ENGINEERING EXPERIMENT STATION
of the Georgia Institute of Technology
Atlanta, Georgia

Informal Progress Report

Project A-430

OZONE - INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

JANUARY 1, 1959 through MARCH 31, 1959

Contract No. AF 41(657)-263

School of Aviation Medicine, USAF
Randolph Air Force Base, Texas

The Welsbach ozonator is capable of producing ozone in concentrations from about 1 to 10 per cent by weight from pure ozygen--depending on the voltage operation, rate of gas flow, and certain other variables. We have found that scrubbing ozone through 400 ml of 0.8 normal sulfuric acid for 20 to 30 minutes produces a near-saturated solution and, as a matter of convenience, we allow this period of time for scrubbing. The final concentration of ozone is then determined by varying the voltage on the ozonator or by diluting the resultant solution. After the ozone solution has been generated it is poured into a glass-stoppered bottle. This particular bottle has a very narrow mouth and the top has been ground smooth so that a tight fit will be obtained when a coverslip is inverted over the open neck. The bottle has a ground glass stopper and special care is necessary to insure that no contaminating or reducing substances are present as these would effectively reduce the ozone concentration. Several methods for ozone analysis have been evaluated in previous investigations. The ferrous-ferric system was selected as the best method for these experimental procedures. This analytical procedure involves the oxidation of the ferrous ion to the ferric ion by ozone:

$$Fe^{++} + O_3 + H_2 \rightarrow Fe^{+++} + O_2 + H_2O$$

Enough ferrous ammonium sulfate in 0.8 normal sulphuric acid is added to the test solution to make a final concentration of approximately 0.5 normal ferrous ammonium sulfate solution. This is back-titrated with potassium permanganate standardized against sodium oxalate. Insofar as this analysis is ordinarily conducted in dilute sulfuric acid solutions, it lends itself particularly well to our experimental procedure and eliminates the necessity of changing the pH during the analytical procedure as would be necessary, for example, with potassium iodide. Another advantage of this analytical method is the sharp end point evidenced in the permanganate titration.

In the dosimetry of the hanging-drop preparations the embryos will be exposed to ozone by inverting the preparations on the cover slip and placing them on the top of the sample bottle, taking pains to insure a complete seal between the cover slip and the ground lip of the bottle. Under these conditions, the concentration of ozone in the gas above the liquid and in contact with the drop containing the embryo preparation may be estimated, based upon the distribution coefficient of ozone at this temperature. After a specified

contact time, the solution will be replaced in the preparation, yolk material added, and observation begun immediately. This particular method of dosimetry seems to be dictated by several considerations: (1) the requirements of the embryo for a balanced physiological solution, (2) the advantage of a short contact time, and (3) the necessity of eliminating reducing materials from the embryo preparations during ozone contact. It has been found in other investigations that ozone is not effective biologically until all reducing materials in solution have been eliminated. The presence of even small amounts of organic or other reducing agents provides marked protection from the ozone effect.

In an attempt to elimate the temperature shock-effect present in the experimental results of other investigators using this material, it was decided to conduct all operations as close to the incubation temperature (38° C) as would be feasible. To this end, a constant-temperature work space was constructed in which all of the preparatory steps and dosimetry could be accomplished. Temperature control was maintained in this inoculation box through thermostat control of the radiant-heating bottom surface. Heat is supplied through a 400-watt radiant-heating wire located beneath a 1/4 inch plate glass surface. This plate glass bottom acts as a continuous heat-radiating surface, and the high heat loss and continuous heat input of this system result in a small amplitude of temperature change while permitting the front of the box to remain open and considerable manipulation to take place through the open front. In operation this box maintains a temperature between 35° and 37° C, care being taken to keep the temperature from going above 38° C. By preparing samples under such conditions and then transferring to the microscope incubator, the temperature shock-effect was eliminated.

A new type of microscope incubator was designed and put into operation which offers several unique advantages. This incubator is an inflated polyvinyl bag through which hot air is forced in a closed circulating system. Forced-draft incubating systems offer good temperature control if there is a large enough temperature differential between the desired incubator setting and room temperature. The flexible polyvinyl bag offers an additional advantage over rigid systems--namely, the microscope controls may be manipulated from the exterior of the bag without breaking into the

closed forced-draft system. This is accomplished by merely pressing in on the bag and manipulating the controls directly through the polyvinyl material. This overcomes one of the more serious drawbacks to a forced-draft system--that of breaking into and disturbing the continuous air flow.

In following the progress of neuroblast cells through mitosis it has been found convenient to use a tape recorder during the actual observations and then to play back the tape at the termination of a run to obtain the data. This results both in better efficiency and undoubtedly reduces some of the experimental bias that would result from a chronological recording of individual cell progress.

Miss Beverly Benton, B.S., has joined the project as a Technical Assistant. Miss Benton has had previous experience with the U. S. Public Health Service and on other project work at the Georgia Institute of Technology.

B. Results

In these preliminary investigations the elimination of temperature shock in the experimental material is considered to be of considerable significance and interest. The presence of this variable in the data of other investigators introduces difficulty in analysis of their data. It superimposes its own effect for a considerable period of time over that of any experimental agent and it is obvious that the elimination of this variable will be a considerable contribution to this particular experimental technique.

The necessity of using reducing-free solutions during ozone dosimetry has been demonstrated by several preliminary experiments. Preliminary data indicate that embryo preparations containing yolk and glucose are many times less sensitive to treatment than embryos in physiological saline. The presence of these substances would necessitate long contact times to produce the desired effects and would introduce additional problems of temperature shock and ozone decomposition during this interval.

III. PLANS FOR THE FUTURE

In the next phase of this program the actual experimental data will be

A-430

ENGINEERING EXPERIMENT STATION
of the Georgia Institute of Technology
Atlanta, Georgia

Informal Progress Report No. 2

Project No. A-430

OZONE-INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

July 1, 1959 through September 30, 1959

Contract No. AF 41(657)-263

School of Aviation Medicine, USAF
Randolph Air Force Base, Texas

I. SUMMARY

During this phase of the program the ozone concentration necessary to produce the desired mitotic inhibition was determined to be in the range 3.5 to 4.5 mg per liter of ozone. Lower concentrations with the same contact time failed to significantly inhibit mitosis. The mitotic inhibition was reversible and went to completion, although delayed. Replicate experiments were run and the data indicate that cells dosed before early prophase (very early prophase, interphase, late telophase, mid-telophase and late telophase) are inhibited and differ significantly from the controls in the increment of time taken to reach the next anaphase. The preliminary information indicates that late interphase - very early prophase is the sensitive stage. At the time of ozone exposure cells more advanced than very early prophase proceeded to anaphase at a time not significantly different from the controls.

II. STATEMENT OF PROGRESS

During this phase of the program, the experimental material was exposed to ozone and the effect on the mitotic mechanism was determined. It was desirable to use a dose such as would produce a reversible inhibition of mitosis. This dose should be sufficient to result in a significant delay in the progression from one stage to another and yet must still permit mitosis to proceed. In addition, the ozone dose to be used must be given quickly enough to permit accurate determination of the particular stage treated, but must be given long enough to allow the gaseous ozone to adequately diffuse in the media and penetrate into the cells. A 5-minute dose time was selected and was used in all of the experimental procedures. Chortophaga embryos were exposed to ozone by inverting the preparations on coverslips and placing them over the top of a sample bottle. A complete seal was maintained between the coverslip and the ground lip of the bottle. The sample bottle contained 200 ml of the ozone solution in a 250-ml-capacity bottle. Under these conditions it may be assumed that the ozone in the solution will come to equilibrium with the experimental media. The embryos were freed as nearly as possible from all adhering yolk material by suspending in a saline-sugar solution with a minimum of extraneous reducing materials. The ozone solution was generated from cylinder oxygen in a Welsbach Model T-23 laboratory ozonator by bubbling the

output stream of ozone-oxygen mixture into 0.8-normal sulfuric acid. The concentration of ozone was regulated by the voltage setting on the ozonator. A 20-minute bubbling time produced a nearly constant ozone concentration for a particular voltage setting.

A series of experiments was run to determine the concentration of ozone necessary to produce a significant inhibition of mitosis. The results are shown in Table I.

TABLE I

DURATION OF MITOSIS AFTER EXPOSURE TO VARIOUS CONCENTRATIONS OF OZONE FOR 5 MINUTES

Ozone Solution (mg/l)	No. Of Cells	Total Mitotic Time (Min.)	Theoretical Time* (Min.)	Difference	P**
0.0 to 1.5	18	3764	3744	20	> .5
1.5 to 2.5	22	4530	4576	46	> .5
2.5 to 3.5	18	3845	3744	101	0.10
3.5 to 4.5	16	5216	3328	1888	< .01

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of FIT. (Fisher)

Under these experimental conditions no effect was detected until the ozone concentration in the dosing bottle was from 3.5 to 4.5 mg per liter of ozone. In this concentration range there was a significant inhibition of mitosis; however, the effect was reversible and mitosis would go to completion, although delayed. This range, 3.5 to 4.5 mg per liter of ozone, was the concentration used in all of the dosimetry experiments. The lower concentrations of ozone undoubtedly represent the "ozone demand" of this particular solution. It was not feasible to work with higher concentrations of ozone because of the excessive duration of mitotic inhibition.

Quantitative information on the effects of ozone on each of the specific stages of mitosis was obtained by replication. To this end preparations were dosed and placed under the microscope, the preparations were mapped and each cell was followed until the time of next anaphase. The total time involved

from the end of dosing to the commencing of observation was 5 minutes; therefore, it is possible to predict the stage dosed. The preliminary results are given in Table II.

TABLE II

TIME TO REACH NEXT ANAPHASE OF CELLS
TREATED WITH 3.5 TO 4.5 MG PER LITER OF OZONE

Stage Treated	No. Cells	Total Time To Next Anaphase (Min.)	Theoretical Time* (Min.)	Difference	P**
Interphase	9	1841	1154	687	< .01
Very early prophase	8	924	824	120	< .01
Early prophase	8	578	544	34	.15
Mid-prophase	7	263	259	4	> .5
Late prophase	10	225	230	5	> .5
Metaphase	5	27	22.5	4.5	> .5
Anaphase	6	1881	1221	660	< .01
Mid-telophase	6	1365	1104	261	< .01
Late telophase	4	937	634	303	< .01

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of FIT. (Fisher)

These data indicate that cells dosed earlier than early prophase (very early prophase, interphase, late telophase, mid-telophase and early telophase) are inhibited by this dose of ozone and differ significantly from controls in the increment of time taken to reach the next anaphase. Cells more advanced at the time of ozone exposure than very early prophase proceed to anaphase at a time not significantly different from the controls.

III. PLANS FOR THE FUTURE

Additional data will be obtained on the time required for cells to reach the next anaphase after dosage with 3.5 to 4.5 mg per liter of ozone. When this information is complete, the individual cells will be studied during the sensitive stages, and their progress from one stage to another followed so that the total effect may be more completely characterized.

Respectfully submitted:

R. H. Fetner, Ph.D.
Project Director

Approved:

Wyatt C. Whitley, Chief
Chemical Sciences Division

ENGINEERING EXPERIMENT STATION
of the Georgia Institute of Technology
Atlanta, Georgia

Informal Progress Report No. 3

Project No. A-430

OZONE-INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

October 1, 1959 through March 31, 1960

Contract No. AF 41(657)-263

School of Aviation Medicine, USAF
Randolph Air Force Base, Texas

I. SUMMARY

In this phase of the program quantitative data were obtained on the progression of neuroblast cells through successive stages of mitosis after ozone exposure in each of the eight stages of mitosis. Cells in anaphase, telophase, interphase and very early prophase were delayed significantly in passing through mid prophase and late prophase stages. Progress through preceding and succeeding stages was the same as control preparations. Thus, exposure to ozone in any of the preprophase stages results in inhibition of mitosis in late prophase, and cells exposed later than the earliest stages of prophase proceed through cell division uninhibited. The sensitive stages to this threshhold concentration of ozone are preprophase stages and the inhibited stage is mid and late prophase. Work is also being done on time lapse cinematography of various cellular phenomena both in control preparations and in those exposed to ozone. Most of the work in the past two months has been concerned with developing techniques and obtaining a backlog of control data which will provide a basis for comparison with the experimental work. The work is being performed on both neuroblast material and human Hela strain in cell culture.

