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# Studies on the biology of the neuroactive amines; a morphologic and pharmacologic approach

Herbert Y. Meltzer  
*Yale University*

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STUDIES ON THE BIOLOGY OF THE  
NEUROACTIVE AMINES  
A MORPHOLOGIC AND PHARMACOLOGIC APPROACH

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HERBERT Y. MELTZER

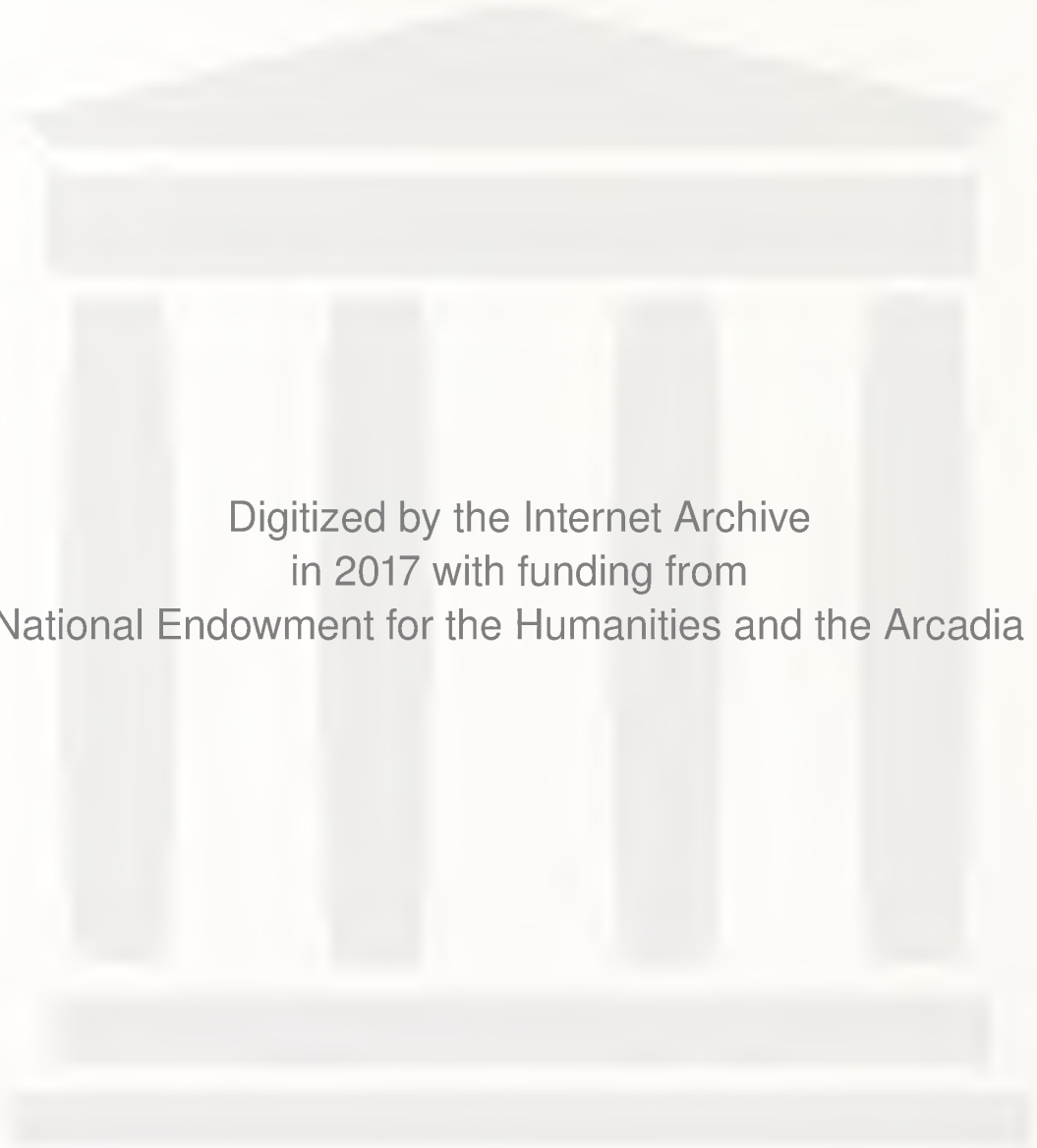
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STUDIES ON THE BIOLOGY OF THE NEUROACTIVE AMINES:  
A MORPHOLOGIC AND PHARMACOLOGIC APPROACH

Herbert Y. Meltzer

A thesis presented to the faculty of the  
Yale University School of Medicine  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Medicine  
1963





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The author gratefully acknowledges the indulgence and support of Drs. R.J. Barnett, N.J. Giarman, D.X. Freedman, and J.P. Green who permitted this young investigator to explore his own ideas in their excellent laboratories. This thesis is dedicated to the memory of my late father, Mr. David Meltzer.

The above is a list of the names of the  
persons who have been appointed to the  
positions of the various departments of  
the Government of the State of New York.  
The names are given in the order in  
which they were appointed, and the  
dates of their appointments are given  
in parentheses. The names of the  
persons who have been appointed to the  
positions of the various departments of  
the Government of the State of New York  
are given in the order in which they  
were appointed, and the dates of their  
appointments are given in parentheses.

# CONTENTS

	<u>Page</u>
PREFACE	1
INTRODUCTION TO MAST CELL STUDIES	3
Outline of Experimental Investigations	4
The Mast Cell	7
Morphology of the Mast Cell	8
The Pharmacology of the Mast Cell	13
Mast Cell Heparin and Its Particulate Nature	13
Mast Cell Histamine and Its Particulate Nature	14
Mast Cell 5-hydroxytryptamine and its Particulate Nature	15
The Release of Mast Cell Amines	15
Other Constituents of Mast Cells	21
Synthesis and Storage of Amines	21
Function of the Mast Cells	22
Reserpine	23
Chlorpromazine	26
The Dunn-Potter Mouse Mastocytoma	27
METHODS	29
Growth of Ascites Cells	29
Growth of Tissue Culture Cells	29
Reserpine Treatment	29
Chlorpromazine Treatment	30
Light and Phase Microscopy	30
Electron Microscopy	30
Isolation of Subcellular Components from the Ascites Cells	31
Isolation of Subcellular Components from the Tissue Culture Cells	33
Determination of 5-HT	34
Determination of Histamine	34
Determination of Heparin	35
Determination of Succinoxidase	35

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	<u>Page</u>
Attempts to Demonstrate Monoamine Oxidase Activity in the Dunn-Potter Mastocytoma	35
Technique of Cotzias and Greenough	35
Technique of Weissbach	36
Attempt to Demonstrate Histaminase Activity in the Dunn-Potter Mastocytoma	36
 RESULTS AND DISCUSSION	
The Ascites Dunn-Potter X-1-C Mastocytoma Cell	37
The Origin of the Mast Cell Granules	38
The Tissue Culture Dunn-Potter X-1-C Mastocytoma Cell	42
Effects of Reserpine on the Tissue Culture Mastocytoma Cells	48
Effects of Chlorpromazine on the Tissue Culture Mastocytoma Cells	58
The Large Granule Fraction of the Tissue Culture Cells	60
Isolation of Mast Cell Granules from the Ascites Cells	61
Monoamine Oxidase and Histaminase	65
 SUMMARY	67
 ELECTRON MICROGRAPHS	70
 INTRODUCTION TO MONOAMINE OXIDASE STUDIES	100
 MATERIAL AND METHODS	110
 EXPERIMENTAL RESULTS	112
Effect of Pyridine Aldoxime Dodecyl iodide (PAD) on the MAO Activity of Homogenates of Liver and Brain of Various Species	112
Effect of PAD on MAO Activity of Washed Rat Liver Mitochondria and Comparison of PAD with other Inhibitors of MAO	113
Effect of Preincubation on the Inhibition of Rat Liver Mitochondrial MAO by PAD	114



	<u>Page</u>
Effect of Dialysis on the Inhibition of Rat Liver Mitochondrial MAO by PAD	115
Effect of PAD on Solubilized MAO	116
Ability of PAD to Inhibit MAO <u>in vivo</u>	117
Effect of PAD on the Levels of 5-HT in Rat Brain	118
Effect of PAD on the Catecholamine Levels of Rat Brain	119
Effect of PAD on 5-hydroxytryptophan decarboxylase of rat kidney <u>in vitro</u>	119
Effect of PAD on 5-HT of Mast Cells	119
Effect of PAD and JB-516 on levels of 5-HT in Rats After Administration of Reserpine	121
Effect on Rats of Pretreatment with PAD followed by Reserpine and Pretreatment with Reserpine Followed by PAD.	122
Effects of Various Quaternary Ammonium Derivatives on MAO <u>in vitro</u>	123
<u>In vivo</u> and <u>In vitro</u> Studies with Thiamine and MAO	123
Effect of Dodecyl Iproniazid on MAO <u>in vitro</u>	124
Ability of Dodecyl Iproniazid Iodide to Inhibit MAO <u>in vitro</u>	125
Effect of Dodecyl Iproniazid on the Levels of 5-HT in Rat Brain	125
DISCUSSION	126
PAD as an Inhibitor of Monoamine Oxidase	126
PAD and Amine Release	131
Dodecyl Iproniazid Iodide	134
The Effect of Thiamine on Monoamine Oxidase	135
SUMMARY	139
BIBLIOGRAPHY	142





PREFACE

The biology of the neuro-active amines has occupied a large portion of the imagination and energy of pharmacologists and physiologists investigating the vertebrate nervous system in the last decade. The basis for this concentration of interest are studies which implicate these amines in neurotransmission and, beyond that, through many derangements in their disposition from synthesis to destruction, in mental and nervous disorders. The investigations described and discussed in this thesis explore certain aspects of this class of compounds and the tissues which possess them.

In brief outline, with several simplifications, the life cycle of a neuro-active amine [e.g., 5-hydroxytryptamine (5-HT), histamine, norepinephrine (NE)] is: synthesis by decarboxylation of its precursor amino acid, storage in membrane-bound granules, release in response to various stimuli, action at the receptor site, and then either degradation by one or more enzymes or reabsorption by a neurosecretory cell for re-use.

The investigations in this work were concerned with: (i) the fine structure of the Dunn-Potter murine mast cell tumor grown in tissue culture and as ascites cells in the mouse peritoneal cavity; the origin of the amine granules of these cells and how they get the amines they contain; the mechanism of amine release by reserpine; the morphologic correlates of chlorpromazine action; the isolation of the amine-containing granules from the ascites mastocytoma cells; the absence of monoamine oxidase (MAO), an enzyme that has

RESULTS

The history of the present study has been  
 - a large number of the investigators and many of the  
 - authors and investigators involved in the work  
 - have been in the past decade. The work for the  
 - construction of the present and future which is the  
 - basis of the present study, beyond that, through way  
 - developments in their disciplines, the results in their  
 - work, in general and various directions. The investigation  
 - described and discussed in this paper is one of the  
 - of this work. It is hoped that the present and future work  
 - in their fields, with several disciplines, the  
 - parts of a two-part series, (I) and (II), (I) and (II)  
 - (I) and (II), (I) and (II), (I) and (II), (I) and (II)  
 - description of the present work, (I) and (II), (I) and (II)  
 - and the other parts, (I) and (II), (I) and (II), (I) and (II)  
 - than by one or more studies or investigations by a researcher  
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 (1) the structure of the present work and call  
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 - the absence of research (I) and (II), or any other

been implicated in the degradation of several biogenic amines, and histaminase, the enzyme which oxidizes histamine, in these cells; (ii) the synthesis and study of a new class of inhibitors of MAO; and (iii) the relationship between the vitamin, thiamine, and MAO. The studies on the mastocytoma cells and the MAO studies will be presented separately for the sake of convenience and clarity.

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INTRODUCTION

The discoveries that histamine is found in the mast cells of a myriad of species,<sup>1</sup> that 5-hydroxytryptamine is found in the mast cells of the rat and mouse,<sup>2,3</sup> and that these amines are largely bound in granules<sup>4</sup> made this cell of great interest to the student of amine biology. It meant that the mast cell, along with the cells of the adrenal medulla and the blood platelets could serve as a model system for the amine biology of the vertebrate nervous system whose complexity makes a direct experimental approach extremely difficult. Thus, information about amine synthesis, destruction, binding, release, regulation, and the influence of hormones and drugs is much more easily obtained from the mast cell than from the vertebrate nervous system. And with the economy of mechanism that nature practices, it seems reasonable to hope that the information obtained from the mast cells would be of value in understanding many of the features of amines in the nervous system. This has indeed proven true. Investigators throughout the world have explored the mast cells with particular attention to their amines. It need not be stressed that the mast cell has great intrinsic interest in its own right.



Outline of Experimental Investigations

The Yale Medical School Department of Pharmacology has been very active in the study of mast cells. The Dunn-Potter mouse mastocytoma<sup>5</sup> which has continued to produce histamine, 5-HT and heparin has been and is being studied by many people in that department. The author felt that these biochemical and pharmacologic studies could be greatly aided if the fine structure of these cells as seen at the level of resolution of the electron microscope could be established, because some of the studies undertaken have obtained a level of sophistication where function could not be divorced from structure. Indeed, even the simpler experiments could be more readily interpreted if the cells' morphology was known, for truly "one (electron microscopic) picture is worth a thousand words."

The Dunn-Potter mastocytoma can be grown as a solid tumor, as ascites cells in the mouse peritoneal cavity, or in tissue culture as a cell suspension. My first endeavour was to establish the fine structure of the ascites cell. It was then decided to use the tissue culture cells for studies using pharmacologic techniques and electron microscopy. The tissue culture cells are a pure population of mastocytoma cells whereas the ascites cells obtained from the mouse peritoneal cavity are really a mixture of mastocytoma cells, fibroblasts and leuokcytes. The tissue culture cells are more readily treated with drugs than the ascites cells and there is no interference from the host animal. The tissue culture cells were found to have a morphology significantly different from



Optical Microscopy of Biological Cells

The basic principle of light microscopy is that light rays are reflected or refracted by the specimen and then collected by the objective and eyepiece lenses to form a magnified image. The resolution of the microscope is limited by the wavelength of light used. The Rayleigh criterion states that two points are just resolved when the central maximum of one diffraction pattern coincides with the first minimum of the other. This means that the minimum distance between two points that can be distinguished is approximately half the wavelength of light. In the visible spectrum, this limits resolution to about 200 nm. Electron microscopy overcomes this limitation by using electrons instead of light. Electrons have a much shorter wavelength (about 0.025 nm for 100 kV) and therefore provide much higher resolution. However, electron microscopy is not suitable for studying living cells because the high vacuum and electron beam would kill them. Fluorescence microscopy is a technique that allows the study of living cells. It involves staining specific structures or molecules with fluorescent dyes. When these dyes are excited by light of a specific wavelength, they emit light of a longer wavelength. This emitted light is then collected and imaged. Fluorescence microscopy provides high contrast and can be used to track the movement of specific molecules in real time. Confocal microscopy is a type of fluorescence microscopy that uses a pinhole to eliminate out-of-focus light, resulting in sharp images of thin optical sections. This allows for 3D reconstruction of the specimen. Phase-contrast microscopy is another technique used for studying living cells. It exploits differences in the refractive index of various cell components to create contrast. This method does not require staining and is suitable for observing cell morphology and motility. Differential interference contrast (DIC) microscopy is another non-staining technique that provides high-contrast images of transparent specimens. It works by converting phase differences into amplitude differences, resulting in a pseudo-color image. Cryo-electron microscopy (cryo-EM) is a technique that allows for the determination of atomic structures of biological macromolecules. It involves freezing the specimen rapidly to preserve its natural state and then imaging it with a transmission electron microscope. Cryo-EM has revolutionized structural biology by allowing the determination of high-resolution structures of proteins and other macromolecules in their native state.

the ascites cells and indeed, from any other mast cell previously described, but I decided to continue my studies with this cell form, fully realizing that it is a very atypical cell. The tissue culture cells have been investigated by Furano<sup>6</sup> who attempted to separate the amine-containing granules from the mitochondria of these cells by density gradient centrifugation. I sought to check his separation by electron microscopy but was unable to reproduce this work. Instead, an unseparated large granule fraction of these cells was observed in the electron microscope.

Several studies were undertaken with drugs which release mast cell 5-HT because it was felt that if the process could be visualized in the electron microscope valuable information about the mechanism of amine release would be forthcoming. It was hoped that such basic questions as whether amine release requires disruption of the cell and release of granules or whether it can occur without loss of cytoplasmic granules or other visible cytologic change could be answered. I hoped to obtain information about the mode of action of the drugs used, reserpine and chlorpromazine.

Of special interest were pictures of ascites mastocytoma cells which show what are thought to be early forms of the mast cell granules. These suggested a new theory for the origin of these granules.

Several studies of these mast cells performed by the author prior to the electron microscope studies will be reported briefly because they are of interest in interpreting some of the electron microscopic findings; (i) separation by density gradient centrifugation of subcellular components,



particularly the amine-containing granules and the mitochondria of the ascites mastocytoma cells; (ii) the demonstration of the absence or extremely low activity in the Dunn-Potter mastocytoma of MAO and histaminase.



The Mast Cell

The discovery of the mast cell is attributed to Paul Ehrlich who, in 1877, while investigating the staining of various tissues with aniline dyes, noted in the connective tissues of various species, granule-containing cells which had the property of staining more violet or redder than the rest of the cells (i.e. metachromasia).<sup>7</sup> He named these cells "Mastzellen" or well-fed cells because their cytoplasm was so densely crammed with granules. Ehrlich described the light microscope morphology, staining properties and distribution of the mast cells, but nothing of note elucidating their physiology or function was accomplished for seventy years. In 1937, Jorpes in Sweden noted that heparin, whose anti-coagulant properties attracted great interest, stained metachromatically with toluidine blue.<sup>8</sup> Jorpes was then able to show that the mast cell content of various tissues was proportional to its concentration of heparin.<sup>1</sup> Another big advance was the finding by Rocha e Silva that after anaphylactic shock both heparin and histamine were released from dog liver.<sup>9</sup> This led to a number of studies which firmly established the presence of histamine in mast cells.<sup>1</sup> Many other constituents of mast cells have since been found, including 5-hydroxytryptamine but the latter only in the mast cells of the mouse and rat.<sup>2,3</sup>

The Cell

The discovery of the cell as a structural unit of life was made in 1665 by Robert Hooke, who, while investigating the structure of various tissues with a primitive microscope, observed that they were composed of various small, square, brick-like structures which he called "cells". The property of staining was first observed in 1828 by Matthias Schleiden and Rudolf Virchow, who established the cell theory. The cell theory states that all organisms are made up of cells, that the cell is the basic unit of structure and function, and that all cells arise from pre-existing cells. The discovery of the cell as a structural unit of life was made in 1665 by Robert Hooke, who, while investigating the structure of various tissues with a primitive microscope, observed that they were composed of various small, square, brick-like structures which he called "cells". The property of staining was first observed in 1828 by Matthias Schleiden and Rudolf Virchow, who established the cell theory. The cell theory states that all organisms are made up of cells, that the cell is the basic unit of structure and function, and that all cells arise from pre-existing cells.

## Morphology of the Mast Cells

The morphology of the mast cell as seen with the light or phase microscope has been reviewed by Asboe-Nansen.<sup>10</sup> Mast cells may vary within wide limits in size and shape depending on many environmental as well as intrinsic factors. They may be flat, spherical, spindle-shaped, stellate or filiform. The rounded mast cells have a diameter of 8-15 microns, while the elongated ones may be twice as long. The nucleus is usually centrally located, about 4-6 microns in size and round or oval. Cells with two nuclei are occasionally seen but segmented nuclei have never been found.

According to Asboe-Hansen, mast cell granules are almost always globular-shaped, rarely oval. Elliptic or rod-shaped granules have been seen and attributed to pressure exerted by adjacent granules. Early investigators found the granules to be 0.2-0.4 microns in size, although one investigator, using freeze-dried mast cells from the rat mesentery found granules ranging in diameter from 0.3-1.0 microns.

Little else could be determined about the morphology of the mast cell, with the light microscope. The first electron micrographs of mast cells and normal mast cell granules were reported by Asboe-Hansen<sup>11</sup> and Kóksal.<sup>12</sup> The techniques of electron microscopy were not very advanced at this time and little new information was obtained from these pictures. Kóksal, however, did demonstrate a definite boundary to the mast cell granules.<sup>12</sup>



Physiology of the Heart

The morphology of the heart cells is similar to that of the skeletal muscle cells.

On these characteristics have been reviewed by Abood and his colleagues.

These cells are very similar to those of the skeletal muscle cells.

They are striated and contain myofibrils as well as sarcoplasmic reticulum.

They are also striated, cylindrical, and branched.

The rounded end cells have a diameter of 10-15 microns.

These cells are elongated and are up to twice as long as wide.

The nucleus is usually centrally located, about 1-2 microns in diameter.

These cells are oval. Cells with two nuclei are occasionally seen but are rare.

They are branched and have been described as having a branching pattern.

According to Abood and his colleagues, these cells resemble the skeletal muscle cells.

They are striated, cylindrical, and branched.

These cells have been described as having a branching pattern.

Adjacent branches are connected by gap junctions.

The branching pattern is similar to that of the skeletal muscle cells.

The branching pattern is similar to that of the skeletal muscle cells.

The branching pattern is similar to that of the skeletal muscle cells.

These cells are similar to those of the skeletal muscle cells.

Little else could be determined about the morphology of these cells.

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The branching pattern is similar to that of the skeletal muscle cells.

The first electron micrographs of mast cells utilizing Palade's osmium tetroxide fixative<sup>13</sup> and thin-sectioning were obtained by Bloom et al. in Sweden.<sup>14</sup> The tissue studied was a dog mastocytoma which contained large amounts of heparin and histamine. The cells had the typical granular appearance of non-neoplastic mast cells. They were about 10 microns in size, round or oval in shape. The nucleus occupied from one-fourth to one-third of the cell volume and varied in shape from round to kidney configuration. The nucleus was surrounded by the usual nuclear membrane and had one or two nucleoli. The cytoplasm was filled with granules about 0.7 microns in size. Mitochondria with the usual fine structure were noted in mast cells for the first time. Some cells had large vacuoles in the cytoplasm. The cell surface had a great number of small filamentous cytoplasmic protrusions about 0.6 microns long and 0.08 microns wide.

Excellent electron micrographs of normal mouse mast cells were published by G.E. Rogers in 1956.<sup>15</sup> Phase microscopy and various histochemical techniques were used to demonstrate that certain granule-containing cells seen in the dorsal skin of three day-old mice were mast cells. The mast cell granules were quite electron dense and irregular in shape. Their size varied from 0.3 to 1.0 microns but were usually 0.5 microns wide. The number of granules per cell varied considerably but unlike most other mast cells that have been studied, did not occupy a large area of the cytoplasm of the cells. Each granule is usually located in a clear area in the cytoplasm which has no definite limiting membrane. The interior of the granule has

The first electron micrograph of a cell is shown in Figure 1. The cell is a typical eukaryotic cell with a nucleus and a large central vacuole. The nucleus is located in the upper right corner of the cell and contains a dense nucleolus. The large central vacuole occupies most of the lower half of the cell. The cell membrane is clearly visible as a thin line surrounding the cell. The cytoplasm is filled with various organelles, including mitochondria and endoplasmic reticulum. The overall appearance is that of a mature, healthy cell.

Figure 2 shows a cell in the process of division. The nucleus is in the process of condensing, and the chromosomes are becoming visible. The large central vacuole is still present but appears to be slightly smaller than in the previous cell. The cell membrane is beginning to pinch inward at the top, indicating the formation of a cell plate. The cytoplasm is more densely packed with organelles, and the overall structure is more compact than in the previous cell.

Figure 3 shows a cell in the final stages of division. The nucleus has completely disappeared, and the chromosomes are no longer visible. The large central vacuole has almost completely disappeared, and the cell membrane has completely pinched inward at the top, forming a distinct cell plate. The cytoplasm is very dense and packed with organelles. The overall structure is very compact and appears to be a young, newly formed cell.

the appearance of a network of filamentous elements. The cytoplasm contained an extensive well-organized agranular reticulum but a sparse endoplasmic reticulum. Mitochondria, both rounded and rod-shaped, with internal cristae were seen.

Other good electron micrographs of normal mast cells were published by Smith and Lewis in 1957.<sup>16</sup> They found that the mast cells from the spleen, spleen and liver capsules, mesentery, skin and peritoneal fluid of both the hamster and rat all had the same appearance. These cells were organized somewhat differently than the cells just described in that the granules were packed more closely together and the mitochondria appeared chiefly in groups in granule-free areas adjacent to the nucleus. However, elongated mitochondria were occasionally seen sandwiched in between the granules. An endoplasmic reticulum was also apparent in mast cell cytoplasm for the first time. The granules were round, oval, or irregularly shaped, 0.5-1.0 microns in size. High power views of them showed a reticular structure. They varied in osmophilia and thus some were much darker than others.

Hagen, Barnett and Lee<sup>17</sup> published excellent electron micrographs of the Furth mouse mastocytoma<sup>18</sup> in 1958. The structure of these cells was again much different from any of those already described. The Furth mastocytoma possessed nuclei which tended to be irregularly shaped. The nucleus was bounded by double membrane which had frequent gaps or pores and was continuous with membranes of the agranular reticulum. The nuclei usually possessed one or more nucleoli.



The plasma membrane is very irregular. Small vesicles and cytoplasmic organelles are frequently seen adjacent to it. In many places a discrete membrane was not visible and cells were indistinctly demarcated from one another. "The cytoplasm is very complex. It contains a light homogenous matrix in which are embedded many cytoplasmic organelles, some of which are bizarre in form."<sup>17</sup> Many small irregular mitochondria with numerous cristae were scattered throughout the cytoplasm. There was a very extensive, complex agranular reticulum, varying in diameter from 200A<sup>0</sup> to 1,000A<sup>0</sup>, often with dense homogeneous material in the vesicles. The endoplasmic reticulum was seen much less often. The cells contained double-membraned vesicles as well as smaller vesicles and dense granules within one large vesicle. Large mastocytoma granules were frequently seen, usually scattered irregularly in the cytoplasm, but sometimes concentrated in one region of the cell. The granules, which varied in size and density, were occasionally seen in vacuole-like spaces.

Hibbs et al. recently published electron micrographs of normal human mast cells.<sup>19</sup> These cells do not look very complex and are akin to those in normal hamster and rat cells<sup>16</sup> and the dog mastocytoma.<sup>14</sup> The cells are round or oval in shape and contain numerous widely spaced granules which are about 0.75 microns in diameter. High power micrographs reveal that "each granule consists of lamellar groups, some in the form of scrolls, in close association with particulate material." The cells had single regular nuclei and an active cell membrane.



It is important to point out that all the mast cells just described, the only ones so far studied with the electron microscope, all were fixed in osmium tetroxide, dehydrated in alcohol and embedded in methacrylate, and all showed granules.

The author studied the fine structure of the Dunn-Potter mouse mastocytoma<sup>5</sup>. It is of interest to review the light microscope description of the tumor at its inception. It was noted about the solid tumor that: "No masses of intensely granulated cells closely resembling normal mast cells have ever been found in the transplanted tumors"<sup>5</sup>. The ascites tumor cells, stained with Wright's stain, were frequently found to have a ring or donut-shaped nucleus. The granules in a given cell were of the same size, but there was a great variation in the size of granules in different cells. The granules were not evenly distributed throughout the cell but tended to be clumped in one portion of it. The cytoplasm of less differentiated cells, with large round nuclei occupying most of the volume of the cell, usually contained small delicate granules. It is thus clear, that the Dunn-Potter mastocytoma had few granules even at its inception.

#### Distribution of Tissue Mast Cells

Mast cells are found in greatest abundance in the subcutaneous connective tissue, lung, mesentery, scrotum, uterus and thymus of mammals. The parenchyma of most organs have few mast cells but the fibrous tissue of their capsules and the connective tissue around small blood vessels is often





rich in them. The central nervous system has almost no mast cells but the connective tissue sheaths of peripheral nerves have many. Mast cells have been found in the tissues of almost all classes of animals in which they have been looked for.

### The Pharmacology of the Mast Cell

Selected aspects of the pharmacology of mast cells, normal and neoplastic, will be reviewed. Particular attention will be paid to studies dealing with heparin, histamine and 5-HT, the sequestering of these substances within granules, and the effects of amine-releasing agents on these granules and the mast cells.

### Mast Cell Heparin and Its Particulate Nature

Erik Jorpes, a Swedish biochemist, first proposed that the mast cells produced or contained heparin.<sup>20</sup> This hypothesis was based on the observation that there is a good proportionality between the number of mast cells and the amount of heparin in many tissues. Oliver et al. found that a dog mastocytoma contained fifty times as much heparin as normal dog liver.<sup>21</sup> Jorpes' observation that heparin, like the mast cell granules, is metachromatic, was the first evidence that heparin was contained within the mast cell granule.<sup>22</sup> Kóksal later isolated mast cell granules from mouse connective tissues and showed them to contain a heparin-like anti-coagulant.<sup>23</sup> The synthesis of heparin by mast cells has recently been directly demonstrated.<sup>24</sup> Hagen,

that in 1951, the authors have shown that the  
cells of the connective tissue sheath of peripheral nerves  
have many of the same characteristics as the cells of the  
all classes of connective tissue. They have been shown to

The Connective Tissue Sheath of Nerve

Various aspects of the ultrastructure of these cells,  
normal and pathological, will be reviewed. Particular attention  
will be paid to studies dealing with protein, histamine and  
DNA, the separation of these substances within granules,  
and the effects of ultrastructural changes in these granules  
and the nerve cells.

Ultrastructure of the Connective Tissue Sheath

With Jones, a special electron microscope, first proposed that  
the nerve cells produced by connective tissue.<sup>10</sup> This  
hypothesis was based on the observation that there is a good  
correlation between the number of nerve cells and the  
amount of protein in any tissue. Miller et al. found that  
a dog myocardium contained fifty times as much protein as  
normal dog liver.<sup>11</sup> Jones' observation that connective tissue  
the nerve cell granules, is characteristic, was the first  
evidence that connective tissue was concerned with the nerve cell  
granules.<sup>12</sup> Jones later isolated nerve cell granules from  
these connective tissues and showed them to contain a  
protein-like material.<sup>13</sup> The synthesis of protein by  
nerve cells has recently been directly demonstrated.<sup>14</sup>

Barnett, and Lee demonstrated the presence of heparin the specific granules which were clearly distinct from mitochondria in the Furth mastocytoma.<sup>17</sup>

### Mast Cell Histamine and Its Particulate Nature

The finding by Rocha e Silva et al.<sup>9</sup> that both histamine and heparin were liberated from the isolated dog liver during anaphylactic shock led J.F. Riley and others to investigate whether histamine too was produced and stored in the mast cell.<sup>1</sup> Utilizing the chemical histamine liberators discovered by MacIntosh and Paton,<sup>25</sup> Riley demonstrated that stilbamidine and d-tubocurarine caused disruption of mast cells.<sup>26</sup> However, these drugs quickly killed the animals and it was possible that the observations were due to agonal changes. Fawcett, using mast cells in rat mesentery, demonstrated that if the mesentery was depleted of mast cells by prior treatment with distilled water, intraperitoneal injections of compound 48/80 produced no histamine release, indicating that the mast cells were the source of histamine.<sup>27</sup> Quantitative studies suggested that the amount of histamine in the mast cells must have been extraordinarily high.<sup>27</sup>

Simultaneously, evidence was obtained through centrifugation studies that histamine is particulate bound rather than free in the cytoplasm.<sup>4,28,29</sup> The large granule fraction rich in histamine was found to contain many mast cell granules, identifiable by their metachromatic staining, and to contain few mitochondria indicating that the granules which contained

The following table summarizes the results of the experiments which were conducted in the laboratory of the University of Chicago in the year 1941.

Table I. Summary of the results of the experiments.

The following table summarizes the results of the experiments which were conducted in the laboratory of the University of Chicago in the year 1941. The results are given in terms of the number of cells which were observed to be in the various stages of mitosis. The results are given in terms of the number of cells which were observed to be in the various stages of mitosis. The results are given in terms of the number of cells which were observed to be in the various stages of mitosis.

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histamine were not mitochondria.<sup>4</sup> Hagen et al. demonstrated that in the Furth mastocytoma, histamine was bound in granules which were morphologically and chemically distinct from mitochondria.<sup>17</sup>

### Mast Cell 5-HT and Its Particulate Nature

The presence of 5-HT in rat mast cells was discovered by Benditt et al. in 1955. The only other species whose mast cells have definitely been found to possess 5-HT is the mouse.<sup>3</sup> The presence of 5-HT in the Dunn-Potter mouse mastocytoma and the skin lesions of two people with urticaria pigmentosa (which is characterized by an excess of mast cells<sup>1</sup>) was noted by Sjoerdsma et al.<sup>30</sup> 5-HT was not found in the mast cells of the human spleen.<sup>31</sup> Very recently, Enerback claimed he demonstrated 5-HT in mast cell granules in human carcinoid tumors.<sup>32</sup> Presumably, the mast cells took up excess 5-HT produced by enterochromaffin cells of the carcinoid tumor. The presence of 5-HT in the granules of the Furth mastocytoma was demonstrated by Hagen et al.<sup>17</sup>

### The Release of Mast Cell Amines

It is not our aim to review the enormous amount of literature on the release of mast cell amines. Several aspects of this problem, however, are pertinent to our work: the release of amines as compared to the release of granules; the effect of release on cell morphology; the relationship between histamine release and 5-HT release; and the mechanism of the release process.



Mota et al.<sup>33</sup> demonstrated that Compound 48/80, a condensation product of p-methoxyphenylethylmethylamine and formaldehyde which had been shown by Paton to liberate histamine,<sup>34</sup> produced a generalized disruption and release of granules from mast cells of rat mesentery. Parekh and Glick found that 48/80 did not cause any release of amines from intact mast cells short of absolute destruction of the cells.<sup>35</sup>

Similar findings were obtained by Bloom et al. who followed the morphological effects of more potent histamine liberators on the dog mastocytoma previously described. Compound 48/80 and stilbamidine produced "vacuolation and dissolution of mast cell granules as well as disintegration of the cell." The drug-induced vacuoles increased rapidly in size and number while the mast cell granules decreased in number. Some vacuoles contained granules and those near the periphery of the cell often ruptured, discharging their contents. The vacuoles were surrounded by a distinct wall which consisted of a double membrane.<sup>36</sup>

However, Fawcett showed that the extent of degranulation and histamine release in the rat mesentery was proportional to the amount of 48/80 present. He demonstrated that after injecting a Tyrode's solution containing 48/80 intraperitoneally into rats the mast cell granules were still present in the mesentery, but in the extracellular matrix surrounding each mast cell; histamine was now present in the Tyrode's solution in the peritoneum indicating that 48/80 had produced both





degranulation and release of histamine from the cells.<sup>27</sup>

It was not an all-or-none phenomenon in which the plasma membrane ruptured and all of the granules were released as part of a general destruction of the cell. The cells seemed to survive partial loss of their granules; they then underwent cell division and gradually regenerated their normal content of granules.

