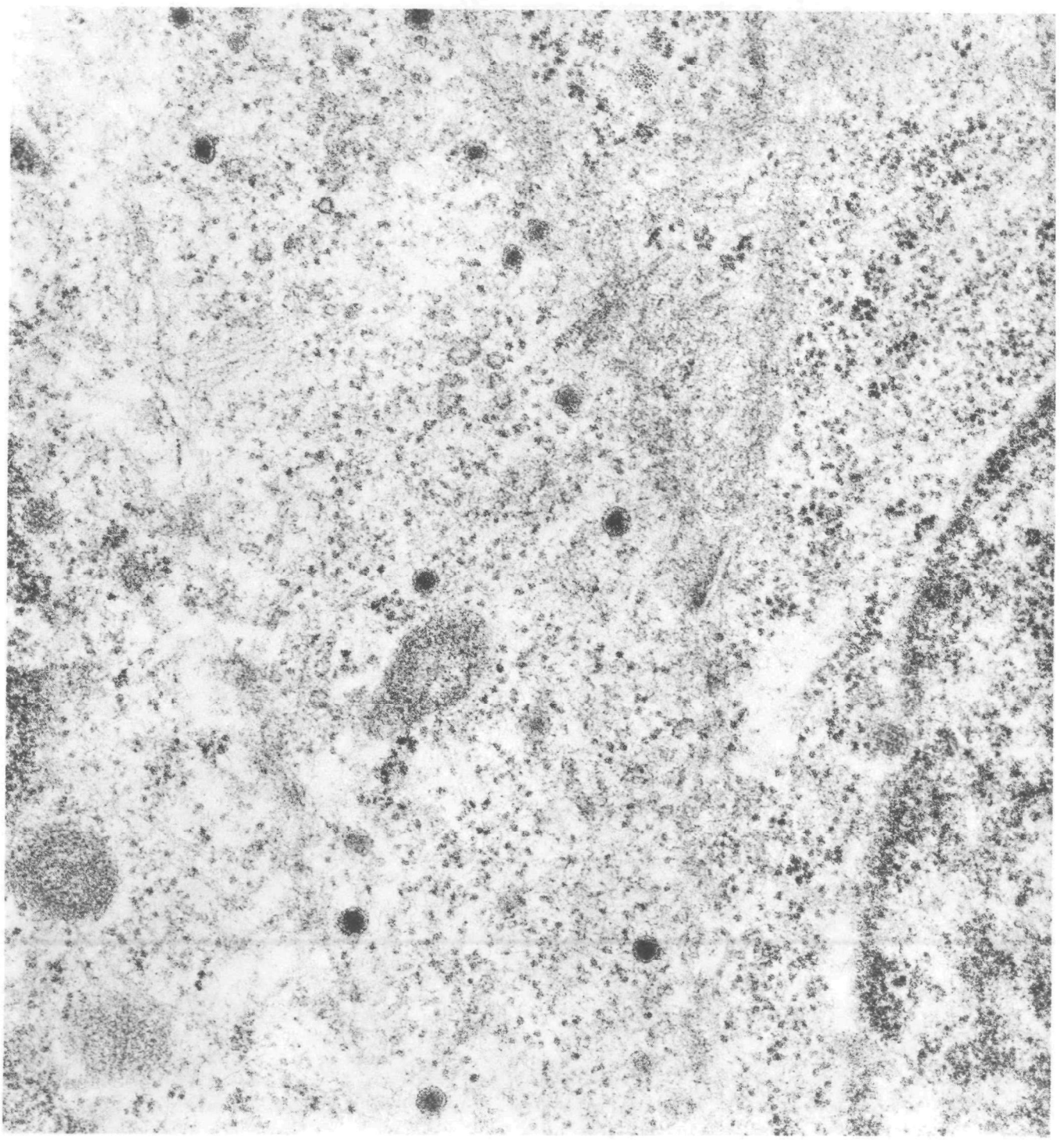
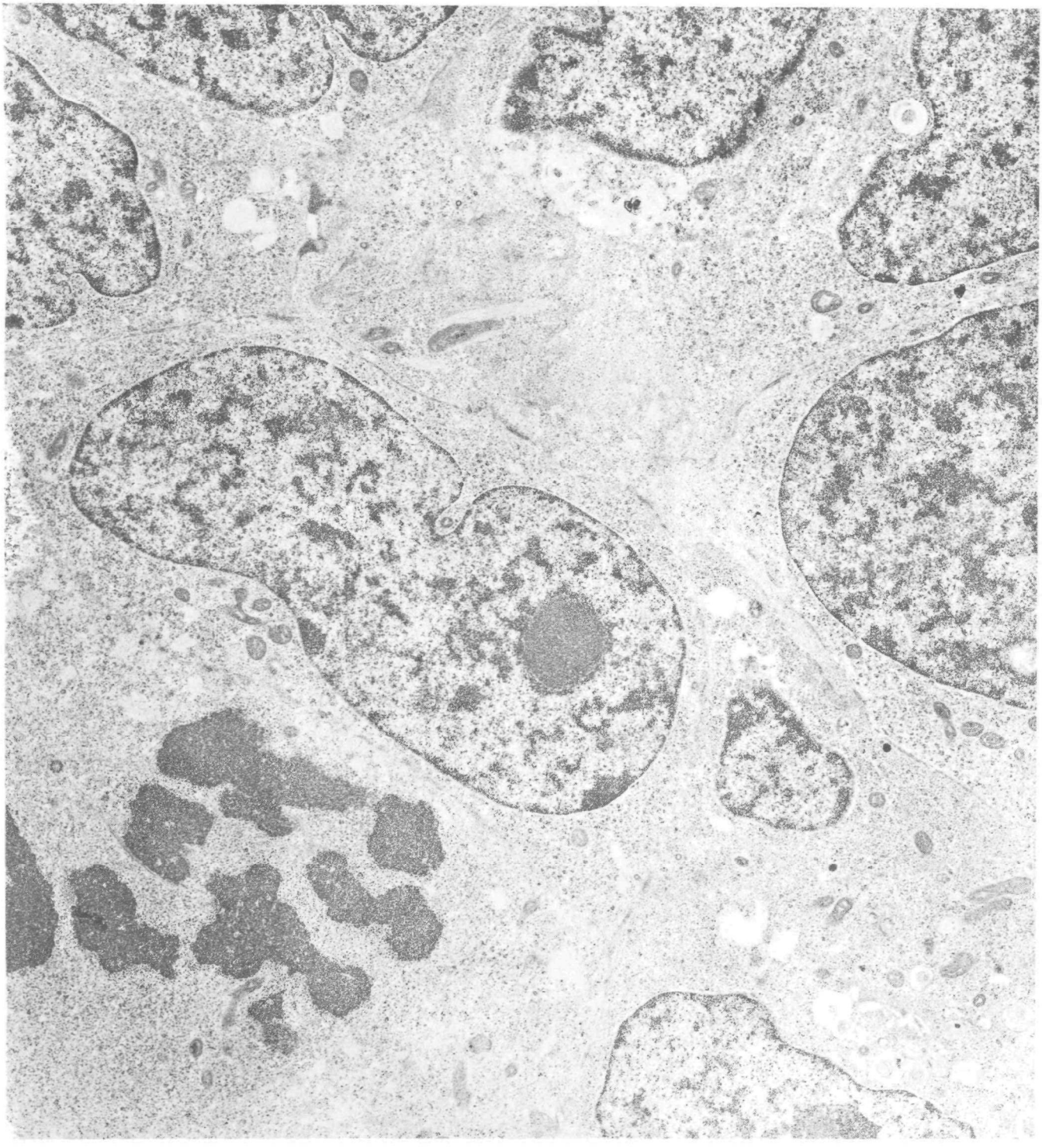


Figure 15



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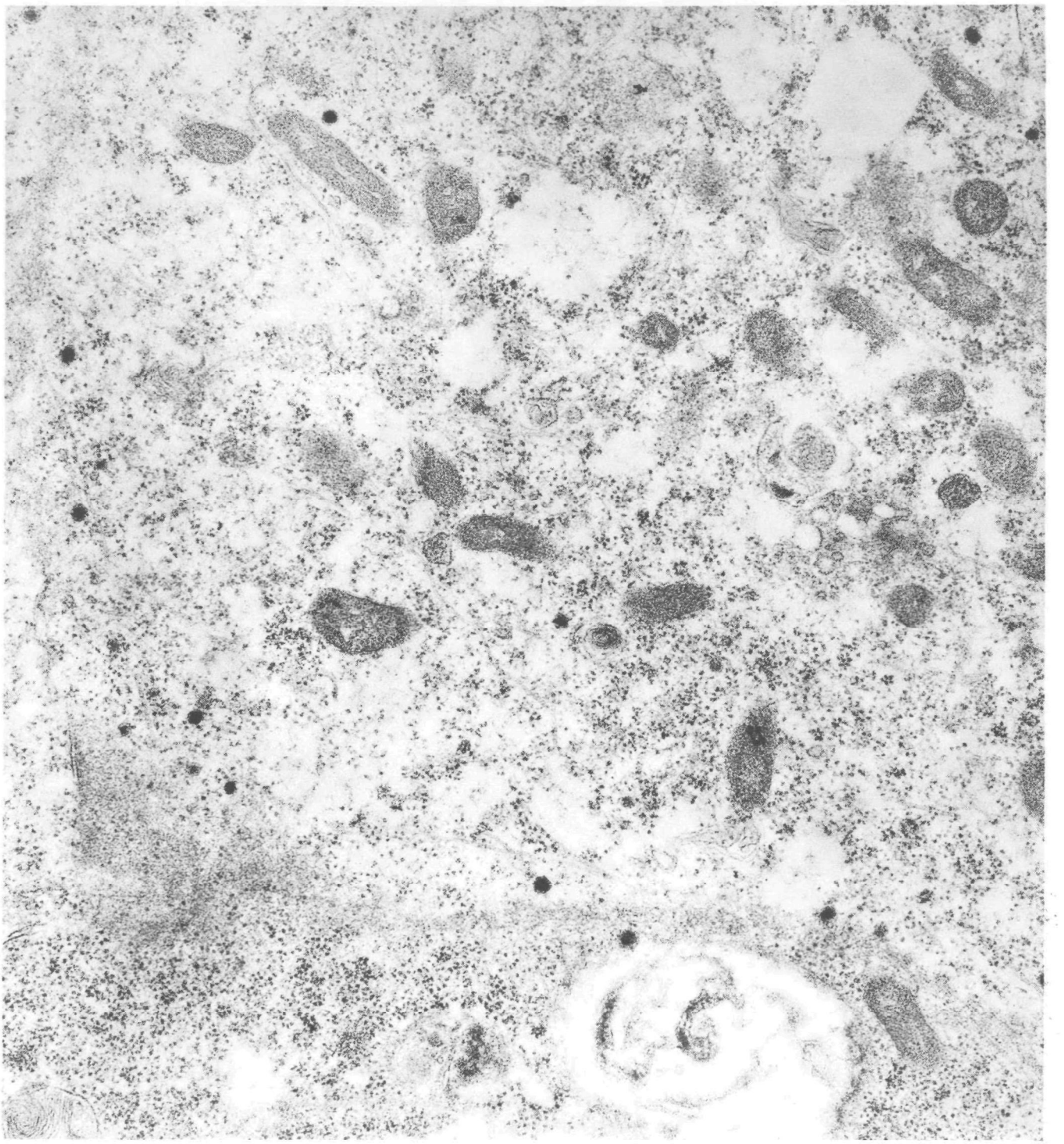

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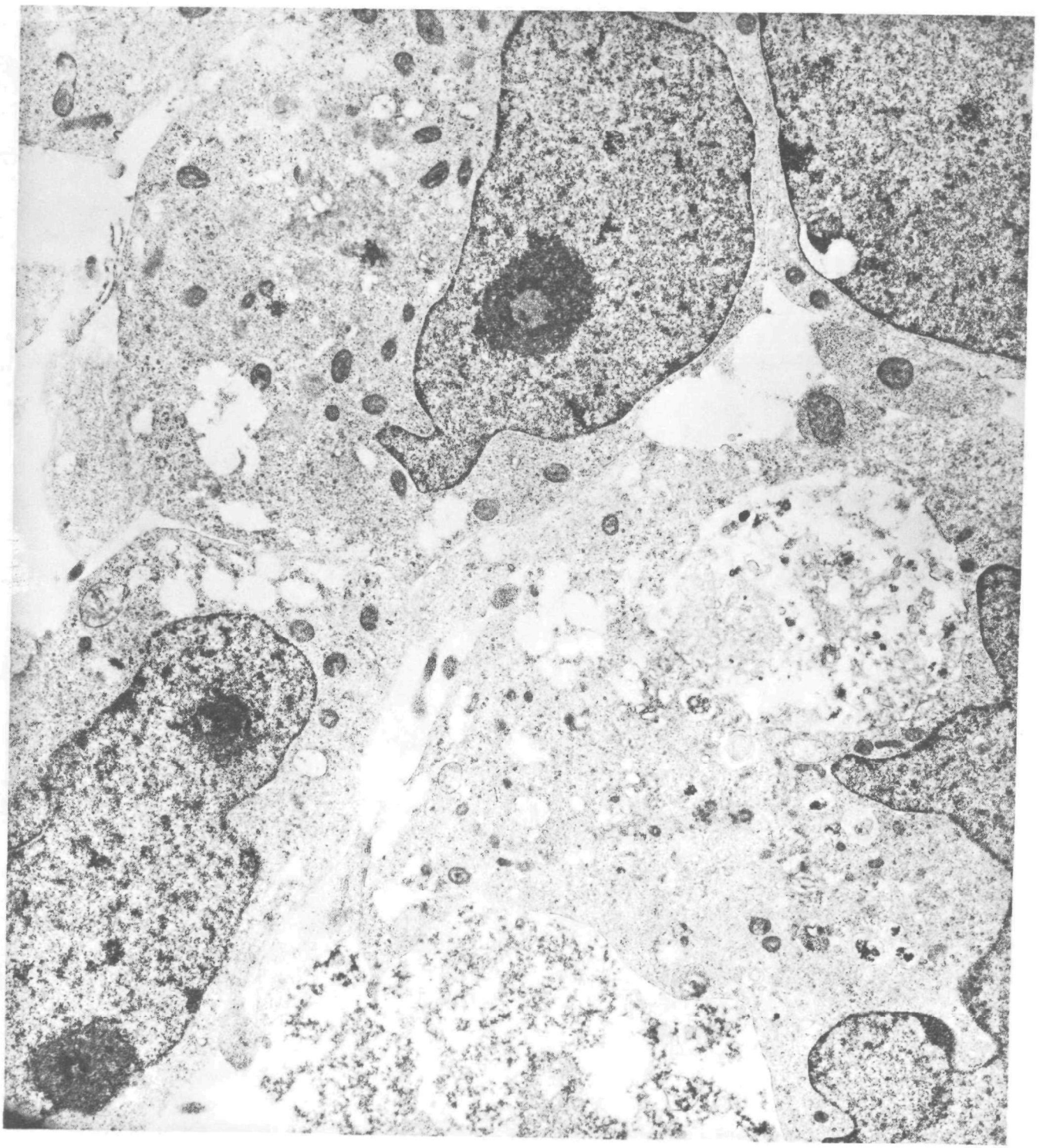

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16. ABSTRACT

An in vitro model utilizing neuroblastoma cell lines was developed for the differentiation of organophosphorus compounds which have the potential for induction of delayed degeneration of peripheral nerves and the spinal cord. IMR-32 neuroblastoma cells, derived from a human tumor, showed specific (3H)-norepinephrine uptake. The greater effect of alkyl over aryl OP compounds suggests a relationship with water solubility, thus equilibrium between adsorption to agar and the surrounding medium or lysosomal contents. The effect of parathion was intermediate, less than that for merphos, DEF and EPN but greater than that for leptophos and TOCP. Electron microscopic observations suggest further studies to define a morphologic lesion peculiar to the clinically delayed neurotoxic OP compounds.

17. KEY WORDS AND DOCUMENT ANALYSIS		
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