II. STATEMENT OF PROGRESS

In this phase of the program individual cells were studied after exposure, and their progress was followed from one mitotic stage to another. The dosimetry procedure has been described in previous reports, and the same procedure was followed in these experiments. In each experiment from one to two cells were followed per preparation, depending upon the number of cells in the particular stage to be studied that could be found in one microscope field. Therefore, the total of six cells used in each experimental group represents from three to six replicate experiments. The cells were exposed to the ozone for 5 minutes and then the time recorded from the beginning of the next successive stage of mitosis and each succeeding stage until the cells entered anaphase. Table I presents the data from those cells exposed in anaphase. The duration of each succeeding stage, up to mid prophase, was

TABLE I
SIX CELLS TREATED IN ANAPHASE

Successive Stages	Total Duration (Minutes)	Expected Duration* (Minutes)	Difference (Minutes)	P**
Mid telophase	153	144	9	>.05
Late telophase	204	198	6	>.05
Interphase	154	142	12	>.05
Very early prophase	147	144	3	>.05
Early prophase	296	276	20	>.05
Mid prophase	192	96	96	<.01
Late prophase	288	96	192	<.01
Metaphase	80	78	2	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

not significantly different from that expected. Mid prophase and late prophase were significantly inhibited; however, once beyond this stage, the cells did proceed through metaphase to anaphase at the expected rate. In Table II, cells were treated in mid telophase. The cells progressed through

TABLE II
SIX CELLS TREATED IN MID TELOPHASE

Successive Stages	Total Duration (Minutes)	Expected Duration* (Minutes)	Difference (Minutes)	P**
Late telophase	212	198	14	>.05
Interphase	141	142	1	>.05
Very early prophase	149	144	5	>.05
Early prophase	273	276	3	>.05
Mid prophase	155	96	59	<.01
Late prophase	293	96	197	<.01
Metaphase	77	78	1	>.05

*Based on control = 208 minutes at 30° C.
**From Table for Goodness of Fit (Fisher).

successive stages at the expected rate until mid prophase and late prophase where there was a significant inhibition, and then proceeded through metaphase to anaphase as in the controls. Cells treated in late telophase (Table III) proceeded as the controls to mid prophase where there was a significant

TABLE III
SIX CELLS TREATED IN LATE TELOPHASE

Successive Stages	Total Duration (Minutes)	Expected Duration* (Minutes)	Difference (Minutes)	P**
Interphase	139	142	3	>.05
Very early prophase	157	144	13	>.05
Early prophase	301	276	25	>.05
Mid prophase	183	96	87	<.01
Late prophase	307	96	211	<.01
Metaphase	82	78	4	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

inhibition in mid prophase and late prophase, and then proceeded at the expected rate to anaphase. Cells treated in interphase (Table IV) also

TABLE IV
SIX CELLS TREATED IN INTERPHASE

Successive Stages	Total Duration (Minutes)	Expected Duration* (Minutes)	Difference (Minutes)	P**
Very early prophase	137	144	13	>.05
Early prophase	294	276	18	>.05
Mid prophase	127	96	31	<.01
Late prophase	307	96	211	<.01
Metaphase	82	78	4	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

proceeded at the expected rate to mid prophase and late prophase where again there was a significant inhibition. Cells treated in very early prophase (Table V) were inhibited in mid prophase and late prophase and then proceeded

TABLE V
SIX CELLS TREATED IN VERY EARLY PROPHASE

Successive Stages	Total Duration (Minutes)	Expected Duration* (Minutes)	Difference (Minutes)	P**
Early prophase	261	276	15	>.05
Mid prophase	133	96	37	<.01
Late prophase	176	96	80	<.01
Metaphase	75	78	3	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

at the expected rate to anaphase. Cells treated in early prophase (Table VI) proceeded to anaphase without inhibition as did cells treated in late prophase and metaphase (Tables VII and VIII).

TABLE VI
SIX CELLS TREATED IN EARLY PROPHASE

Successive Stages	Total Duration (Minutes)	Expected Duration* (Minutes)	Difference (Minutes)	P**
Mid prophase	107	96	11	>.05
Late prophase	112	96	16	>.05
Metaphase	72	78	6	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

TABLE VII
SIX CELLS TREATED IN MID PROPHASE

Successive Stages	Total Duration (Minutes)	Expected Duration* (Minutes)	Difference (Minutes)	P**
Late prophase	91	96	5	>.05
Metaphase	82	78	4	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

TABLE VIII
SIX CELLS TREATED IN LATE PROPHASE

Successive Stages	Total Duration (Minutes)	Expected Duration* (Minutes)	Difference (Minutes)	P**
Metaphase	73	78	5	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

Cells exposed to ozone, at the threshhold concentration, in the stages of anaphase, telophase, interphase and very early prophase, will be significantly inhibited in passing through mid prophase and late prophase, and then will reach anaphase at the expected rate. The cells are sensitive during these times and this sensitivity is expressed by an inhibition during the late stages of prophase or conversely, the sensitivity is expressed by a delay in entering metaphase.

Work is being done on time lapse cinematography, primarily in control preparations. Sixteen-millimeter Kodak Plus X negative film has been used; development of it with Kodak D-19 has provided the best contrast. A backlog is being obtained of control data and film footage on trypsinized Chortophaga cells and the human Hela strain in cell culture to be used as a basis for comparison with ozone-exposed material.

III. PLANS FOR THE FUTURE

When a sufficient backlog of control data has been obtained, these same materials will be exposed to ozone and a frame-by-frame analysis will be made to determine the effect that ozone exposure has on various cellular phenomena.

Respectfully submitted,

Robert H. Fetner, Ph.D.
Project Director

Approved:

Wyatt C. Whitley, Chief
Chemical Sciences Division

Georgia Institute of Technology
STATE ENGINEERING EXPERIMENT STATION
Atlanta, Georgia

FORMAL PROGRESS REPORT

PROJECT NO. A-430

RESEARCH AND REPORTS ON OZONE-INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

- o - o - o - o -

CONTRACT NO. AF 41(657)-263

SCHOOL OF AVIATION MEDICINE
UNITED STATES AIR FORCE
RANDOLPH AIR FORCE BASE, TEXAS
R&D PROJECT NO. 7758-130

- o - o - o - o -

JUNE 30, 1959

Georgia Institute of Technology
STATE ENGINEERING EXPERIMENT STATION
Atlanta, Georgia

FORMAL PROGRESS REPORT

PROJECT NO. A-430

RESEARCH AND REPORTS ON OZONE-INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

- o - o - o - o -

CONTRACT NO. AF 41(657)-263

SCHOOL OF AVIATION MEDICINE
UNITED STATES AIR FORCE
RANDOLPH AIR FORCE BASE, TEXAS
R&D PROJECT NO. 7758-130

- o - o - o - o -

JUNE 30, 1959

FORMAL PROGRESS REPORT, PROJECT A-430

TABLE OF CONTENTS

This Report Contains 7 Pages

I. ABSTRACT

The ozonator has been put into operation and the analytical technique selected which will best fit into the experimental procedure. Various dosimetry methods for the tissues have been investigated and an effective system worked out which will permit various concentrations of ozone to be brought into contact with the specimen without altering the sensitive media environment necessary for the normal functioning of the tissues. A controlled temperature work area has been designed and constructed which offers a unique advantage for this type investigation. A controlled temperature microscope incubator has also been constructed. Through the use of these two pieces of equipment an important advance has been made in this particular experimental procedure by the elimination of temperature shock-effect. This will prevent the mitotic cycling induced by sudden temperature variations and will result in a better analysis of data. The time lapse moving picture apparatus has been set up and is functional. Preliminary sets of photographs of the living material are being used to investigate the feasibility of using this technique for all the experimental runs as well as the more detailed study of the individual disassociated cells. We have completed moving into our new Radioisotopes and Bioengineering Building.

II. PROGRESS

A. Literature

A recent report by Brinkman and Lamberts (1) described the effects of ozone on the blood enzyme systems after ozone inhalation in mammals. Correspondence with these investigators has emphasized the difficulty of explaining the persistence of ozone or an ozone decomposition product for a long enough period to produce this effect. The results, however, are reproducible and suggest that there must be some physiologically active intermediate product involved. A recent paper by E. J. Fairchild of the Public Health Service was brought to our attention. This paper, intitled "Protection Against Lethal Effects of Ozone and Nitrogen Dioxide by

- - - - -

(1) Brinkman, R. and Lamberts, H. B., "Ozone As A Possible Radiomimetic Gas." Nature 181, 1202-1203 (1958).

Sulphur Compounds:, was presented at the fifty-second annual meeting of the Air Pollution Control Association held at Los Angeles, California from June 22nd to June 25th. Although the text of Dr. Fairchild's work is not available as yet, a reference was made specifically to our ozone research programs and data here at Georgia Tech.

B. Experimental Work

A Welsbach Model T23 Laboratory ozonator has been obtained and set up in the laboratory. Ozone is generated at room temperature from cylinder oxygen and is scrubbed successively through a 0.8 normal sulfuric acid solution and then through a 2 per cent potassium-iodide trap. The ozone to be used for dosimetry will be that present as dissolved ozone in the 0.8 normal sulfuric acid solution. It has been shown in previous investigations that ozone is reasonably stable under such conditions--at least the rate of decomposition will be minimized and will be less than 10 per cent over the period of the experiment. The concentration of ozone present in such a solution is a function of the distribution coefficient between the ozone and the gas immediately above it. This in turn is determined by the temperature. At or close to room temperature (23°C) the distribution coefficient for ozone is about 0.24. This means that at saturation the ozone concentration will be about one quarter of that present in the gas which is being bubbled through it. The Welsbach ozonator is capable of producing ozone in concentrations from about 1 to 10 per cent by weight from pure oxygen--depending on the voltage operation, rate of gas flow, and certain other variables. We have found that scrubbing ozone through 400 ml of 0.8 normal sulfuric acid for 20 to 30 minutes produces a near-saturated solution and, as a matter of convenience, we allow this period of time for scrubbing. The final concentration of ozone is then determined by varying the voltage on the ozonator or by diluting the resultant solution. After the ozone solution has been generated it is poured into a glass-stoppered bottle. This particular bottle has a very narrow mouth and the top has been ground smooth so that a tight fit will be obtained when a cover slip is inverted over the open neck. The bottle has a ground glass stopper and special care is necessary to insure that no contaminating or reducing substances are present as these would effectively reduce the ozone concentration. Several methods for ozone analysis have been evaluated in

previous investigations. The ferrous-ferric system was selected as the best method for these experimental procedures. This analytical procedure involves the oxidation of the ferrous ion to the ferric ion by ozone:

$$Fe^{++} + O_3 + H_2 \longrightarrow Fe^{+++} + O_2 + H_2O$$

Enough ferrous ammonium sulfate in 0.8 normal sulphuric acid is added to the test solution to make a final concentration of approximately 0.5 normal ferrous ammonium sulfate solution. This is back-titrated with potassium permanganate standardized against sodium oxalate. Insofar as this analysis is ordinarily conducted in dilute sulfuric acid solutions, it lends itself particularly well to our experimental procedure and eliminates the necessity of changing the pH during the analytical procedure as would be necessary, for example, with potassium iodide. Another advantage of this analytical method is the sharp end point evidenced in the permanganate titration.