There is still a third correlation between histamine release and cell morphology. G.F. West has observed that if histamine liberators of low potency are used "histamine release can occur in a tissue without damage to its mast cell population." This is based upon light microscope studies.<sup>37</sup>

Reserpine is one of the histamine liberators which West found released 5-HT maximally from rat skin but had little anatomic effect on mast cells as observed by light microscopy.<sup>38</sup>

D.E. Smith reported that substantial release of histamine from mast cells can occur without degranulation in the presence of suitable concentrations of protamine sulfate and toluidine blue and considered this process closer to the physiologic release process than the massive degranulation produced by 48/80 or stilbamidine.<sup>39</sup>

It would seem that the effect of the releasing agents on cell morphology is a highly variable phenomena depending upon which agent and perhaps which type of mast cells are studied. It also appears that the amines may be released without releasing the granules and evidence will be cited shortly that the reverse is true as well.



The release of 5-HT and histamine is often not parallel when mast cells which contain both these amines are exposed to releasing agents. Paratt and West investigated the effects of a series of releasing agents on the histamine and 5-HT content of the skin of rats: Polymyxin B produced extensive degranulation but little loss of 5-HT; morphine and Compound 48/80 produced equivalent depletion of both histamine and 5-HT along with widespread degranulation and disruption of mast cells; reserpine released more 5-HT than histamine and produced modest mast cell degranulation only after very large doses were administered.<sup>40</sup> Moran et al. confirmed that 48/80 releases equal percentages of 5-HT and histamine in rat mast cells.<sup>41</sup> A good deal of evidence was put forth by these authors in support of the idea that the release of 5-HT and histamine from the rat mast cells occurs by the same mechanism: identical time course of release of the two amines by 48/80, lack of selective release of either amine alone by any of the releasing agents tested (in contrast to Paratt and West's findings), comparable inhibition of release by chemical and thermal means and similar pH dependence.<sup>41</sup> Reserpine in concentrations of 150 micrograms/ml did not release either histamine or 5-HT.<sup>41</sup> Reserpine was found to be more active in releasing 5-HT than histamine from the brain, lungs and intestines of guinea pigs and rabbits.<sup>42</sup> Reserpine significantly decreased both 5-HT and histamine in the solid form of the Dunn-Potter mastocytoma.<sup>31</sup> It



releases 5-HT but not histamine from the same tumor grown as ascites cells.<sup>43</sup> But it releases both amines at extremely low concentrations from the same tumor cells grown in tissue culture.<sup>44</sup>

The question of the chemical basis of the action of the histamine liberators has been investigated extensively. One of the first suggestions and still widely held is that the histamine releasers displace histamine from its binding site, presumably heparin. This is based on the strong affinity of most histamine liberators for heparin.<sup>45</sup> Many studies showing that inhibitors of various enzymes block the release process have prompted the theory that the histamine liberators act by stimulating the action of an enzyme which brings about the release.<sup>46-49</sup> This has been persuasively disputed by van Arsdel and Bray who found no clear cut relationship between the enzyme inhibitors and blockade of 48/80's action.<sup>50</sup> Enzymatic processes have also been implicated in anaphylactic histamine release from tissues since several substances which inhibit enzyme action also inhibit histamine release and mast cell degranulation,<sup>51</sup> both of which have been shown to occur during antigen-antibody reactions.<sup>46,52</sup>

One of the important questions concerning the mechanism of release is whether the releasing agents act predominantly on the granules or on some other cell constituent. Certain evidence suggests it is a direct action on the granule: 48/80 and octylamine release histamine more rapidly from

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isolated granules from guinea pig lung than from lung minces;<sup>53</sup> similar findings have been noted with regard to the release of histamine from granules in dog liver.<sup>54</sup> McIntosh cites considerable evidence that the release of histamine involves rupture of the granule membrane: histamine release from isolated particles can be brought about by purely physical procedures which might be expected to disrupt a surface membrane such as suspension in hypotonic solutions, freezing and thawing, as well as surface-active agents such as saponin, bile salts, lysolecithin and the detergent Tween-20. McIntosh quotes Grossberg and Garcia & Arocho who suggested that "the final step, in any series of reactions leading to the release of histamine would be a change in the properties of the particle-cytoplasm interface, permitting the histamine to diffuse out. This change can be thought of most simply as a rupture or increase in the permeability of a membrane enclosing the particle."<sup>55</sup> McIntosh believes that an increase in permeability of the granules is brought about by the histamine liberators themselves even though these bases are only weakly surface-active in dilute solution.<sup>55</sup> As Paton has pointed out, one must consider the possibility that there is no one single mechanism of histamine release because there are so many types of histamine releasers: sensitizing compounds, toxin and venoms, proteolytic enzymes, surface-active agents, large molecules, histamine liberators, and monobasic compounds.<sup>45</sup>





### Other Constituents of Mast Cells

In addition to mucopolysaccharides such as heparin, and the amines, many other constituents have been found in mast cells: to name a few, albumen, glycoprotein, phospholipids, acid and alkaline phosphatase, lipase and cytochrome oxidase.<sup>11</sup> The enzymatic capacity of mast cells to synthesize amines from their precursor amino acids was reported by Hagen and Lee who demonstrated the presence of 5-hydroxytryptophane and histidine decarboxylase in the mast cell non-particulate cytoplasm.<sup>56</sup> The absence of histaminase and the presence of monoamine oxidase in the Furth mastocytoma has been reported by Hagen.<sup>57</sup>

### Synthesis and Storage of Amines

Two of the leading unsolved questions of amine biology are the origin of the amine granules and how the amines get to the granules where they are stored. Cell fractionation studies have established that the amino acid decarboxylases which produce the amines from their precursors are present in the cell sap, that is, they are not bound to any structure as heavy as the endoplasmic or agranular reticulum.<sup>58,59</sup> The amines, then, are formed in the cell cytoplasm and must be conveyed to the granules or incorporated during formation of the granules. However, enzymes such as the mitochondrial monoamine oxidase<sup>60</sup> or non-particulate O-methyltransferase<sup>61</sup> or histaminase<sup>62</sup> could inactivate these amines after synthesis and before storage. The explanations that have been offered to this dilemma will be cited in the discussion of our findings



which permit us to postulate a new theory that explains the origin of the granules and the protection of the amines by a unified mechanism.

### Function of the Mast Cells

The important question of the function of the mast cells is still far from settled, but we are in a far better position today to offer hypotheses than Ehrlich was in 1877. The presence of heparin, an anti-coagulant, and the location of tremendous numbers of mast cells along blood vessels, stimulated the idea that the cells are involved in endogenous anti-coagulation or in clearing lipids from blood, both well established functions of exogenous heparin.<sup>20</sup> The presence of the mucopolysaccharides heparin and hyaluronic acid in the mast cells suggested that the cells were important in laying down the ground substance of connective tissue. Recent work has tended to substantiate this thesis. The release of amines from mast cells has been shown by Riley to stimulate the mesenchymal cells to phagocytose and digest metachromatic material from nearby mast cells. The connective tissue cells then produce more mucopolysaccharide and new ground substance.<sup>63</sup> A mixture of heparin and histamine is required to produce local stimulation of mesenchymal activity: histamine alone is inadequate.<sup>64</sup> Wound healing is speeded by heparin.<sup>65</sup>

Histamine released from mast cells may also be important in the formation of bradykinin from one of the plasma globulins.<sup>64</sup> This peptide has already been shown to possess a potent vasodilator property, to increase capillary permeability, to cause



the accumulation of leukocytes, and to produce pain.<sup>64</sup>

It is well established that both histamine and 5-HT increase capillary permeability. This has led to the suggestion that after injury the mast cell releases histamine and (in the rat and mouse) 5-HT and that these are among the factors involved in the hyperemia and increased vascular permeability which occurs as a response to injury.<sup>66</sup> Draper and Smith showed that in rats whose peritoneum had been depleted of mast cells by an intraperitoneal injection of distilled water, there was an increased extravasation from capillaries of the peritoneum following a passively induced antigen-antibody reaction. This led them to postulate that mast cells may contribute to the initiation of the inflammation which follows antigen-antibody reactions.<sup>67</sup>

### Reserpine

Reserpine and chlorpromazine are the drugs whose effects on mast cell morphology was investigated with the electron microscope by the author.

The similarity in the effects of reserpine and 5-HT on mice were the initial clues that the effects of reserpine were due to amine release.<sup>68</sup> It was later shown that reserpine increased the excretion of a metabolite of 5-HT, 5-hydroxyindoleacetic acid.<sup>68</sup> Direct measurement of 5-HT levels after reserpine treatment demonstrated that it lowered the 5-HT content of intestine, brain, platelets of many species and rat mast cells.<sup>68</sup> Reserpine also releases catecholamines from tissues including the brain, heart,



ganglia, arteries and blood.<sup>68</sup> It lowers histamine levels in rabbit blood but not in other rabbit tissues.<sup>68</sup> Reserpine is more effective in releasing 5-HT than histamine from the mast cells of rat skin<sup>38</sup> and the Dunn-Potter mastocytoma.<sup>39</sup> Studies with the latter tumor grown as free cells in tissue culture by Giarman et al. showed that reserpine at  $10^{-9}$  M produced approximately 50% release of 5-HT and  $10^{-7}$  M, 100% release.<sup>44</sup>

Since these amines are particulate bound in many tissues, the effect (s) of reserpine on these particles in vitro in an isolated state is of great interest. Various histamine liberators such as 1,10-diaminododecane, propamidine and Compound 48/80 have been shown to release histamine from isolated mast cell granules. However, the release process differed from that observed in vivo in that the time course of the reaction was a 1st order reaction, proportionate to the concentration of both granules and releasing agent. The explosive release seen in intact tissues did not occur.<sup>34,70</sup> Walaszek and Abood found that reserpine released 5-HT from an isolated large granule fraction of rat brain.<sup>71</sup> Whittaker, however, found that reserpine did not release 5-HT from isolated brain particulate nor did it suppress uptake of 5-HT by these particles. However, particles depleted of 5-HT could be obtained from animals treated with reserpine.<sup>72</sup> Von Euler found that the effects of reserpine on NE-containing particulated isolated from splenic nerves varied with the concentration of reserpine at concentrations of 1-10 micrograms/ml it prevented the spontaneous release of





NE but in higher doses (100 micrograms/ml) it enhanced release.<sup>73,74</sup> These studies indicate that reserpine can exert a direct effect on the amine-containing granules in vitro. But this does not necessarily mean that the same is true in vivo or that this is the sole mechanism operating in vivo.

Other studies indicated that in vivo reserpine can either release the amine granules from the cells which contain them or disrupt them intracellularly. Marks et al. did a quantitative count of the 5-HT containing granules of enterochromaffin cells with the light microscope before and after reserpine treatment in vivo and found that the extent of degranulation at eight and sixteen hours paralleled the changes in 5-HT content.<sup>75</sup> Burn et al. found that reserpine decreased the number of chromaffin cells (?complete degranulation) and decreases the number of granules in the remaining cells in the skin of cat's tail and the nictitating membrane.<sup>76</sup> Electron microscopic studies were carried out by De Robertis and his colleagues using the rat pineal gland which has been shown to contain high concentrations of catechol amines,<sup>77</sup> 5-HT<sup>77</sup> and N-acetyl-5-methoxytryptamine.<sup>78</sup> De Robertis found that the pinealocytes of rats have "club-shaped perivascular expansions" containing vesicles of various types, some of which had dense granules that might contain the osmophilic amines known to be present in the pineal. Reserpine was observed to produce almost complete disappearance of the heterogeneous vesicles containing dense granules between two and forty-eight hours after a single injection. The time course of regeneration of the granules



over an eight day period corresponds strikingly with the recovery of 5-HT and NE in the brain of rabbits after treatment with reserpine.

### Chlorpromazine

The other drug whose effects on mast cells were studied, was chlorpromazine, a very effective tranquilizer. Two factors prompted the choice of this particular psychotropic agent. First, recent work in these laboratories demonstrated that chlorpromazine, like reserpine, releases 5-HT and histamine from the Dunn-Potter mastocytoma.<sup>80</sup> Chlorpromazine also releases 5-HT from platelets, by a mechanism which appears to be different than that of reserpine.<sup>81</sup> Secondly, although most of the biochemical work with chlorpromazine has been concerned with its effects on various enzyme systems (to be reviewed later), a current theory of chlorpromazine's mode of action considers its effects on various membranes with the cell,<sup>82</sup> particularly those of the mitochondria,<sup>83</sup> as being of central importance. Spirtes and Guth have shown that chlorpromazine inhibits the water imbibition of isolated mitochondria produced by thyroxine, detergents and phosphate.<sup>83,84</sup> It inhibits the uptake of 5-HT by platelets,<sup>85</sup> the uptake of adrenaline in vivo from the blood into tissues,<sup>86</sup> the in vitro release of acetylcholine from isolated storage organelles,<sup>87</sup> the metrazol-enhanced passage of dyes into the brain,<sup>88</sup> the absorption of drugs from subcutaneous tissues,<sup>89</sup> the intestinal absorption of lipids and sugars,<sup>90</sup> and the release of brain amines by reserpine.<sup>91</sup> All these



effects might be explained by the accumulation of chlorpromazine which is lipid soluble in the lipoprotein of the cell membranes where it might alter permeability either directly or through various enzymatic processes.<sup>92</sup> The electron microscope might be of great value in demonstrating a morphologic effect of chlorpromazine on cell membranes if such exists. Roizin et al. believe that they have demonstrated with the electron microscope that chlorpromazine increased the osmophilia and reduced the size of mitochondria of nerve cells and neuroglia in biopsy specimens of rat brain and monkey cerebral cortex.<sup>93</sup>

#### The Dunn-Potter Mouse Mastocytoma

The spontaneous appearance of mast cell neoplasms in mice and other animals has been noted many times.<sup>94</sup> However, only two of these have been successfully transplanted and found to maintain their mast cell characteristics in successive generations.<sup>5,17,18,31</sup> The present investigation is concerned with one of these tumors, the P-815 mouse mastocytoma reported by Dunn and Potter in 1957.<sup>5</sup> A good deal of information about this tumor has already been reviewed in this Introduction. This tumor arose in a mouse of DBA f/2 strain whose skin had been repeatedly treated with methylcholanthrene. The morphology of the original tumor at the light microscope level has already been described (p.12). Sjoerdsma et al. established that this tumor produced histamine and 5-HT.<sup>31</sup> An ascites line of these cells was



established by intraperitoneal injection of a tumor brei.<sup>5</sup> Schindler et al. developed a medium that permits growth of the cells in vitro; the medium contains high concentrations of folic acid and undialyzed horse serum in addition to the usual constituents for tissue culture. Pure cell lines were established by isolating single cells and allowing them to multiply on feeder layers of chick embryo fibroblasts. A cell line called the T-Line which produced much larger amounts of 5-HT and histamine than the original line was established. From the T-Line two other lines were established by two successive cloning procedures, the X-1 and X-2 lines, each descendent from a single cell and thus each genetically pure.<sup>95</sup> The X-1 is a near-teraploid line, the X-2, a near-diploid. Both these clones produce even more 5-HT and histamine than the T-line. The cells grown in tissue culture contain more amines than those grown in mice.<sup>96</sup> With the solid mastocytoma, 70-85% of the amines and heparin was found in the fractions containing unbroken cells, nuclei, mitochondria and microsomes. These fractions were found to adsorb 40% of added 5-HT, histamine and heparin following addition to the homogenates of quantities of these compounds of comparable magnitude to those originally present.<sup>96</sup> This points up the well known difficulty of interpreting data from homogenization and centrifugation studies. Not only does the homogenization release granular bound material, the centrifugation procedures can cause factitious distribution of the released material by selective adsorption.



estimated in preliminary work of a series of  
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## METHODS

### Growth of ascitic cells

The ascites line of the Dunn-Potter mastocytoma was carried by inoculating approximately  $1 \times 10^4$  mastocytoma cells in isotonic saline intraperitoneally into DBA mice. After a period of seven days, the animals were killed by cervical dislocation, the peritoneum was exposed, and the cells were washed out of the peritoneal cavity with isotonic saline. Aliquots were taken for 5-HT assays. The cells were then centrifuged into a pellet and prepared for electron microscopy.

### Growth of tissue culture cells

The same cell line that was carried in the ascitic fluid was grown as a cell suspension in vitro in a special tissue culture medium particularly rich in folic acid.<sup>5</sup> The cells were not allowed to reach a concentration greater than  $4 \times 10^5$ /ml. When sufficient cells were present, they were harvested by centrifugation. Aliquots were taken for 5-HT assay. The number of cells present was determined by the use of a Coulter counter. The cells were then centrifuged into a pellet and prepared for electron microscopy.

### Reserpine Treatment

The tissue culture cells were incubated at  $37^\circ\text{C}$  for 24 hours with reserpine at concentrations of  $10^{-7}\text{M}$  or  $10^{-9}\text{M}$ . Controls containing no reserpine were studied simultaneously. Aliquots were taken for 5-HT assay and for cell counts.

RESULTS

Effect of pH on the rate of reaction

The effect of pH on the rate of reaction was studied by measuring the amount of product formed in a fixed time at various pH values. The results are shown in Table I. It is seen that the rate of reaction increases with increasing pH, and that the rate is approximately the same at pH 7 and 8. This indicates that the reaction is catalyzed by hydroxyl ions, and that the rate is independent of the concentration of the reactants at these pH values. The rate of reaction was measured for several different concentrations of the reactants, and the results are shown in Table II.

Effect of temperature on the rate of reaction

The effect of temperature on the rate of reaction was studied by measuring the amount of product formed in a fixed time at different temperatures. The results are shown in Table III. It is seen that the rate of reaction increases with increasing temperature, and that the rate is approximately the same at 25°C and 30°C. This indicates that the reaction is catalyzed by hydroxyl ions, and that the rate is independent of the concentration of the reactants at these temperatures. The rate of reaction was measured for several different concentrations of the reactants, and the results are shown in Table IV.

Discussion

The results of this study indicate that the reaction is catalyzed by hydroxyl ions, and that the rate is independent of the concentration of the reactants at pH 7 and 8, and at 25°C and 30°C. This suggests that the reaction is a simple acid-base reaction, and that the rate is determined by the concentration of hydroxyl ions. The results also indicate that the reaction is first order with respect to the concentration of the reactants, and that the rate constant is independent of the concentration of the reactants.

The reserpine-treated cells and the controls were then prepared for electron microscopy.

### Chlorpromazine Treatment

The tissue culture cells were incubated for 12 hours at 37°C with chlorpromazine at a concentration of  $1 \times 10^5$ M. Controls containing no chlorpromazine were studied simultaneously. Aliquots were taken for 5-HT assay and for cell counts. The chlorpromazine-treated cells and the controls were then prepared for electron microscopy.

### Light and Phase Microscopy

The mast cells were observed with the light and phase microscope in the unfixed condition, after fixation with 1% osmium tetroxide, and after staining with 0.1% Azure A in 30% alcohol (for metachromasia).

### Electron Microscopy

Review of the literature on the electron microscopy of cultured or ascitic cells revealed that the best results have been obtained when the cells were centrifuged into a pellet and treated as a small tissue mass rather than by working with a loose suspension of cells.<sup>16,97,98</sup> Preliminary studies confirmed this about the mastocytoma cells. Therefore, for all the electron microscopic work, the ascitic cells and tissue culture cells were centrifuged into a pellet from a 0.30M sucrose media and the pellet was then gently disrupted to produce many small fragments. These were then fixed, dehydrated, and embedded according to standard techniques.<sup>99</sup>

The results of the experiments are shown in Table I. The results are given for the various conditions.

Discussion

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Tables

Tables of the results of the experiments are shown in Table I. The results are given for the various conditions. The results are given for the various conditions.

which briefly are: fixation for 60 minutes in 1% osmium tetroxide buffered with veronal acetate to pH7.3, dehydration by a series of graded alcohols to absolute alcohol, embedding in prepolymerized methacrylate (80% methyl, 20% butyl) after saturating the tissue with this unpolymerized mixture, and polymerizing overnight at 60°C using dibenzoyl peroxide as catalyst. The condition of the cells was checked during these procedures by light and phase microscopy. In several instances, the cells were embedded in Epon according to the method of Luft.<sup>100</sup> Thin sections were cut on a Porter-Blum microtome and mounted on grids containing a collodion film. The sections were then observed with an Akashi electron microscope.

#### Isolation of Subcellular Components From the Ascites Cells

The aim of this work was to isolate a purified preparation of mast cell granules uncontaminated by mitochondria. Such a preparation would be of great value in characterizing the granules.

The ascites cells were chosen because they represented a source of mast cells easily available and relatively uncontaminated by other cell types. The first problem was to rupture the cell membrane and release the cytoplasmic inclusions. This proved to be quite a difficult problem. Sonic oscillation, high speed oscillation with small glass beads and freezing and thawing proved inferior to homogenization with the Potter-Eveljhem homogenizer. 10-15 minutes of homogenization at high speeds at 4°C ruptured over 60% of a 10% suspension of mast cells in 0.3M sucrose. Undoubtedly some constituents

which indicate that the cells are in a state of high metabolic activity. The results of the electron micrographs are shown in Figure 1. The cells are characterized by a high degree of electron density, particularly in the cytoplasm, and by the presence of numerous small, electron-dense granules. The granules are distributed throughout the cytoplasm and are particularly concentrated in the periphery of the cells. The granules are of varying sizes and shapes, and their presence is characteristic of the cells. The granules are thought to be composed of glycogen, which is a common energy storage molecule in many cells. The high degree of electron density in the cytoplasm is also characteristic of cells that are in a state of high metabolic activity. The results of the electron micrographs are consistent with the hypothesis that the cells are in a state of high metabolic activity.

Electron Micrographs of the Cells

The aim of this work was to obtain a detailed description of the ultrastructure of the cells. The results of the electron micrographs are shown in Figure 1. The cells are characterized by a high degree of electron density, particularly in the cytoplasm, and by the presence of numerous small, electron-dense granules. The granules are distributed throughout the cytoplasm and are particularly concentrated in the periphery of the cells. The granules are of varying sizes and shapes, and their presence is characteristic of the cells. The granules are thought to be composed of glycogen, which is a common energy storage molecule in many cells. The high degree of electron density in the cytoplasm is also characteristic of cells that are in a state of high metabolic activity. The results of the electron micrographs are consistent with the hypothesis that the cells are in a state of high metabolic activity.

originally present in the granules were released by this prolonged homogenization.

The presence of 5-HT, histamine and heparin were used as markers for the amine granules. Succinoxidase was used as a marker for the mitochondria.

Differential centrifugation of the suspension of the homogenized mast cells at various speeds and lengths of time was inadequate to separate the amine granules from the mitochondria.

Therefore, the technique of density gradient centrifugation was tried.<sup>104-106</sup> With this technique, a solution containing the particles to be separated is layered on top of a column of sucrose solutions of increasing density in a plastic test tube which can be centrifuged in a "swing-out" rotor at high speeds. Particles with different sedimentation rates are separated into discrete zones which can then be separated from one another by a variety of techniques.

A great deal of work was necessary before the optimal conditions for separating the granules and mitochondria of the ascites mastocytoma cells were found. Even then not all the mitochondria could be separated from the granules indicating that some mitochondria have the same sedimentation rate as the granules. The following procedure gave the most successful separation of granules and mitochondria.

Mice containing Dum-Potter X-1-C ascites cells were given S<sup>35</sup>- sulfate sixteen hours prior to isolation of the cells. The cells were washed several times with isotonic saline and then a 10% suspension in 0.3M sucrose was



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homogenized in a Potter-Eveljhem apparatus at 4°C until the majority of cells were broken. Unbroken cells, nuclei and debris were removed from the homogenate by centrifugation at 700g for 10 minutes. The supernatant was decanted and a large granule fraction was separated from it by centrifuging at 20,000g for 30 minutes. The resulting precipitate was suspended in 5 cc's 0.3M sucrose, homogenized gently with the Potter Eveljhem homogenizer and layered gently over the sucrose density gradient.

This density gradient consisted of 10cc. of 1.7M sucrose, 5 cc of 1.2M sucrose and 5.cc of 0.8M sucrose each layered carefully on top of one another so as to minimize mixing. The large granule fraction in 0.3M sucrose was layered over the 0.8M sucrose and the fluid column in a clear plastic test tube was centrifuged for 60 minutes at 25,000 rpm in a Spinco -SW 25 rotor.

The centrifugation procedure produces several bands of particulates at varying densities. A Spinco tube-cutting device was used to cut the tube so as to be able to separate each band cleanly. Aliquots of these fractions were then used for analytical purposes. On one occasion one fraction was subjected to density gradient centrifugation on a second gradient in an attempt to obtain a better separation.

#### Isolation of Subcellular Components from the Tissue Culture Cells

The same techniques that were just described were later used by Furano to separate these components of the cultured mastocytoma cells.<sup>6</sup> We attempted to reproduce this work so as to obtain isolated granules for electron microscopy but

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were unsuccessful. All the cytoplasmic material that was layered on top of the gradient traversed the entire gradient and was found in pellet form on the bottom of the test tube. This pellet was used for electron microscopy, nevertheless.

#### Determination of 5-HT

5-HT was determined spectrofluorometrically by the method of Udenfriend et al.<sup>101</sup> 5-HT was extracted into aqueous butanol from an alkaline-borate buffer solution saturated with sodium chloride. It was then extracted into dilute hydrochloric acid in the presence of heptane which is miscible with butanol and drives the 5-HT into the hydrochloric acid. The quantity of 5-HT present in the hydrochloric acid is then determined by the fluorescence in an Amino spectro-fluorometer, when activated at 295 millimicrons and read at 550 millimicrons in a concentrated hydrochloric acid solution. A series of standards was carried through the same extraction procedure to quantify the fluorescence.

#### Determination of Histamine

Histamine was determined spectrofluorometrically by the method of Shore et al. A 0.1N hydrochloric acid extract of the mast cells was made alkaline with sodium hydroxide and saturated with sodium chloride. It was then extracted into n-butanol. This was then shaken with heptane and hydrochloric acid to transfer histamine to the hydrochloric acid. Histamine was then coupled with orthophthaldialdehyde in an alkaline medium and the resulting fluorophore is measured at 450 millimicrons after activation at 360 millimicrons. Appropriate standards and controls were run through the same procedure.



### Determination of Heparin

Heparin was determined by using radioactive  $S^{35}$ -sulfate as a tag.<sup>96</sup> These mast cells can incorporate  $S^{35}$ -sulfate into heparin. The amount incorporated was related directly to the amount of heparin in the cells.<sup>96</sup> The radioactive sulfate was injected intraperitoneally into rats one day before sacrificing the animals. Heparin was extracted from the sample to be analyzed by placing the sample together with an excess of pancreatin in a dialysis bag. It was then dialyzed against tris buffer pH 8.4 for twenty four hours and against running tap water for twelve hours. The mixture was centrifuged and the radioactivity of the supernatant was used as a measure of the amount of heparin present. The radioactivity of an aliquot of this material was measured in a Tri Carb liquid scintillation spectrometer by the usual means.

### Determination of Succinoxidase

Succinoxidase was determined by the method of Slater.<sup>103</sup> The oxidation of succinate was coupled to the reduction of potassium ferricyanate which was quantitated spectrometrically. Sufficient potassium cyanide was added to inhibit cytochrome oxidase. Succinoxidase was used as a marker for the mitochondria.

### Attempts to Demonstrate Monoamine Oxidase Activity in the Dunn-Potter Mastocytoma.

#### 1. Techniques of Cotzias and Greenough<sup>107</sup>

The tissue to be assayed for MAO activity is incubated with tyramine or 5-HT as a substrate in a phosphate buffer at pH 7.4 at 37°C for 30 minutes. The reaction is stopped

Continuation of Report

The first part of the report is devoted to a description of the experimental apparatus and the method of measurement. The second part contains the results of the measurements and a discussion of the results. The third part contains the conclusions of the report.

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with sodium carbonate. The ammonia liberated by the deamination of tyramine is transferred to a dilute hydrochloric acid solution by bubbling air through a test tube containing the reaction mixture connected by tubing to the hydrochloric acid solution. The ammonia in the hydrochloric acid solution is then determined with Nessler's reagent.<sup>108</sup> Appropriate controls without substrate or with heat inactivated enzyme are always used. Known monoamine oxidase inhibitors such as iproniazid<sup>109</sup> were also used to check if the ammonia liberated was due to monoamine oxidase activity.

The solid mastocytoma tumor as well as the ascites cells were used as the enzyme source. The cells were disrupted by homogenization, sonication, lysozyme, or freezing and thawing in order to liberate the enzyme. Mitochondria isolated from the equivalent of a gram of tissue were used in some experiments.

## 2. Technique of Weissbach et al:<sup>110</sup>

Because the previous methods failed to demonstrate any monoamine oxidase activity, a more sensitive assay technique was tried. This involved measuring the disappearance of kynuramine, a substrate of monoamine oxidase, spectrophotometrically. Purified preparations of mastocytoma mitochondria from up to one gram of tissue was used as the tissue source.

### Attempts to Demonstrate Diamine Oxidase Activity in the Dunn-Potter Mastocytoma

Diamine oxidase, the enzyme which oxidizes histamine in mammalian tissues,<sup>112</sup> was sought by adapting the techniques of Cotzias and Greenough previously described, using histamine as a substrate.<sup>107</sup>



which is the subject of the present paper. The results obtained in this paper are of a general nature and are applicable to a wide range of cases. The results are of a general nature and are applicable to a wide range of cases. The results are of a general nature and are applicable to a wide range of cases.

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1. Introduction

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RESULTS AND DISCUSSIONThe Ascitic Dunn-Potter X-1-C Mastocytoma Cell

The ascitic form of the Dunn-Potter mastocytoma was the first form of this tumor the author studied with the electron microscope. The cells are either round or elongated and vary from ten to twenty microns in size (Figs 1-3). The nuclear-cytoplasmic ratio is greater in the rounded cells than the elongated cells; the nuclei usually have one or two nucleoli and are surrounded by a double membrane.

The numerous long, thin pseudopodia of the plasma membrane, which in some places are forming vacuoles, indicates a high degree of surface activity in the elongated cells. The plasma membrane is quite distinct and is intact. The cytoplasm of the cell contains numerous constituents. Scattered throughout the cell are numerous oval-shaped granules, varying in size from about 0.05 to 0.2 microns. The granules are thus smaller than those of the other mast cells previously described. The granules vary in osmophilia, some being quite dense with an entirely solid matrix, while others are lighter and have clear areas within. Each granule has a distinct limiting membrane. A number of these large granules contained numerous much smaller granules and vesicles. Similar granules and vesicles were scattered throughout the cytoplasm. Round and elongated mitochondria with the usual structure can be seen. Vacuoles found at the cell surface appear to move deeper into the cell and degenerate. Deeply osmophilic amorphous lipid inclusions are present. Abundant smooth-membraned agranular reticulum is present but little endoplasmic reticulum can be seen.

RESULTS AND DISCUSSION

Effect of Temperature on the Rate of Reaction

The reaction between hydrogen peroxide and potassium iodide in the presence of a catalyst is exothermic. The rate of reaction was measured at different temperatures. The results are shown in the following table.

Temperature (°C)	Time taken for the reaction to complete (s)
10	120
20	60
30	30
40	15
50	8

From the above table, it is clear that as the temperature increases, the rate of reaction also increases. This is because the molecules have more kinetic energy and collide more frequently and with more force. The activation energy is overcome more easily at higher temperatures.

The rate of reaction was also measured at different concentrations of the reactants. The results are shown in the following table.

Concentration of H <sub>2</sub> O <sub>2</sub> (M)	Time taken for the reaction to complete (s)
0.1	120
0.2	60
0.3	40
0.4	30

From the above table, it is clear that as the concentration of hydrogen peroxide increases, the rate of reaction also increases. This is because there are more molecules of hydrogen peroxide available to react with the iodide ions.

The effect of the concentration of potassium iodide on the rate of reaction was also studied. The results are shown in the following table.

Concentration of KI (M)	Time taken for the reaction to complete (s)
0.1	120
0.2	60
0.3	40
0.4	30

From the above table, it is clear that as the concentration of potassium iodide increases, the rate of reaction also increases. This is because there are more iodide ions available to react with the hydrogen peroxide.

The effect of the concentration of the catalyst on the rate of reaction was also studied. The results are shown in the following table.

Concentration of Catalyst (M)	Time taken for the reaction to complete (s)
0.1	120
0.2	60
0.3	40
0.4	30

From the above table, it is clear that as the concentration of the catalyst increases, the rate of reaction also increases. This is because there are more catalyst molecules available to provide an alternative pathway for the reaction.

The ascites mastocytoma cells, even though they possess granules are quite different in appearance from normal mouse mast cells,<sup>15</sup> (p.9), rat mast cells,<sup>16</sup> (p.10), human mast cells<sup>19</sup> (p.11), and even the Furth mouse mastocytoma.<sup>17</sup> (p.10). They resemble somewhat the dog mastocytoma cell described by Bloom et al (p.9) in their general configuration, distribution of granules, and active plasma membrane.

Thus, electron microscopy reveals that there is a tremendous species variation in mast cells although **this** is not as evident in light microscope studies. Indeed, the ascites mast cells look like "typical" mast cells with the light microscope in that they appear full of metachromatic granules other than in the area occupied by the nucleus. The explanation of the difference between the light and electron microscope appearance may be that in the electron microscope we look at a two-dimensional view of very thin horizontal sections which contain granules in only a few areas whereas in the light microscope we see the "composite of a series of thin sections" which collectively contain granules in all areas of the horizontal planes contained in the thin sections so that when viewed from above, as in the light microscope, one gets the impression that the non-nuclear cytoplasm is occupied only by granules. This presupposes that the rest of the material in the cell is essentially transparent.

#### The Origin of the Mastocytoma Granules

Two of the most interesting and important unanswered questions of amine biology concern the origin of the amine-containing granules and how the amines get from the cell

The analysis of the data shows that the  
 results are in good agreement with the  
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 are shown in Figure 1, and the solid line  
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The experimental results are shown in  
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 data points are in good agreement with  
 the theoretical predictions. The error  
 bars are included for each data point.  
 The overall fit is very good, indicating  
 that the theoretical model is valid.

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cytoplasm where they are synthesized from their precursor amino acids by the decarboxylating enzymes<sup>57,58</sup> to the granules where they are stored and yet avoid degradation by the various cytoplasmic or mitochondrial enzymes capable of inactivating these compounds.<sup>59,61</sup> It has been suggested that the amines in the granules are those which have been able to escape degradation,<sup>111</sup> a seemingly wasteful mechanism, or that the amines are bound in some fashion which prevents them from being destroyed while passing from clear cytoplasm to granules.<sup>112</sup>

Pictures of the ascites mastocytoma cells and certain of the atypical granule-containing tissue culture cells provide the basis for a new theory of the origin of the granules of these cells and how they come to contain amines. As previously mentioned, these cells contain an extensive agranular reticulum (which includes the Golgi apparatus) consisting mainly of tubules and sacs seen sometimes in profile as linear arrays parallel to one another but mostly in cross section as microvesicles and microgranules (which are interpreted as microvesicles containing protein, heparin and amines (Fig. 1-3, 13,14). In some areas of the cell, these microvesicles were closely associated with one another but not membrane-bound (Fig.3,G2, G3). These were most commonly seen in the region of the Golgi apparatus (Fig. 3). Elsewhere there are membrane-bound particulates, of the same size and shape as mature amine granules (Figs. 1,2) which contain microvesicles and microgranules (Fig. 3, G1; Fig. 14, G1). In other particulates of this general nature, the microvesicles and micro-granules



appear to be clumping together (Fig. 3, G1; Fig. 13, G1; Fig. 14, G2, G3). In the final stage of this process, which I believe to be that of granular maturation, the microgranules and microvesicles have coalesced within the membrane-delimited area. The membrane of the final granule is probably a dilated part of the agranular Golgi reticulum which has detached itself from this system.

I further propose that the amines, after being synthesized in the clear cytoplasm gain access to the lumen of the vesicles and cisternae of the Golgi reticulum, part of which is then incorporated into the granules as well as used to form their limiting membrane in the manner just described. This would protect the amines from any degradative enzymes in the mitochondria or cell sap as the amines become granular bound. Green and Day have shown that 30% of the 5-HT and histamine of the solid Dunn-Potter mastocytoma was found in the microsomal fraction.<sup>96</sup> This could represent amines in transit to the granules if the proposed theory is correct.

The granules of normal mouse mast cells have been noted to have a "filamentous structure,"<sup>15</sup> those of normal rat and guinea pig mast cells, a "reticular structure,"<sup>16</sup> and those of normal human mast cells, to consist of "lamellar groups."<sup>19</sup> It has been claimed that the inner part of the granules of the adrenal medulla is in the form of very small granules.<sup>113</sup> The studies of Ball cited by Hagen and Barnett<sup>114</sup> demonstrate that the granules of the adrenal medulla possess an enzyme characteristic of the microsomes, which are partly made up of agranular reticulum.<sup>115</sup> This led them to speculate that the



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protein matrix of the adrenal medullary granules originates in the endoplasmic reticulum (where all proteins are synthesized). Hagen, Barnett and Lee noted that the large mastocytoma granules of the Furth mastocytoma occurred in vacuole-like spaces in close relationship to the vesicular system of agranular reticulum. They suggest that this "may indicate a probable role of the reticulum in the development of the granules or the laying down of its contents."<sup>17</sup>

Numerous studies with other cell types which produce granules containing secretory material other than amines, suggest that their granules also originate in, if not from, the agranular reticulum, with which the agranular reticulum is in direct continuity. Wellings, Siegel, Dalton and Felix in a series of papers have extensively studied melanocytes in this regard, demonstrating the progressive maturation of melanin granules within the agranular reticulum.<sup>116-119</sup> Similar mechanisms have been claimed for plasma cells<sup>120</sup> and lactating mouse mammary cells.<sup>121</sup> A morphological and functional association of the reticulum of the pancreatic acinar cell in the development of zymogen granules has been described by Siekevitz and Palade.<sup>122</sup>

This hypothesis that the granules of the Dunn-Potter mastocytoma cells are made from segments of agranular reticulum containing sequestered amines synthesized in the free cytoplasm may be considered an instance of Essner's and Novikoff's thesis that; "Cytoembranes are in a dynamic state of flux, movement and transformation in the living cell and smooth surfaced derivatives of the endoplasmic reticulum become fashioned into the Golgi membranes as the Golgi membranes are

The first part of the report deals with the general situation of the country and the progress of the work done during the year. It is followed by a detailed account of the various projects and the results achieved. The report concludes with a summary of the work done and the prospects for the future.

1951

The second part of the report deals with the work done during the year. It is divided into several sections, each dealing with a different aspect of the work. The first section deals with the work done in the field of research and the results achieved. The second section deals with the work done in the field of education and the results achieved. The third section deals with the work done in the field of social work and the results achieved.

1952

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being refashioned into those that delimit secretory vacuoles."<sup>123</sup>

Whether this mechanism is a common one for making amine granules cannot yet be answered. The studies cited concerning the fine structure of the granules of other mast cells<sup>15,16,19</sup> and the adrenal<sup>113</sup> and the chemical nature of the adrenal granules<sup>114</sup> suggests that it may be a widely employed system.

This hypothesis is readily testable by looking for additional enzymatic activities and constituents common to both the granules and the agranular reticulum of the ascites cells, both of which can be isolated in reasonably pure states by procedures which the author developed. These studies are in progress. Electron microscopic autoradiography with radioactive histamine, 5-HT or heparin would be a very elegant way to test the hypothesis.

#### The Tissue Culture Dunn-Potter X-1-C Mastocytoma Cell

It was thought that distinct advantages would accrue from working with cells from tissue culture rather than with ascites cells aspirated from the mouse peritoneal cavity. The aspirate is really a mixture of neoplastic mast cells, fibroblasts and leukocytes. It was thought that the cultured cells would provide pure populations of identical mast cells which could be readily obtained in large quantities. Also the tissue culture cells could be easily treated with known concentrations of drugs for known periods of time without the interference provided by the mouse. Similar considerations have made the tissue culture cell the most frequently investigated form of this mastocytoma.

It was thus of great interest to learn that, unlike the ascites cells, the tissue culture cells contained few or no

184. The first part of the paper deals with the general principles of the theory of the structure of the cell. It is shown that the cell is a complex system of interacting parts, and that the structure of the cell is determined by the interaction of these parts. The second part of the paper deals with the structure of the cell in the case of a specific example. It is shown that the structure of the cell is determined by the interaction of the parts of the cell, and that the structure of the cell is determined by the interaction of the parts of the cell.

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granules. (Figs. 4-7). While several tissue culture cells definitely possessed granules (Fig. 8), the great majority possessed no cytoplasmic particulates that could be called granules. Those granules that were present in the cultured cells were much smaller than those of the ascitic cells (Fig. 8). This is the only cell that has been described which is functionally a mast cell but which contains no granules. Whether or not it should be called a mast cell is a question of semantics. But the fact that amines are not in granules is obviously of paramount importance in interpreting experiments on amine biology in these cells.

The tissue culture cells have a relatively undifferentiated structure. They have one or two irregular nuclei with one or more large nucleoli, abundant mitochondria, an extensive agranular reticulum, and pinocytotic vesicles. In these respects, save for more mitochondria and the absence of granules and vacuoles, they are quite like the ascites cells.

The absence of granules in the tissue culture cells sets them apart from the ascites cells and belies their descent from the same cell. The ascites cell line as presently maintained is frequently renewed from the tissue culture cells. Clearly the environment in which these cells divide and grow exerts a great influence on the cells phenotype, on which part of its genetic inheritance is expressed.

The X-1-C cell line is derived from a single cell and is presumably genetically pure. This has led investigators to believe that a population of these tissue culture cells was homogeneous. But, with the electron microscope, many atypical cells were seen.

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As was mentioned, a few cells were seen which were identical in all respects, save for the appearance of membrane bound inclusions of smaller size than mitochondria, but lacking an internal structure, which could be amine granules or degenerated mitochondria (Fig. 8). Just why these cells and no others produce granules cannot yet be answered.

A few cells were seen which contained bizarre "multi-vesiculated dense bodies" which look very much like the lysosomes described by de Duve (Fig. 9).<sup>124</sup> These were seen very infrequently in the ordinary tissue culture mastocytoma cells.

The most interesting atypical cell was seen in a reserpine-treated group of cells. The basic morphology of this cell could not be ascribed to the effects of reserpine since it was the only one of tens of thousands of reserpine-treated cells that had this appearance. This cell bore a striking resemblance to the normal mouse mast cells described by Rogers (p. 9, Figs. 10-12). Both cells possess about the same number of granules, each surrounded by a clear area and located at the periphery of the cell. The cytoplasm of both cells is relatively undifferentiated. In the reserpine-treated mastocytoma cell, some of the granules are being released through the cell membrane. This will be discussed shortly. The similarities between this "atypical" neoplastic mouse mast cell and the normal mouse mast cell prompt the speculation that the genetic material of the neoplastic cell line still possesses the information to produce a cell near-normal in appearance on occasion.



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Another very interesting variant which was seen only once was a cell three or four times as large as large as any other cell, with four nuclei, and a cytoplasm densely packed with granules of varying sizes and density, vesicles and mitochondria. (Figs. 13, 14). Significantly, this tissue culture cell which possessed granules demonstrated what I interpret as early forms of granules, made up of agranular reticulum, and identical to those seen more frequently in the ascites cells.

A number of cells were seen whose cytoplasm was occupied mainly by large clear area which could have been granules with a sparse matrix. (Fig. 15). These cells resembled the normal rat and hamster mast cells described by Smith and Lewis.<sup>16</sup>

It can be stated with conviction that the failure to see granules in most of the cultured cells in electron micrographs is not an artifact of preparation. The same technique of osmium tetroxide fixation, alcohol dehydration and embedding in methacrylate or epon have been successfully used by all investigators who have published electron micrographs of mast cells.<sup>14-17,19</sup> The same technique demonstrated granules in the ascites Dunn-Potter mastocytoma cells. In addition, the fine structure of the tissue culture cells was excellently preserved.

It is possible, but very unlikely, that all the amines are present in the granules of the few cells with granules and that the great majority of cells possess neither granules

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or amines. This would necessitate an enormously high concentration of amines in these granules, approximately 100,000 times the concentration of amines in the ascites tumor cells based on the amine concentration of each cell type, the percentage of cells with granules and the number of granules per cell.

It is clear then that most of the amines in these cells are not granular bound unless they are present in mitochondria. Certain evidence makes this unusual possibility at least worth considering, even though every amine-containing cell studied to date has amine granules which are distinct from mitochondria.<sup>111</sup> First, these cells are unusually rich in mitochondria; whereas the ascites cells possess few mitochondria and many granules, the reverse is true of the tissue culture cells. Second, density gradient centrifugation of the tissue culture cell large granule fraction, which sedimented between 700g and 10,000g in thirty minutes and contained 53% of the 5-HT of the cultured mast cells gave results compatible with the amines being in the mitochondria: 49% of this fraction's 5-HT was in a sub-fraction which contained 60% of its succinoxidase activity (located in the mitochondria)<sup>118</sup> while another 43% of the 5-HT was found in a sub-fraction which contained 24.5% of the succinoxidase activity.<sup>(6)</sup> Thus, most of the amines and succinoxidase were in the same sub-fractions implying that they might well be located in the same sub-cellular particle. My attempts to check this by electron microscopy will be reported and discussed in full, later, but it should be mentioned here that they are consistent with the proposition that the amines are located in

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the mitochondria. The data on the cultured cells just presented should be contrasted with the author's studies using density gradient centrifugation of the large granule fraction of the ascites cells which were done several years before the work just presented. With the ascites cells, the author was able to obtain one sub-fraction of the large granule fraction which contained 56% of the total succinoxidase activity and no 5-HT at all, while another sub-fraction had 100% of the 5-HT and histamine and only 28% of the succinoxidase activity. This is at least suggestive that in the ascites cells there are amine storage granules which are not mitochondria and indeed it is the ascites cells which do contain many granules when viewed in the electron microscope. Some mitochondria have been found to contain phosphatides and cerebroside sulfate, both of which have been implicated in amine binding.<sup>111</sup> The chief objection to 5-HT being stored in mitochondria would be that the enzyme which oxidizes 5-HT, monoamine oxidase (MAO) is a mitochondrial enzyme.<sup>125</sup> The author's prolonged studies with sensitive MAO assay techniques failed to find any MAO activity in the Dunn-Potter mastocytoma. The same is true for histaminase, the enzyme which oxidizes histamine. Thus, both these amines could be stored in mitochondria, whereas this would be impossible for the usual cell which contains biogenic amines and the enzymes which degrade them.

It is very hard to conceive of the mitochondria being used to store amines. As was mentioned, all amine-containing cells studied to date have amine granules distinct from

the mitochondria. The fact that the electron spin resonance (ESR) spectra of the mitochondria should be compared with the ESR spectra of the whole cell is due to the fact that the mitochondria contain a large amount of iron-sulfur proteins which are known to be ESR active. The ESR spectra of the mitochondria are very similar to those of the whole cell, and this is due to the fact that the mitochondria contain a large amount of iron-sulfur proteins which are known to be ESR active. The ESR spectra of the mitochondria are very similar to those of the whole cell, and this is due to the fact that the mitochondria contain a large amount of iron-sulfur proteins which are known to be ESR active.

III

mitochondria. The role of the mitochondria in energy metabolism hardly makes it suitable to act as a storage particle. The mechanism of amine storage I just postulated for the Dunn-Potter ascites cells would be ruled out. Why the tissue culture cells should store their amines in mitochondria is not clear. Much more work will have to be done to settle this problem. At this point, all we can say is that the experimental evidence available concerning the tissue culture cells is consistent with the possibility that in these cells the mitochondria contain histamine and 5-HT but that past experience with other amine-containing cells makes this a highly unlikely possibility.

It is possible that the amines or any complexes they may be bound in, are free in the cytoplasm. Furano's finding of the particulate nature of the amines of these cells<sup>6</sup> may be spurious and represent cytoplasmic amines adsorbed on particles after homogenization. The absence of monoamine oxidase and histaminase in these cells which I demonstrated would permit the amines to be non-sequestered.

#### Effects of Reserpine on the Tissue Culture Mastocytoma Cells

It was a difficult decision to decide between using the ascites cells which possess granules and the tissue culture cells which possess essentially none to investigate the process of amine release with reserpine. I decided to use the tissue culture cells because this is the cell that several other investigators are using to investigate the release process



The first of the documents in this  
 category is a letter from the  
 Department of Justice dated 1/20/50  
 to the Honorable Earl Warren in which  
 the Bureau advised that it had  
 obtained a copy of the letter  
 to the Honorable Earl Warren dated  
 1/19/50 from the Honorable Earl  
 Warren to the Honorable Earl Warren  
 dated 1/19/50. This letter, which  
 is a copy of the letter from the  
 Honorable Earl Warren to the  
 Honorable Earl Warren dated 1/19/50  
 is being furnished to you for  
 your information.

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 from the Honorable Earl Warren to  
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 1/19/50. The letter from the  
 Honorable Earl Warren to the  
 Honorable Earl Warren dated 1/19/50  
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ATTACHED TO THIS REPORT IS A COPY OF  
THE LETTER FROM THE HONORABLE EARL WARREN  
DATED 1/19/50 TO THE HONORABLE EARL WARREN

It was a letter from the  
 Honorable Earl Warren to the  
 Honorable Earl Warren dated 1/19/50  
 which is being furnished to you  
 for your information. I decided to  
 the Honorable Earl Warren dated 1/19/50  
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with biochemical and pharmacologic techniques. The opportunity to correlate their data with morphologic changes in the cell was decisive.

After being treated with reserpine at concentrations of  $10^{-7}$  for twenty-four hours, the tissue culture mastocytoma cells were found to contain less than 0.1 gamma of 5-HT per  $10^8$  cells, less than 1% of the control levels. Similarly, the cells had lost the greater part of their histamine content. The cells treated with  $10^{-9}$ M reserpine had approximately 50% of their original levels of 5-HT and histamine at the end of this time.<sup>69</sup> When these cells were looked at with the electron microscope after suitable preparation, a number of different reactions to the drug treatment were noted. They may represent different stages of the release process so that all cells at one time may have gone through the stages seen. The cells were observed only at the end of twenty-four hours when there was no further measurable release at both concentrations of the drug studied. It is obviously of interest to study the cells at earlier times when the process is not yet complete. Thus, the cells to be described are cells who have already released their amines, some perhaps with the cellular changes that brought about still present, others with the cell not showing any unusual features, but lacking amines.

The outstanding change in cell morphology noted was the development of varying numbers of "multivesiculated dense bodies" in a large percentage of the cells. (Figs. 16,17). Some cells possessed as many as thirty of these bodies (Fig.17).

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They are 0.2 microns or oval in shape, and characteristically densely osmophilic with internal discrete clear areas. The appearance of these bodies suggests that they are lysosomes, the enzyme-containing particulates de Duve discovered in liver and subsequently found in a number of the tissues.<sup>124</sup> The multivesiculated dense bodies were almost never found in the untreated cells. More were found in the cells treated with  $10^{-9}$  M than in those treated with  $10^{-7}$  M reserpine, perhaps because the release process was incomplete at the lower concentration.

The chemical nature of these particles together with what is known about amine-binding and release suggests that the appearance of these particles in reserpine-treated, amine-releasing cells is not simply coincidental; rather evidence can be marshalled for the theory that reserpine brings about amine release in these mastocytoma cells by "inducing" the formation of lysosomes, whose enzymatic components could serve as the actual means of bringing about amine release.

First, let us consider the nature of the lysosomes. Their discovery is credited to de Duve who found he could separate by centrifugation a subcellular fraction from liver, rich in acid phosphatase and poor in mitochondrial enzymes.<sup>124</sup> These particles have since been found to contain at least ten hydrolases which have an acid pH optimum: acid phosphatase, the cathepsins A, B, and C, phosphoprotein phosphatase, mannosidase, acid ribonuclease, acid deoxyribonuclease, B-galactosidase, B-glucuronidase and arylsulfatase A and B.<sup>124</sup>

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the people of the United States.

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As will be indicated, several of these enzymes are strong candidates for a role in the release of amines.

The lysosomes of the liver are fairly polymorphic. Some "appear to be solid and are surrounded by a single membrane; others show one or more internal cavities, sometimes lined with a broad layer of denser material, or contain clumps of such material." Most of them have a fine granular structure. Lysosomes of other tissues have somewhat different size and shape. They have been found in practically all tissues.<sup>126</sup> If we are correct in considering the "multivesiculated dense bodies" as lysosomes, this will be the first identification of these structures in mast cells. Enzymatic studies are in progress to definitely identify these bodies as lysosomes. These cannot be reported as yet. However, additional morphologic evidence will be presented subsequently which tends to support their identification as lysosomes.

To understand the proposal that lysosomal enzymes are important in amine release, it is first necessary to review some of what is known about amine binding. The most information available on this subject pertains to the catecholamines stored in the granules of the adrenal medulla. All evidence points to an analagous mechanism existing in all amine-containing cells, including the mast cells.<sup>111</sup>

Hillarp et al. has shown that in the adrenal medulla the sympathomimetic amines are found in granules<sup>127</sup> which others have shown are bounded by a membrane.<sup>128,113</sup> Hillarp showed that amines, adenosine phosphates, proteins and lipids represent practically the entire content of solids in the

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granules.<sup>129</sup> He and his colleagues also showed that the granules of the adrenal medullary cells store catechol amines and ATP in nearly equivalent amounts<sup>130,131</sup> and that stimulation of the medulla in vivo causes a drop in the ATP that is proportional to the decrease in catechol amines.<sup>132,133</sup> Sheep treated with reserpine released amines and adenosine phosphates from their adrenal medulla at the same rate.<sup>134</sup> Hillarp believed he had demonstrated ATPase activity in the adrenal medullary granules.<sup>135</sup> This finding was disputed by others who claimed Hillarp's granule fraction was contaminated with mitochondria and that pure fractions of granules contain no ATPase.<sup>136</sup> Nevertheless, the presence of ATPase in the granules is still an open question because of the difficulty of demonstrating this enzyme. Hillarp suggested that amine release is brought about by the dephosphorylation of ATP to which the amines are probably bound, by the ATPase of the granules which is activated, either directly or indirectly, by the agent which triggers release.<sup>135</sup>

The lysosomal enzymes might work independent of, or together with granular ATPase or other intragranular mechanisms of release. Let us consider an independent mode of action first. The action of the lysosomal enzymes might be to degrade the anion which the amines are bound to - in the mast cell this is probably either heparin or ATP.<sup>1,111</sup> The lysosomes possess both sulfatases and phosphatases<sup>124</sup> although it has not been proven that heparin and ATP are



The first part of the document is a list of names and titles, including:
   
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substrates of these enzymes. In the tissue culture mastocytoma cells where the amines may be in the cytoplasm, the action of these enzymes on the amine complexes could occur directly; in cells where amines are in granules, the enzymes would first have to enter the granules. The lysosomal enzyme (s) might increase the permeability of, or otherwise alter strategic membranes with the cell, perhaps either the granule membrane or the cell membrane. These membranes are basically lipoprotein in nature and the lysosomes possess the necessary equipment (phosphatases, proteinases) to bring about their disruption. After disruption, amine release may occur simply by diffusion because a previously impermeable barrier has now been removed, or contact of lysosomal degradative enzymes and the anions bound to the amines may be promoted. Another possibility is that disruption of the granule membrane may change conditions with the granule, such as the pH or the concentration of various ions within the granule and thus modify the activity of the enzymes present in these granules. As has already been mentioned, there is evidence for an ATPase in the granules.<sup>126,135</sup> One of the important questions regarding the significance of granule ATPase in the release of amines was the mechanism by which it might be activated at the time of release. The change in local conditions that might occur as the result of the action of lysosomal enzymes may be the answer.

It might be argued that the appearance of lysosomes in these cells is an indication of cell death because the lysosomes

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have been implicated in this process.<sup>119</sup> Against this is the finding that the reserpine-treated cells will resynthesize their amines, divide and grow when placed in fresh media.

It is clear that no such lysosomal-mediated explanation of the mode of action of reserpine is possible when one is dealing with isolated amine particles in vitro. But it is not at all necessary that these two phenomena have a common mechanism. It has already been pointed out that in vitro reserpine possesses certain properties such as an inhibition of amine release which are never seen in vivo.

It is quite possible, of course, that the increase in lysosomes following reserpine treatment, has nothing to do with amine release. Other morphologic differences between some few reserpine-treated cells and untreated cells were seen. These will now be described.

At the very outset of these experiments on the neoplastic mouse mast cells, I hoped to see the process of granule release in reserpine-treated cells. The almost invariable absence of granules in these highly dedifferentiated cells generally frustrated this desire. How surprising it was, then, to find one cell, which had been treated with reserpine, in which a number of granules were passing through or had already traversed the cell membrane, and whose general morphology bore a striking resemblance to that of a normal mouse mast cell as described by Rogers (Figs. 10-12 p.9).<sup>14</sup> A possible interpretation would be that this cell had regained some of its normal characteristics and was responding as a normal granule-filled mast cell would, that is, with

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granule release. Some reserpine-treated typical tissue culture cells were seen which had cytoplasmic inclusions passing through the cell membrane (Fig. 18). This was occasionally seen in untreated cells. I am presently investigating the effects of reserpine on the ascite mastocytoma cells which do possess granules.

Reserpine treatment produced a group of cells which suggested another mode of amine release. These cells had large membrane-bound areas with a very sparse matrix. (Fig. 19). In several of these cells, these areas were located at the cell surface with a partially disrupted segment of the plasma membrane as one of its borders. Other such areas were often seen deeper within these cells. It is possible that discharge of cellular contents had taken place at the cell surface, leaving behind amorphous membrane-bound inclusions that then detached themselves from the cell surface to migrate back into the middle of the cell. What makes this of particular interest is that De Robertis and Vaz Ferreira described a similar occurrence in the adrenal medulla of rabbits after stimulation of the splanchnic nerve.<sup>137</sup> Their observations suggested that the secretion of the catechol amines involved an increase in size but a decrease in electron density of the granules and their attachment to the cell surface. The granules then released their contents at the cell surface and were left as folded membranes attached to the cell surface. Furthermore, Wellings and De Ome observed that in lactating mouse mammary cells vacuoles



containing milk protein granules appeared to open at the cell surface and release their contents into the alveolar lumen. Similar methods of secretion have been observed in many other tissues. An analogous process might be happening in the tissue culture mastocytoma cell, differing in that a whole region of the cell enclosed within a membrane, probably part of the agranular reticulum is being discharged. If the amines are located in the cytoplasm of these cells this would effectively accomplish amine release. This cell type was not seen in any of the untreated cells. It is unlikely that it represents vacuole formation at the cell surface, although a secondary result of such a process might be imbibition of extracellular fluid.

At least a half of the cells seen after treatment with both  $10^{-7}M$  and  $10^{-9}M$  showed no morphologic difference from untreated cells, despite the fact that the cells treated with  $10^{-7}M$  possessed less than 1% of their normal amine concentration. This indicates that release can occur without any visible change in the cells morphology, or that the changes which brought about or accompanied release in these cells was no longer evident. It is impossible to make a choice between these two interpretations, although the latter possibility seems more likely.

It would be nice to fit all these findings into a single interpretation that would encompass them all. Since the untreated cells are clearly polymorphic, we are not really required to do so. Reserpine may act differently on



The first part of the report is devoted to a general  
 description of the country and its resources. It  
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 various industries and occupations. The  
 next section deals with the population and  
 the social conditions of the people. The  
 final part of the report is a summary of  
 the findings and conclusions.

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each different cell type present. This clearly seems to be the case in the cells with granules where reserpine produced release of the granules. It is thus possible that these findings, the induction of what appear to be lysosomes, the release of granules, and the release of cell cytoplasm from membrane-bound areas are phenomena seen only in these atypical neoplastic cells. Nevertheless, they do suggest new experiments which should and can be done on other tissues such as the adrenal medulla and the central nervous system.

Some suggestions may be offered about the origin of and additional activities of the "multivesiculated dense bodies" which both tend to support that they are lysosomes and indicate how reserpine promotes their formation. At the same time they contribute in a small way to what is known about the origin of and the activity of the lysosomes. In Figs. 16 and 17, in the midst of areas packed with the multivesiculated dense bodies, we see membrane-bound vacuoles filled with additional segments of membrane and clumps of osmophilic matter. They appear to be early stages of the osmophilic dense bodies. In Fig. 17, we see the dense bodies seeming to ingest mitochondria. This phenomenon has been described previously by Novikoff<sup>138</sup> and the resulting inclusion has been called a "cytolysome".<sup>19</sup> These micrographs suggest that the lysosomes are formed in dilations of the agranular reticulum, as suggested by Novikoff<sup>140</sup> by ingesting cellular material and that once formed



the lysosomes can incorporate other cell contents such as mitochondria.

Effects of Chlorpromazine on the Dunn-Potter Tissue Culture Mastocytoma Cells

Until recently most of the experimental studies of the biochemical basis for chlorpromazine's central nervous system depressant or tranquilizing action were concerned with its activity as an inhibitor of respiratory enzymes or oxidative phosphorylation.<sup>141</sup> In vitro effects could be demonstrated on these systems only by using concentrations of the drug hundreds of times greater than those achieved in vivo.<sup>141</sup> Recent experiments which were reviewed in the Introduction (p. 91-92) have led to the hypothesis that the primary effect of chlorpromazine is on various cell membrane phenomena,<sup>82</sup> particularly those of the mitochondria.<sup>83-93</sup>

Studies with the mast cells treated with chlorpromazine at  $10^{-5}$  M for twelve hours demonstrate a morphologic derangement of mitochondrial structure which seems to result from the action of this drug. (Figs. 20-22). In the untreated mastocytoma cells the outer limiting membrane of the mitochondria is smooth contoured and continuous, whereas the inner one folds inward periodically to form the incomplete septa that form the cristae. The two leaves of the crista usually run straight and parallel and are separated by a narrow interspace of uniform width (Figs. 4-7, 23).<sup>142</sup> The mitochondria of the chlorpromazine treated cells frequently have highly disrupted outer membranes. Cristae are

the following are the principal points of the  
report.

Results of the Investigation

The first part of the report deals with the  
general principles of the method used in the  
investigation. It is shown that the method is  
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it is possible to obtain quantitative results  
from the observations. The second part of the  
report deals with the experimental results. It  
is shown that the method is applicable to a  
wide range of cases and that it is possible to  
obtain quantitative results from the observations.  
The third part of the report deals with the  
discussion of the results. It is shown that  
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quantitative results from the observations.  
The fourth part of the report deals with the  
conclusions. It is shown that the method is  
applicable to a wide range of cases and that  
it is possible to obtain quantitative results  
from the observations.

seldom perpendicular to the limiting mitochondrial membrane but are instead parallel or come off at irregular angles. Some mitochondria appear compartmentalized by crista running in a number of directions. Other mitochondria consist of whorls of membranes, like the myelin of a nerve sheath. There are numerous elongated unusually shaped mitochondria in the treated cells. There are very few normal appearing mitochondria in most of the treated cells (Figs. 20-22). This is to be contrasted with the appearance of "multi-vesiculated dense bodies" in about a quarter of the cells after reserpine treatment.

The morphological effects of chlorpromazine on mitochondrial membranes could be due to the same chemical changes which produce inhibition of mitochondrial swelling in hypotonic solutions, etc. (p. 91-92), <sup>85-93</sup> or the reverse could be true and the chemical effects might be due to the morphological effects which have some other origin. These morphological effects could explain the inhibition of mitochondrial respiratory enzymes which are known to be dependent upon an intact mitochondrial structure and to involve a series of enzymes located in precise order along the cristae.<sup>143</sup> The bizarre mitochondrial forms produced by chlorpromazine should interfere with these delicate functions to a great extent. On the other hand, the enzymatic inhibition could be the cause of the morphologic aberrations. Regardless, this work has established an

The first part of the paper is devoted to a general discussion of the problem of the existence of a solution of the system of equations (1) in the case of a linear operator. It is shown that the existence of a solution is guaranteed if the operator is self-adjoint and the right-hand side of the system is orthogonal to the null space of the operator.

In the second part of the paper, the problem of the existence of a solution of the system of equations (1) is considered in the case of a nonlinear operator. It is shown that the existence of a solution is guaranteed if the operator is monotone and the right-hand side of the system is orthogonal to the null space of the operator.

The third part of the paper is devoted to a general discussion of the problem of the existence of a solution of the system of equations (1) in the case of a nonlinear operator. It is shown that the existence of a solution is guaranteed if the operator is monotone and the right-hand side of the system is orthogonal to the null space of the operator.

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effect of chlorpromazine on mitochondrial membranes that is well suited for experimental analysis.

Giarman has established that this dose of chlorpromazine release significant amounts of 5-HT from the tissue culture cells.<sup>144</sup> As was discussed previously, it is possible that the tissue culture cells store their amines in mitochondria. If so, the release of amines by chlorpromazine may be related to the mitochondrial structural changes just discussed as cause or effect. The increased disruption of the plasma membrane of the mastocytoma cells may also be involved. There was no evidence of lysosome formation, granule release or release of cytoplasm in the chlorpromazine-treated cells, as was seen in the reserpine-treated cells.

#### The Large Granule Fraction of the Tissue Culture Cells

A large granule fraction was isolated from the tissue culture cells after homogenization in 0.3M sucrose. This fraction was layered on a density gradient according to the method of Furano<sup>6</sup> but no separation was achieved. The only tissue evident after centrifugation was on the bottom of the centrifuge tube. This material was prepared for electron microscopy which revealed that it consisted largely of distorted mitochondria (Fig. 24). Very few granules were noted. It is clear that these cells possess very few if any granules.





Isolation of Mast Cell Granules from the  
Ascites Cells

The purpose of this work was to isolate the amine and heparin containing granules of these cells so that accurate chemical studies of their nature could be carried out. These studies were carried out before electron microscopy revealed that the ascites cells, but not the tissue culture cells possessed such granules, so the choice was a fortuitous one.

Initially, the cells were vigorously homogenized with the Potter-Eveljhem apparatus, followed by removal of unbroken cells, nuclei and debris by centrifugation. A large granule fraction is then sedimented from this cell and nucleus free homogenate by centrifugation and the distribution of amines, heparin and succinoxidase between the large granule fraction and supernatant determined. The results of one experiment are given in Table 1. They are representative of many similar experiments.

# THE HISTORY OF THE UNITED STATES

The history of the United States is a story of growth and change. From the first settlers to the present day, the nation has evolved through various stages of development. The early years were marked by exploration and the establishment of colonies. The American Revolution led to the birth of a new nation, and the subsequent years saw the expansion of territory and the growth of industry. The Civil War was a pivotal moment in the nation's history, leading to the abolition of slavery and the strengthening of the federal government. The 20th century brought significant social and economic changes, including the rise of the industrial revolution and the emergence of the United States as a global superpower.

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62  
Table 1

The Distribution of Histamine, 5-HT and Heparin in Fractions Derived from Mastocytoma Cells by Differential Centrifugation

	Nucleus-free Homogenate	Large Granule Fraction	Supernatant
Histamine	63	53	15
5-HT	180	66	51
Heparin	100,000	67,500	31,500
Succinoxidase	7,600	5,400	1,750

Histamine and 5-HT are expressed as micrograms. Heparin is expressed as total counts. Succinoxidase is expressed as change in optical density under the assay conditions described in the section on Methods.

This demonstrates that the greater part of the histamine, heparin and succinoxidase is particulate in nature. Only about 35% of the 5-HT of the nucleus free homogenate was found in the large granule fraction. Some 28% was in the supernatant. 37% of the 5-HT could not be accounted for and is presumed to be non-enzymatically destroyed since, as will be discussed shortly, no enzyme system capable of degrading 5-HT has ever been found in these cells. Values for the amount of 5-HT which could be recovered in the particulate fraction varied between 30 and 45% in numerous experiments. But the 37% of the 5-HT of the nucleus free homogenate which could not be accounted for, as well as some or all of the supernatant 5-HT might originally have been in the granules. Undoubtedly, much 5-HT is released from the granules during the arduous process of homogenization needed to disrupt these cells. The 5-HT must be more easily

The following table shows the results of the analysis of the data obtained from the experiments conducted during the period from 1950 to 1952.

Experiment No.	Time (min)	Temperature (°C)	Yield (%)
1	10	100	85
2	20	100	80
3	30	100	75
4	40	100	70

The results of the analysis show that the yield of the product decreases as the reaction time increases. This is due to the fact that the reaction is reversible and the product is unstable at high temperatures.

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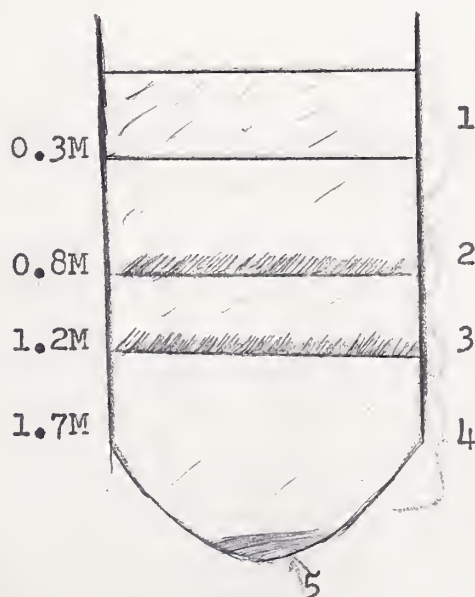
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removable by mechanical methods from the granules than heparin or histamine. Hagen, Barnett and Lee have shown the granules of the Furth mastocytoma easily lose their amines in isolation procedures.<sup>17</sup> Of the 5-HT of the nucleus-free homogenate which could be accounted for either in the particulate form or in the supernatant, 60% was particulate (Table 1).

The large granule fraction is then subject to density gradient centrifugation as previously described. After centrifugation, two distinct bands of tissues are evident; (Text Fig. 1) the first is at the border of the 0.8M and 1.2M sucrose solutions and the second is at the border of the 1.2M and 1.8M sucrose solutions. There is a small precipitate at the bottom of the tube.

Text Fig. 1



Five fractions are taken with the aid of the tube cutting device already described and aliquots analyzed for 5-HT, histamine, heparin and succinoxidase. The results are described in Table 2.

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Table 2

Distribution of 5-HT, Histamine, Heparin and Succinoxidase in the Five Fractions Obtained From Density Gradient Centrifugation of the Large Granule Fraction.