In the dosimetry of the hanging-drop preparations the embryos will be exposed to ozone by inverting the preparations on the cover slip and placing them on the top of the sample bottle, taking pains to insure a complete seal between the cover slip and the ground lip of the bottle. Under these conditions, the concentration of ozone in the gas above the liquid and in contact with the drop containing the embryo preparation may be estimated, based upon the distribution coefficient of ozone at this temperature. After a specified contact time, the solution will be replaced in the preparation, yolk material added, and observation begun immediately. This particular method of dosimetry seems to be dictated by several considerations: (1) the requirements of the embryo for a balanced physiological solution, (2) the advantage of a short contact time, and (3) the necessity of eliminating reducing materials from the embryo preparations during ozone contact. It has been found in other investigations that ozone is not effective biologically until all reducing materials in solution have been eliminated. The presence of even small amounts of or other reducing agents provides marked protection from the ozone effect.

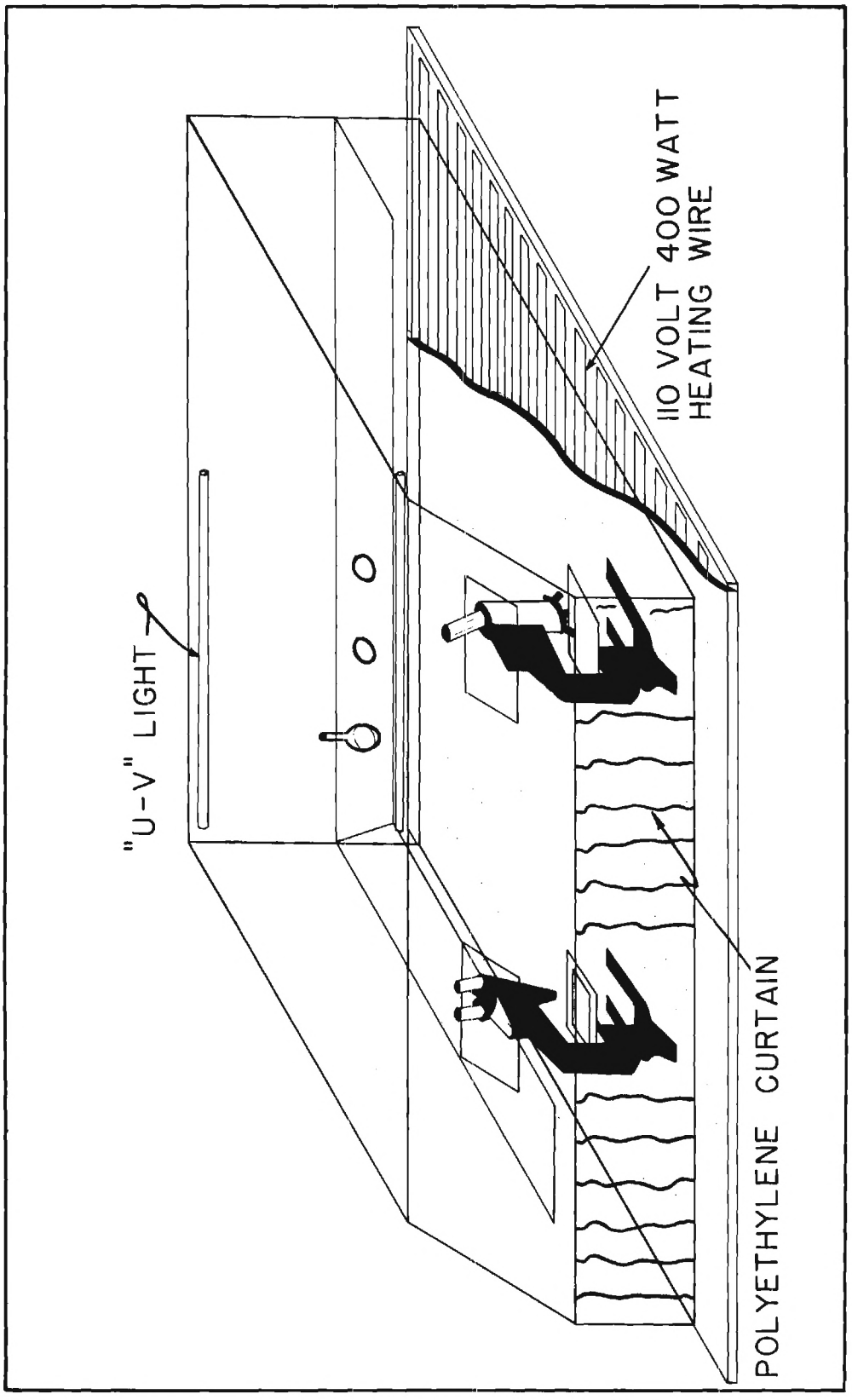
In an attempt to eliminate the temperature shock-effect present in the experimental results of other investigators using this material, it was decided to conduct all operations as close to the incubation temperature (38°C) as would be feasible. To this end, a constant-temperature work space was constructed in

which all of the preparatory steps and dosimetry could be accomplished (Figure 1). Temperature control was maintained in this inoculation box through thermostat control of the radiant-heating bottom surface. Heat is supplied through a 400-watt radiant-heating wire located beneath a 1/4 inch plate glass surface. This plate glass bottom acts as a continuous heat-radiating surface, and the high heat loss and continuous heat input of this system result in a small amplitude of temperature change while permitting the front of the box to remain open and considerable manipulation to take place through the open front. In operation this box maintains a temperature between 35° and 37°C, care being taken to keep the temperature from going above 38°C. By preparing samples under such conditions and then transferring to the microscope incubator, the temperature shock-effect was eliminated.

A new type of microscope incubator was designed and put into operation which offers several unique advantages. This incubator is an inflated polyvinyl bag through which hot air is forced in a closed circulating system. Forced-draft incubating systems offer good temperature control if there is a large enough temperature differential between the desired incubator setting and room temperature. The flexible polyvinyl bag offers an additional advantage over rigid systems--namely, the microscope controls may be manipulated from the exterior of the bag without breaking into the closed forced-draft system. This is accomplished by merely pressing in on the bag and manipulating the controls directly through the polyvinyl material. This overcomes one of the more serious drawbacks to a forced-draft system--that of breaking into and disturbing the continuous air flow.

In following the progress of neuroblast cells through mitosis it has been found convenient to use a tape recorder during the actual observations and then to play back the tape at the termination of a run to obtain the data. This results both in better efficiency and undoubtedly reduces some of the experimental bias that would result from a chronological recording of individual cell progress.

The feasibility of using time lapse photography in the routine experimental procedure is being investigated. The reduced visibility necessitated by the thick tissue involved, which precludes the use of phase contrast optics, requires


110 VOLT 400 WATT
HEATING WIRE
"U-V" LIGHT
POLYETHYLENE CURTAIN

a very precise control of film resolution if this technique is to be possible. Some progress has been made in this direction, but it is not definite as yet whether this will be possible. Should this technique prove feasible, it will permit a greater accuracy and flexibility in the recording the duration of the various mitotic stages as well as the progression from stage to stage. The first ozone exposure experiments have emphasized the susceptibility of the mitotic process to this agent. A pronounced effect is produced by very low concentrations and short contact intervals. Cells in metaphase appear to be most sensitive, producing a considerable chromosome stickiness as the cells progress into anaphase. The preliminary experimental runs have enabled us to determine the range of ozone concentration and contact times which can be used in our experimental procedures.

We have completed moving into our new quarters in the Radioisotopes and Bioengineering Building. The better facilities and increased space should greatly facilitate the conduct of this contract. See attached diagram.

III. FUTURE PLANS

The work is proceeding as per proposals and no variations are seen at this time.

IV. PERSONNEL

There has been no change in personnel.

V. FINANCIAL REPORT

For the period 31st December through 30th June, the following expenditures were made.

Personnel Services	$ 4,975.22
Materials and Supplies	620.15
Equipment	2,796.38
Overhead	2,835.88
TOTAL	$11,227.63

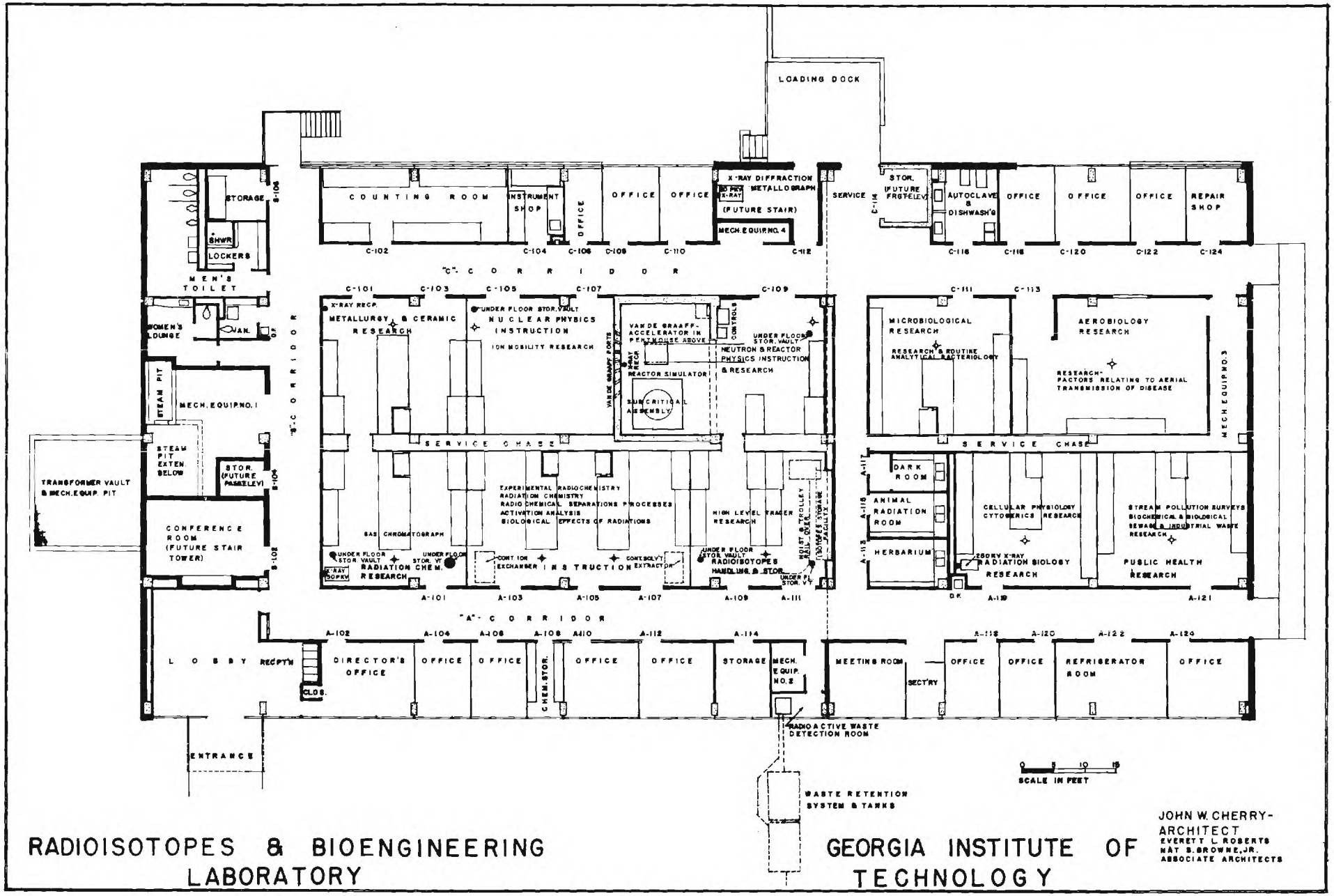
A voucher in this amount has been submitted.