Fraction	5-HT		Histamine		Heparin		Succinoxidase	
	Units	%	Units	%	Units	%	Units	%
1	0	0	0	0	15,000	26	700	16
2	0	0	0	0	0	0	2400	56
3	63	100	12	100	30,000	53	1100	28
4	0	0	0	0	7,600	13	0	0
5	0	0	0	0	4,500	8	0	0

5-HT and histamine are expressed as micrograms. Succinoxidase is expressed as change in optical density under the assay conditions already described. Heparin is expressed as total counts.

All of the 5-HT and histamine and 53% of the heparin is in fraction three, which is the material which did not sediment below 1.7M sucrose. This fraction contained 28% of the succinoxidase activity as well. Fraction 2 contained 56% of the succinoxidase activity and no 5-HT, histamine, or heparin.

Fraction 2 was resuspended over a second density gradient consisting of 2.5cc 0.8M sucrose, 5.0cc 1.5M sucrose, 7.5cc 1.6M sucrose and 5.0cc of 1.7M sucrose and centrifuged for an hour at 25,000g. A single band was evident at the level of the 1.5M sucrose layer. This was found to contain nearly all of the histamine, heparin,



Table 1 shows the results of the regression analysis. The dependent variable is the logarithm of the average number of children per woman. The independent variables are the logarithm of the average number of children per woman in the previous generation, the logarithm of the average number of children per woman in the previous generation squared, and the logarithm of the average number of children per woman in the previous generation cubed.

Variable	Mean	Standard Deviation	Standard Error	t-Statistic	Probability >  t
ln(X)	2.00	0.50	0.05	4.00	0.0001
ln(X)^2	4.00	1.00	0.10	4.00	0.0001
ln(X)^3	8.00	1.50	0.15	5.33	0.0000
ln(Y)	2.00	0.50	0.05	4.00	0.0001
ln(Y)^2	4.00	1.00	0.10	4.00	0.0001
ln(Y)^3	8.00	1.50	0.15	5.33	0.0000

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5-HT and succinoxidase that had been layered over the gradient indicating no further separation was achieved by the second gradient.

This should not be constructed as an indication that in the ascites cells, the amine-containing particles are identical with some of the mitochondria. Had this second gradient produced a fraction with succinoxidase activity, but containing no amines, we could have definitely ruled out the identity of these particles. The only fair interpretation of the data is that there is in these cells a population of mitochondria which behaves identically to amine-containing particulates during certain centrifugation procedures. Electron microscopy of the intact cells suggests that the amine granules and mitochondria are different. Electron microscopy of the isolated fractions will be done shortly and should help settle the problem. At the present stage of purification, the amine-containing particles in fraction 3 are not sufficiently uncontaminated by mitochondria to permit the precise studies needed to characterize the granules chemically. Other techniques will be employed to improve the separation.

#### Monoamine Oxidase and Histaminase (Diamine Oxidase)

The attempts to detect the presence of these enzymes by liberation of ammonia from substrates such as tyramine, 5-HT and histamine by the method of Cotzias and Greenough<sup>107</sup> met with no success. In a few experiments on monoamine oxidase, small amounts of ammonia were produced, but the production

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Summary of the work done during the year

The work done during the year has been divided into three main sections. The first section is devoted to a general survey of the work done during the year. The second section is devoted to a detailed account of the various projects undertaken. The third section is devoted to a summary of the results obtained.

was not inhibited by known monoamine oxidase inhibitors such as iproniazid. No evidence for histaminase was found.

The sensitive spectrophotometric assay for monoamine oxidase<sup>110</sup> failed to reveal any enzyme present in mitochondria from large numbers of the ascites, as well as the solid tumor cells. It could be calculated from the number of cells used that if they do possess this enzyme, they contain less than 1/300th the amount present in mouse liver.

The absence of both MAO and histaminase in these cells is to be contrasted with the presence of MAO and absence of histaminase in the Furth mastocytoma.<sup>17</sup> It would be very interesting to determine if normal mast cells possess these enzymes. Green points out that cells which contain biogenic amines, almost invariably contain enzymes which inactivate them.<sup>111</sup> Thus, these amines must normally be protected from the degradative enzymes, usually by granule sequestration. The absence of these enzymes in the Dunn-Potter mastocytoma cells allows the cell wide latitude in its handling of the amines, and it thus is less surprising that the tissue culture form of this tumor possesses no granules, but may store its amines either in the mitochondria or the free cytoplasm as previously discussed. No catabolism of histamine or 5-HT by any enzyme system has ever been found in this tumor, though it has been extensively looked for.<sup>30,43</sup> This opens up the interesting question of what prevents the cells from continually accumulating amines.

and the following is a list of the names of the  
persons who were present at the meeting.  
Name

The meeting was held on the 10th of  
the month of June, 1900, at the residence of  
Mr. J. H. Smith, 123 Main Street, New York  
City. The following persons were present:  
Name

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Name

SUMMARY

1. The basic structure of the Dunn-Potter mastocytoma X-1-C cells grown in tissue culture and the mouse peritoneum (Ascitic cells) as seen in the electron microscope, is described. These cells are 7-15 millimicrons in size, possess in common 1-2 nuclei usually irregular in shape, a plasma membrane, a well-developed agranular reticulum, but sparse endoplasmic reticulum and lipid inclusions. The ascites cells possess large vacuoles which do not appear in the tissue culture cells.
2. The ascites cells possess varying numbers of oval granules about 0.05 and 0.2 microns. They have few mitochondria.
3. The tissue culture cells possess few or no granules and large numbers of mitochondria. The possible storage of amines in these cells in mitochondria is discussed. The polymorphism of the tissue culture cells is described.
4. A new theory for the origin of the amine granules and how they get the amines they contain, is proposed and supported. Collections of microvesicles and micogranules of the agranular reticulum appear to be reorganizing within dilations of the agranular reticulum into mature granules. Chemical assays and evidence from the literature are advanced in supports of this interpretation.
5. It is proposed that the amines after synthesis by the cytoplasmic decarboxylases are secreted into the granular reticulum which then is formed into the granules.

1. The first objective of the present study is to determine the effect of the concentration of the reactants on the rate of the reaction. This is done by measuring the rate of reaction at different concentrations of the reactants while keeping the other conditions constant. The results show that the rate of reaction increases with the increase in the concentration of the reactants.

2. The second objective is to determine the effect of the temperature on the rate of the reaction. This is done by measuring the rate of reaction at different temperatures while keeping the other conditions constant. The results show that the rate of reaction increases with the increase in the temperature.

3. The third objective is to determine the effect of the presence of a catalyst on the rate of the reaction. This is done by measuring the rate of reaction with and without a catalyst while keeping the other conditions constant. The results show that the rate of reaction increases with the presence of a catalyst.

4. The fourth objective is to determine the effect of the surface area of the reactants on the rate of the reaction. This is done by measuring the rate of reaction with different surface areas of the reactants while keeping the other conditions constant. The results show that the rate of reaction increases with the increase in the surface area of the reactants.

5. It is concluded that the rate of reaction is affected by the concentration of the reactants, the temperature, the presence of a catalyst, and the surface area of the reactants. The rate of reaction increases with the increase in the concentration of the reactants, the temperature, the presence of a catalyst, and the surface area of the reactants.

6. Treatment of the tissue culture cells with reserpine at  $10^{-7}M$  and  $10^{-9}M$  for 24 hours, which releases approximately 100% and 50% of the histamine and 5-HT of these cells respectively results in the formation of "osmophilic dense bodies" in many of the cells. It is proposed that these osmophilic dense bodies are lysosomes. A number of possible mechanism whereby the lysosomes might bring about amine release are discussed.

7. The release of granules in an unusual reserpine-treated cell which strikingly resembled normal mouse mast cells is described.

8. The possible relationship to amine release of certain membrane-enclosed clear areas of cytoplasm in the reserpine-treated cells is discussed.

9. Gross derangement of the structure of the mitochondria of chlorpromazine-treated tissue culture cells is illustrated.

10. The morphology of the large granule fraction of the tissue culture cells is described. It consists mainly of mitochondria.

11. The separation of the mitochondria and amine granules of the tissue culture cells by density gradient centrifugation is described. One fraction contained 56% of the mitochondrial enzyme activity and no histamine, heparin



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or 5-HT. Another fraction contained 100% of the 5-HT and histamine, 53% of the heparin, but only 28% of the mitochondrial enzyme activity.

12. The absence of monoamine oxidase and histaminase in the Dunn-Potter mastocytoma is discussed.

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Fig. 1: A typical ascites Dunn-Potter mastocytoma cell showing the granules (G) which are easily distinguishable from the mitochondria (M). The nucleus (N) is in the upper left-hand corner. Vesicles of the agranular-reticulum (AR) are scattered throughout the cytoplasm. The highly active cell surface ingesting extracellular fluid is apparent. (X15,000).

Fig. 2: High magnification picture of ascites mastocytoma cell showing membrane-bound granules (G) some of which can be seen to be made up of still smaller granules and vesicles. Nucleus (N) with a double membrane and abundant agranular reticulum (AR) are apparent. (X35,000).

Fig. 3: An ascites mastocytoma cell. In the cytoplasm adjacent to the nucleus (N) are aggregates of microgranules and microvesicles (G1, G2, G3). The vesicles in G1 are clearly enclosed within a membrane. The similarity between these microgranules and microvesicles and those of the agranular reticulum (AR) (see also Fig. 2) is clear. In G1, G2, and G3, some of the microbodies appear to have clumped together, producing areas resembling the mature granules seen in Fig. 2. G1, G2 and G3 could be early forms of the mast cell granules, thus indicating the agranular reticulum as the origin of the granules. (X30,000).



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Fig. 1

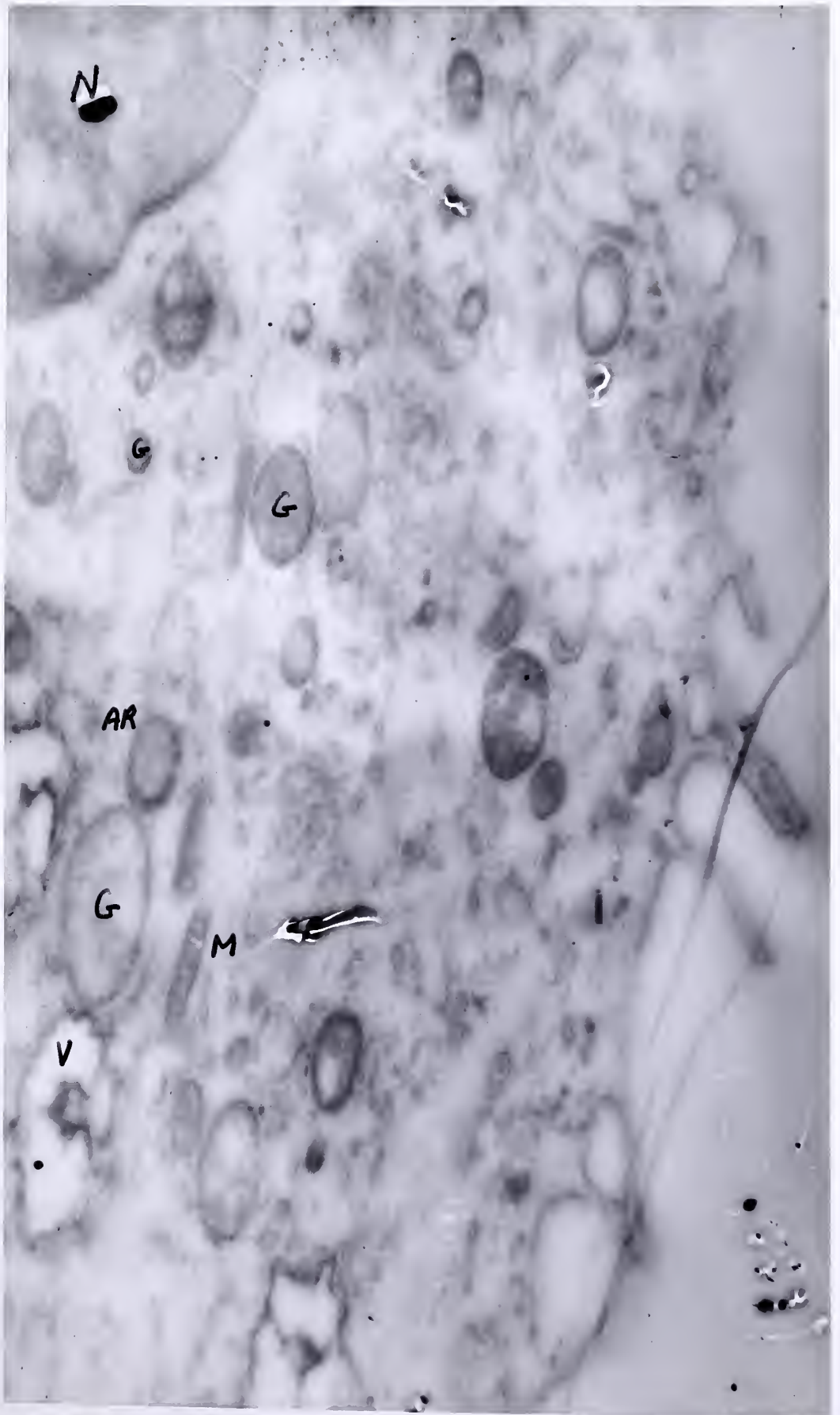






Fig. 2

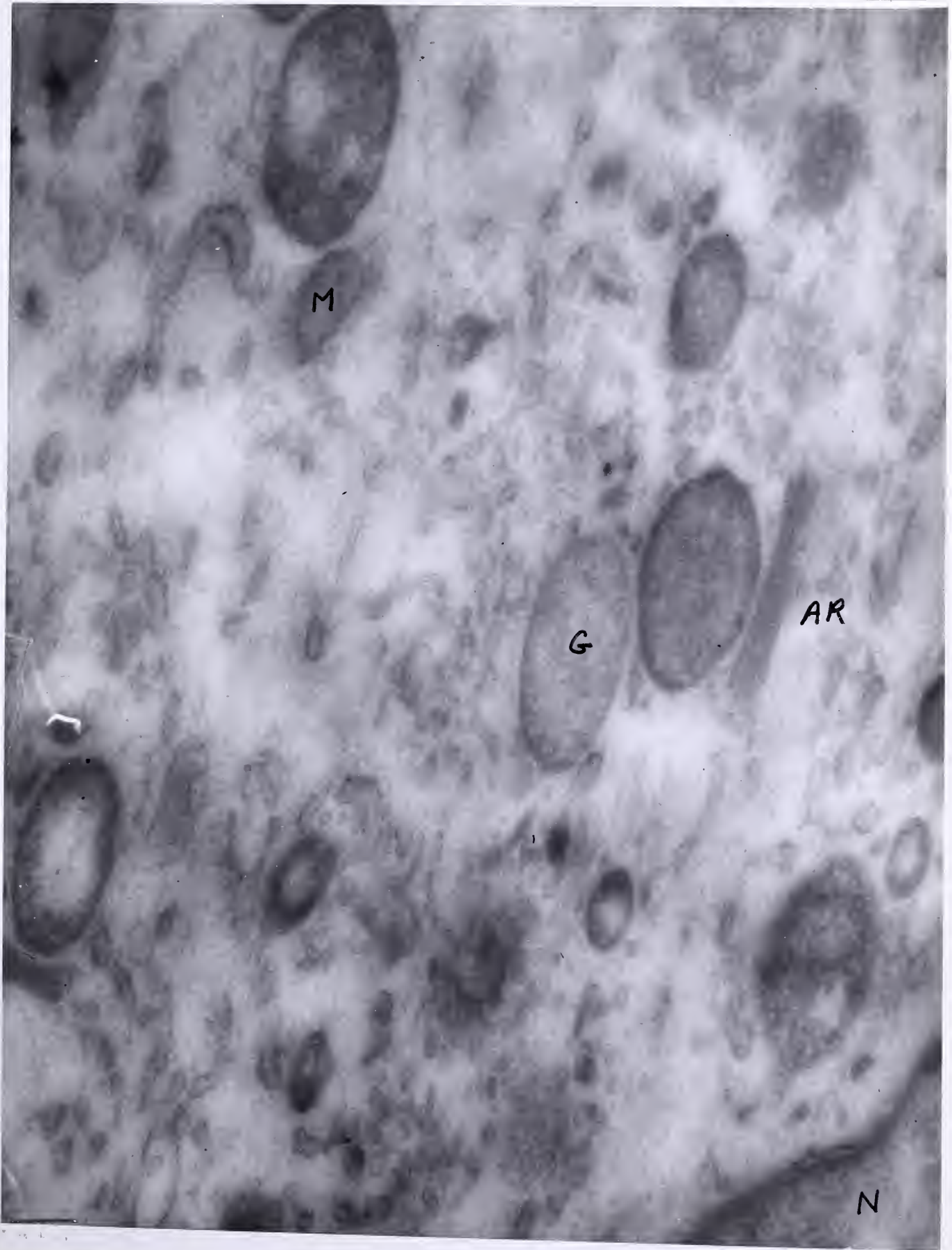
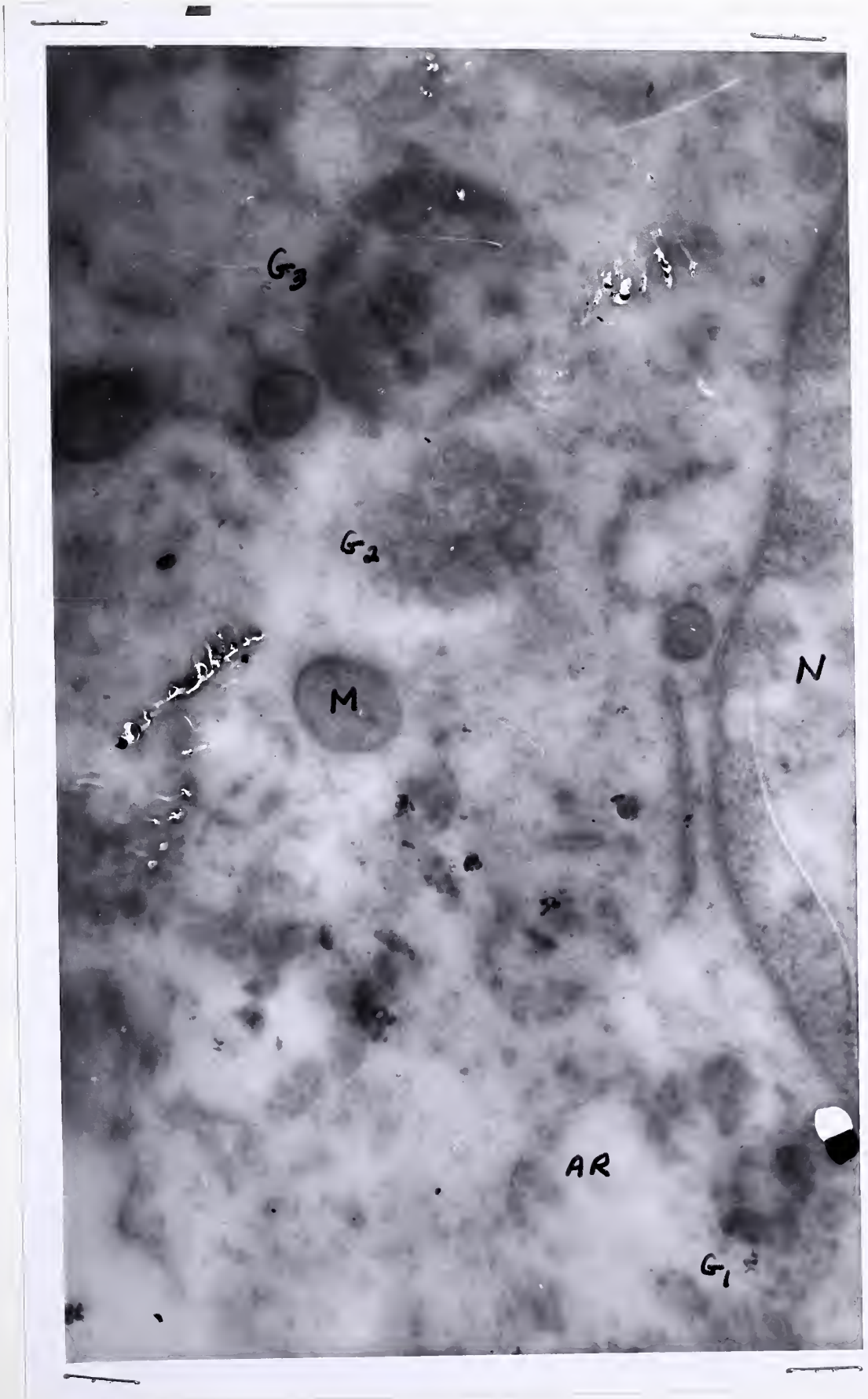




Fig. 3





Figs. 4, 5: Two typical tissue culture mastocytoma cells. Nucleus (N) with double membrane (NM) is evident as are a great many mitochondria (M). A few cytoplasmic inclusions seem to lack cristae and could be either degenerated mitochondria or granules (A). The border (B) between two adjacent cells is evident in Fig. 5, as is a large lipid body (Li). (Fig. 4 X18,000; Fig. 5, X16,000).

Fig. 6: One of the few tissue culture cells in which the endoplasmic reticulum (ER) was seen.

Fig. 7: High power picture of tissue culture mast cell with cytoplasm filled with numerous mitochondria (M), agranular reticulum (AR), and two lipid bodies (Li). Note absence of granules. (X43,000).

Fig. 8: Several tissue culture cells closely adherent to one another with small, membrane-bound granules (G) scattered through the cytoplasm and easily distinguishable from the mitochondria (M).

Fig. 1. The diagram shows the structure of the molecule of the polymer obtained from the reaction of the monomer with the catalyst. The structure is shown in the form of a chain of repeating units. The units are connected by covalent bonds. The structure is shown in the form of a chain of repeating units. The units are connected by covalent bonds. The structure is shown in the form of a chain of repeating units. The units are connected by covalent bonds.

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Fig. 4. The diagram shows the structure of the molecule of the polymer obtained from the reaction of the monomer with the catalyst. The structure is shown in the form of a chain of repeating units. The units are connected by covalent bonds. The structure is shown in the form of a chain of repeating units. The units are connected by covalent bonds.

Fig. 4

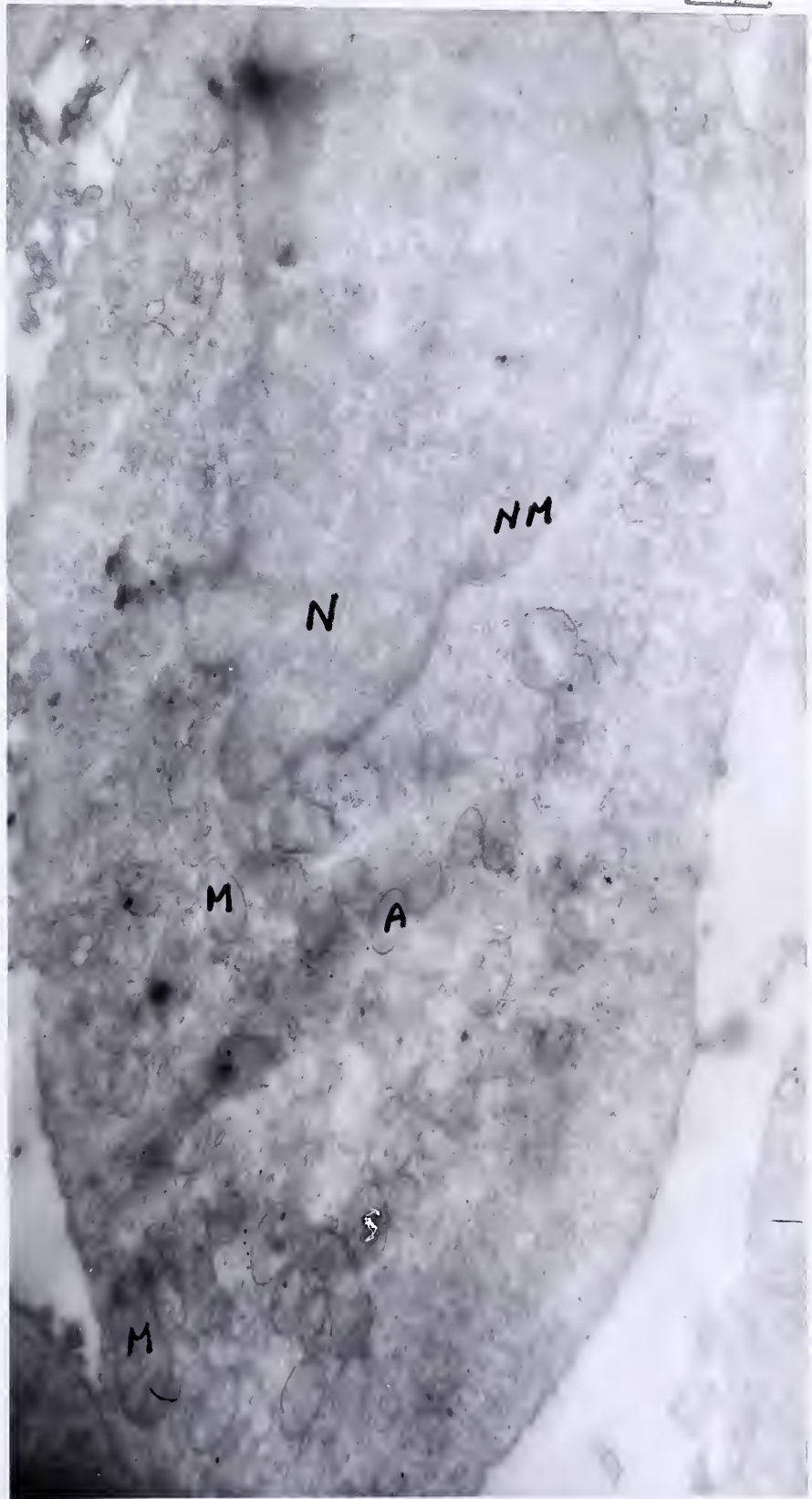
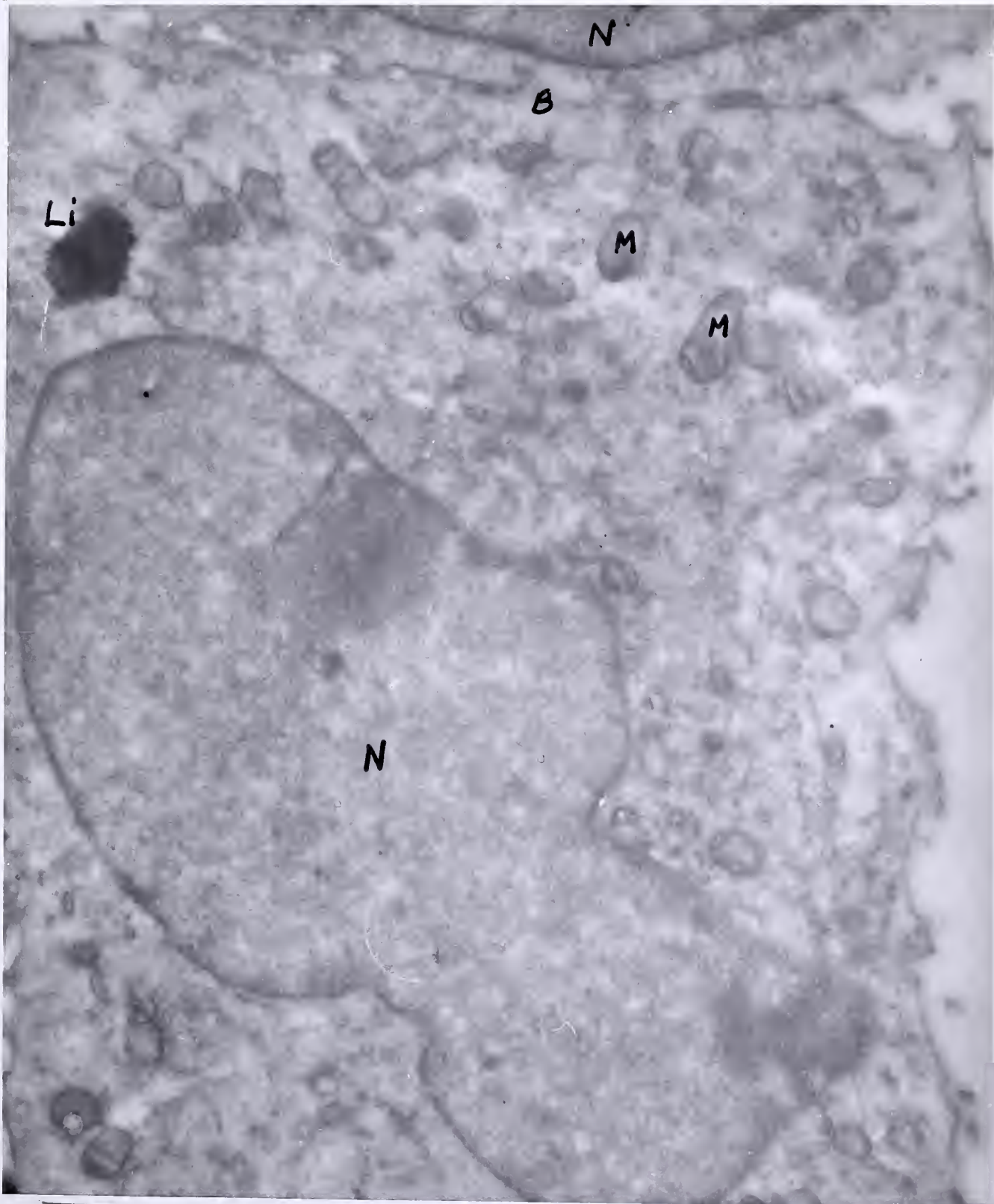




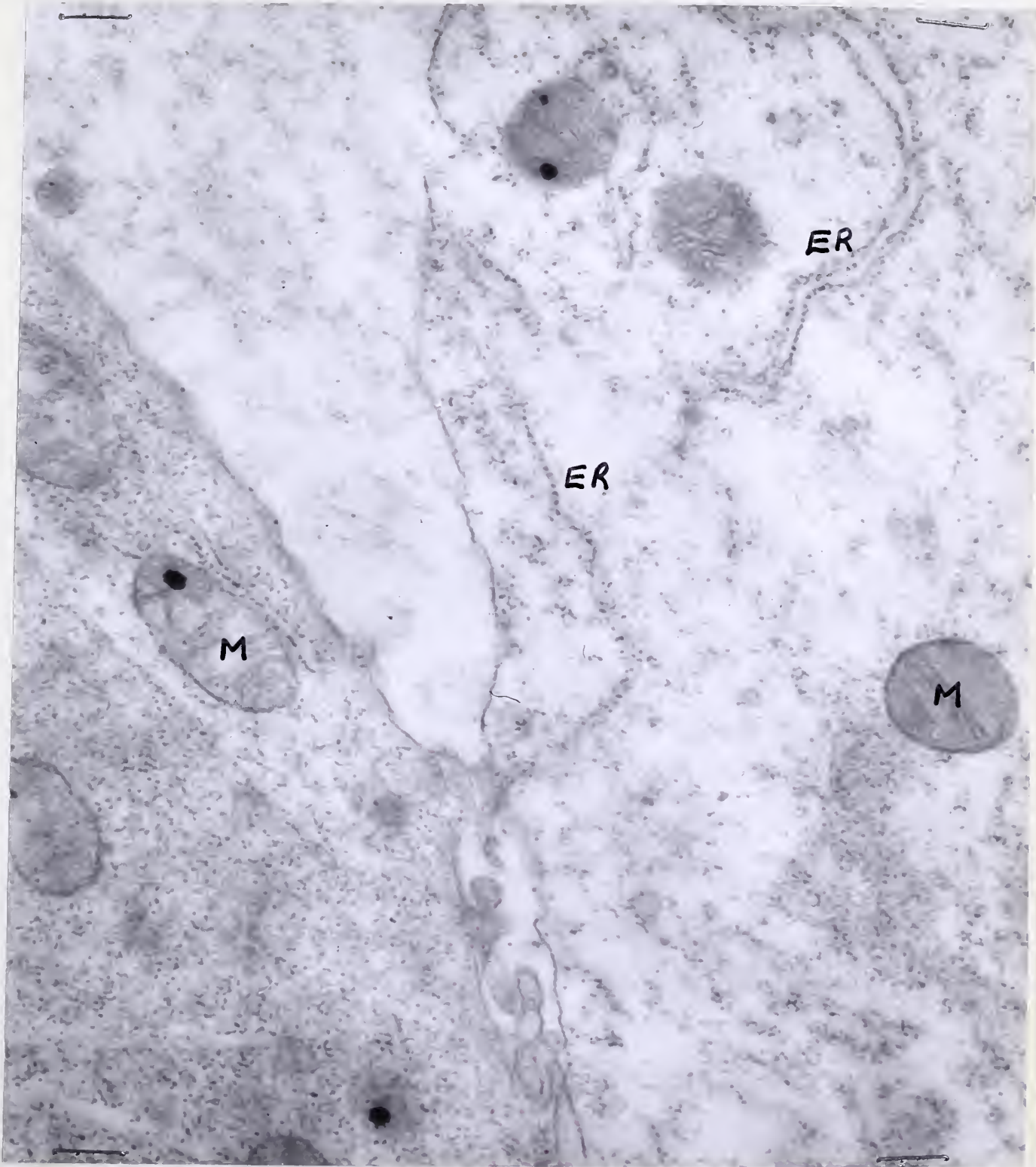


Fig. 5





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Fig. 6



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Fig. 7

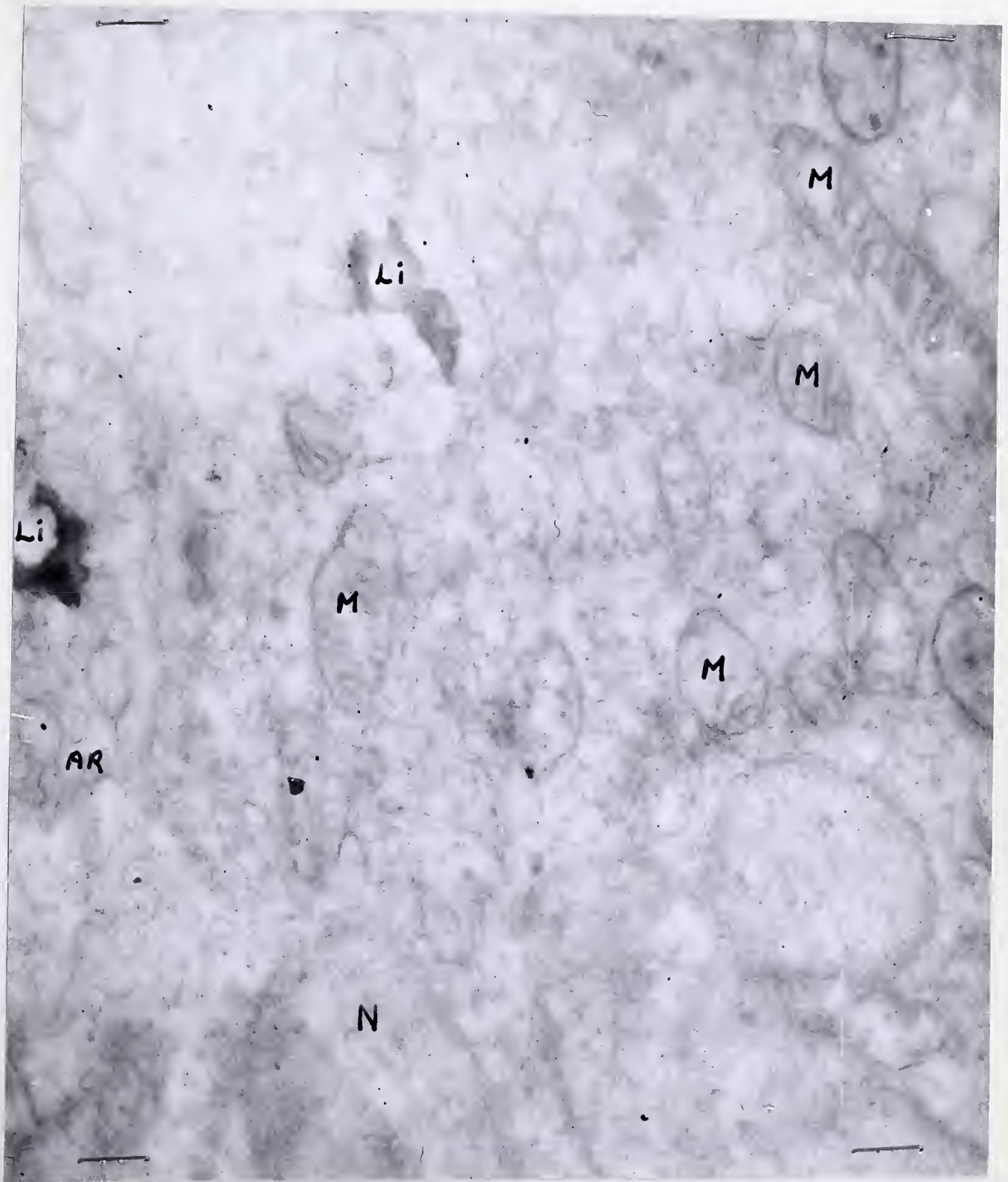




Fig. 8

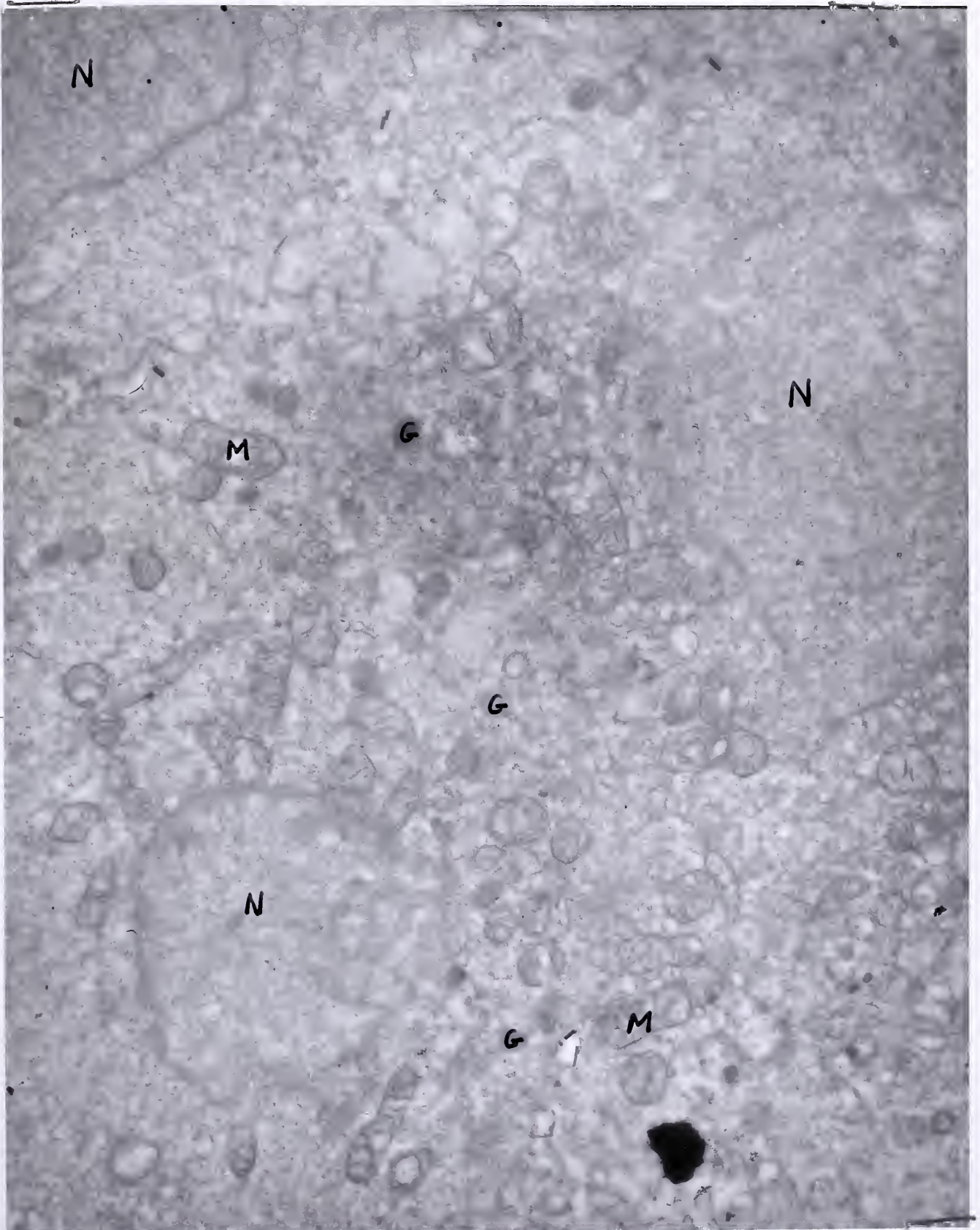






Fig. 9: A tissue culture cell with many very bizarre inclusions (L). They resemble somewhat the vacuolated dense bodies (probably lysosomes) seen after reserpine treatment of these cells. (X13,000).

Fig. 10: A tissue culture mastocytoma cell treated with reserpine. Note the granules (G), some of which are within the cell and surrounded by a clear area of cytoplasm, and other granules which have been or being extruded at the cell surface in the upper right-hand corner. No nucleus is present but this may be due to the plane of sectioning. Mitochondria (M) and lipid bodies (L) are present. Note the difference between this cell and the other tissue culture cells (Figs. 4-7) and the similarity to a normal mouse mast cell (Fig. 12). (X12,000).

Fig. 11: A high power shot of the same cell seen in Fig. 10. The granules (G) are seen more clearly as well as the extrusion of granules at the cell surface. (X30,000).

Fig. 12: A normal mouse mast cell. Note how few granules are present and the clear area around "each granules." Nucleus (N) and mitochondria are apparent. Compare with Fig. 10 (X7,000).

Item 10: A balance sheet as at the end of the year 1950, showing the following figures: (1) Assets, \$100,000; (2) Liabilities, \$50,000; (3) Capital, \$50,000.

Item 11: A balance sheet as at the end of the year 1951, showing the following figures: (1) Assets, \$120,000; (2) Liabilities, \$60,000; (3) Capital, \$60,000.

Item 12: A balance sheet as at the end of the year 1952, showing the following figures: (1) Assets, \$150,000; (2) Liabilities, \$75,000; (3) Capital, \$75,000.

Item 13: A balance sheet as at the end of the year 1953, showing the following figures: (1) Assets, \$180,000; (2) Liabilities, \$90,000; (3) Capital, \$90,000.

Fig. 9

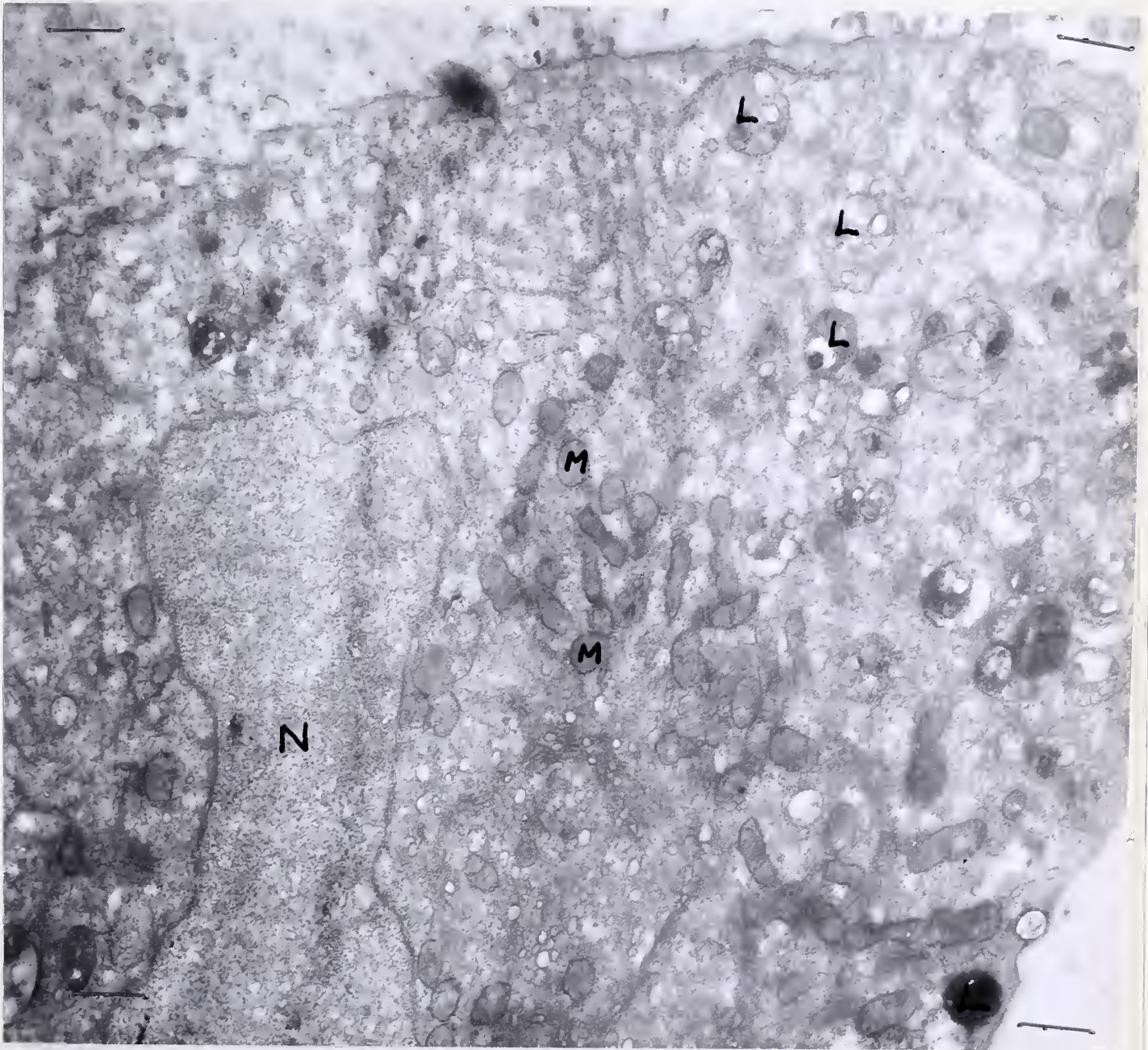
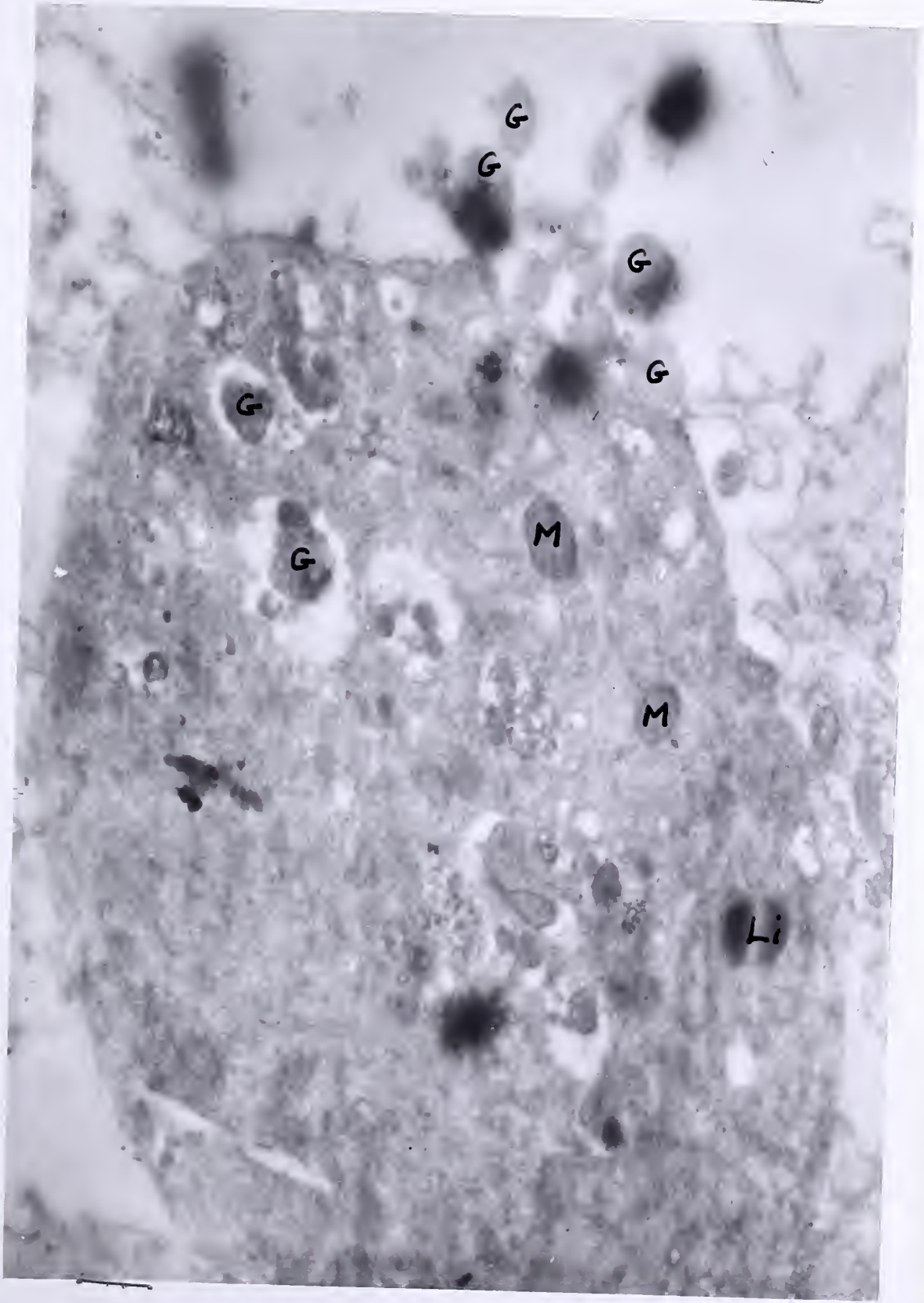




Fig. 10





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Fig. 11

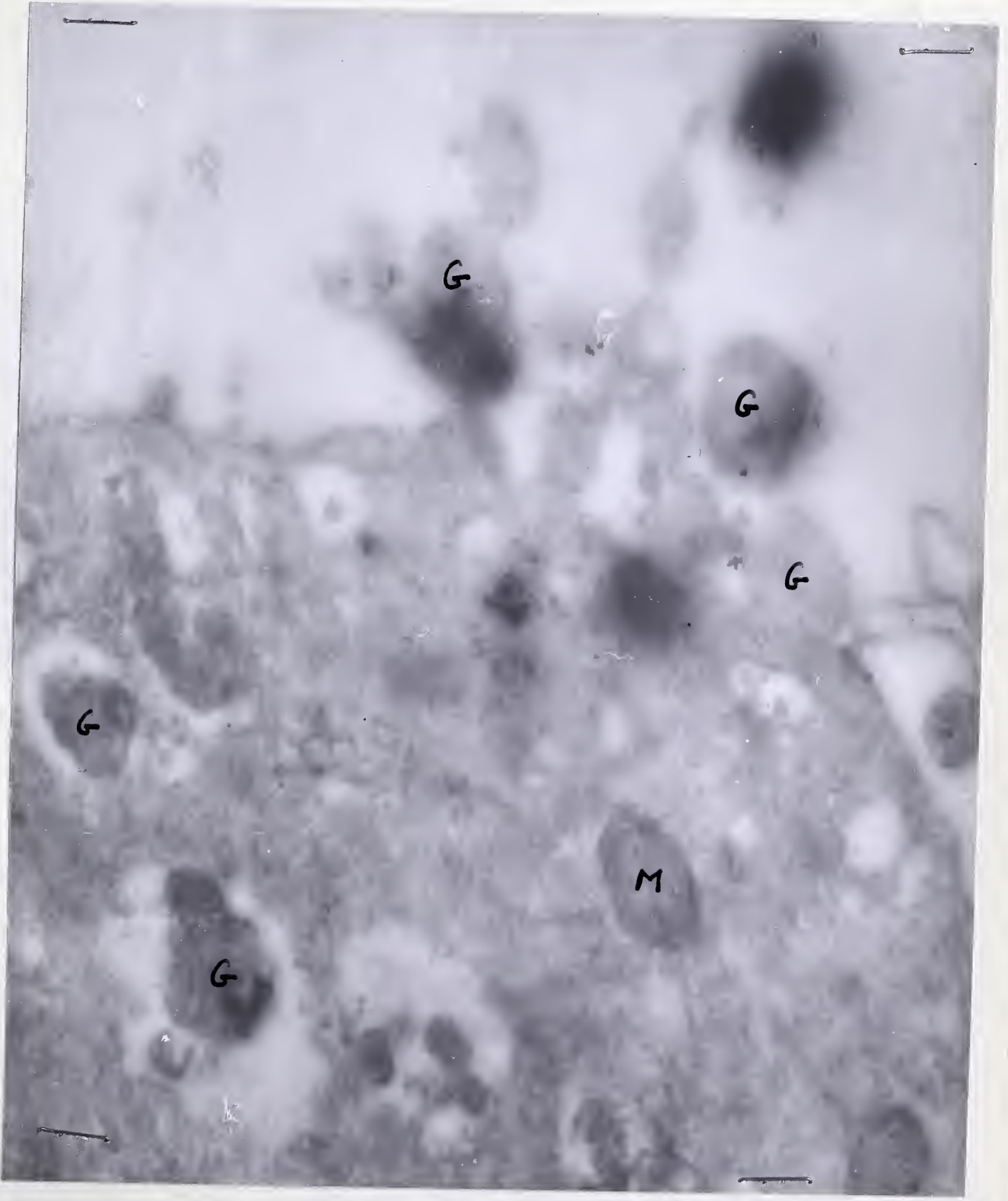






Fig. 12

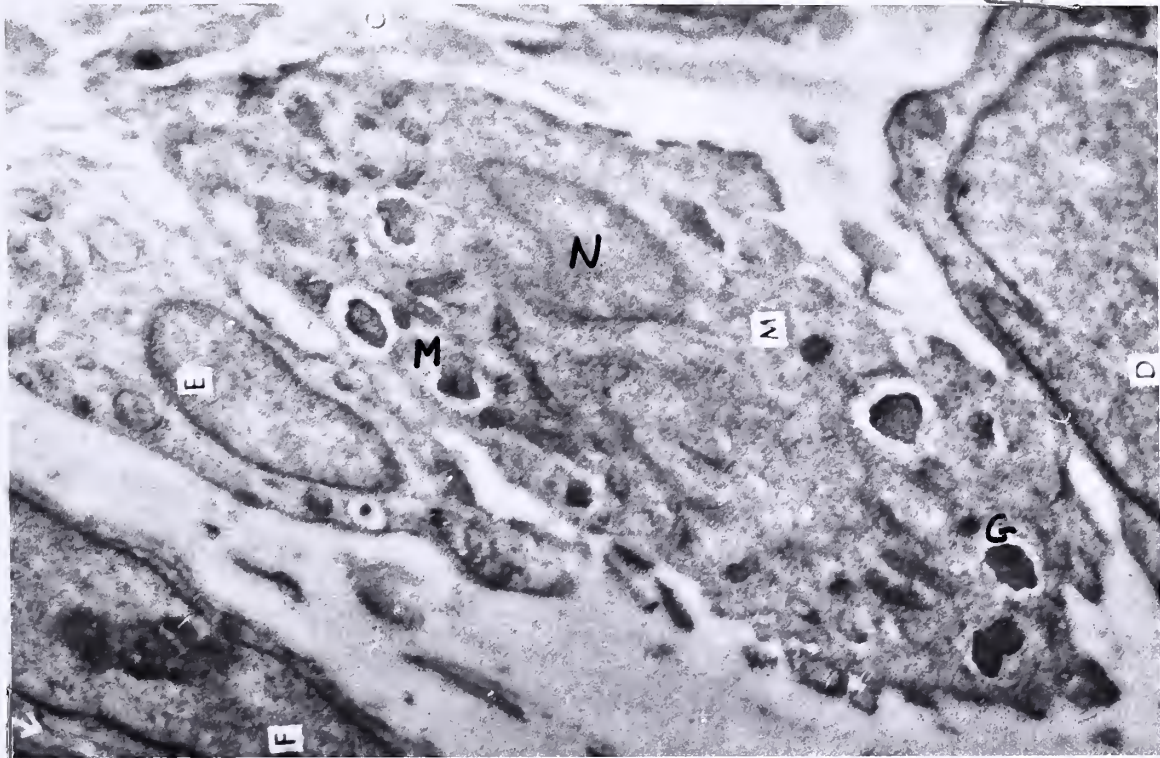




Fig. 13: Portion of an unusual mastocytoma cell, much larger than any other cell, with four nuclei (N). Mitochondria (M), granules (G), and lipid bodies (Li) are seen. Many of the granules are made up of microgranules (G<sub>1</sub>, G<sub>2</sub>, G<sub>3</sub>). Note the similarity between these granules and those of the ascites cell. (Figs. 2, 3). Small granules (SG) are scattered throughout the cytoplasm. (X12,000).

Fig. 14: Another region of the same cell seen in Fig. 13. G<sub>1</sub>, G<sub>2</sub> and G<sub>3</sub> are granules whose fine structure is clearly evident. G<sub>4</sub> is a mature granule. Numerous small granules, mitochondria, and agranular reticulum are scattered throughout the cytoplasm. (X12,000).

Fig. 14: An unusual tissue culture cell with large clear areas in the cytoplasm and two large lipid inclusions (Li). The clear areas (G) probably represent granules. Mitochondria (M) are located between the granules. (X18,000).



Fig. 13

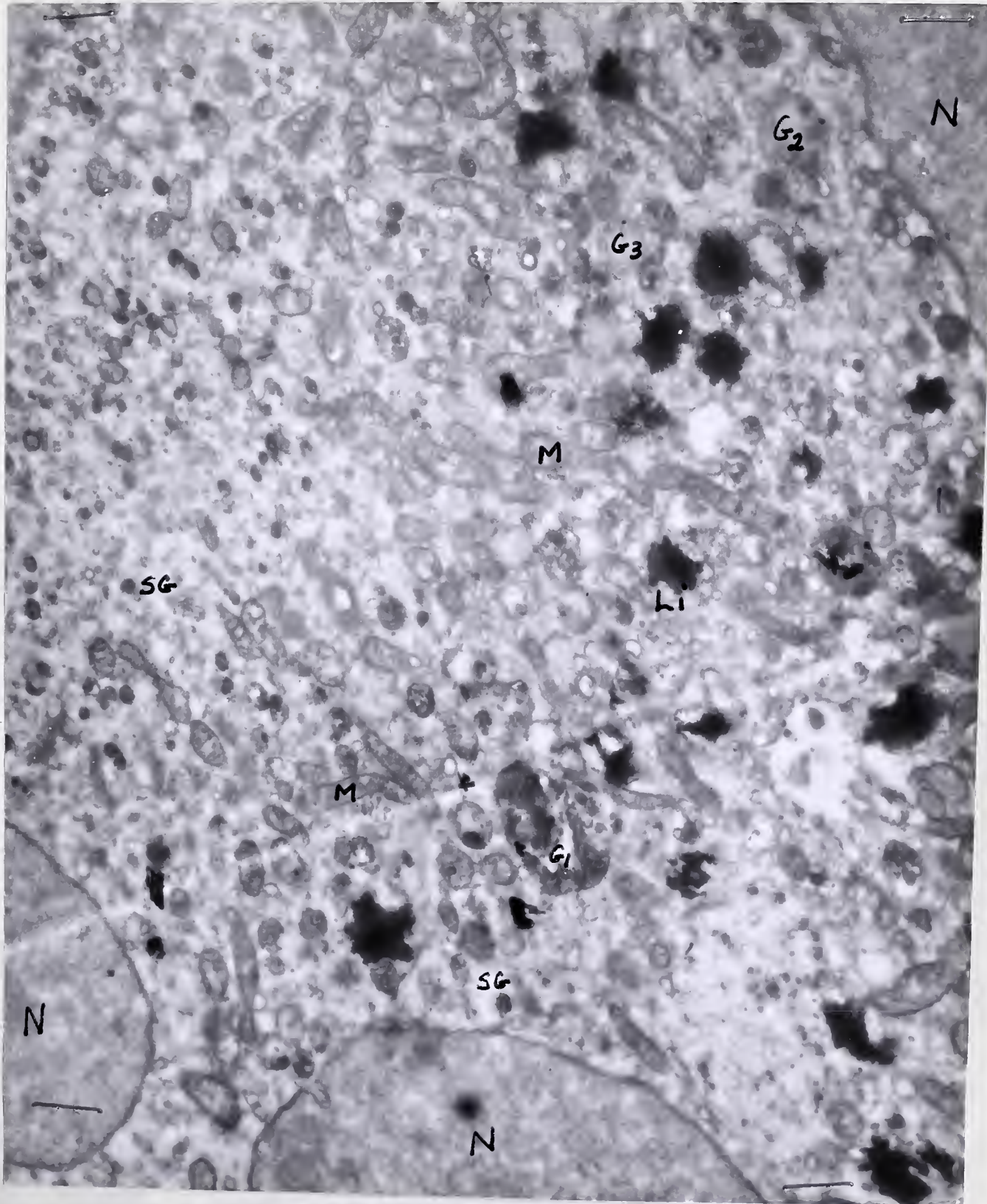




Fig. 14

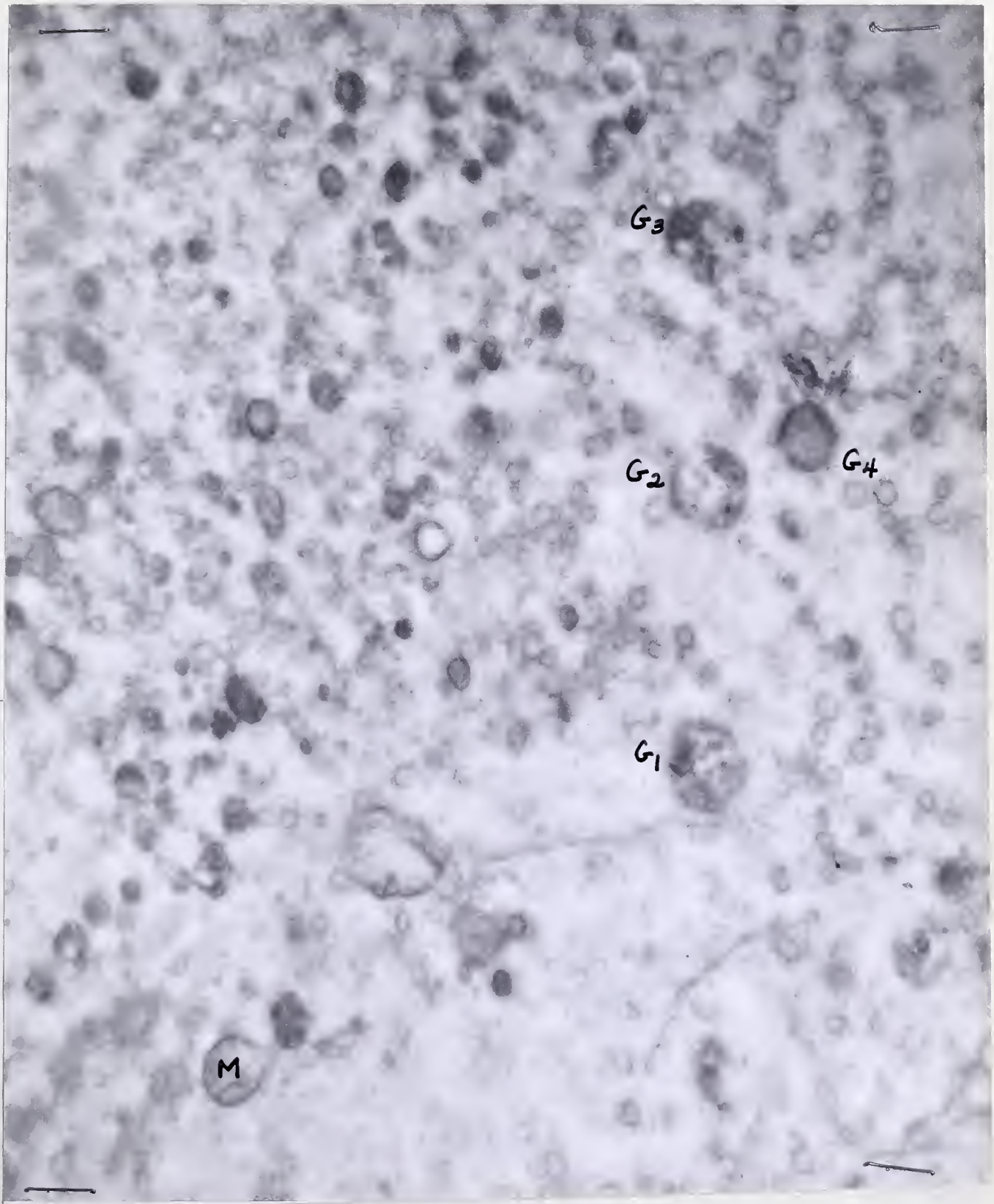






Fig. 15





Fig. 16: Portion of a tissue culture cell treated with reserpine at  $10^{-9}$ M for 24 hours. Numerous vacuolated dense bodies are seen (L). Large vacuoles (V) containing smooth membranes and osmophilic matter are evident. The vacuolated dense bodies could be formed from the material in these vacuoles. The cell membrane is intact. (X15,000).

Fig. 17: Another cell filled with vacuolated dense bodies (L). The relationship of these to mitochondria (M) is evident at the areas where the mitochondria are marked. The mitochondria appear to be undergoing dissolution in connection with the formation or activity of the vacuolated dense bodies. (X15,000).

Fig. 18: Two tissue culture cells treated with reserpine at  $10^{-9}$ M for 24 hours. The arrow indicates a granule which is being extruded from or budded from one of the cells. A granule (G) is apparent in the second cell. (X20,000).

Fig. 19: Tissue culture cell treated with reserpine at  $10^{-9}$ M for 24 hours. Membrane-bound amorphous cytoplasmic areas (I-IV) at the cell surface and deeper within the cytoplasm may have been produced by discharge of cytoplasmic constituents at the cell surface. Gaps in plasma membrane are evident in areas I, IV, V. (X15,000).