Respectfully submitted,

Robert H. Fetner
Project Director

Approved:

Wyatt C. Whitley, Chief
Chemical Sciences Division



RADIOISOTOPES & BIOENGINEERING LABORATORY

GEORGIA INSTITUTE OF TECHNOLOGY

JOHN W. CHERRY-ARCHITECT
EVERETT L. ROBERTS
NAT S. BROWNE, JR.
ASSOCIATE ARCHITECTS

FORMAL PROGRESS REPORT

PROJECT NO. A-430

RESEARCH AND REPORTS ON OZONE-INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

- o - o - o - o -

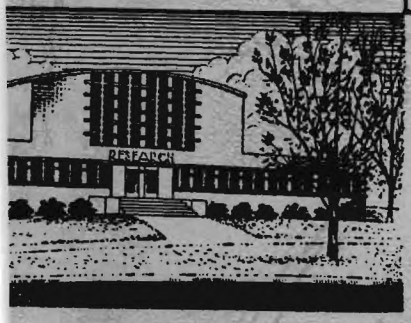
CONTRACT NO. AF 41(657)-263

SCHOOL OF AVIATION MEDICINE

UNITED STATES AIR FORCE
RANDOLPH AIR FORCE BASE, TEXAS
R&D PROJECT NO. 7758-130

- o - o - o - o -

JUNE 30, 1960



Engineering Experiment Station
Georgia Institute of Technology
Atlanta, Georgia

ENGINEERING EXPERIMENT STATION
of the Georgia Institute of Technology
Atlanta, Georgia

FORMAL PROGRESS REPORT

PROJECT NO. A-430

RESEARCH AND REPORTS ON OZONE-INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

- o - o - o - o -

CONTRACT NO. AF 41(657)-263

SCHOOL OF AVIATION MEDICINE

UNITED STATES AIR FORCE
RANDOLPH AIR FORCE BASE, TEXAS
R&D PROJECT NO. 7758-130

- o - o - o - o -

JUNE 30, 1960

TABLE OF CONTENTS

I. ABSTRACT

The first objective of this program, to determine the effects of ozone on mitosis has been completed. Data were obtained on the progression of neuroblast cells through successive stages of mitosis after ozone exposure in each of the eight stages of mitosis. It was found that cells exposed in anaphase, telophase, interphase and very early prophase were delayed significantly in passing through mid prophase and late prophase stages. Progression through preceding and succeeding stages was the same as control preparations. Thus exposure to ozone in any of the pre-prophase stages results in inhibition of mitosis in late prophase and cells exposed later than the earlier stages of prophase proceed through the next cell division uninhibited. The sensitive stages to the threshold concentration of ozone are pre-prophase and the inhibited stage is mid and late prophase. In the second phase of the program considerable footage of film has been obtained of time lapse cinematography of both neuroblast cells and human Hela cells in tissue culture. Films have been obtained at various speeds---1, 5, 15 and 60 frames per minute on the study of the metabolic cells and a time sequence of 15 frames per minute in the analysis of mitosis. A method has been developed for cover slip culture of Hela cells which permits ozone dosimetry without interference with the normal growth processes.

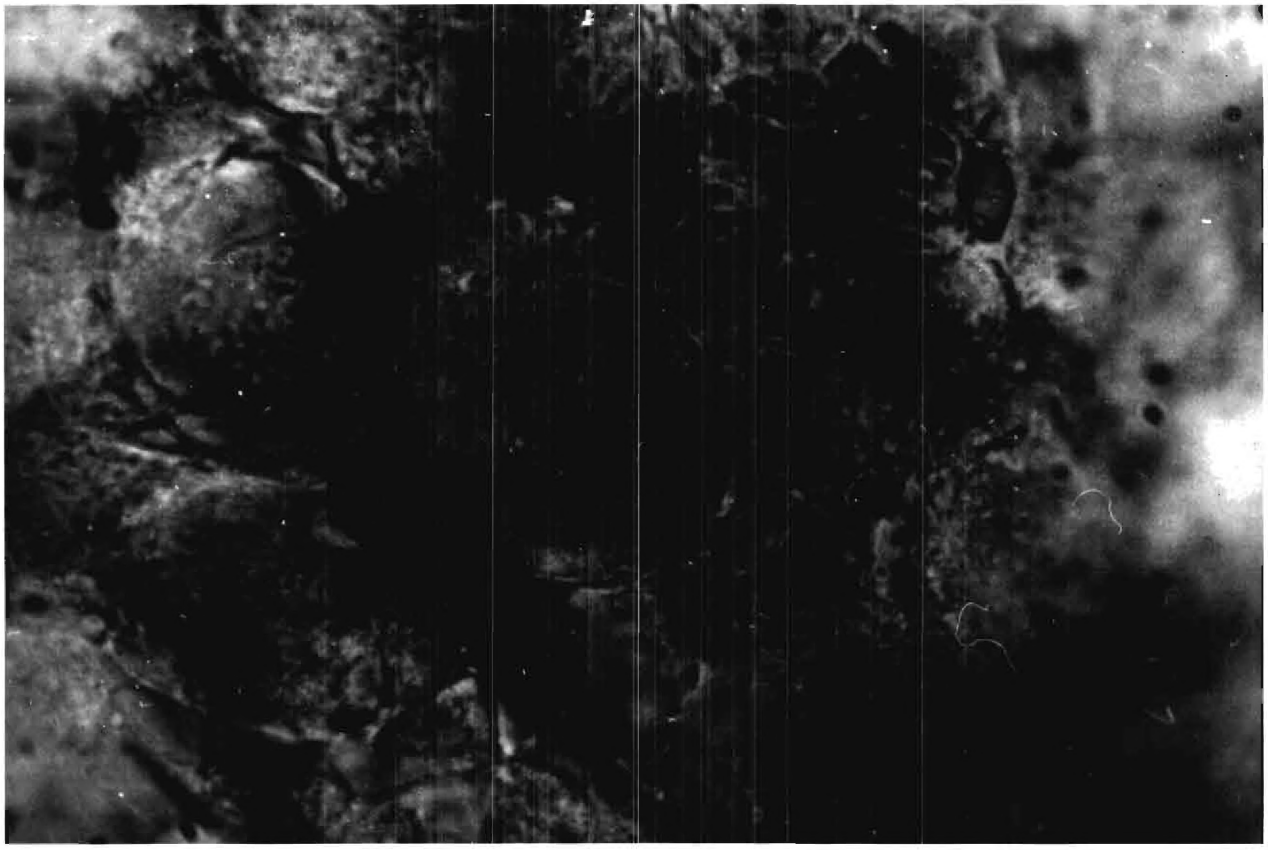
II. STATEMENT OF PROGRESS

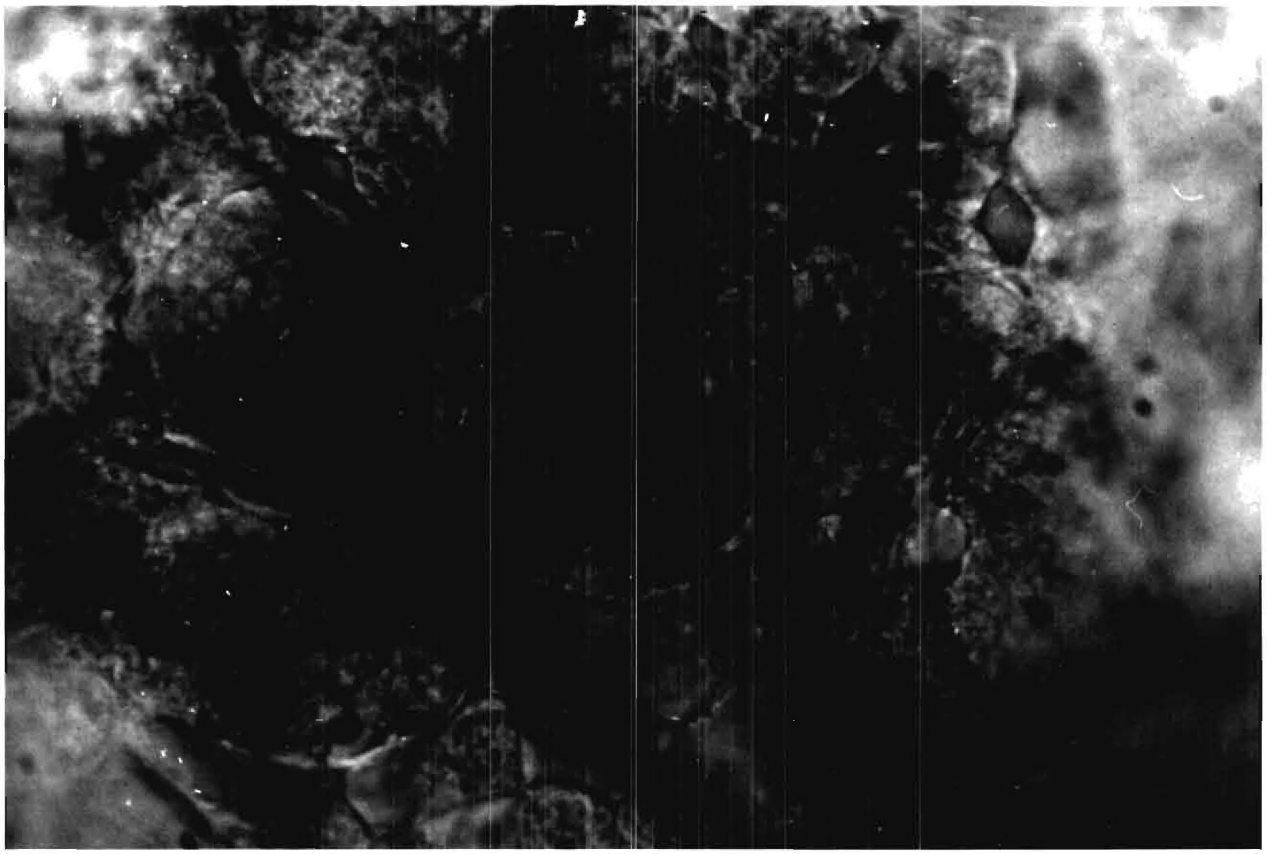
A. Experimental Work

A complete sequence of mitosis in the neuroblast cells of Chortophaga embryos is presented on the following pages. To the best of our knowledge

PLATE I. At Beginning of sequence, cell A in mid prophase cell B in late prophase.

PLATE II. Twelve minutes later, cell A is in late prophase nuclear outline becoming irregular due to the protrusion of the chromosomes. Cell B still in late prophase. Cell C in early prophase.





this is the only such sequence which has been made available. Because the thickness of the specimen precludes effective use of phase contrast optics, these pictures were made using bright field microscopy. The sequence is of the same field in a Chortophaga preparation and covers a period of 208 minutes at 38° C. In the description of the stages which follows cell A (indicated in the legends which accompany each photograph) is the cell which was maintained in focus. Other cells, however, are described as they appear in focus to demonstrate various other stages of division. A description of each plate follows:

Plate I

At the initiation of this sequence cell A is in mid prophase. This stage is described as beginning "when the chromosome threads can be followed from one part of the nucleus to another."[1] In all stages of prophase the nucleus has a homogeneous core, approximating a somewhat doughnut shape although it is not visible in this photograph because the focus of the microscope is above the core area. This stage of mitosis lasts 16 minutes and ends when the chromosomes are considerably more condensed. Cell B is in late prophase, the next stage of division.