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Fig. 16

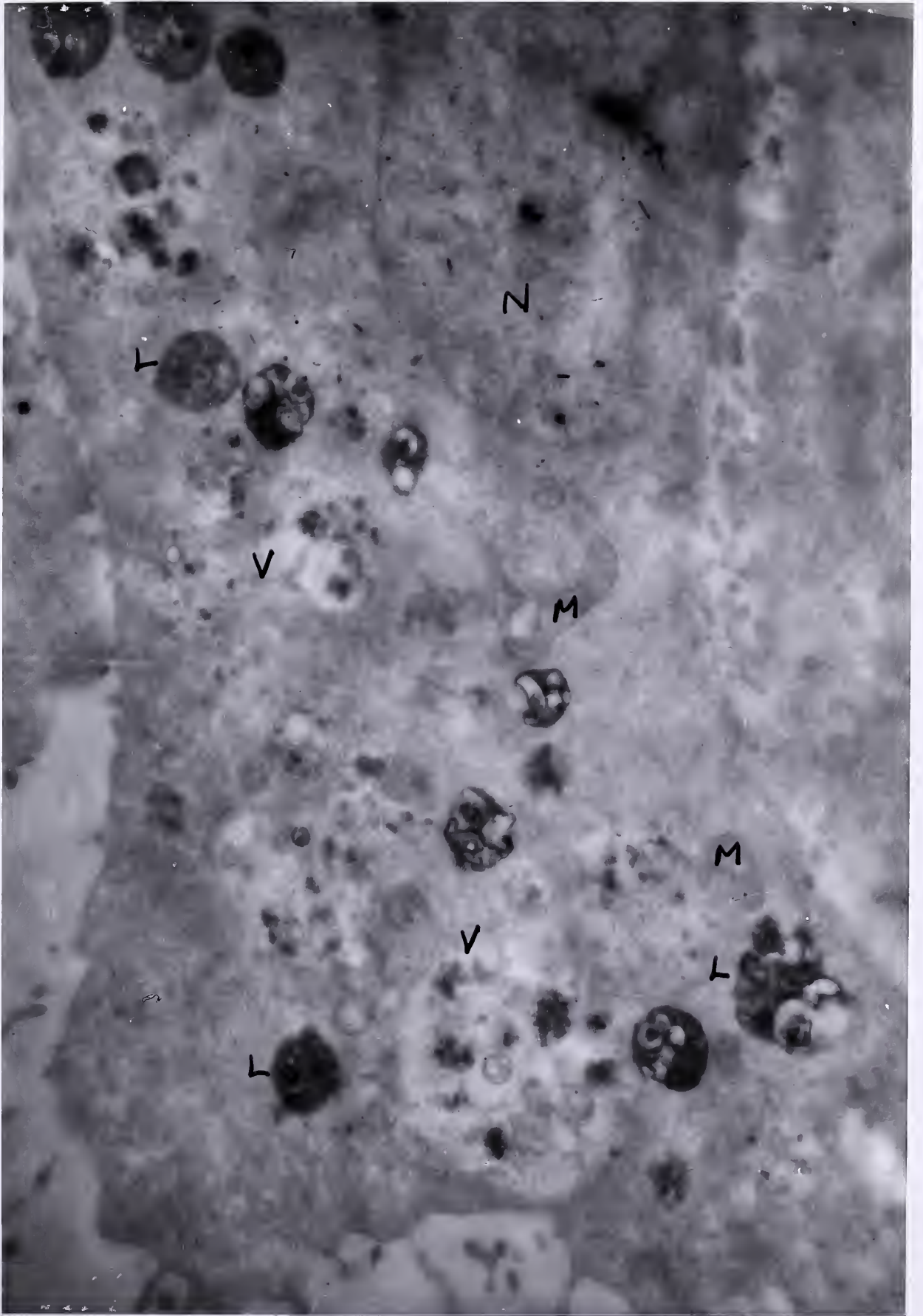




Fig. 17

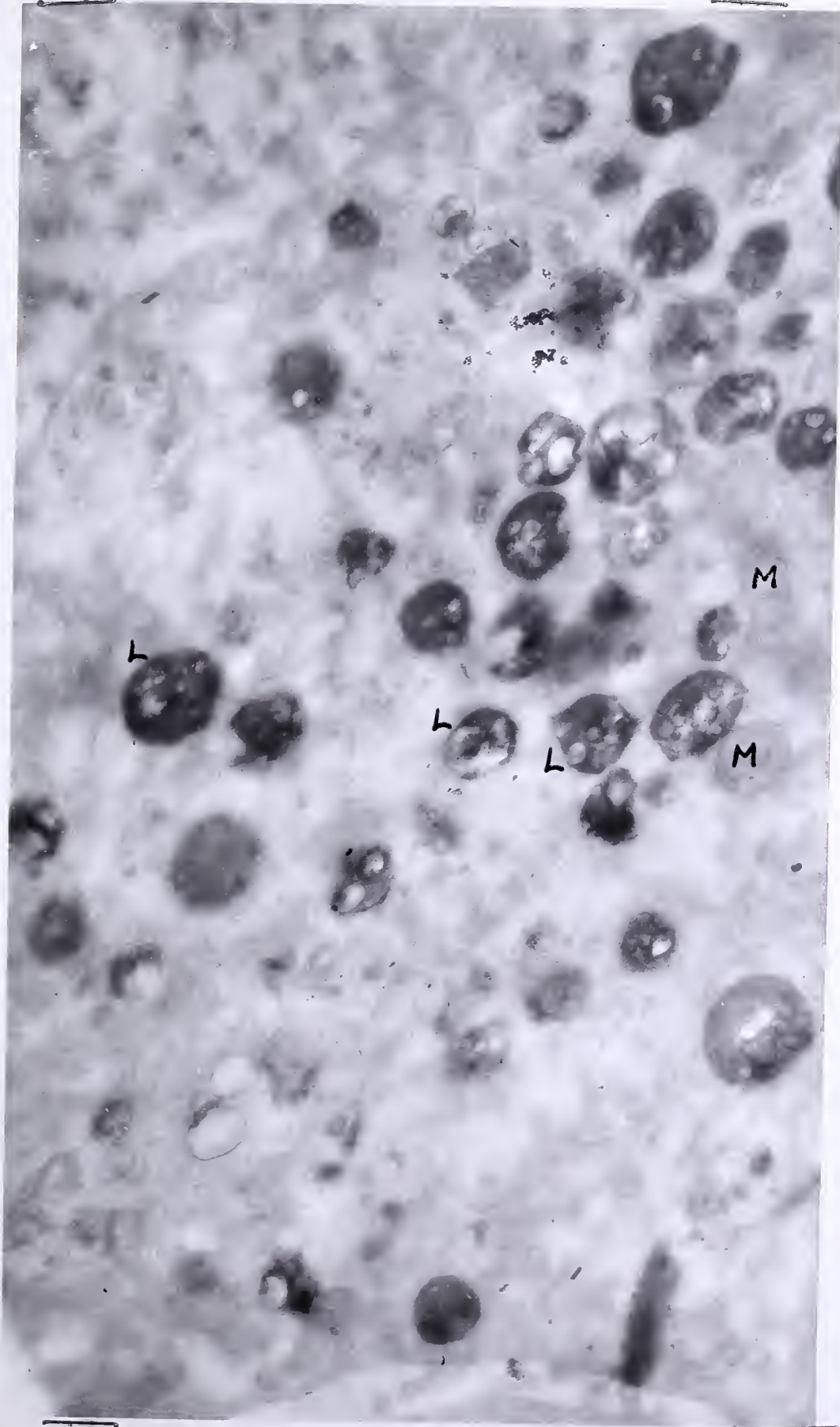






Fig. 18

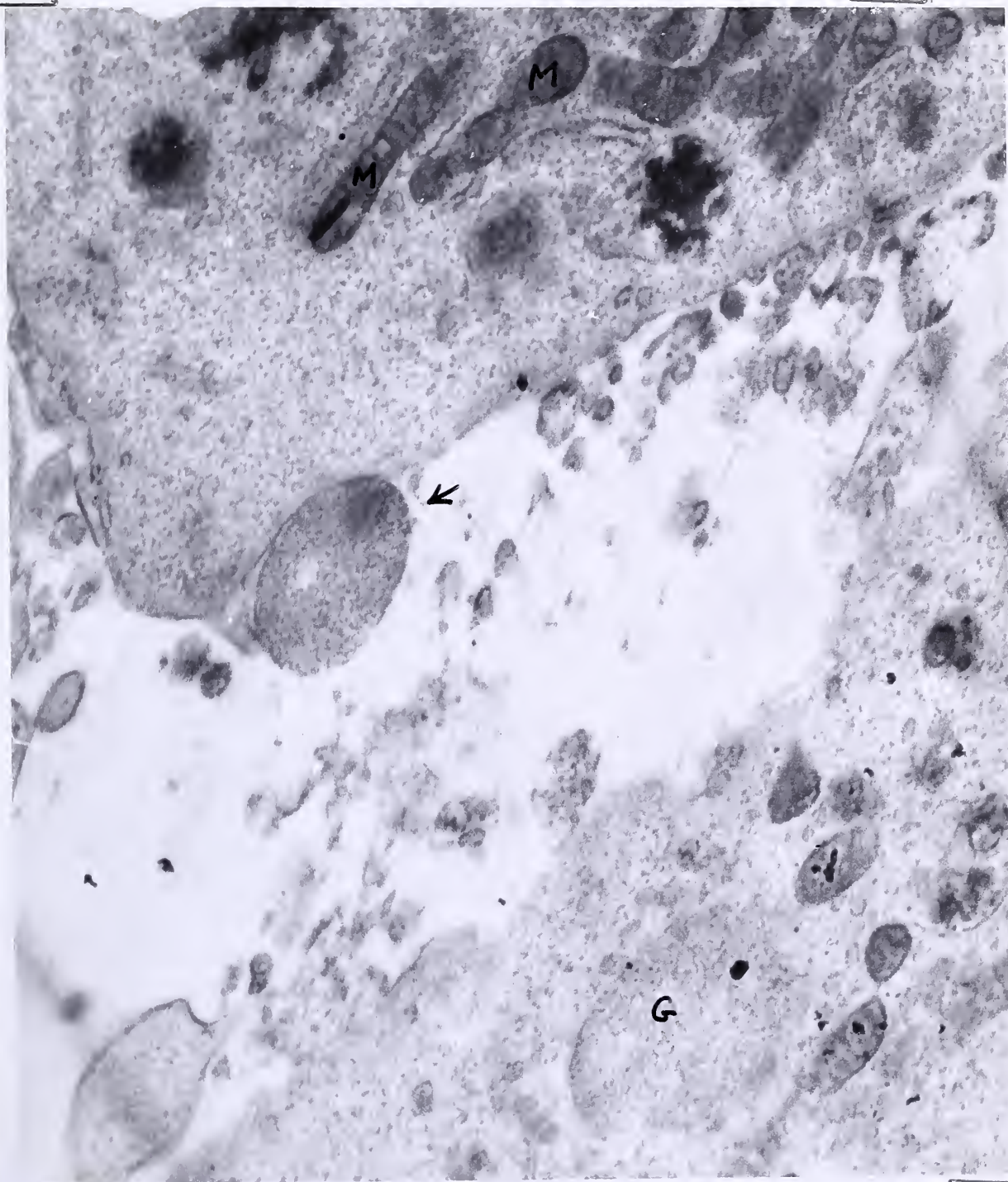
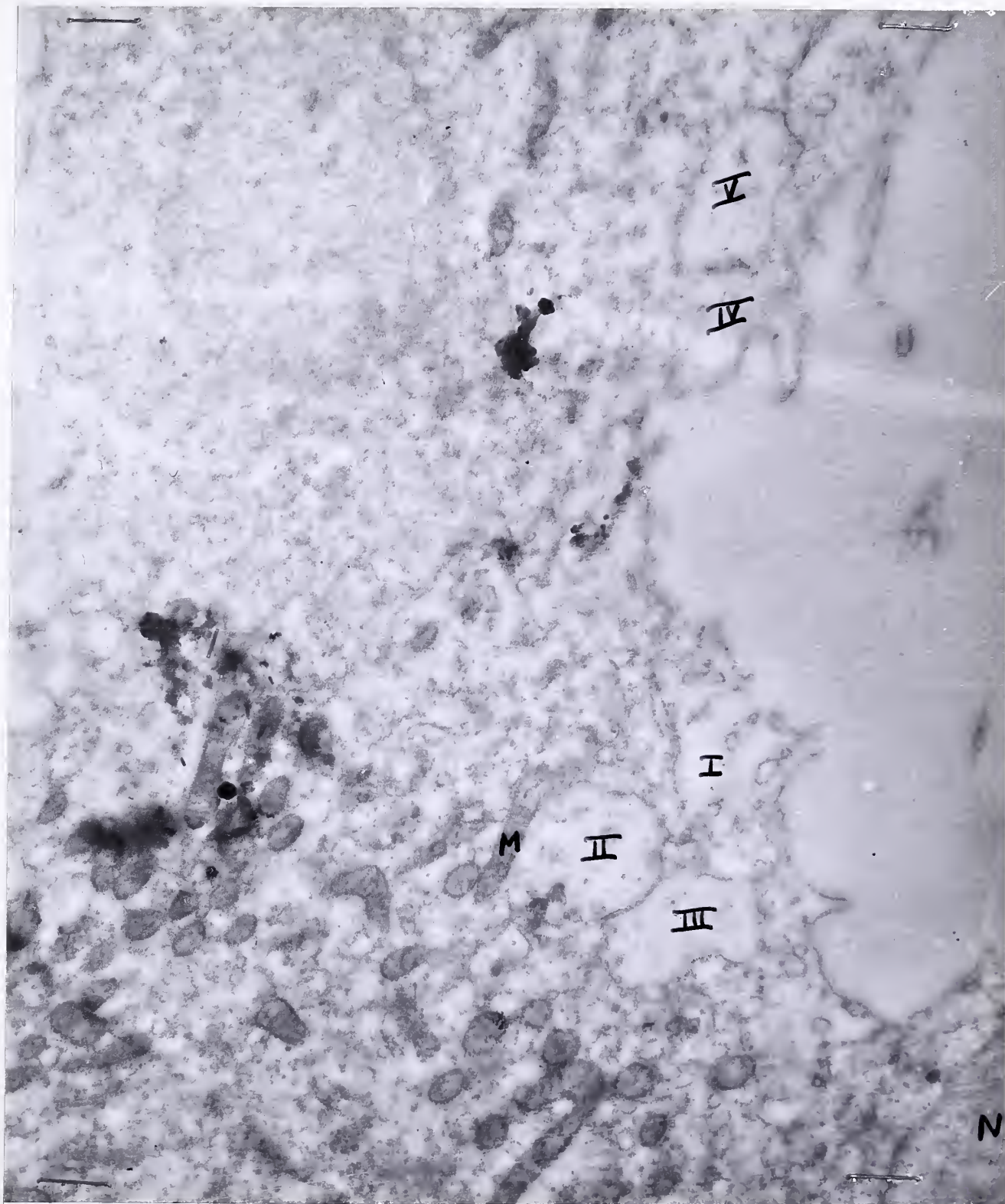




Fig. 19



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Figs. 20-22: Tissue culture cells treated with chlorpromazine at  $10^{-5}M$  for 12 hours. Practically all the mitochondria present have grossly distorted internal structures. Note that the outer membrane of some of the mitochondria is partially absent as well. Some of the mitochondrial membranes have a whirled configuration. (Fig. 20, X20,000; Fig. 21, X25,000; Fig. 22, X15,000).

Fig. 23: Normal mitochondria of tissue culture cells for purposes of comparison with cells treated with chlorpromazine. Note two parallel membranes of the cristae which are perpendicular to the outer mitochondrial membrane. See also Figs. 4-8. (X30,000).

Fig. 24: The large granule fraction from the ascites cells consisting mainly of battered mitochondria. A few granules are evident elsewhere amongst the degenerated membranes (X20,000).

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Fig. 20

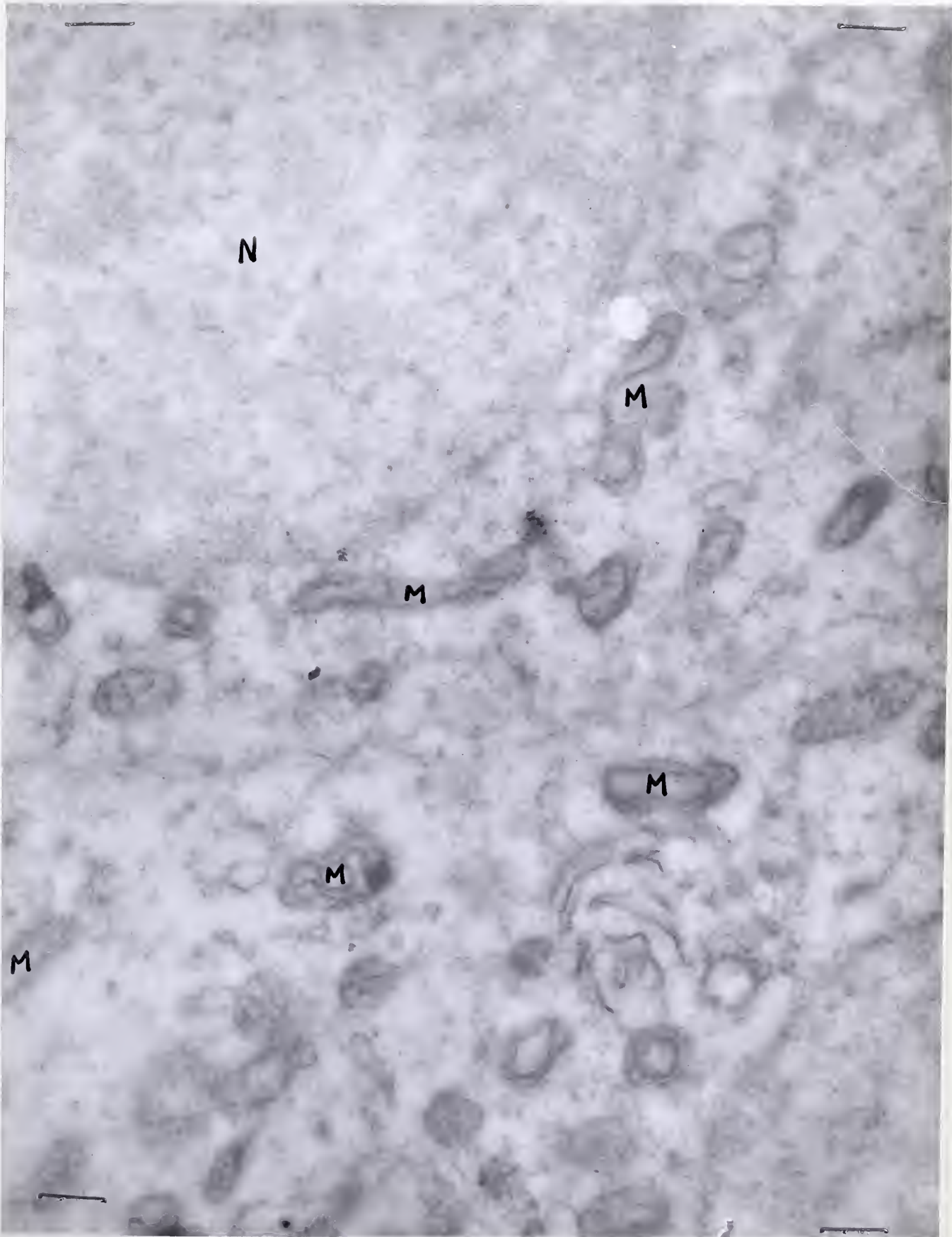






Fig. 21

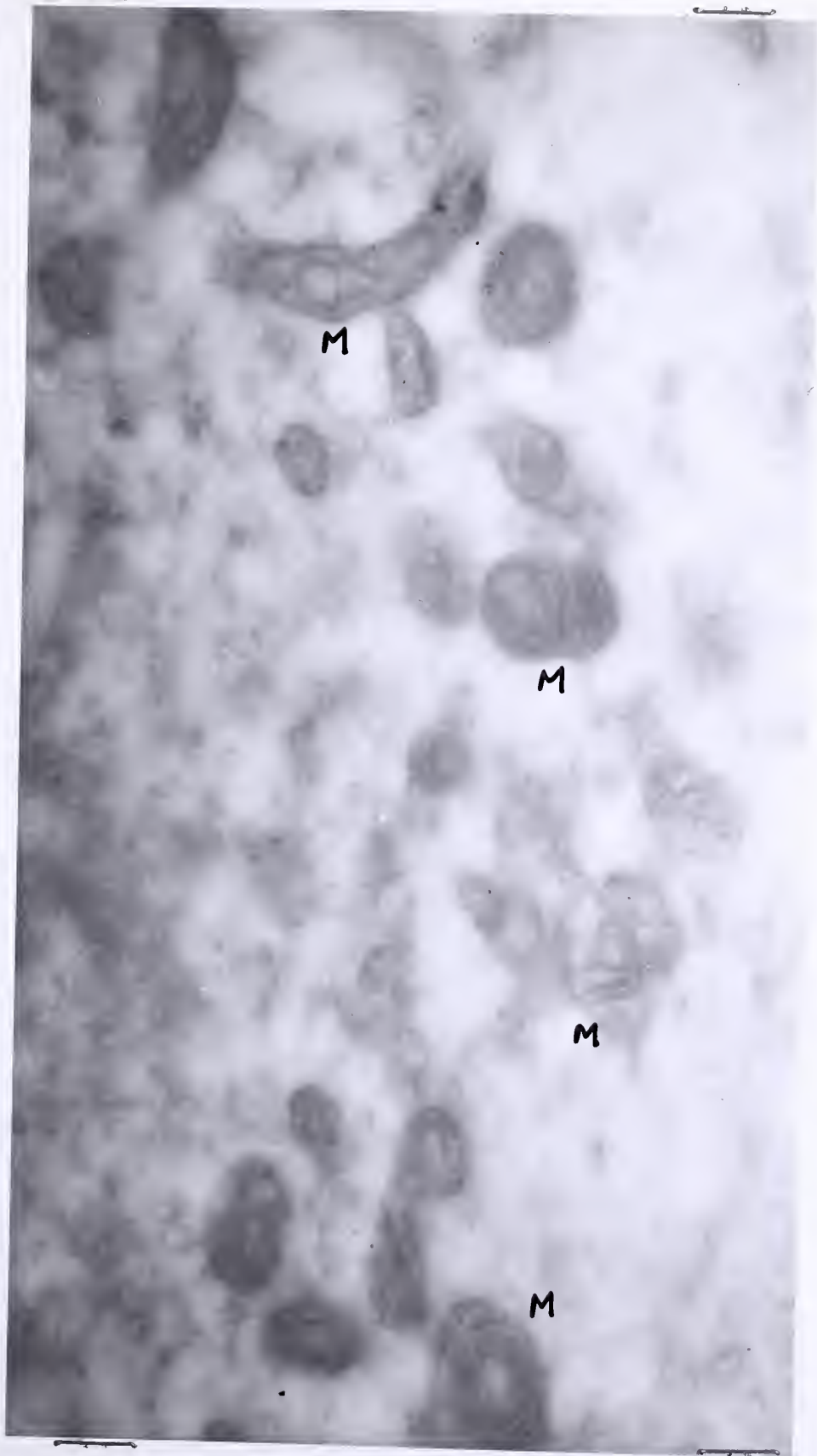




Fig. 22

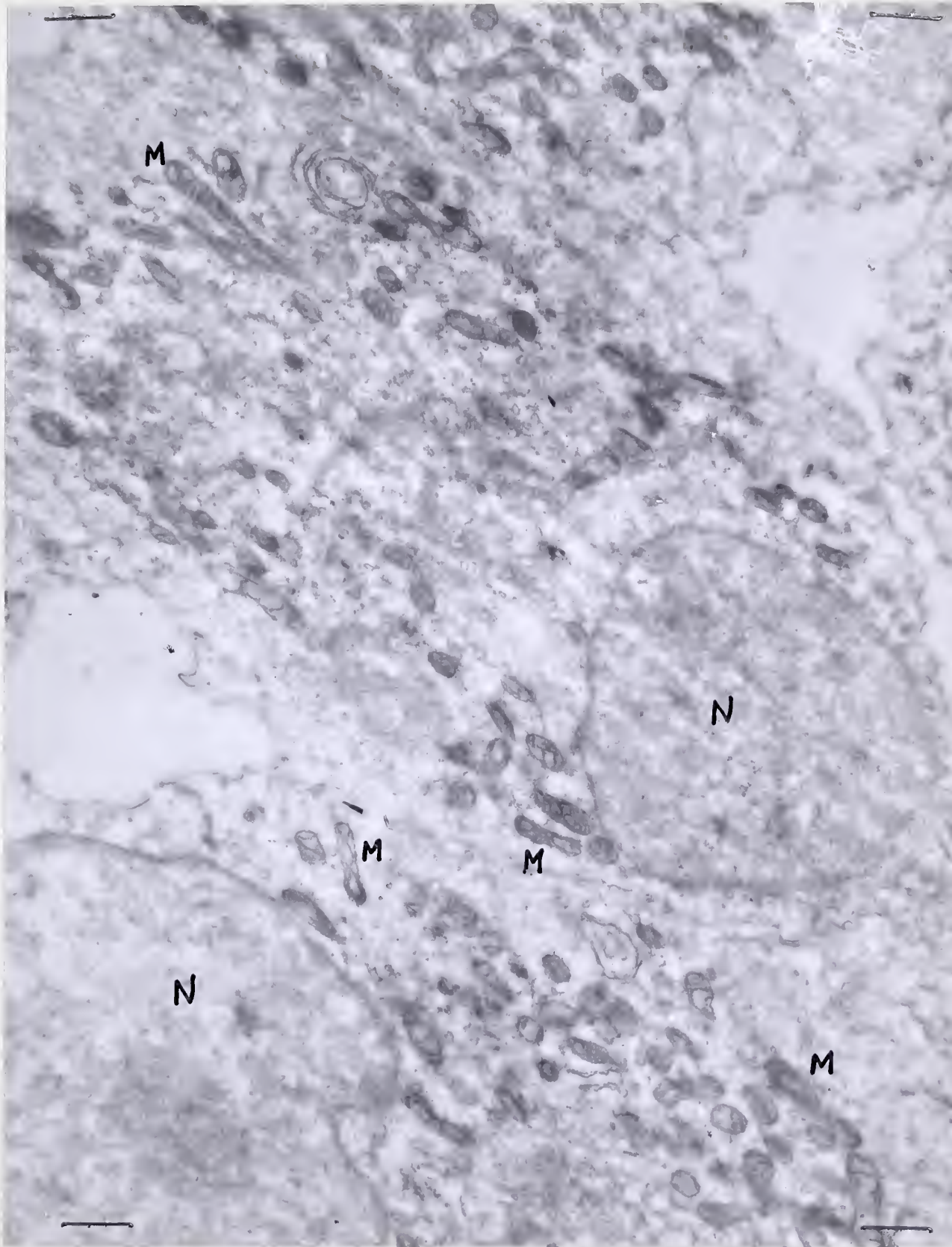




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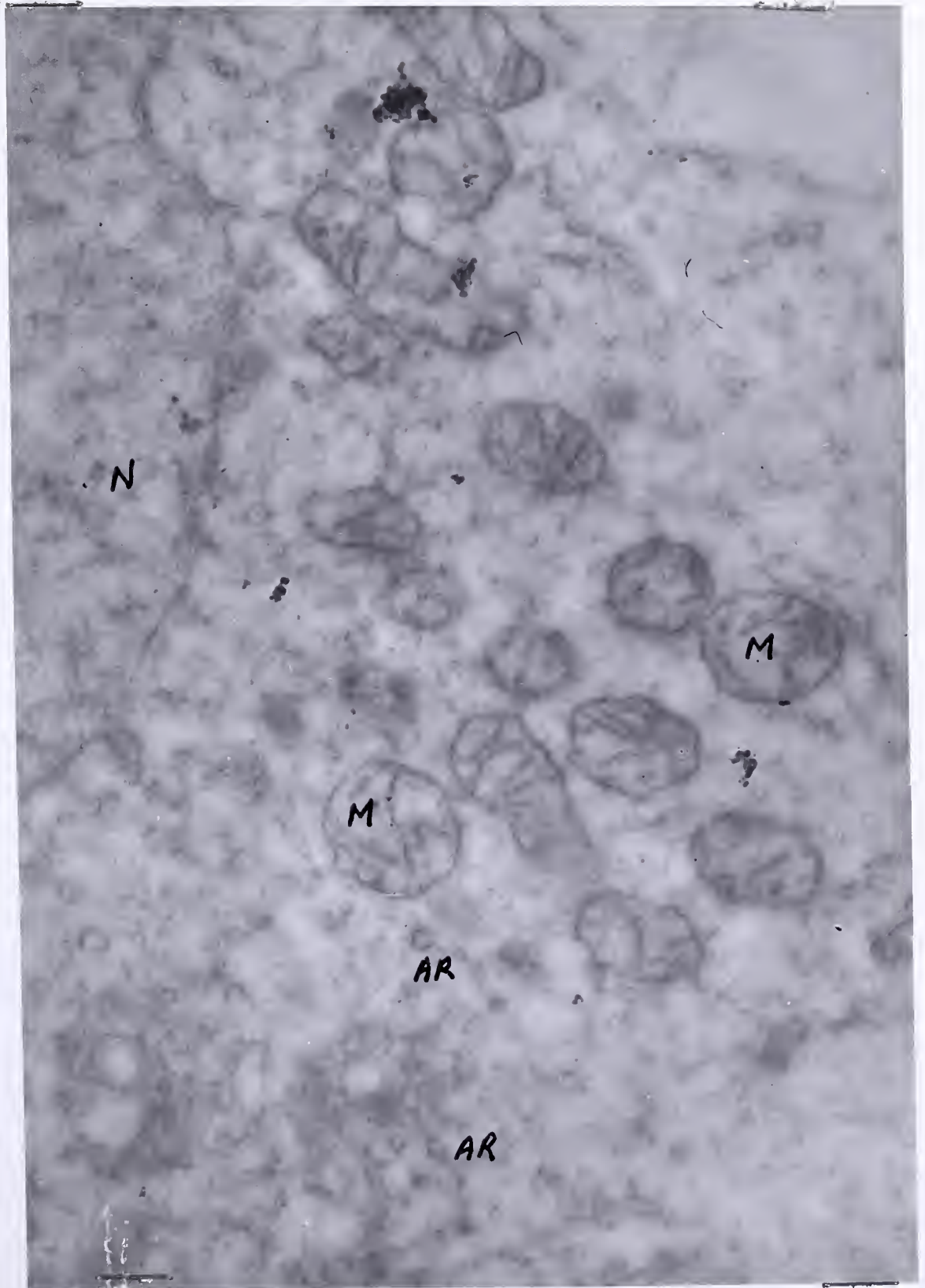
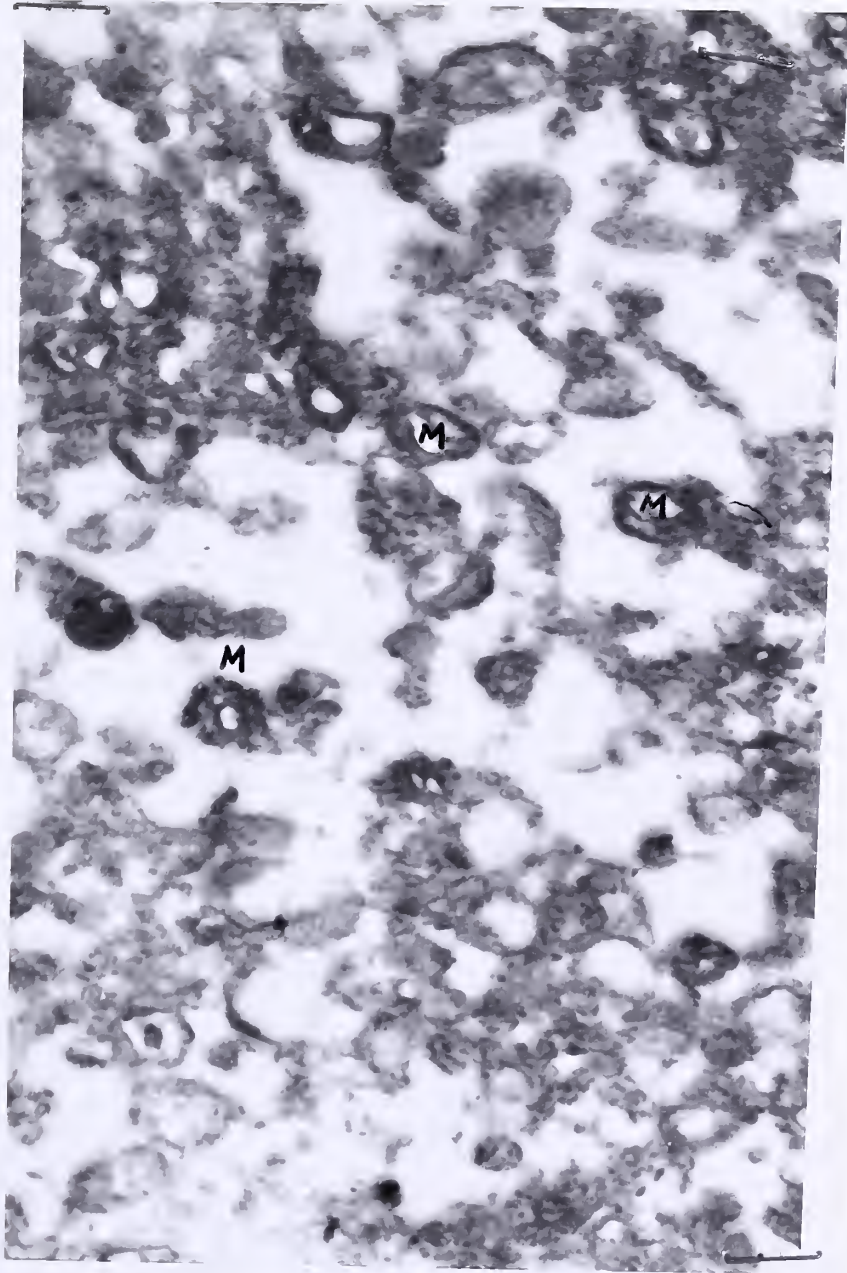




Fig. 24







INTRODUCTION

The first evidence for the oxidative deamination of amines by living tissues was the discovery in 1928 of a liver enzyme which oxidized tyramine.<sup>145</sup> Further studies in the 1930's on the enzymes which metabolize amines through oxidative deamination led to the concept that there was a single enzyme which degraded diverse monoamines such as tyramine, the catecholamines, and aliphatic amines, and another which oxidized diamines such as putrescine, the former being named monoamine oxidase (MAO), and the latter, diamine oxidase.<sup>146-148</sup> It was the idea that MAO could be related to central nervous system function that made it such an interesting enzyme to investigate. In 1940, Mann and Quastel observed that amphetamine inhibited MAO in vitro and suggested that the stimulant effect of amphetamine might be due to inhibition of MAO in vivo by blocking the formation of certain amine degradative products which they believed had a depressant effect.<sup>149</sup> However, no inhibition could be demonstrated in vivo after administration of sufficient amphetamine to produce stimulation. The next stimulus to work in this area was the finding by Zeller et al.,<sup>150</sup> that isoniazid and iproniazid, "hydrazines" that were active as

Iproniazid

anti-tuberculous agents and which clinically had the "side-effect" of being central stimulants,<sup>151</sup> were potent inhibitors

ANNEXURE

The following is a list of the names of the persons who have been appointed as members of the committee to inquire into the activities of the persons mentioned in the list of names given in the Annexure to the report of the committee on the subject mentioned above.

The names of the persons appointed as members of the committee are as follows:—

1. Mr. [Name]

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of MAO in vitro and in vivo. Subsequent studies demonstrated that reserpine pretreatment produced marked excitation rather than depression when given to animals pretreated with iproniazid and that it no longer possessed its capacity to lower brain 5-HT and catecholamine levels in these animals.<sup>152,153</sup> These observations led to the testing of iproniazid as an antidepressant.<sup>154</sup> The success of this clinical trial and the assumption that the mode of action of iproniazid was the inhibition of MAO attracted a great many investigators to this area. Since this finding, hundreds of basic and clinical studies of MAO and its inhibitors have been carried out in an effort to explore the relationship of this enzyme, its substrates, and its inhibitors, with mental illness, affective states such as depression and stimulation, angina pectoris and many other conditions.

A possible explanation of the anti-depressant action of the monoamine oxidase inhibitors (MAOI) was soon forthcoming with the finding that the MAOI caused the accumulation of brain 5-HT and catecholamines, presumably by preventing their breakdown.<sup>155-157</sup> The accumulation of these amines which are believed to function as neurotransmitters,<sup>158</sup> was, and is believed by some to exert an anti-depressant effect per se. It should be mentioned that rigidly controlled clinical studies on the efficacy of the MAOI as anti-depressants have not always yielded unequivocal results; and because of the toxicity of these compounds, they are now used infrequently as compared to imipramine (Tofranil) which is not a MAOI and which most studies

The first part of the report deals with the general situation of the country and the progress of the work done during the year. It is followed by a detailed account of the various projects and schemes which have been carried out. The report then goes on to discuss the financial position of the organization and the results of the various committees and sub-committees. Finally, it concludes with a summary of the work done and a list of the names of the members of the organization.