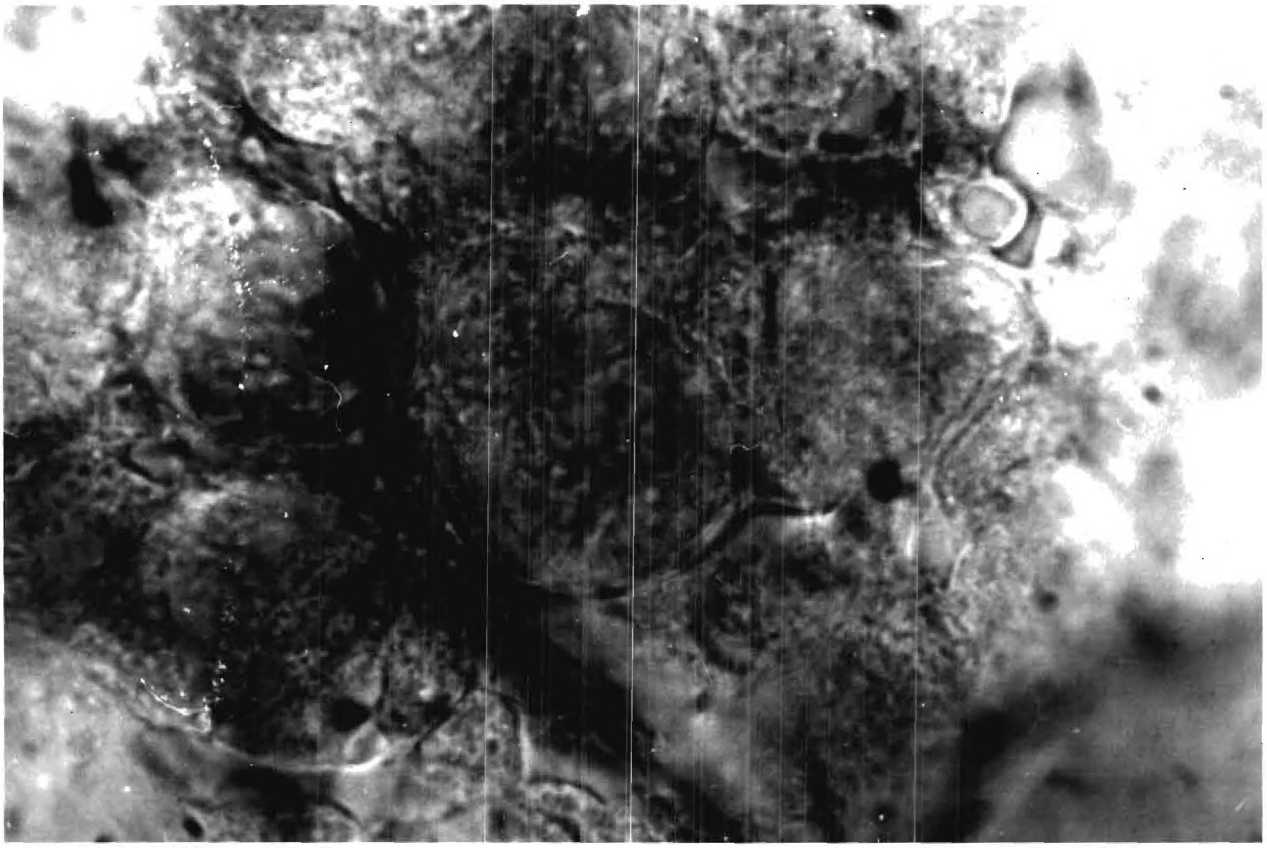
Plate II

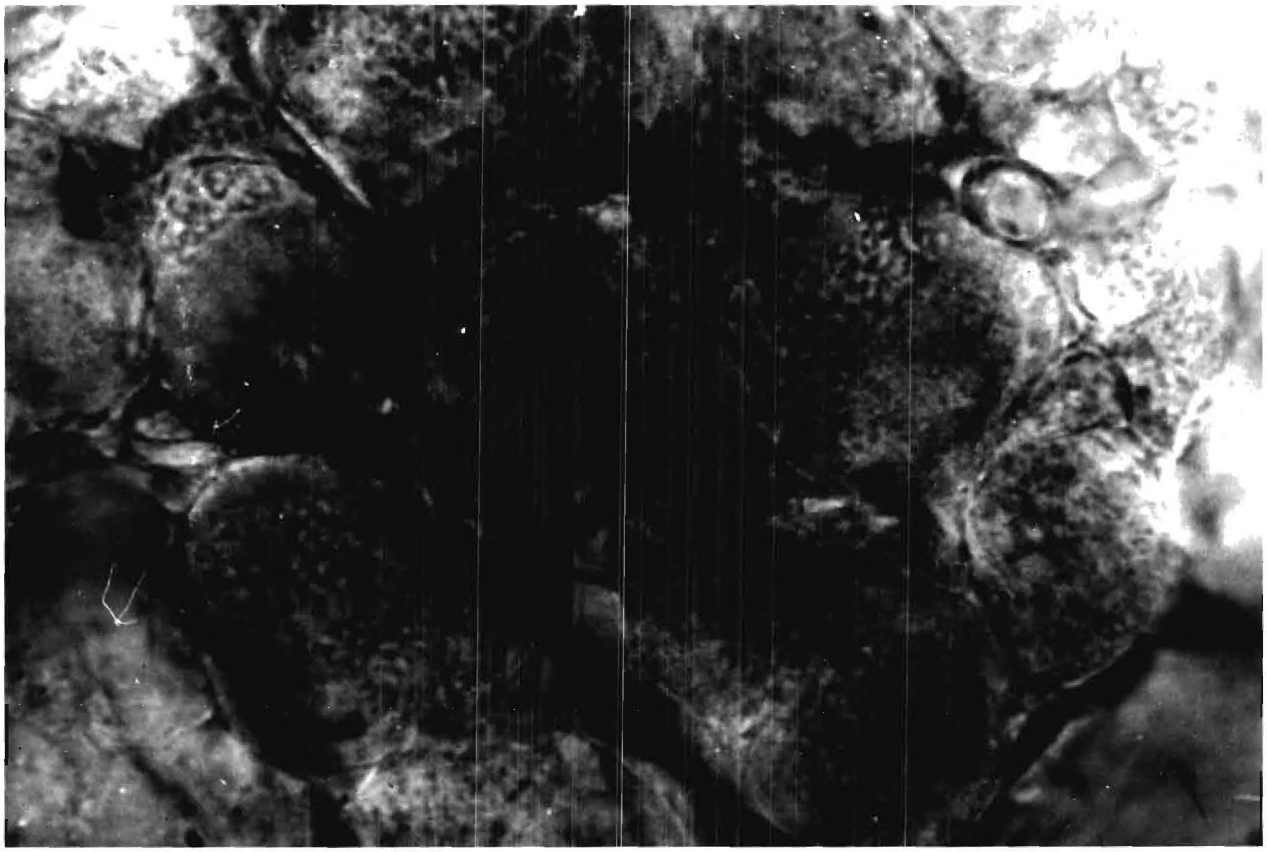
Twelve minutes later cell A is now in late prophase. This stage is described as beginning "when about seven chromosomes are seen near the nuclear membrane in one-fourth of the optical section of the nucleus."[1] Also in this stage the nuclear membrane or the outline of the nucleus

(1) St. Amand, 1956, Radiation Research 5.

PLATE III. Thirteen minutes later cell A still in late prophase, Cell B in prometaphase; the nuclear membrane having disappeared and the cell assuming a spherical shape.

PLATE IV. Five minutes later, cell A has passed through prometaphase and is in the early stages of metaphase. The chromosomes are becoming orientated on the equatoral plate and are almost at their maximum constriction (minimum length). The cell is sherical. Cell B is slightly more advanced in metaphase. Cell C in mid prophase; this nucleus is doughnut shaped with the focus intersecting the nuclear core. Cell D is in late prophase and demonstrates the concave - convex cell shape.





becomes irregular as it conforms to the shape of the increased size of the chromosomes. Cell B shown in this plate is slightly more advanced than cell A and the entire cell has begun to round up and has lost its hemispherical shape. This indicates the end of very late prophase. The duration of late prophase (including very late prophase) is 16 minutes. Cell C is in early prophase.

Plate III

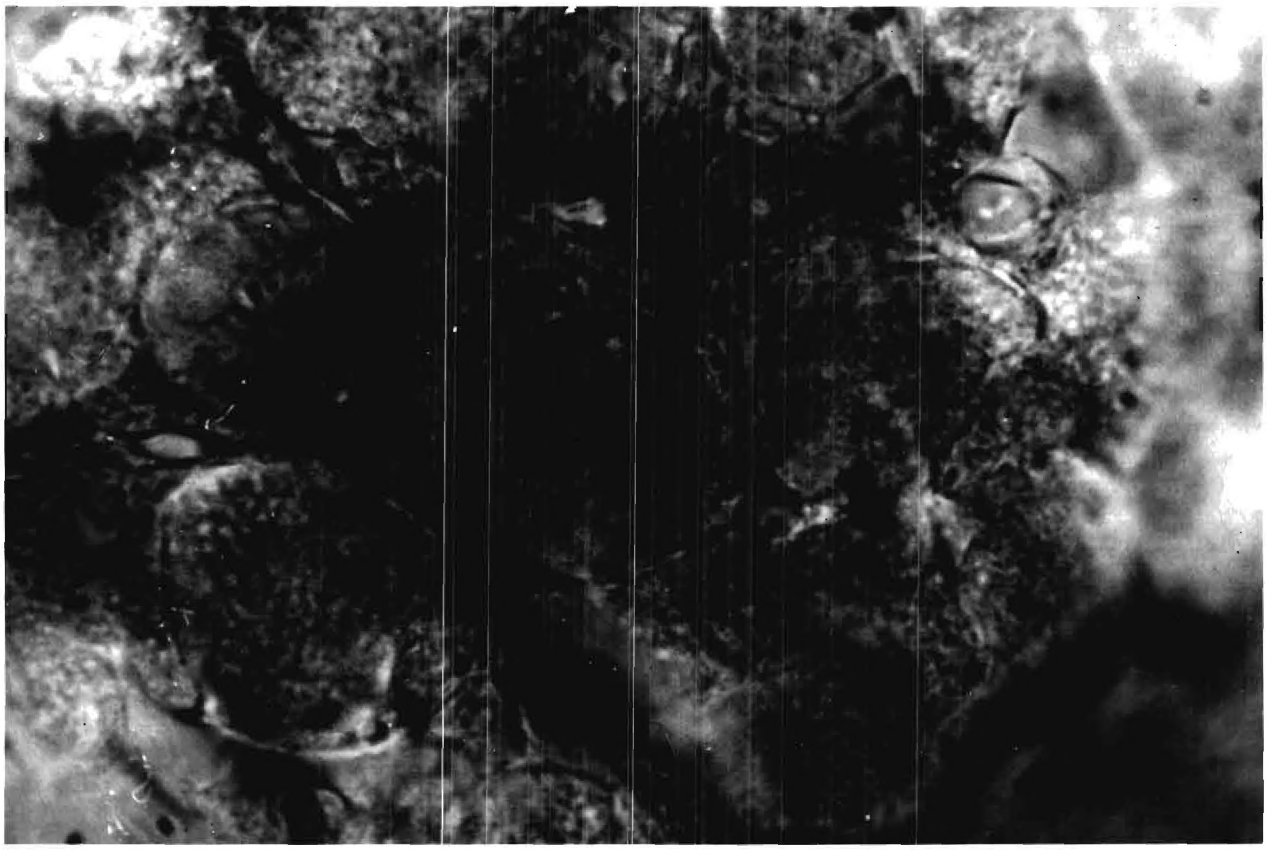
Cell A is in the terminal phase of late prophase and the nuclear membrane appears to be in the process of breaking down or disappearing in the lower right hand quadrant of the nucleus. The late prophase stage ends with the disappearance of the nuclear membrane. Cell B, while not in good focus, is in the prometaphase stage---the nuclear membrane having disappeared entirely and the chromosomes not yet having become orientated on the equatorial plate.

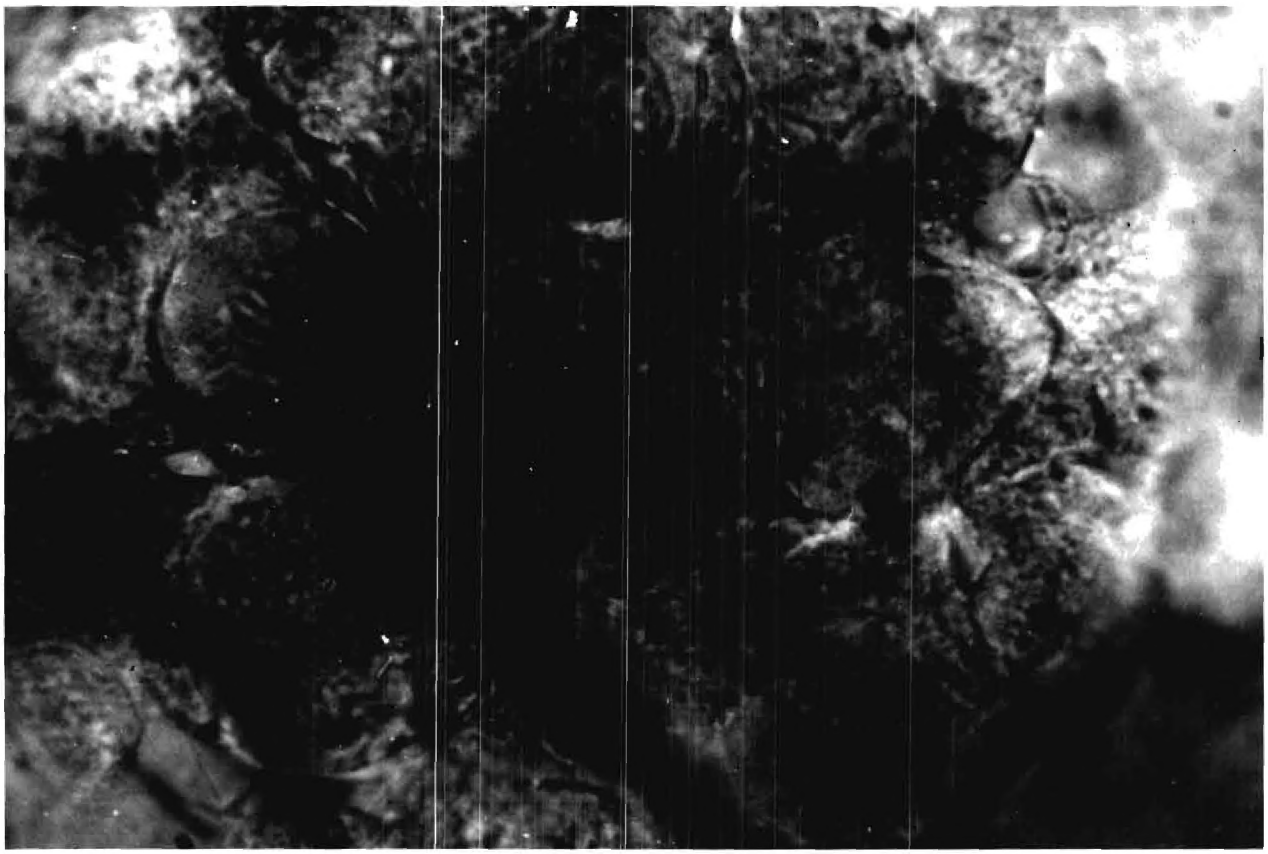
Plate IV

Cell A is now in the metaphase stage having passed through prometaphase or the period from the disappearance of the nuclear membrane to the orientation of the chromosomes on the equatorial plate. The prometaphase condition lasts only 4 minutes and has taken place between the photograph of Plate III and Plate IV. In the metaphase condition the spindle mechanism has reached maximum development and the centric ends of the chromosomes lie on the equatorial plate. A clear zone is visible in cell A on both sides of the equatorial plate. This zone remains free of the mitochondria which are visible in the cytoplasm at this stage because of the gelation of the protoplasmic mass. They are obscured in other preparations because of the blurring effect produced by Brownian movement;

PLATE V. Five minutes later Cell A in metaphase; cell B at end of metaphase; end view of chromosomes demonstrate double nature. Cell C in mid prophase. Cell E in late telophase.

PLATE VI. Five minutes later both cells A and B in early anaphase. Cell C in late prophase. Cell E shows late telophase nuclear granules, focus at periphery of nuclear membrane.





however, during metaphase the mitochondira are immobilized in the gelated cytoplasm. Cell A represents a side view of the metaphase figure and cell B a slightly tilted view. A polar view of the metaphase plate presents a typical "wagon wheel" type of metaphase with the central zone free of chromosomes. Cell C clearly shows the central core through a prophase nucleus which is evident because the plane of focus intersects the core area. Also, in this plate cell B shows the concave-convex shape of the prophase cell. This is typical of these cells and enables one to anticipate the direction of the next mitosis because the daughter cells are budded alternately from each lobe of the concave surface.

Plate V

Cell A is still in metaphase, the cleared ozone or centrosome is clearly evident in the lower right hand corner of the cell. The mitochondria are still visible as discrete bodies. Cell B, in which the focus is at the distal ends of the chromosomes, demonstrates the double nature of the chromosome bodies. This indicates the initiation of anaphase. The metaphase stage has a duration of 9 minutes. Cell C has progressed to mid prophase.

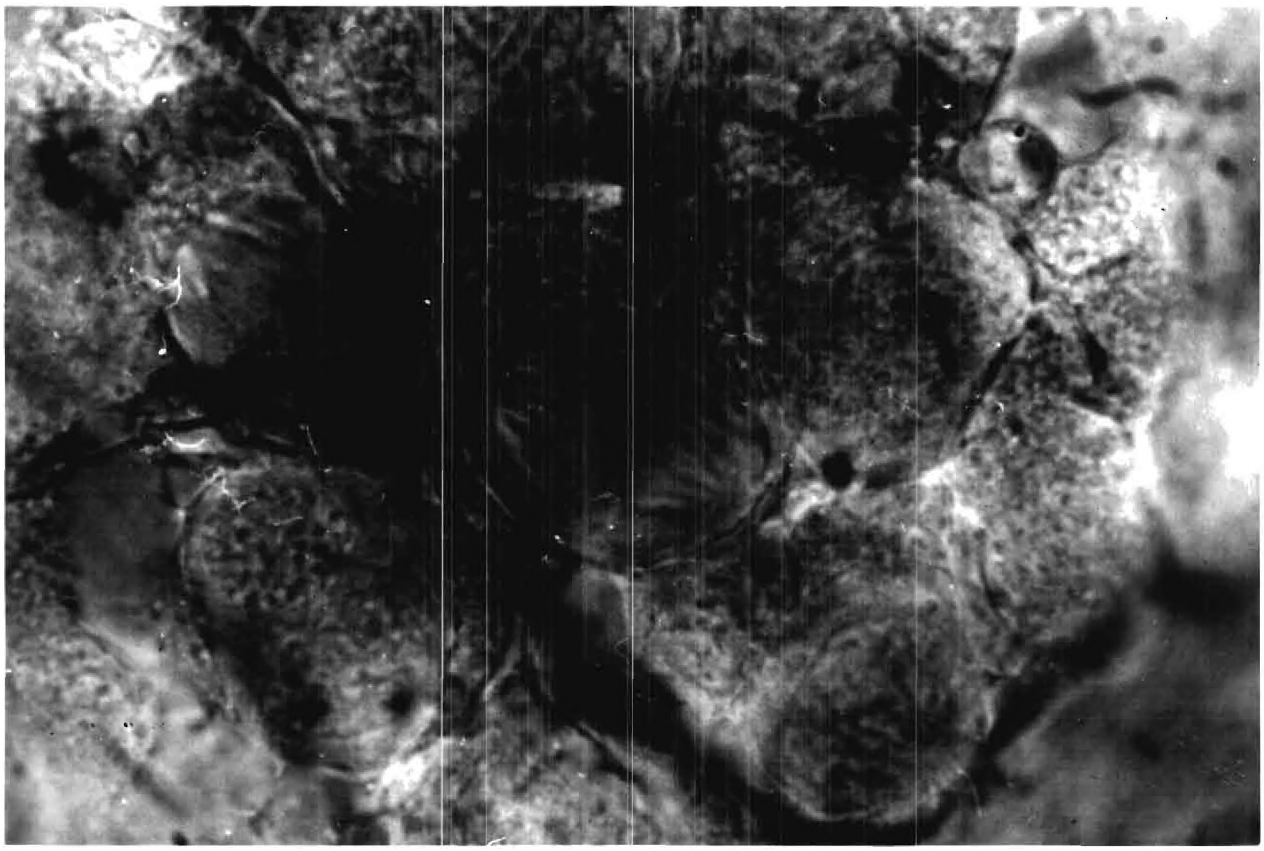
Plate VI

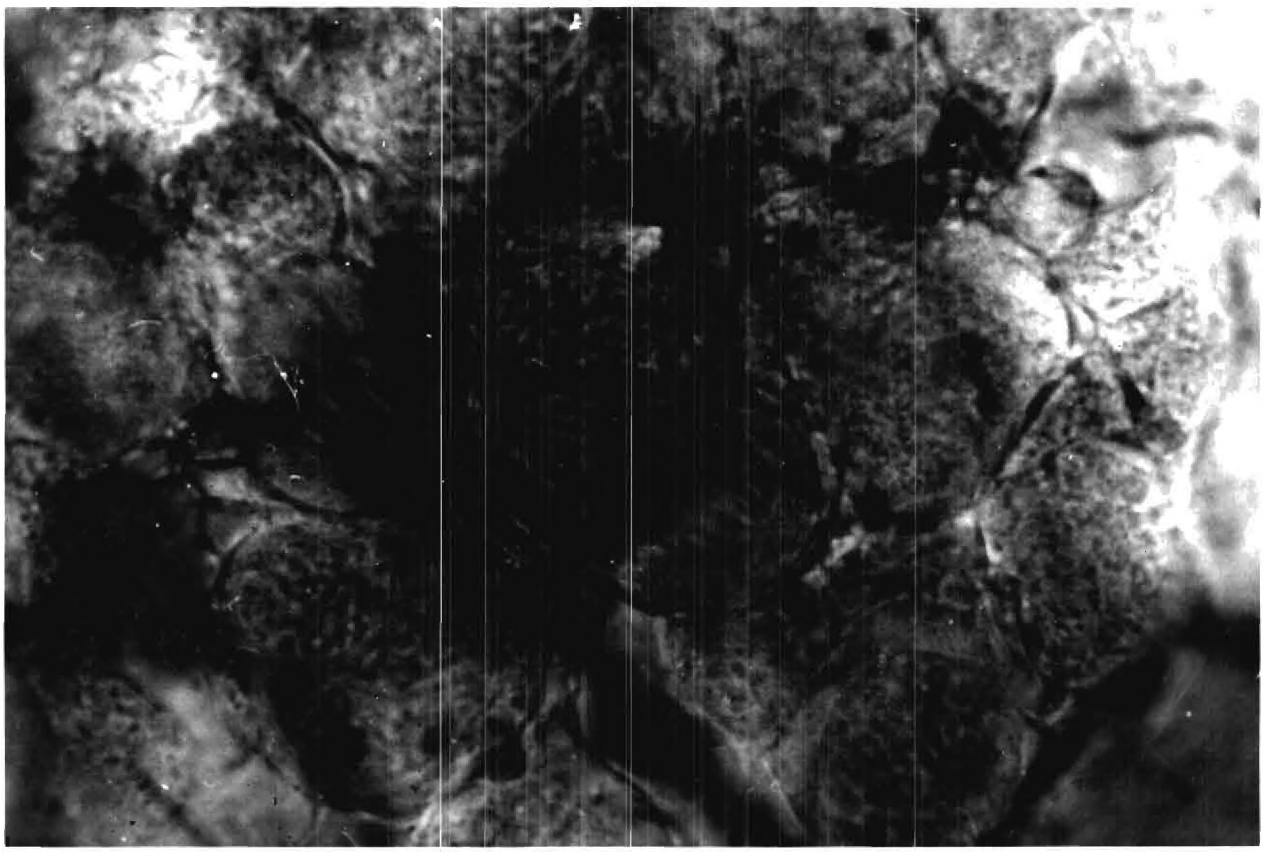
Cell A has begun anaphase, initiated by the separation of the proximal ends of the chromatids. The double nature of the chromosomes may be seen by viewing their distal ends.

Cell B is in a more advanced stage of anaphase, the proximal ends

PLATE VII. Two minutes later, both Cells A and B in anaphase; in both cells daughter chromosomes are pressed against cell membrane at point where daughter will bud, while maternal chromosomes are orientated more centrally in what will become mother cell. Cell C in late prophase.