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have shown to be a more effective and less toxic mood elevator).<sup>159</sup>

The multiplicity of effects of the hydrazines on other enzymes and on various parameters of amine biology other than just accumulation of amines, have greatly complicated the explanation of the anti-depressant action of these compounds. The hydrazines have been shown to inhibit diphosphopyridine nucleotidase,<sup>160</sup> spermine oxidase,<sup>161</sup> guanidine deaminase,<sup>162</sup> succinic dehydrogenase,<sup>163</sup> histaminase,<sup>164</sup> and even the decarboxylase which brings about amine synthesis from precursor amino acids (by interfering with coenzyme of the decarboxylase, pyridoxal - 5' - phosphate).<sup>165, 166</sup> The MAOI potentiates the action of various pharmacologic agents,<sup>167</sup> including the barbiturates.<sup>168</sup> The hydrazines have been shown to inhibit the release by reserpine of 5-HT from rat brain,<sup>169</sup> as well as inhibiting the release of NE from various storage sites.<sup>170</sup> Schanberg and Giarmen have shown that phenylisopropyl-hydrazine produces an increase in the proportion of amines which are "bound" (within granules) rather than free (in the cytoplasm). Iproniazid increases the amount of both forms of the amine proportionately and does not change its distribution.<sup>171</sup> Dubnich et al.<sup>172</sup> have shown that 5-HT levels in rat brain continue to increase in response to doses of phenethylhydrazine which exceeded the dose required for the complete inhibition of MAO. This was thought to be due to the blockade of amine release. Others have shown that the various MAOI increase the amount of rat brain adenosine triphosphate and



suggest this augmented energy supply is the reason for the anti-depressant effect.<sup>173</sup> Gey and Pletscher have shown that MAOI can produce increased blood lactic acid levels.<sup>174</sup> There are many studies which demonstrate peripheral effects of the MAOI such as sympathomimetic effects,<sup>175</sup> ganglionic blockade<sup>176, 177</sup> (but see also<sup>77</sup>) and adrenergic blocking action.<sup>172a</sup>

Despite the embarrassing wealth of findings, the most reasonable explanation of the mood-elevating effect of the hydrazines, is the inhibition of MAO and blockade of amine-release, leading to the accumulation of brain 5-HT and possibly other amines. The main evidence in favor of this choice is that many compounds which inhibit MAO seem to have an anti-depressant effect irrespective of their structure, although there is no correlation between in vitro and in vivo studies. (Usdin and Usdin examined an exhaustive series of psychotropic compounds for MAO inhibition in vitro. They found some but not all hallucinogens, tranquilizers and anti-depressants were MAOI in vitro and concluded: "No obvious relationship between psychotropic action and MAO inhibition can be discerned."<sup>173a</sup> As previously mentioned, however, studies in vitro are of limited value in this field because of a host of factors, such as metabolism of the agent in vivo and regional specialization of the brain with regard to such properties as biochemistry and permeability barriers). Additional evidence for the relationship between elevated 5-HT levels and anti-depression is that other modes of elevating the brain amines such as administer-



The first part of the paper is devoted to the study of the asymptotic behavior of the solutions of the system (1) as  $\epsilon \rightarrow 0$ . It is shown that the solutions of (1) converge to the solutions of the system (2) in the sense of the weak convergence of measures.

In the second part of the paper, we study the asymptotic behavior of the solutions of the system (1) as  $\epsilon \rightarrow 0$  in the case of a non-convex domain. It is shown that the solutions of (1) converge to the solutions of the system (2) in the sense of the weak convergence of measures.

In the third part of the paper, we study the asymptotic behavior of the solutions of the system (1) as  $\epsilon \rightarrow 0$  in the case of a domain with a boundary of class  $C^1$ . It is shown that the solutions of (1) converge to the solutions of the system (2) in the sense of the weak convergence of measures.

In the fourth part of the paper, we study the asymptotic behavior of the solutions of the system (1) as  $\epsilon \rightarrow 0$  in the case of a domain with a boundary of class  $C^1$ . It is shown that the solutions of (1) converge to the solutions of the system (2) in the sense of the weak convergence of measures.

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In the tenth part of the paper, we study the asymptotic behavior of the solutions of the system (1) as  $\epsilon \rightarrow 0$  in the case of a domain with a boundary of class  $C^1$ . It is shown that the solutions of (1) converge to the solutions of the system (2) in the sense of the weak convergence of measures.

ing the precursors of the catecholamines, dihydroxyphenylalanine, or the precursor of 5-HT, 5 hydroxytryptophan, are associated with central stimulation.<sup>174a,175a</sup> (Bonny-castle et al. have shown that a variety of central depressant drugs, including sedatives, hypnotics, analgesics, fixed and volatile anesthetics, produced a significant elevation of rat brain 5-HT. His data suggested that the 5-HT elevation was secondary to the central nervous system depression, not the cause of it).<sup>176a</sup> Shore<sup>177a</sup> noted a temporal relationship between the pharmacological effects of iproniazid and an increase in the levels of brain 5-HT and NE. The many studies relating amines to behavioral changes induced by other drugs such as reserpine are also in favor of this hypothesis. Indeed, the knowledge that iproniazid, an antidepressant, raised 5-HT levels in the brain, while reserpine, a tranquilizer, lowered 5-HT in brain, stimulated great interest in the study of brain biochemistry and pharmacology.

To compound the interest in the MAOI, studies show their efficacy in relieving angina pectoris<sup>178, 179</sup> their hypotensive effect,<sup>180</sup> their anti-convulsant effect,<sup>181</sup> their anti-inflammatory effect,<sup>182,183</sup> an analgesic effect,<sup>184</sup> and even an anti-fertility action.<sup>185</sup> They are clearly a fascinating group of compounds.

In most cases, only the hydrazines have been studied in these experiments. Whether or not the many other classes of compounds which inhibit MAO and which are not hydrazines

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 The fourth part of the document contains a detailed  
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possess these properties as well, remains to be investigated. Among the other classes of inhibitors of MAO which have been reported, are agents which combine with sulfhydryl groups,<sup>186</sup> aliphatic alcohols,<sup>187</sup> chlorpromazine,<sup>188</sup> the harmala alkaloids,<sup>189</sup> p-tolyl ether of choline,<sup>190</sup> trans-2-phenylcyclopropylamine (tranylcypromine),<sup>191</sup>  $\alpha$ -methyl- and  $\alpha$ -ethyltryptamine (etryptamine),<sup>192</sup> N-benzyl-N-methyl-2-propynylamine (pargyline),<sup>193</sup> N-methyl- $\alpha$ -methyltryptamine,<sup>194</sup> and others. (The work to be described here on quaternary nitrogen compounds as inhibitors of MAO was begun when only the hydrazines were established as MAOI and enthusiasm for MAOI as anti-depressants was at its height).

Certain properties of the hydrazines as MAOI need to be pointed out to place in proper context some of the author's studies of the quaternary nitrogen compounds. The hydrazines and tranylcypromine require preincubation with MAO in order to exert their maximum inhibitory effect, whereas the harmala alkaloids do not require preincubation. The hydrazines and tranylcypromine are irreversible inhibitors once they have had prolonged contact with MAO, manifested by the fact that extensive dialysis does not diminish the extent of inhibition. The harmala alkaloids are reversible, competitive inhibitors. Both of these classes of compounds will raise brain 5-HT and catecholamine levels in various organs (e.g. in brain and heart) of many species, including the rat.<sup>189, 195, 196</sup>

Studies of these inhibitors in vitro have invariably been done on particulate MAO because it has never been entirely

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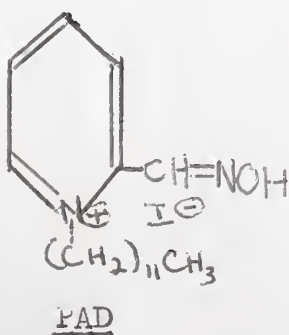
In the second section, the author outlines the various methods used to collect and analyze the data. This includes both manual and automated processes. The manual process involves reviewing each entry individually, while the automated process uses software to identify patterns and anomalies.

The third part of the document provides a detailed breakdown of the results. It shows that there is a significant correlation between the variables being studied. This finding is supported by statistical analysis and is consistent with previous research in the field.

Finally, the document concludes with a summary of the key findings and offers recommendations for future research. It suggests that further studies should focus on refining the data collection methods and exploring the underlying causes of the observed trends.

freed from its mitochondrial matrix. In one study, MAO solubilized with the detergent isoctylphenoxypolyethoxyethanol was used.<sup>197</sup> In the presence of this solubilizing agent, MAO activity will not sediment despite centrifugation conditions sufficient to bring down microsomes. (Recently MAO has been obtained from homogenate subjected to sonic oscillations in a form which did not sediment after centrifugation at 35,000g for 30 minutes without the presence of a detergent).<sup>198</sup>

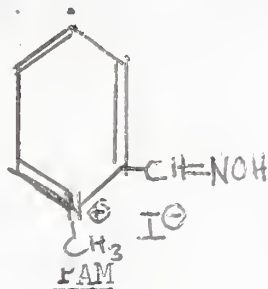
While investigating new inhibitors of MAO, the author discovered that various compounds which contained a quaternary nitrogen atom, usually in the form of a quaternary pyridinium moiety, were effective MAOI. At first, it seemed that the possibility of this class of compounds being of clinical or even of investigative use, was slight because of the evidence that highly ionic molecules cannot penetrate the blood-brain barrier.<sup>199</sup> However, it seemed likely that a long-chain N-alkyl group would enhance the lipid solubility of the quaternary pyridinium molecule and thus allow it to penetrate the blood-brain barrier, which is known to be most permeable to compounds of high lipid solubility.<sup>200</sup> With this in mind, pyridine aldoxime dodecylidide (PAD), which has a quaternary pyridinium nucleus with a long chain N-alkyl radicle, was investigated as a possible inhibitor of MAO.



The first part of the manuscript, which is the most important, contains a detailed description of the author's life and his work. The second part is a collection of letters and documents. The third part is a list of the author's works. The fourth part is a list of the author's correspondents. The fifth part is a list of the author's friends. The sixth part is a list of the author's enemies. The seventh part is a list of the author's admirers. The eighth part is a list of the author's detractors. The ninth part is a list of the author's supporters. The tenth part is a list of the author's opponents. The eleventh part is a list of the author's associates. The twelfth part is a list of the author's acquaintances. The thirteenth part is a list of the author's contacts. The fourteenth part is a list of the author's relations. The fifteenth part is a list of the author's connections. The sixteenth part is a list of the author's ties. The seventeenth part is a list of the author's bonds. The eighteenth part is a list of the author's links. The nineteenth part is a list of the author's connections. The twentieth part is a list of the author's relations. The twenty-first part is a list of the author's contacts. The twenty-second part is a list of the author's acquaintances. The twenty-third part is a list of the author's contacts. The twenty-fourth part is a list of the author's acquaintances. The twenty-fifth part is a list of the author's contacts. The twenty-sixth part is a list of the author's acquaintances. The twenty-seventh part is a list of the author's contacts. The twenty-eighth part is a list of the author's acquaintances. The twenty-ninth part is a list of the author's contacts. The thirtieth part is a list of the author's acquaintances. The thirty-first part is a list of the author's contacts. The thirty-second part is a list of the author's acquaintances. The thirty-third part is a list of the author's contacts. The thirty-fourth part is a list of the author's acquaintances. The thirty-fifth part is a list of the author's contacts. The thirty-sixth part is a list of the author's acquaintances. The thirty-seventh part is a list of the author's contacts. The thirty-eighth part is a list of the author's acquaintances. The thirty-ninth part is a list of the author's contacts. The fortieth part is a list of the author's acquaintances. The forty-first part is a list of the author's contacts. The forty-second part is a list of the author's acquaintances. The forty-third part is a list of the author's contacts. The forty-fourth part is a list of the author's acquaintances. The forty-fifth part is a list of the author's contacts. The forty-sixth part is a list of the author's acquaintances. The forty-seventh part is a list of the author's contacts. The forty-eighth part is a list of the author's acquaintances. The forty-ninth part is a list of the author's contacts. The fiftieth part is a list of the author's acquaintances.



PAD was conceived and synthesized by Wilson and Ginsburg<sup>201</sup> to obtain a more lipid-soluble analog of pyridine aldoxime methiodide (PAM)<sup>202</sup> which was designed and shown by Wilson<sup>203</sup> to be



an extraordinarily effective reactivator of cholinesterase inhibited by organophosphorous compounds such as diisopropyl-fluorophosphate. Wilson reasoned that because of the N-alkyl radicle PAD would be more likely than PAM to penetrate the blood-brain barrier and to reactivate organophosphorous-inhibited brain cholinesterase. PAM itself has been shown to enter the brain<sup>204</sup> although no similar direct evidence is available that PAD can pass the blood-brain barrier. (The studies presented in this paper strongly suggest that PAD and related compounds gain entrance into rat brain). PAD (in conjunction with atropine) has been shown to be an effective antidote in poisoning, due to organophosphorous cholinesterase inhibitors.<sup>205</sup> In addition to reactivating cholinesterase, PAD can also inhibit cholinesterase, block conduction at the node of Ranvier, increase the permeability of nerve fibers to sodium ions<sup>206</sup> and depolarize resting nerve fibers.<sup>207</sup> Some of these effects have been attributed to the reaction of PAD with the postulated acetylcholine receptor protein along the nerve axon.<sup>206</sup> The work presented here shows that, in addition to these effects on acetylcholine





metabolism and on the proteins associated with it, PAD exerts two important effects on the metabolism of 5-HT: (i) inhibition in vitro of MAO, the enzyme that is mainly responsible for the catabolism of 5-HT; and (ii) release of 5-HT from brain and mast cells in which 5-HT may be bound to particulate components. Most of these studies with PAD have been published. <sup>209, 210</sup>

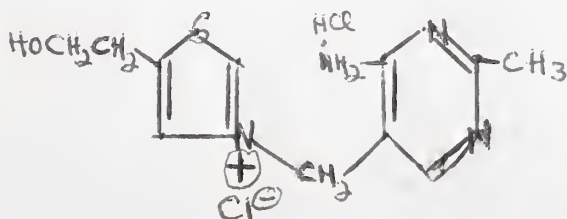
The studies with PAD indicated it would be very interesting to investigate dodecyl iproniazid iodide, the long-chain N-alkyl derivative of iproniazid. This



Dodecyl Iproniazid Iodide

compound would have two functional groups that could inhibit MAO, at least in vitro, as well as being able to release 5-HT. Synthesis of this compound was accomplished in the Lederle Laboratories and a small amount was graciously supplied to the author.

Early in the study of the effect of quaternary nitrogen compounds on MAO, the author felt that it might be profitable to investigate the role of thiamine in MAO because thiamine has a quaternary nitrogen in its thiazole moiety.



Thiamine Hydrochloride

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Preliminary studies with thiamine - deficient animals revealed brain MAO to be elevated, which could come about if thiamine or a metabolite of it were natural endogenous MAOI. The elevation of brain MAO in thiamine-deficient rats was independently confirmed and published by Gal and Drewes<sup>211</sup> who, whoever did not speculate on any role thiamine might play as a MAOI. A few experiments and an intensive literature search which unearthed a number of relevant findings enabled the author to publish some speculations about the effect of thiamine on MAO.<sup>212</sup> These will be detailed in the Discussion section of this thesis.



## MATERIALS AND METHODS

5-HT creatinine sulfate and dl-5-hydroxytryptophan (5-HTP) were obtained from the California Corporation for Biochemical Research. Kynuramine was obtained from the Regis Chemical Company; decamethonium, hexamethonium, PAM, and cetyl pyridinium iodide were purchased from the K & K Chemical Company. Stigmonene bromide was a gift of the Warner-Chilcott Company. Iproniazid phosphate was a gift from Hoffman-LaRoche, Inc. Catron (JB-516 was a gift from Lakeside Laboratories. Hamaline was a gift of Professor Nicholas Giarman of the Yale Department of Pharmacology. Thiamine and thiamine pyrophosphate were purchased from Schwartz Biochemicals, as was diphosphopyridine nucleotide. Succinylcholine, d-tubocurarine, neostigmine and N-methyl nicotinamine were obtained from various commercial sources. PAD was synthesized by the method of Wilson and Ginsburg.<sup>60</sup> Long-chain alkyl pyridinium compounds were synthesized by the method of Knight and Shaw.<sup>213</sup> Dodecyl iproniazid iodide was synthesized by the Lederle Laboratories; the author is very grateful to Dr. Paul Bell, Director of Biochemical Research, Lederle Laboratories, for supplying this compound.

5-HT was determined spectrophotometrically by the method of Udenfriend et al.<sup>101</sup> Numerous experiments were performed to show that PAD does not interfere with the determination of 5-HT by this method. Catecholamines were determined by a spectrofluorometric techniques according to the method



of Bertler et al.<sup>214</sup> MAO activity was assayed spectrophotometrically.<sup>110</sup> All incubations were for 10 minutes within the housing of a Beckman DU spectrophotometer. The results obtained were occasionally checked by another method, involving the liberation of ammonia.<sup>107,108</sup> 5-HTP decarboxylase was determined by the technique of Gaddum and Giarman.<sup>215</sup> Mitochondria were prepared from rat liver according to the method of Hogeboom,<sup>216</sup> and frozen at -20°C until needed. Each milliliter of the mitochondrial suspension used contained the mitochondria from 1 g of liver (wet weight). MAO from such mitochondrial preparations was "solubilized" by the method of Zeller et al.<sup>217</sup> All compounds were administered to animals intraperitoneally. The rats used were males of approximately 150 -g weight (Sprague-Dawley strain). The mice were DBA/2 males weighing about 20g. The mast cells used were the Dunn-Potter murine mastocytoma, ascites form.<sup>96</sup> PAD was dissolved in a solution containing NaHCO<sub>3</sub>, 1 mg/ml. In all experiments involving PAD, the control animals were given the NaHCO<sub>3</sub> solution.



The first part of the report deals with the general situation in the country. It is noted that the economy is showing signs of recovery, but that there are still many problems to be solved. The government is taking steps to improve the situation, but more work is needed.

The second part of the report deals with the situation in the various regions. It is noted that the situation is not uniform, and that there are still many problems in some of the regions. The government is taking steps to improve the situation, but more work is needed.

The third part of the report deals with the situation in the various sectors of the economy. It is noted that there are still many problems in some of the sectors, but that there are also some signs of recovery. The government is taking steps to improve the situation, but more work is needed.

The fourth part of the report deals with the situation in the various social groups. It is noted that there are still many problems in some of the social groups, but that there are also some signs of improvement. The government is taking steps to improve the situation, but more work is needed.

The fifth part of the report deals with the situation in the various international relations. It is noted that there are still many problems in some of the international relations, but that there are also some signs of improvement. The government is taking steps to improve the situation, but more work is needed.

EXPERIMENTAL RESULTS(1) Effect of PAD on the MAO activity of homogenates of liver and brain of various species.

Table 1 demonstrates that PAD can inhibit the MAO of both liver and brain of a number of species. Because of the crudeness of these preparations, it is not possible to say whether MAO of brain or liver is more susceptible to inhibition by PAD.

TABLE 1. EFFECT of PAD on the MAO activity of LIVER and BRAIN HOMOGENATES of RAT, RABBIT, GUINEA PIG, and MOUSE

Species	% Inhibition of MAO			
	<sup>Liver</sup> 10 <sup>-4</sup> M*	10 <sup>-5</sup> M	<sup>Brain</sup> 10 <sup>-4</sup> M	10 <sup>-5</sup> M
Rat	90	25	41	16
Rabbit	69	20	60	20
Guinea Pig	100	20	40	12
Mouse	66	18	45	15

The brains and livers of adult animals killed by decapitation were excised, homogenized in 4 vol of cold distilled water, and filtered through cheesecloth; 0.2 ml of brain homogenate and 0.1 ml of liver homogenate were used for each assay of MAO activity. All assays were done in duplicate with less than 5% variation.

\* Concentrations of PAD.

PROBABILITY

1. A die is thrown. Find the probability of getting a number less than 4.

Solution: Let S be the sample space.

$S = \{1, 2, 3, 4, 5, 6\}$   
Let A be the event that the number is less than 4.  
 $A = \{1, 2, 3\}$

$n(S) = 6$  and  $n(A) = 3$

$\therefore P(A) = \frac{n(A)}{n(S)} = \frac{3}{6} = \frac{1}{2}$

2. A card is drawn from a well-shuffled deck of 52 cards. Find the probability of getting a king or a queen.

Solution: Let S be the sample space.

$S = \{1, 2, 3, \dots, 52\}$

Card	Number	Color	Suit
King	13	Black	Spade
King	14	Red	Heart
King	15	Black	Club
King	16	Red	Diamond
Queen	17	Black	Spade
Queen	18	Red	Heart
Queen	19	Black	Club
Queen	20	Red	Diamond

$n(S) = 52$   
Let A be the event that the card is a king or a queen.  
 $A = \{13, 14, 15, 16, 17, 18, 19, 20\}$   
 $n(A) = 8$

$\therefore P(A) = \frac{n(A)}{n(S)} = \frac{8}{52} = \frac{2}{13}$

(2) Effect of PAD on MAO activity of washed rat liver mitochondria and comparison of PAD with other inhibitors of MAO.

Table 2 shows that the inhibition of MAO in vitro, obtainable with PAD, is substantial. As can be seen in Table 3, PAD is less potent than is JB-516 (Catron, one of the most potent hydrazines used clinically) as an inhibitor of MAO in vitro, but is more potent than iproniazid. Table 3 also indicates that a number of long-chain N-alkyl quaternary pyridine compounds can inhibit MAO in vitro.

TABLE 2. EFFECT of PAD on MAO activity of Washed RAT LIVER MITOCHONDRIA in vitro

Concentration of PAD	% Inhibition of MAO activity
5 x 10 <sup>-4</sup> M	100
5 x 10 <sup>-5</sup> M	75
10 <sup>-5</sup> M	40
4 x 10 <sup>-6</sup> M	20

Preincubation of enzyme and PAD was not performed. Four determinations at each concentration of inhibitor were performed with less than 5% variation.

TABLE 3. COMPARISON of PAD and Related Compounds with Phenylisopropyl-hydrazine and iproniazid as MAO inhibitors in vitro

Inhibitor (10 <sup>-5</sup> M)	% Inhibition
JB-516	100
PAD	40
Isonicotinic acid dodecyl iodide	40
Dodecyl pyridine iodide	25
Tetradecyl pyridine iodide	20
Cetyl pyridine iodide	20
Iproniazid	5

JB-516 and iproniazid were preincubated with the washed rat liver mitochondria for 15 min. before kynuramine was added. Four assays with each inhibitor were performed with less than 5% variation.

The first part of the report deals with the general situation of the country and the progress of the work done during the year. It is followed by a detailed account of the work done in each of the various departments. The report concludes with a summary of the work done and a statement of the progress made.

The second part of the report deals with the financial statement of the year. It shows the income and expenditure of the various departments and the balance sheet at the end of the year.

Department	Income	Expenditure	Balance
Administration	1000	800	200
Education	2000	1800	200
Health	1500	1300	200
Public Works	1200	1000	200
Other	800	700	100
<b>Total</b>	<b>6500</b>	<b>5600</b>	<b>900</b>

The third part of the report deals with the work done in each of the various departments. It shows the progress made in each of the various departments and the results of the work done.

The fourth part of the report deals with the work done in each of the various departments. It shows the progress made in each of the various departments and the results of the work done.

Department	Work Done	Results
Administration	1000	200
Education	2000	200
Health	1500	200
Public Works	1200	200
Other	800	100
<b>Total</b>	<b>6500</b>	<b>900</b>

The fifth part of the report deals with the work done in each of the various departments. It shows the progress made in each of the various departments and the results of the work done.

(3) Effect of preincubation on the inhibition of rat liver mitochondrial MAO by PAD.

PAD, unlike hydrazine-derived inhibitors of MAO, does not require preincubation to exert its maximal inhibitory action on MAO. Table 4 gives the results of an experiment showing that preincubation of MAO with PAD produced no effect on the inhibition obtained.

TABLE 4. EFFECT of PREINCUBATION on the INHIBITION of MITOCHONDRIA by PAD

Concentration of PAD	MAO activity No preinc.	( $\Delta$ O.D./10 min) Preinc.
$4 \times 10^{-5}M$	0.172	0.170
	0.100	0.100

The substrate, kynuramine, was added to the reaction mixture prior to the addition of MAO, or 30 min. after MAO. In both series, reactions were run in duplicate with and without PAD.

The following information is for your information only.

It is requested that you provide the following information:

- 1. Name of the person or organization to whom the information is being provided.
- 2. Address of the person or organization.
- 3. Telephone number.
- 4. Date of the information.
- 5. Name of the person providing the information.

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(4) Effect of dialysis on the inhibition of rat liverMitochondrial MAO by PAD.

The hydrazine inhibitors irreversibly inhibit MAO, while harmaline is a reversible inhibitor.<sup>189</sup> The inhibition due to PAD is not reversible by extensive dialysis, despite the fact that no preincubation is necessary. The results given in Table 5 show that, in an experiment comparing harmaline and PAD, only the harmaline inhibition of MAO was reversible.

TABLE 5. Effect of Dialysis on PAD and Harmaline Inhibition

Mitochondrial MAO		
Inhibitor	$\Delta$ O.D./10 min/1 No dialysis	ml of mixture Dialysis
PAD ( $1.5 \times 10^{-3}$ M)	0.130	0.110
Harmaline ( $1.5 \times 10^{-3}$ M)	0.0	0.0
	0.0*	0.60

Of the mitochondrial suspension, 0.4 ml was added to 7.6 ml of a solution of  $\text{NaHCO}_3$ , 1 mg/ml. The same amount of mitochondria also was added to 7.6 ml of the  $\text{NaHCO}_3$  solution which contained PAD at a concentration of  $1.5 \times 10^{-3}$  M. Each mixture was divided in half. One-half was dialyzed for 24 hr. against 3 changes of 4 l of distilled water at  $4^\circ\text{C}$ ; the other half was left in a test tube at  $4^\circ\text{C}$ . After 24 hr. 1-0 ml. of each mixture was test for MAO activity. The same experiment was performed simultaneously with harmaline at the same concentration ( $1.5 \times 10^{-3}$  M). These experiments were performed twice with less than 5% variation.

\*MAO activity could not be determined because harmaline absorbed too strongly at 360 mu at this concentration; however, harmaline has been reported to inhibit MAO completely at concentrations much lower than this.<sup>189</sup>



The following information is provided for your reference. The data presented here is based on the most recent measurements available. It is important to note that the values are subject to experimental uncertainties and may vary slightly from those reported in the literature.

The first set of data shows the results of the initial experiment. The measured values are consistent with the theoretical predictions within the limits of experimental error. The second set of data, obtained under different conditions, shows a similar trend, further supporting the validity of the model.

The following table summarizes the key parameters and their values.

Parameter	Value	Uncertainty
$\alpha$	0.12	$\pm 0.01$
$\beta$	0.05	$\pm 0.005$
$\gamma$	0.01	$\pm 0.001$
$\delta$	0.005	$\pm 0.0005$

The data presented in the table above are the result of a series of experiments conducted over a period of several months. The experimental setup was carefully calibrated to ensure the highest possible accuracy. The results show a clear correlation between the measured values and the theoretical model, indicating that the model is a good representation of the physical system being studied.

It is worth noting that the uncertainty in the measurements is relatively small, particularly for the parameters  $\alpha$  and  $\beta$ . This suggests that the experimental technique used is highly precise and reliable. The consistency of the results across different experimental conditions further strengthens the confidence in the findings.

The following conclusions can be drawn from the data presented here. First, the measured values are in excellent agreement with the theoretical predictions. Second, the experimental technique used is highly accurate and reliable. Finally, the results provide strong evidence in support of the theoretical model.

(5) Effect of PAD on solubilized MAO.

It might be argued that, because of its detergent-like structure, PAD exerts a nonspecific solubilizing action on MAO. This was tested by observing the effect of PAD on MAO solubilized with isooctylphenoxypolyethoxyethanol. At three different concentrations of PAD, the percentage inhibition of the solubilized enzyme and of that in the mitochondrial suspension were the same (see Table 6) although the total activity of the solubilized enzyme was only one-fifth as great. This is in contrast with the findings of Cotzias *et al.*<sup>197</sup> who noted an increase in enzyme activity after solubilization.

TABLE 6. EFFECT of PAD on "Solubilized" MAO, as compared with the Mitochondrial MAO

Concentration of PAD	% Inhibition of mitochondrial MAO	% Inhibition of solubilized MAO
$2.5 \times 10^{-4}$	100	100
$5.0 \times 10^{-5}$	74	74
$1.0 \times 10^{-5}$	40	40

Cutscum was added to the mitochondrial suspension to make a 5% solution. It was shaken intermittently in the cold for 4 hr and then centrifuged at 100,000 x g for 1 hr. The supernatant fraction was decanted and tested for MAO activity and inhibition by PAD. This was compared with the activity and inhibition obtained with another aliquot of the mitochondrial MAO. All assays were done in duplicate with less than 5% variation.

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(6) Ability of PAD to inhibit MAO *in vivo*.

PAD was administered intraperitoneally in various regimens to rats which were then sacrificed at varying times after the last dose. The range of dosages was from 10 to 16 mg/kg for 1-7 days, and the elapsed time after the last administration of PAD was 1-5 hr. Brains and livers were treated as in (1). Little difference could be detected by this method in the activity of the homogenates on MAO of control and treated animals, whereas animals given comparable dosages of the hydrazine inhibitors showed extensive or complete inhibition of MAO.

(7) Effect of PAD on the levels of 5-HT in rat brain.

A sensitive indicator of MAO inhibition, *in vivo*, is an increase in the brain level of 5-HT. It was therefore of interest to determine whether PAD could raise this level. The results, shown in Table 7, indicate that PAD lowered rat brain levels of 5-HT when these were determined 4 hours after the last administration of PAD; 24 hours after a dose that produced extensive lowering of 5-HT in brain, the level had returned to normal.



TABLE 7. Effect of PAD on 5-HT levels in Brain

PAD (mg/kg)	No. of days administered	Change (% +)
1. 30	1	-15 + 2
2. 15	7	-36 + 4
3. 20	2	-40 + 4
4. 50	2	-64 + 5
5. 65	1	No change 24 hr after injection

Drugs were given intraperitoneally. Each group consisted of 6 male rats. In the first 4 experiments the treated animals and controls were killed by exsanguination 4 hr after PAD and their brains homogenized in 5 ml of 0.1 N HCl. In the fifth experiment the animals were killed 24 hr after administration of PAD.

Department of Mathematics, University of California, Berkeley

Year	Number of Students	Faculty
1950	100	10
1955	150	15
1960	200	20
1965	250	25
1970	300	30
1975	350	35
1980	400	40
1985	450	45
1990	500	50
1995	550	55
2000	600	60
2005	650	65
2010	700	70
2015	750	75
2020	800	80

The following table shows the number of students and faculty members in the Department of Mathematics from 1950 to 2020. The number of students has increased steadily over the years, while the number of faculty members has also increased, but at a slower rate than the number of students. This suggests that the department has been able to maintain a high level of quality and research output while also expanding its size.

(8) Effect of PAD on the catecholamine levels of the rat brain.

No effect on rat brain catecholamines was observed when high doses of PAD were given intraperitoneally for either short or long periods.

(9) Effect of PAD on 5-hydroxytryptophan decarboxylase of rat kidney in vitro.

PAD,  $10^{-3}M$ , produced no significant inhibition of rat kidney 5-hydroxytryptophan decarboxylase.

(10) Effect of PAD on 5-HT of mast cells.

The ascites mast cells of the Dum-Potter mouse mastocytoma contains 5-HT which behaves as though it were bound in granules in pharmacologic experiments.<sup>218</sup> These cells are more suitable for studies on amine release than are the non-nucleated platelets that often are used. Table 8 shows that PAD and other long-chain quaternary pyridinium compounds, but not PAM, released 5-HT from mast cells. JB-516 also caused a similar release.



ARTICLE 10. -

SECTION 10.01

It is the policy of the State of Texas to encourage the development and use of the State's natural resources and to protect the State's environment from pollution and degradation.

ARTICLE 11. -

SECTION 11.01

The State shall encourage the development and use of the State's natural resources and to protect the State's environment from pollution and degradation.

ARTICLE 12. -

The State shall encourage the development and use of the State's natural resources and to protect the State's environment from pollution and degradation.

SECTION 12.01

TABLE 8. Release of 5-HT from Mast Cells

Drug (0.5 mg/ml)	% Release
	20
PAD	100
Tetradecyl pyridine iodide	100
Cetyl pyridine iodide	100
PAM	20
JB-516	60

The mast cells were removed from DBA/2 mice by aspiration of the peritoneal contents, and suspended in Hank's medium.<sup>219</sup> Duplicates were incubated in 20-ml beakers at 37° for 2 hr in a Dubnoff shaker, with and without inhibitors dissolved in Hank's medium.<sup>8</sup> Each beaker contained approximately 10<sup>8</sup> cells. After incubation the cells were centrifuged and the supernatant fractions decanted. The cells were washed with more Hank's medium, centrifuged, and the 2 supernatant fractions combined. The 5-HT content of both the cells and the supernatant fractions were measured spectrophotofluorometrically. There was less than 5% variation in the duplicates.



(11) Effect of PAD and JB-516 on levels of 5-HT in rats after administration of reserpine.

It is conceivable that the release of 5-HT by PAD in the rat brain masked some inhibition of MAO that otherwise would have been detected through increased levels of 5-HT. It was thought that the administration of PAD, after maximal release of 5-HT by reserpine, might then permit the detection of MAO inhibition through the accumulation of newly synthesized 5-HT. Table 9 shows that inhibition of MAO could not be demonstrated in vivo even by this method whereas that caused by JB-516 is readily apparent.

TABLE 9. 5-HT Levels after Reserpine followed by either

PAD or JB-516		
Compound administered	5-HT levels ( $\mu$ g)	Range
Reserpine	0.09	0.08-0.10
Reserpine + PAD	0.09	0.08-0.10
Reserpine + JB-516	0.46	0.43-0.49

Eighteen rats were given 4 mg of reserpine per kg; 6 of these were given 15 mg of PAD per kg. and 6 were given 10 mg of JB-516 per kg. 5 hr after the reserpine. The 5-HT levels at 1 hr after the last injection were determined.

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(12) Effect on rats of pretreatment with PAD followed by reserpine, and pretreatment with reserpine followed by PAD.

Pretreatment of rodents with an inhibitor of MAO can reverse the sedative effects of reserpine.<sup>175</sup> Since PAD possesses some of the properties of each of these compounds, it was of interest to observe the performance of PAD in both these roles. As can be seen in Table 10, the behavior of PAD was not typical of an inhibitor of MAO, since it did not reverse the sedative effects of reserpine when given as pretreatment; rather, it delayed them. On the other hand, PAD did not act like reserpine, since it did not produce hyperactivity in rats pretreated with JB-516.

TABLE 10. Effect on Rats of Pretreatment with PAD followed by reserpine, and of pretreatment with JB-516 followed by PAD

Drug administered	Behavioral observations
Reserpine	Sedation in 20 min
PAD followed by reserpine	Sedation in 60 min
JB-516 followed by reserpine	Enhanced alertness and activity
JB-516 followed by PAD	No change from untreated animals
JB-516	No change from untreated animals

Each group consisted of 6 animals; these were kept in individual cages. Observations were continued for 4 hr. All doses (mg/kg) were: reserpine 5; PAD, 50; JB-516, 20. PAD was given for 2 days, the last dose 4 days prior to reserpine or 30 min after JB-516. JB-516 was given 30 min before PAD or reserpine.



(13) Effects of various quaternary ammonium derivatives on MAO in vitro.

Of many readily available compounds tested, decamethonium (85% inhibition at  $10^{-3}$ M), hexamethonium (25% at  $10^{-3}$ M), and thiamine (70% at  $10^{-2}$ M) exhibited weak inhibitory actions. Choline, acetylcholine, PAM, diphosphopyridine nucleotide, N-methyl nicotinamide, succinylcholine, d-tubocurarine, stigmonene bromide, and neostigmine were inactive. Several short-chain N-alkyl derivatives of heterocyclic nitrogen compounds were also good inhibitors in vitro.

(14) In vivo and in Vitro Studies with Thiamine and MAO.

As mentioned previously, studies by the author and Gal and Drewes<sup>211</sup> demonstrated that in thiamine-deficient rats the MAO-activity of brain and intestine is increased. However, rats given thiamine, 70mg/kg subcutaneously, and sacrificed after 2 hours, when thiamine pyrophosphate levels in liver are elevated,<sup>220</sup> showed no inhibition of the MAO-activity of liver, brain, and intestine, as compared with untreated controls. Studies in vitro showed that thiamine at a concentration of  $10^{-2}$ M inhibits the MAO of rat liver mitochondria 70%, whereas thiamine pyrophosphate is slightly less effective.