PLATE VIII. One minute later cells A and B in late anaphase. In Cell B the daughter chromosomes are beginning to extrude into a cell. Cell C in late prophase. Cell D in late prophase.





having achieved a considerable separation.

Cell E is of particular interest because it demonstrates very clearly the nuclear granules visible during the late telophase stage. The smaller size of the nucleus is also evident.

Plate VII

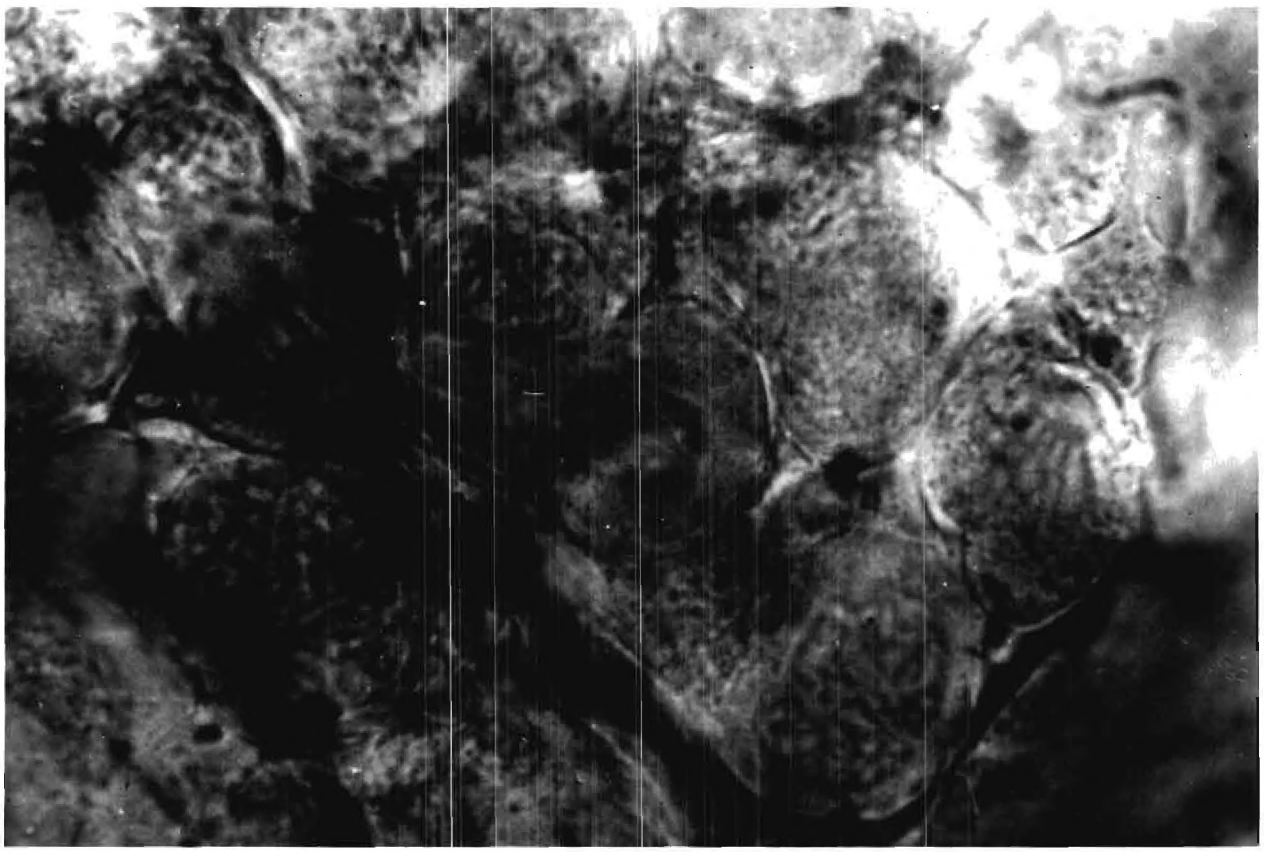
Two minutes later the anaphase figures have considerably advanced, the chromosomes being well separated in both cell A and in cell B, which is more advanced than cell A; the left hand set of chromosomes have migrated to the cell membrane while the right hand group of chromosomes has become more centrally orientated in the cell. In both of these cells the set of chromosomes to the right will remain in the mother cell, and the left set will push against the cell membrane and bud out into the daughter ganglion cell. In cell A, most of the chromosomes have completely separated; however, there is one, a long pair, in the upper right of the cell in which two sister chromosomes are apparently still in conjunction.

Plate VIII

This photograph taken one minute later demonstrates the rapidity with which the anaphase stage is accomplished. The separation of the chromosomes during anaphase is very rapid, being completed when the cleavage furrow appears to be complete. This entire stage of mitosis requires 9 minutes. Cell A demonstrates the budding out of that portion of the cell which will become the daughter ganglion cell, and cell B demonstrates the uneven division of the cell. In cell B, as in cell A, the central orientation of the chromosomes in the mother cell is plainly shown in contrast to the orientation of the daughter chromosomes in the budding

PLATE IX. Two minutes later Cell A in late anaphase, Cell B in early telophase. Cells C and D in late prophase.

PLATE X. Two minutes later both Cells A and B in early telophase. Cell E in late telophase.



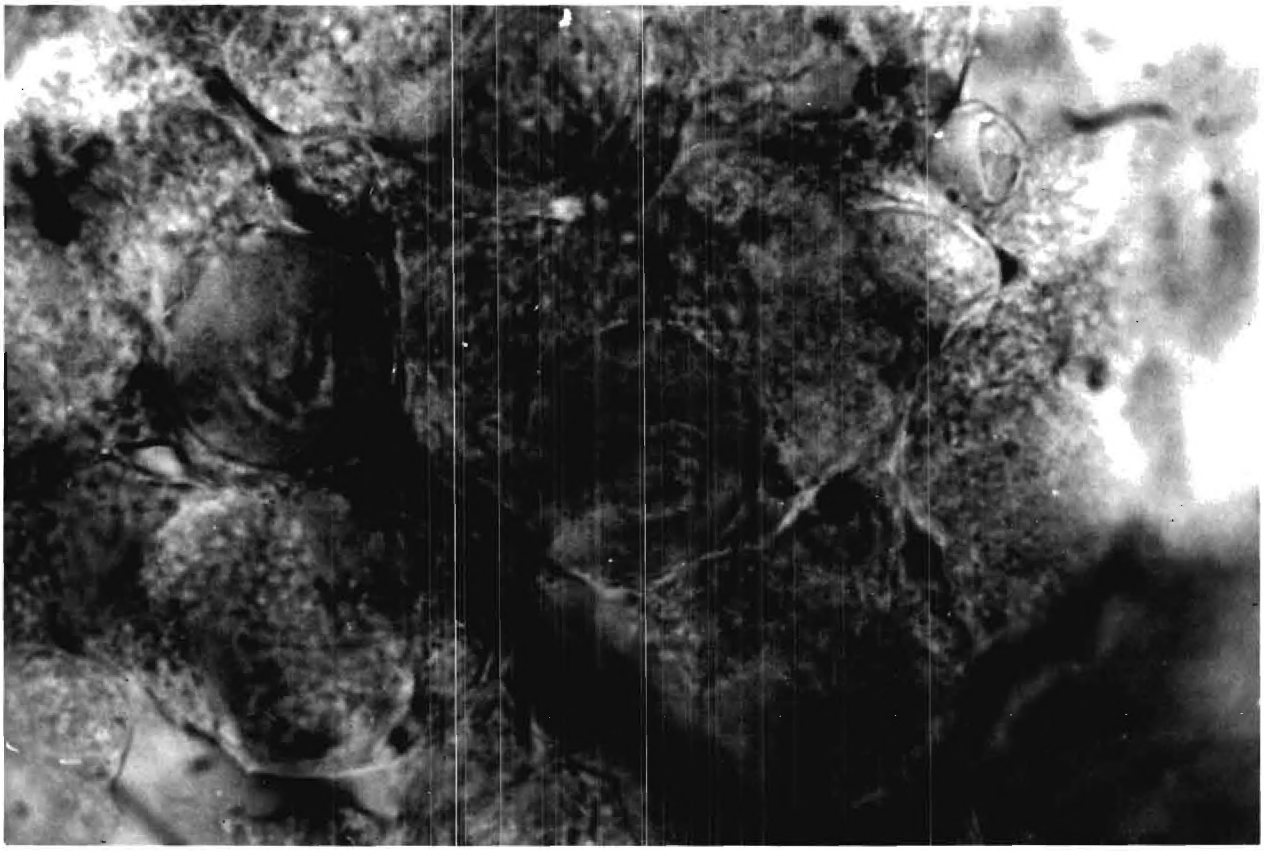
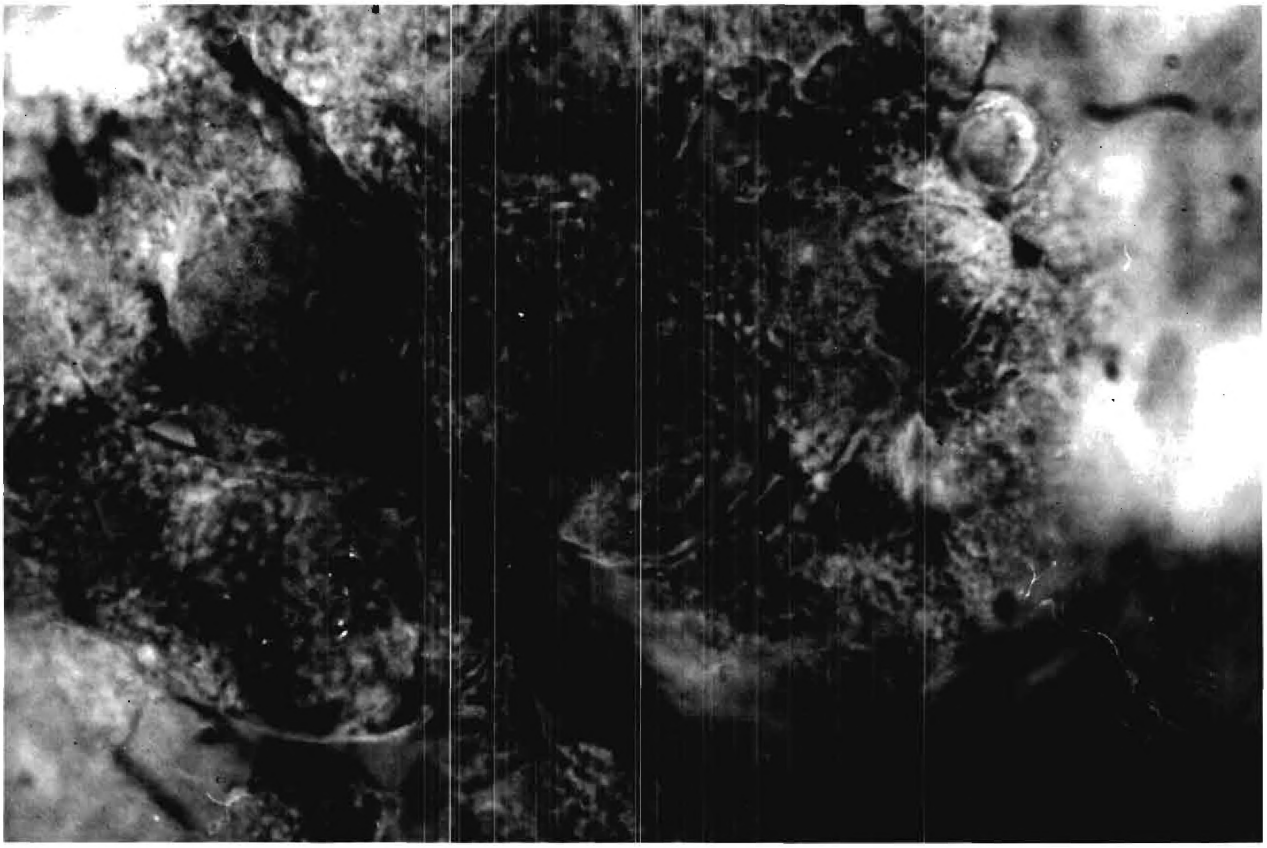
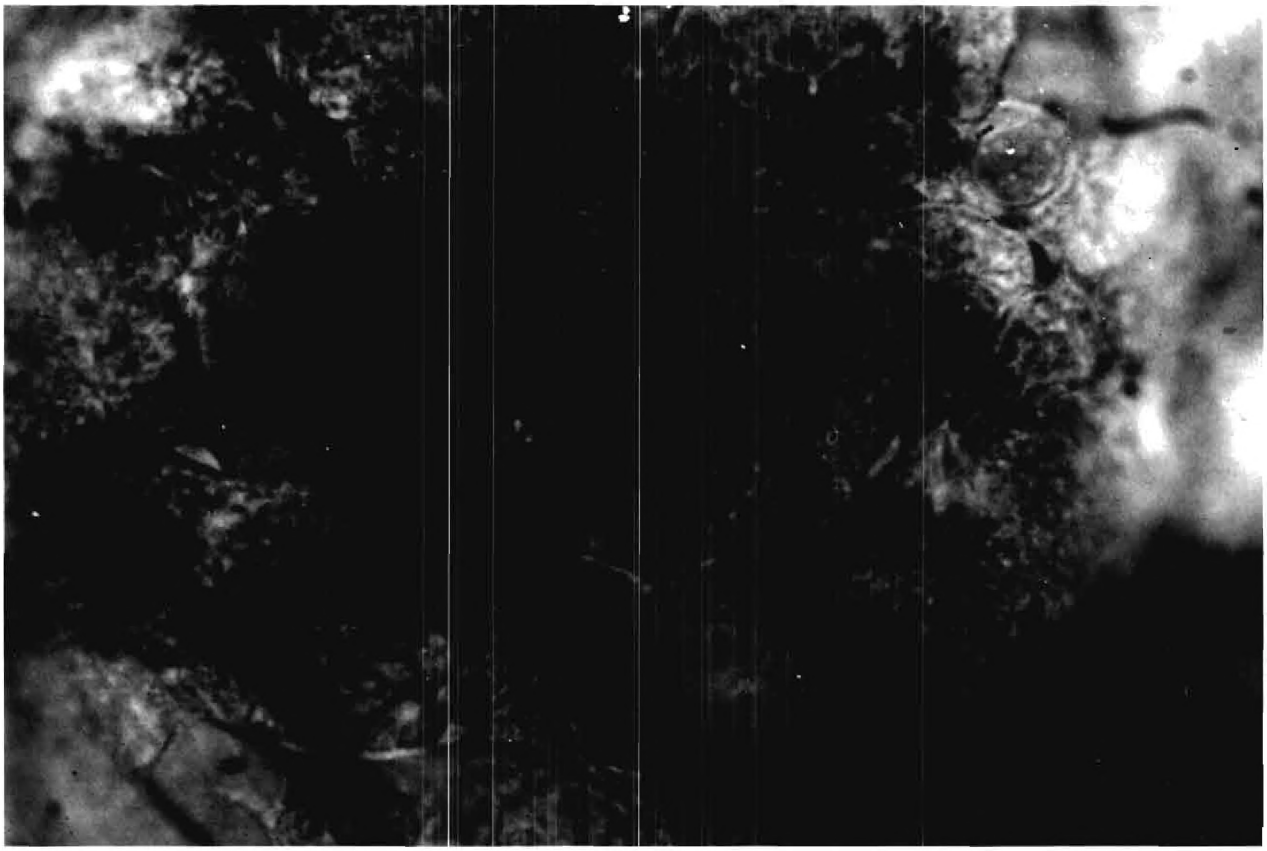