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(15) Effect of Dodecyl Iproniazid Iodide on MAO  
in vitro.

Table 11 demonstrates that dodecyl iproniazid iodide is an effective inhibitor of rat liver mitochondrial MAO without any preincubation, but that is an appreciably more effective inhibitor after preincubation.

Table 11. Effect of Dodecyl Iproniazid Iodide on MAO Activity of Washed Rat Liver Mitochondria With and Without Preincubation

Concentration of Dodecyl Iproniazid Iodide	% Inhibition of MAO	
	No Preinc.	Preinc.
$1 \times 10^{-4}M$	45%	95%

The substrate, kynuramine, was added to the reaction mixture which contained dodecyl iproniazid iodide prior to the addition of mitochondrial MAO, or 30 minutes after the MAO. In both series, reactions were run in duplicate with and without dodecyl iproniazid iodide.

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(16) Ability of Dodecyl Iproniazid Iodide to Inhibit MAO in vitro.

Dodecyl iproniazid iodide, 20 mg/kg, was given intraperitoneally to rats which were sacrificed four hours later. Liver specimens were treated as in (1). Dodecyl iproniazid iodide produced a 70% inhibition of rat liver MAO under these conditions.

(17) Effect of Dodecyl Iproniazid Iodide on the Levels of 5-HT in Rat Brain.

Dodecyl iproniazid iodide, 20 mg/kg, was given intraperitoneally to 6 rats which were sacrificed four hours later. Brains were excised and treated as in (7). Dodecyl iproniazid iodide increase 5-HT levels by 50% under these conditions.

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DISCUSSIONPAD as an Inhibitor of Monoamine Oxidase

These studies demonstrate that the long-chain N-alkyl quaternary pyridinium compounds have a unique series of effects on brain amines and on MAO. PAD was studied extensively as a prototype of this class, but some of the experiments performed demonstrate that these properties are probably possessed by most or all of the whole class of compounds.

PAD ranks among the most potent MAO inhibitors in vitro when related to published comparative data from studies with iproniazid,<sup>47-53</sup> and the author's studies with PAD, iproniazid, and other MAOI. PAD irreversibly inhibited MAO in vitro, without preincubation. This sets it apart from the "hydrazines," which required preincubation, and from the harmala alkaloids, which are reversible inhibitors. Dodecyl iproniazid iodide, which was designed as a combination of a hydrazine MAOI and a long-chain N-alkyl pyridinium compound, was found to be an effective inhibitor without preincubation, but to be an even more effective inhibitor after preincubation. This could be due to two separate modes of MAO inhibition: that attributable to the N-alkyl pyridinium moiety, not requiring any preincubation and accounting for most of the 45% inhibition due to the hydrazine grouping requiring preincubation and accounting for the additional 50% inhibition noted after 30 minutes preincubation. As mentioned above, the potency

THE HISTORY OF THE UNITED STATES

The history of the United States is a story of growth and change. It begins with the first settlers who came to the shores of the Atlantic Ocean. They found a land of opportunity and freedom. Over the years, the colonies grew and became more independent. They fought for their rights and eventually won the right to be a free and sovereign nation. The United States has since become a world leader in many fields, including science, technology, and the arts.

The story of the United States is a story of progress and achievement. It is a story of a people who have built a great nation from a small group of settlers. The United States has shown the world that it is possible to create a society based on freedom, equality, and justice. The United States has led the world in many ways, and it continues to do so today. The United States is a land of hope and opportunity, and it is a land that has inspired people all over the world. The United States is a great nation, and it is proud to be a part of it.

of PAD as an in vitro inhibitor is considerable, and it is not unreasonable to predict that more potent inhibitors of MAO, containing a quaternary pyridinium nitrogen, can be synthesized.

Every attempt to demonstrate inhibition of MAO in vivo with these compounds was unsuccessful: (i) There was no decrease in the MAO activity of liver and brain homogenates of PAD-treated animals; (ii) 5-HT levels in brain decreased rather than increased after treatment with PAD; (iii) PAD produced no increase in the 5-HT levels of rats pretreated with reserpine to release nearly all of the 5-HT initially present, thus eliminating the possibility that release of 5-HT by PAD was obscuring a simultaneous accumulation due to inhibition of MAO. Rats given JB-516, a potent MAOI, readily accumulated 5-HT under the same conditions indicating that after the initial release by reserpine and degradation by MAO, newly formed 5-HT would accumulate, though its binding might be impaired by the reserpine. In the PAD-treated animals no accumulation was noted, probably because MAO was not inhibited. (iv) Finally, there was no reversal of the reserpine-induced depression in rats pretreated with PAD.

There are several questions to be considered in seeking an explanation for the absence of in vivo-inhibition of MAO. Can PAD penetrate the rat blood-brain barrier and reach the cells of the central nervous system? Can PAD



The first part of the paper is devoted to a general discussion of the problem of the existence of solutions of the system of equations (1) under the assumption that the functions  $f_i(x)$  are continuous and bounded in the domain  $D$ .

In the second part we consider the case when the functions  $f_i(x)$  are piecewise continuous and bounded. We show that in this case the system (1) has a solution in the domain  $D$  if and only if certain conditions are satisfied.

In the third part we study the question of the stability of the solutions of the system (1) with respect to the initial conditions. We show that the solutions are stable if the matrix  $A(x)$  is negative definite in the domain  $D$ .

In the fourth part we consider the case when the functions  $f_i(x)$  are piecewise continuous and bounded, and the matrix  $A(x)$  is piecewise constant. We show that in this case the system (1) has a solution in the domain  $D$  if and only if certain conditions are satisfied.

In the fifth part we study the question of the stability of the solutions of the system (1) with respect to the initial conditions. We show that the solutions are stable if the matrix  $A(x)$  is negative definite in the domain  $D$ .

In the sixth part we consider the case when the functions  $f_i(x)$  are piecewise continuous and bounded, and the matrix  $A(x)$  is piecewise constant. We show that in this case the system (1) has a solution in the domain  $D$  if and only if certain conditions are satisfied.

In the seventh part we study the question of the stability of the solutions of the system (1) with respect to the initial conditions. We show that the solutions are stable if the matrix  $A(x)$  is negative definite in the domain  $D$ .

enter the various types of cells of the rat liver? If penetration does occur, was the amount of PAD administered adequate to produce detectable inhibition?

The evidence for the ability of PAD and other quaternary pyridiniums to penetrate the rat blood-brain barrier is the decrease in the 5-HT levels which PAD produces, and the increase in brain levels of 5-HT which dodecyl iproniazid iodide produces. The release of 5-HT from mast cells in vitro by PAD supports the hypothesis that the release is mediated directly by this agent rather than in some indirect manner. There is the possibility that the releasing effect is produced by less PAD than is required for the inhibitory effect on MAO, so that sufficient PAD enters the brain to produce the former but not the latter effect. The exclusion of PAD from the rat liver is another possibility that cannot yet be dismissed, although it is not a very attractive one. The demonstration of the inhibition of liver MAO by dodecyl iproniazid iodide makes this very unlikely.

The question of adequate dosage is less troublesome, as can be shown by comparing PAD with iproniazid. After rats were given a single dose of iproniazid of  $5 \times 10^{-5}$  moles/kg body weight, MAO activity determined subsequently in vitro did not return to normal for 5 days.<sup>221</sup> In the most vigorous attempt to demonstrate MAO inhibition by PAD,  $1.4 \times 10^{-4}$  moles/kg of PAD was given to each of the six rats daily for



seven days, and no inhibition of MAO of brain or liver was detected in any rat, despite the evidence presented herein that PAD, like iproniazid, is an irrevocable inhibitor, and that the in vitro-assay used in these experiments<sup>110</sup> is more sensitive than is the one formerly in use.<sup>221</sup> Another possible explanation is that PAD is rapidly detoxified in vivo but not in vitro by the mitochondria. Thus, in vivo, PAD which reached amine-containing granules before reaching mitochondria could exert its releasing action, whereas the mitochondrial detoxification would prevent PAD from inhibiting MAO, a mitochondrial enzyme. If so, analogs of PAD that are not rapidly inactivated in vivo might be synthesized, and a useful MAOI might result. However, recent studies of the metabolism of PAM in man<sup>222</sup> and in lower animals<sup>223</sup> revealed that the drug is excreted largely unchanged, although small amounts of a number of other metabolites were noted. Whether the same holds true for PAD remains to be established.

Perhaps the best explanation for the difference found in vitro and in vivo is the recent work by Aebi.<sup>224</sup> Aebi examined mitochondrial MAO in various states of preservation of the structure of mitochondria and showed that the enzyme behaved differently under each set of circumstances. The activity of MAOI was also influenced by the morphologic state of the mitochondria. The effectiveness of the MAOI studied, varied with the intactness of the mitochondria



leading Aebi to conclude that it was very difficult to predict what results would be obtained in the intact state (in vivo) from studies on mitochondria that were to some extent disrupted (in vitro). Pletscher and Gey<sup>225</sup> have noted many other compounds which inhibit MAO in vitro but not in vivo. Werke et al.<sup>226</sup> have recently reported that N-benzyl -N' - isopropylhydrazine inhibits MAO in vivo but not in vitro and speculate that many effective inhibitors have probably been missed because most screening methods for new MAOI are of an in vitro nature.

The weak ability of hexamethonium to inhibit MAO is of some interest in the light of the recent demonstration that inhibitors of MAO can produce ganglionic blockade.<sup>176</sup> This is not to suggest that the effects of hexamethonium are due to MAO inhibition; rather, it is meant to point up the reciprocity between these two activities that might lead to an exploration of other similarities. It is interesting, moreover, that hexamethonium potentiated the effects of 5-HT in some experiments with the isolated guinea pig ileum.<sup>227</sup> Could this have been due to MAO inhibition?

In the same light, the actions of PAD on both acetylcholinesterase and acetylcholine receptors, and on MAO may be reiterated. It would be of interest to determine whether the influences of PAD on the release of 5-HT and on the activity of MAO are of any importance for understanding the observations of Dettbarn and Wilson already alluded to.<sup>206,207</sup>

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It is worthy of note that chlorpromazine<sup>228</sup> and tolazoline<sup>229</sup> also inhibit both MAO and cholinesterase.

#### PAD and Amine Release

In experiments to determine whether PAD could increase 5-HT levels as would be expected of a MAOI, it was noted that instead of increasing 5-HT levels, PAD actually produced a decrease in the amount of the amine. The extent of lowering was found to be dependent upon the dosage of PAD. That 5-HT release accounted for the decrease in 5-HT levels noted in Table 7 is indicated by the inability of PAD to inhibit 5-HTP decarboxylase and the ability of PAD to produce similar changes in the 5-HT levels of mast cells (Table 8).

In the experiments with mast cells, the 5-HT which sedimented with the cells and which was present in the supernatant fluid was determined with and without treatment of the cells with PAD, other quaternary pyridinium ions, etc. All the 5-HT was recovered in either the supernatant fluid or the cell mass. The quaternary pyridiniums, including PAD, effected a transfer of the 5-HT from the cells to the supernatant fluid. This strongly suggests release of the amine.

The amine-releasing action of PAD was an unexpected finding and added a new dimension of interest to the study of this compound. Firstly, because the mechanism of many of the clinically and experimentally valuable actions of



Section 101

The first part of the document discusses the general principles of the law. It states that the law is based on the principle of justice and equity. The law is designed to protect the rights of individuals and to ensure that they are treated fairly. The law is also designed to promote the public good and to maintain the peace and order of the community. The law is based on the principle of the rule of law, which means that everyone is subject to the law and that no one is above the law. The law is also based on the principle of the separation of powers, which means that the powers of the government are divided among the executive, legislative, and judicial branches. The law is also based on the principle of the protection of individual rights, which means that the government is limited in its power to interfere with the rights of individuals. The law is also based on the principle of the promotion of the public good, which means that the government has a duty to promote the welfare of the community. The law is also based on the principle of the maintenance of peace and order, which means that the government has a duty to maintain the peace and order of the community. The law is also based on the principle of the protection of the environment, which means that the government has a duty to protect the environment for the benefit of present and future generations. The law is also based on the principle of the promotion of the economy, which means that the government has a duty to promote the growth and development of the economy. The law is also based on the principle of the protection of the consumer, which means that the government has a duty to protect the interests of consumers. 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the rauwolfia alkaloids, particularly reserpine has been thought to be related to amine-release,<sup>230-232</sup> It is likely that study of other amine-releasing agents can help illuminate this matter. PAD is particularly useful in this regard, because it is a matter of heated dispute whether it is through 5-HT or catecholamine depletion that reserpine brings about its actions. This problem has been approached by using drugs such as tetrabenazine,<sup>233</sup> ~~10-methyl-meta-tyrosine~~,<sup>234</sup> conditions such as reserpine plus hypothermia,<sup>235</sup> or a diet deficient in 5-hydroxy-tryptophan<sup>236</sup> to deplete selectively either the catecholamines or 5-HT and to determine whether any correlation between behavior and cerebral change in one or the other amines could be obtained. At this point, there is good evidence in both camps, (perhaps an indication that neither amine is involved). Nevertheless, the studies reported here indicate that PAD can be of use in this problem. No other agent has been reported to the author's knowledge, which depletes bring 5-HT but not the catecholamines, although it has many other effects, as previously noted. This selective action of PAD could be exploited to elucidate the physiologic import of 5-HT depletion. Qualitatively, animals treated with doses of PAD which depleted 65% of their 5-HT seemed no less active and alert than controls who had been treated identically, except for not receiving PAD. The studies detailed in Table 10 indicated that PAD pretreatment delayed, not hastened the onset of sedation

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in rats given small doses of reserpine. Also rats pre-treated with phenylisopropylhydrazine (JB-516), an effective MAOI, were alerted and activated by reserpine, whereas a high dose of PAD had no effect on animals pre-treated with phenylisopropylhydrazine. These differences in the effects of PAD and reserpine, although admittedly subjective and in need of quantitative verification, indicate that if amine-release is the means by which reserpine produces behavioral effects, it is mediated via change in catecholamines, rather than 5-HT. (It is of course possible, that it is another amine, or no amine at all, through which reserpine operates). The second reason for being interested in the amine-depleting action of PAD is that it is the only compound which has been shown to both release brain amines and inhibit MAO, Tranylecypromine, one of the MAOI mentioned previously, has been shown to release norepinephrine from the hearts of both rats and cats,<sup>237</sup> but it has not been observed to release brain amines. Iproniazid can diminish the monoamines in the cat hypothalamus but this was thought to be a non-specific effect.<sup>238</sup> It is of interest that PAD's effect on 5-HT binding is not long-lasting. Twenty-four hours after a large dose of PAD, 5-HT levels return to normal.

If a quaternary pyridinium molecule were synthesized which could inhibit brain MAO in vivo as well as release brain 5-HT, it might turn out to have unique behavioral and possibly therapeutic effects since it is by no means

The first part of the report is devoted to a general

description of the project and its objectives.

The second part contains a detailed description of the

methodology used in the study, including the

data collection and analysis procedures.

The third part presents the results of the study,

including the statistical analysis and the

interpretation of the findings.

The fourth part discusses the implications of the

study and provides recommendations for future

research in this area.

The fifth part concludes the report and summarizes

the main findings and conclusions.

The sixth part contains the references used in the

study, and the seventh part contains the

appendices, including the questionnaires and

the data tables.

The eighth part contains the index and the

list of figures and tables.

The ninth part contains the acknowledgments and

the author's contact information.

The tenth part contains the abstract and the

summary of the report.

The eleventh part contains the

concluding remarks and the

final thoughts of the author.

The twelfth part contains the

appendices and the

certain that it is through catecholamine release rather than through 5-HT release by which reserpine acts, or that 5-HT depletion has no behavioral effect. Studies on the treatment of depression in man with a combination of reserpine and iproniazid, i.e., with an amine releaser and a MAOI, have indicated that this combination has particular value.<sup>239</sup>

#### Dodecyl Iproniazid Iodide

The studies with dodecyl iproniazid iodide showed that it possessed the properties that were predicted by the author. The inhibition of MAO in vitro without preincubation was probably due to the N-alkyl group bonded to the pyridine nitrogen atom, whereas the supplemental inhibitory potency noted after preincubation was probably due to the hydrazine moiety. Studies in vivo demonstrated that it is a potent inhibitor of liver MAO (70% inhibition at 20 mg/kg) and that it raises brain 5-HT levels. This implies that it inhibits brain MAO also, or prevents the release of brain 5-HT. It could also release brain 5-HT despite the 50% elevation of 5-HT over control values because the simultaneous inhibition of MAO would prevent its destruction so it might accumulate in its "released" state. There were many interesting experiments to do with this compound, but unfortunately only a very small amount was available to the author and the method of synthesis was not available.



The effects of PAD and dodecyl iproniazid iodide on rat brain imply that both these agents can pass the blood-brain barrier and provide additional evidence for Wilson's idea<sup>201</sup> that long-chain N-alkyl radicals can be used to enhance the ability of highly ionized molecules to penetrate this barrier.

#### The Effect of Thiamine on Monoamine Oxidase

In a recent publication,<sup>211</sup> Gal and Drewes demonstrated that in thiamine-deficient rats, the MAO-activity of the brain and intestine is increased, as compared to the activity of rats fed ad libitum with a normal laboratory diet. These workers suggested that the increase might be related to the stress theory of Selye. However, it is proposed by the author, that, in vivo, thiamine, which has a quaternary nitrogen in its thiazole moiety, is either itself an inhibitor of MAO, or more likely, is metabolized via a pathway which produces another quaternary nitrogen compound which is an inhibitor of MAO. Thus, the increased MAO-activity in the thiamine-deficient animals might be accounted for by a diminution in the amount of this thiamine-like inhibitor. The MAO-inhibition caused by this thiamine-like compound also might explain the hypotension and ganglionic blockade which thiamine produces in man and experimental animals.<sup>240,241</sup> Gertner has shown that MAOI block ganglionic transmission in cats and dogs,<sup>176</sup> while hypotension in man has been noted with the administration of MAOI.<sup>180</sup>



The first part of the document is a letter from the Secretary of the State to the Governor, dated the 1st day of January, 1862. The letter is addressed to the Governor and is signed by the Secretary of the State. The letter contains the following text:

Dear Sir: I have the honor to acknowledge the receipt of your letter of the 29th inst. in relation to the application of the State of New York for the admission of the State of New York to the Union. I have the honor to inform you that the same has been referred to the Committee on the subject, and they have reported in favor of the admission of the State of New York to the Union. I have the honor to inform you that the same has been referred to the Committee on the subject, and they have reported in favor of the admission of the State of New York to the Union.

THE STATE OF NEW YORK

IN SENATE, January 1st, 1862.

REPORT

OF THE

COMMISSIONERS OF THE LAND OFFICE

FOR THE YEAR 1861.

ALBANY: PUBLISHED BY HENRY RILEY, STATE PRINTER, 1862.

The following is a list of the contents of the report:

CHAPTER I. GENERAL STATEMENT OF THE LANDS BELONGING TO THE STATE.

CHAPTER II. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1861.

CHAPTER III. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1862.

CHAPTER IV. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1863.

CHAPTER V. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1864.

CHAPTER VI. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1865.

CHAPTER VII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1866.

CHAPTER VIII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1867.

CHAPTER IX. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1868.

CHAPTER X. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1869.

CHAPTER XI. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1870.

CHAPTER XII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1871.

CHAPTER XIII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1872.

CHAPTER XIV. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1873.

CHAPTER XV. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1874.

CHAPTER XVI. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1875.

CHAPTER XVII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1876.

CHAPTER XVIII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1877.

CHAPTER XIX. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1878.

CHAPTER XX. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1879.

CHAPTER XXI. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1880.

CHAPTER XXII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1881.

CHAPTER XXIII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1882.

CHAPTER XXIV. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1883.

CHAPTER XXV. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1884.

CHAPTER XXVI. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1885.

CHAPTER XXVII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1886.

CHAPTER XXVIII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1887.

CHAPTER XXIX. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1888.

CHAPTER XXX. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1889.

CHAPTER XXXI. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1890.

CHAPTER XXXII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1891.

CHAPTER XXXIII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1892.

CHAPTER XXXIV. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1893.

CHAPTER XXXV. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1894.

CHAPTER XXXVI. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1895.

CHAPTER XXXVII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1896.

CHAPTER XXXVIII. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1897.

CHAPTER XXXIX. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1898.

CHAPTER XL. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1899.

CHAPTER XLI. STATEMENT OF THE LANDS BELONGING TO THE STATE, AS OF THE 1st JANUARY, 1900.

Experiments presented here show that thiamine and thiamine pyrophosphate in vitro are weak inhibitors of MAO: at  $10^{-2}$  M thiamine inhibits the MAO of rat liver mitochondria 70%, a result of little significance with respect to the proposed mechanism in vivo. Furthermore, rats given 70 mg of thiamine per kg subcutaneously, and sacrificed after two hours, when thiamine pyrophosphate levels in liver are elevated,<sup>220</sup> showed no inhibition of the MAO-activity of liver, brain and intestine as compared with untreated controls. However, this does not constitute a refutation of the proposed theory, if the rate of formation of the postulated thiamine metabolite proceeds maximally with the amount of thiamine provided by a normal diet.

Substantial indirect evidence can be provided for the theory. Although in higher animals most of the thiamine which is absorbed is excreted unchanged, the fate of the thiamine which is degraded, is largely unknown.<sup>242</sup> Molluscs, carp, and some other lower species possess an enzyme, thiaminase,<sup>243</sup> which catalyses the reaction of thiamine with a variety of basic compounds with loss of the 4-methyl-5-hydroxyethyl-thiazole portion, and the formation of other quaternary compounds derived from the pyrimidine portion.<sup>243</sup> No similar enzyme has been found in mammalian tissue, but only small amounts may be present. Such a reaction could produce a potent quaternary inhibitor of MAO. It is significant that Minz<sup>244</sup> and von Muralt,<sup>245,246</sup> using labeled thiamine, has shown that, on excitation of a peripheral nerve,

THE HISTORY OF THE UNITED STATES

The first part of the book is devoted to a general history of the United States from its discovery by Columbus in 1492 to the present time. It covers the early years of settlement, the struggle for independence, the formation of the Constitution, and the expansion of the country to the Pacific Ocean. The author discusses the political, economic, and social developments that have shaped the nation over the centuries.

The second part of the book is a detailed account of the American Civil War, from 1861 to 1865. It describes the causes of the war, the military campaigns, and the ultimate victory of the Union. The author also examines the impact of the war on the nation's economy and society, and the role of the war in the development of the modern United States.

The third part of the book is a study of the American West, from the early days of exploration to the present. It discusses the discovery of gold, the settlement of the frontier, and the development of the West as a major part of the United States. The author also examines the role of the West in the nation's history and the impact of the West on the rest of the country.

The fourth part of the book is a study of the American South, from the early days of settlement to the present. It discusses the development of the plantation system, the struggle for independence, and the impact of the Civil War on the South. The author also examines the role of the South in the nation's history and the impact of the South on the rest of the country.

The fifth part of the book is a study of the American Midwest, from the early days of settlement to the present. It discusses the development of the prairie, the settlement of the frontier, and the impact of the Civil War on the Midwest. The author also examines the role of the Midwest in the nation's history and the impact of the Midwest on the rest of the country.

The sixth part of the book is a study of the American Northeast, from the early days of settlement to the present. It discusses the development of the industrial revolution, the settlement of the frontier, and the impact of the Civil War on the Northeast. The author also examines the role of the Northeast in the nation's history and the impact of the Northeast on the rest of the country.

The seventh part of the book is a study of the American Northwest, from the early days of settlement to the present. It discusses the development of the frontier, the settlement of the frontier, and the impact of the Civil War on the Northwest. The author also examines the role of the Northwest in the nation's history and the impact of the Northwest on the rest of the country.

The eighth part of the book is a study of the American Southwest, from the early days of settlement to the present. It discusses the development of the frontier, the settlement of the frontier, and the impact of the Civil War on the Southwest. The author also examines the role of the Southwest in the nation's history and the impact of the Southwest on the rest of the country.

The ninth part of the book is a study of the American West Coast, from the early days of settlement to the present. It discusses the development of the frontier, the settlement of the frontier, and the impact of the Civil War on the West Coast. The author also examines the role of the West Coast in the nation's history and the impact of the West Coast on the rest of the country.

The tenth part of the book is a study of the American South West, from the early days of settlement to the present. It discusses the development of the frontier, the settlement of the frontier, and the impact of the Civil War on the South West. The author also examines the role of the South West in the nation's history and the impact of the South West on the rest of the country.

metabolites of thiamine appear, which are as yet unidentified. Other studies have shown that excitation of the vagal nerve can release thiamine into the heart.<sup>247-250</sup>

Four quaternary ammonium ions, one of them a quaternary pyridinium molecule have been found in the giant nerve fibers of the squid.<sup>251</sup> One of these might be the postulated inhibitor; if so, this would provide a mechanism for preventing the oxidation of any neurohumor which is a substrate for MAO.

The hypotension and ganglionic blockade produced by thiamine and by known inhibitors of MAO is suggestive that these compounds are related. This action of thiamine is unrelated to its properties as coenzyme.<sup>240</sup> This blockade is also different in kind from that produced by hexamethonium,<sup>241</sup> as is that caused by the inhibitors of MAO.<sup>176</sup>

A final argument which may be offered is that in thiamine-deficiency state in man (beri-beri), the initial symptoms are emotional - depression, apathy, and disinterest,<sup>252,253</sup>

a syndrome a physician might now treat with an inhibitor of MAO, which indeed would be only replacement therapy, if thiamine administration to thiamine-deficient organisms does in fact produce an MAO inhibitor.

It is interesting that thiamine-deficient rats also show increased cholinesterase activity<sup>116</sup> and diamine oxidase activity,<sup>117</sup> while thiamine is also a weak inhibitor of these enzymes, in vitro. It has been proposed

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that thiamine inhibits both of these enzymes in vivo.<sup>254,255</sup>

Thus, there is both precedent for the theory presented and a wider scope for a mechanism which would delay the catabolism of neurohumors such as acetylcholine, histamine, serotonin, and the catecholamines.



SUMMARY OF PART TWO

1. Pyridine aldoxime dodecyl iodide (PAD) was found to inhibit the monoamine oxidase (MAO) of homogenates of rat, rabbit, guinea pig, and mouse brain and liver in vitro. This is the first demonstration that a quaternary nitrogen compound can inhibit MAO. Numerous other such compounds were also found to inhibit MAO in vitro.
2. PAD is a more powerful MAO inhibitor in vitro than iproniazid which at one time was widely used in the treatment of depression.
3. No preincubation of enzyme and inhibitor is necessary and the inhibition is irreversible. This sets it apart from other MAO inhibitors.
4. PAD is also an effective inhibitor of "solubilized" MAO.
5. PAD is not an effective MAO inhibitor in vivo. Possible explanations for this discrepancy between in vivo and in vitro results are discussed.
6. PAD, like reserpine, was found to lower the 5-hydroxytryptamine (5-HT) levels in rat brain, but has no effect on the catecholamine levels. It also releases 5-HT from the Dunn-Potter mouse mastocytoma ascites cells.



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7. PAD does not inhibit rat kidney 5-hydroxytryptophane decarboxylase in vitro.
8. These findings suggest that PAD exerts a selective releasing action on bound 5-HT. It is the only compound reported which selectively depletes 5-HT but not the catecholamines.
9. PAD treatment produced no accumulation of rat brain 5-HT after initial depletion of 5-HT with reserpine whereas 5-HT did accumulate after treatment with a hydrazine type MAO inhibitor under the same conditions. This is a more sensitive indication that no MAO inhibition occurs in vivo.
10. Animals given sufficient PAD to deplete their 5-HT stores do not have the appearance of animals treated with reserpine. In addition, animals pretreated with phenylisopropylhydrazine (PIH), and inhibitor of MAO, and then given PAD do not have the appearance of animals pretreated with PIH and then given reserpine.
11. Decamethonium, hexamethonium and thiamine are also weak inhibitors of MAO in vitro.
12. The reported increase in brain MAO activity of thiamine-deficient rats is interpreted as an indication that thiamine or probably one of its metabolites is an MAO inhibitor in vivo. Relevant data from the literature is offered in support of this idea.

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13. Dodecyliproniazid iodide was conceived of as a molecule combining the MAO inhibitory properties of a hydrazine and the 5-HT releasing properties of a quaternary nitrogen compound. It was found to inhibit MAO in vitro with both the hydrazine moiety and the quaternary nitrogen moiety. It was also found to release rat brain 5-HT in vivo. This is the first compound to both inhibit MAO in vivo and release brain 5-HT and might have unique psychopharmacologic properties.



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2. In the second section, the author outlines the various methods used to collect and analyze data. This includes both qualitative and quantitative approaches, as well as the use of advanced statistical tools to identify trends and patterns.

3. The third part of the document focuses on the implementation of these findings. It provides a detailed plan of action, including specific tasks, responsibilities, and timelines for each phase of the project.

4. The fourth section addresses the challenges and risks associated with the proposed strategy. It offers practical solutions and contingency plans to mitigate potential obstacles and ensure the successful execution of the plan.

5. Finally, the document concludes with a summary of the key points and a call to action. It encourages all stakeholders to work together to achieve the organization's goals and maintain a commitment to excellence and innovation.

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- 1. Introduction . . . . . 10
- 2. Theoretical Framework . . . . . 15
- 3. Methodology . . . . . 20
- 4. Results . . . . . 25
- 5. Discussion . . . . . 30
- 6. Conclusion . . . . . 35
- 7. References . . . . . 40
- 8. Appendix . . . . . 45
- 9. Bibliography . . . . . 50
- 10. Index . . . . . 55

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- 1. Introduction ..... 1
- 2. Theoretical Framework ..... 2
- 3. Methodology ..... 3
- 4. Results ..... 4
- 5. Discussion ..... 5
- 6. Conclusion ..... 6
- 7. References ..... 7
- 8. Appendix ..... 8
- 9. Bibliography ..... 9
- 10. Index ..... 10

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1. Introduction	1
2. Theoretical background	2
3. Methodology	3
4. Results	4
5. Discussion	5
6. Conclusion	6
7. References	7
8. Appendix	8
9. Bibliography	9
10. Index	10
11. Glossary	11
12. Acknowledgements	12
13. Author's note	13
14. Contact information	14
15. Declaration of interest	15
16. Funding	16
17. Data availability	17
18. Ethics approval	18
19. Informed consent	19
20. Author contributions	20
21. Correspondence	21
22. Additional information	22
23. Supplementary material	23
24. References	24
25. Index	25
26. Glossary	26
27. Acknowledgements	27
28. Author's note	28
29. Contact information	29
30. Declaration of interest	30
31. Funding	31
32. Data availability	32
33. Ethics approval	33
34. Informed consent	34
35. Author contributions	35
36. Correspondence	36
37. Additional information	37
38. Supplementary material	38
39. References	39
40. Index	40
41. Glossary	41
42. Acknowledgements	42
43. Author's note	43
44. Contact information	44
45. Declaration of interest	45
46. Funding	46
47. Data availability	47
48. Ethics approval	48
49. Informed consent	49
50. Author contributions	50
51. Correspondence	51
52. Additional information	52
53. Supplementary material	53
54. References	54
55. Index	55
56. Glossary	56
57. Acknowledgements	57
58. Author's note	58
59. Contact information	59
60. Declaration of interest	60
61. Funding	61
62. Data availability	62
63. Ethics approval	63
64. Informed consent	64
65. Author contributions	65
66. Correspondence	66
67. Additional information	67
68. Supplementary material	68
69. References	69
70. Index	70
71. Glossary	71
72. Acknowledgements	72
73. Author's note	73
74. Contact information	74
75. Declaration of interest	75
76. Funding	76
77. Data availability	77
78. Ethics approval	78
79. Informed consent	79
80. Author contributions	80
81. Correspondence	81
82. Additional information	82
83. Supplementary material	83
84. References	84
85. Index	85
86. Glossary	86
87. Acknowledgements	87
88. Author's note	88
89. Contact information	89
90. Declaration of interest	90
91. Funding	91
92. Data availability	92
93. Ethics approval	93
94. Informed consent	94
95. Author contributions	95
96. Correspondence	96
97. Additional information	97
98. Supplementary material	98
99. References	99
100. Index	100

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1. Introduction 1

2. Theoretical Framework 2

3. Methodology 3

4. Results 4

5. Discussion 5

6. Conclusion 6

7. References 7

8. Appendix 8

9. Bibliography 9

10. Index 10

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The third part of the document focuses on the results of the analysis. It shows that there is a clear trend in the data, which is consistent with the initial hypothesis. This finding is significant as it provides strong evidence for the proposed model.

Finally, the document concludes with a summary of the findings and some recommendations for future research. It suggests that further studies should be conducted to explore the underlying causes of the observed trends.

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1. Introduction . . . . . 1

2. Theoretical Framework . . . . . 2

3. Methodology . . . . . 3

4. Results . . . . . 4

5. Discussion . . . . . 5

6. Conclusion . . . . . 6

7. References . . . . . 7

8. Appendix . . . . . 8

9. Bibliography . . . . . 9

10. Index . . . . . 10

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1. Introduction	1
2. Literature Review	2
3. Methodology	3
4. Results	4
5. Discussion	5
6. Conclusion	6
7. References	7
8. Appendix	8
9. Bibliography	9
10. Index	10
11. Glossary	11
12. Acknowledgements	12
13. Author's Note	13
14. Contact Information	14
15. Declaration of Interest	15
16. Funding	16
17. Data Availability	17
18. Ethics Approval	18
19. Conflicts of Interest	19
20. Supplementary Materials	20
21. Correspondence	21
22. Peer Review History	22
23. Copyright	23
24. Reprints and Permissions	24
25. Distribution	25
26. Open Access	26
27. Terms and Conditions	27
28. Disclaimer	28
29. Privacy Policy	29
30. Final Statement	30

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. . . . .	131
. . . . .	132
. . . . .	133
. . . . .	134
. . . . .	135
. . . . .	136
. . . . .	137
. . . . .	138
. . . . .	139
. . . . .	140
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. . . . .	144
. . . . .	145
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. . . . .	150
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. . . . .	152
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. . . . .	156
. . . . .	157
. . . . .	158
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. . . . .	161
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. . . . .	163
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. . . . .	167
. . . . .	168
. . . . .	169
. . . . .	170
. . . . .	171
. . . . .	172
. . . . .	173
. . . . .	174
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. . . . .	176
. . . . .	177
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. . . . .	185
. . . . .	186
. . . . .	187
. . . . .	188
. . . . .	189
. . . . .	190
. . . . .	191
. . . . .	192
. . . . .	193
. . . . .	194
. . . . .	195
. . . . .	196
. . . . .	197
. . . . .	198
. . . . .	199
. . . . .	200

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1. Introduction	1
2. Literature Review	10
3. Methodology	20
4. Results	30
5. Discussion	40
6. Conclusion	50
7. References	60
8. Appendix	70
9. Bibliography	80
10. Glossary	90
11. Index	100
12. Summary	110
13. Acknowledgements	120
14. About the Author	130
15. Contact Information	140
16. Disclaimer	150
17. Copyright	160
18. Privacy Policy	170
19. Terms and Conditions	180
20. Final Remarks	190







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