PLATE XI. After 3 minutes Cells A and B still in early telophase. Cell E in late telophase.

PLATE XII. Three minutes later Cells A and B in mid telophase, nucleoli have become visible as small refractive spheres. Cell E in late telophase.





ganglion cell.

Plate IX

Cell A is in late anaphase, the daughter cell having reached its maximum extrusion and the cleavage furrow is beginning to form between the two cells. After the cleavage furrow is complete, the daughter cell comes back and rests tightly against the mother cell. Cell B, which is slightly advanced of cell A, has completed anaphase, the cleavage furrow having completely separated the two cells and the daughter ganglion cell having returned to a position adjacent to the mother cell.

Plate X

Both cell A and cell B are in early telophase. This stage commences when the cleavage furrow appears complete and terminates when the nucleoli become visible. It is a very short stage, of 6 minutes duration.

Plate XI

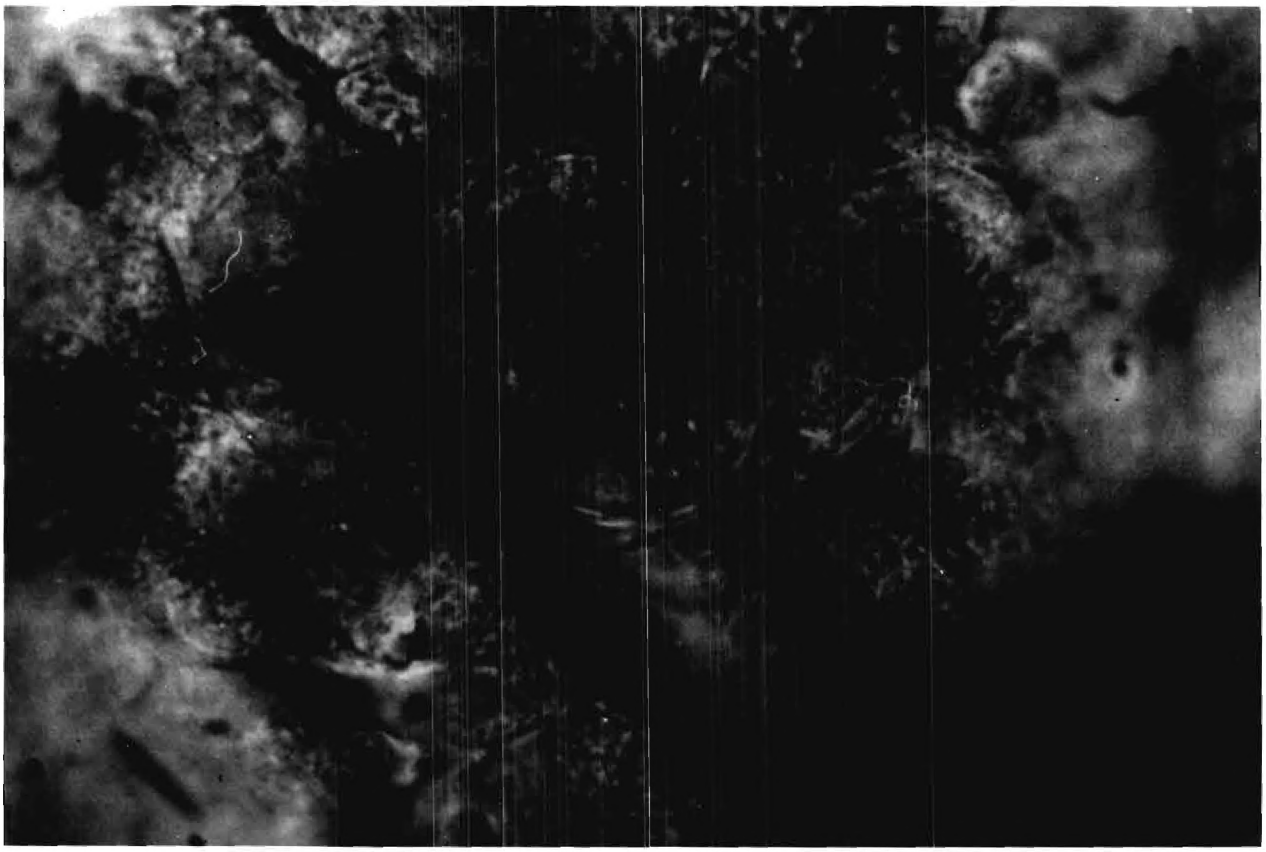
Three minutes later than the previous picture cell A is still in early telophase. The nucleus, however, has rounded up considerably into a definitely formed body. Cell E again is in late telophase.

Plate XII

Cell A is in mid telophase as in cell B. The nucleoli have become visible in the nucleus as small refractive spheres; both cells are still nearly spherical.

PLATE XIII. Four minutes later Cells A and B in mid telophase, nucleoli increasing in size.

PLATE XIV. Eight minutes later Cells A and B in mid telophase, nucleoli have attained maximum spherical size.



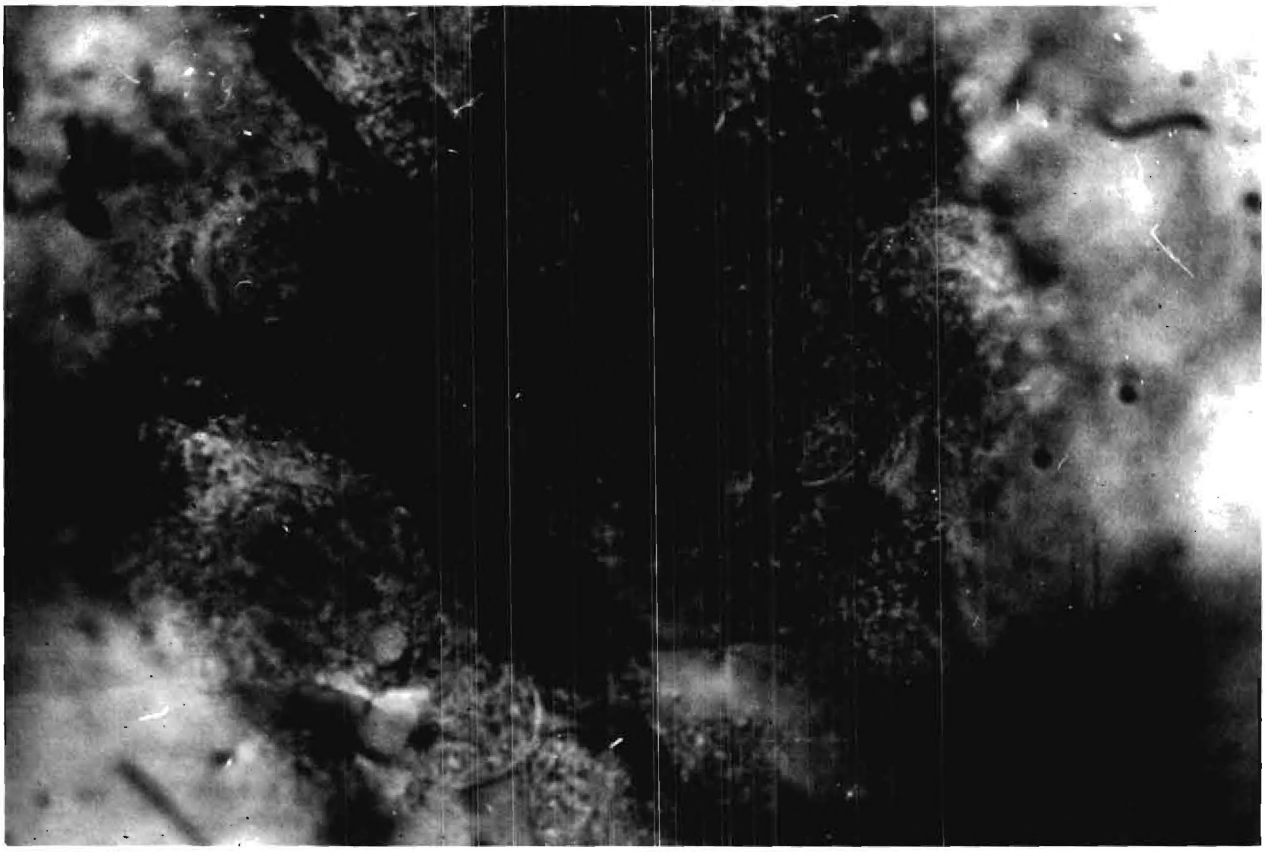
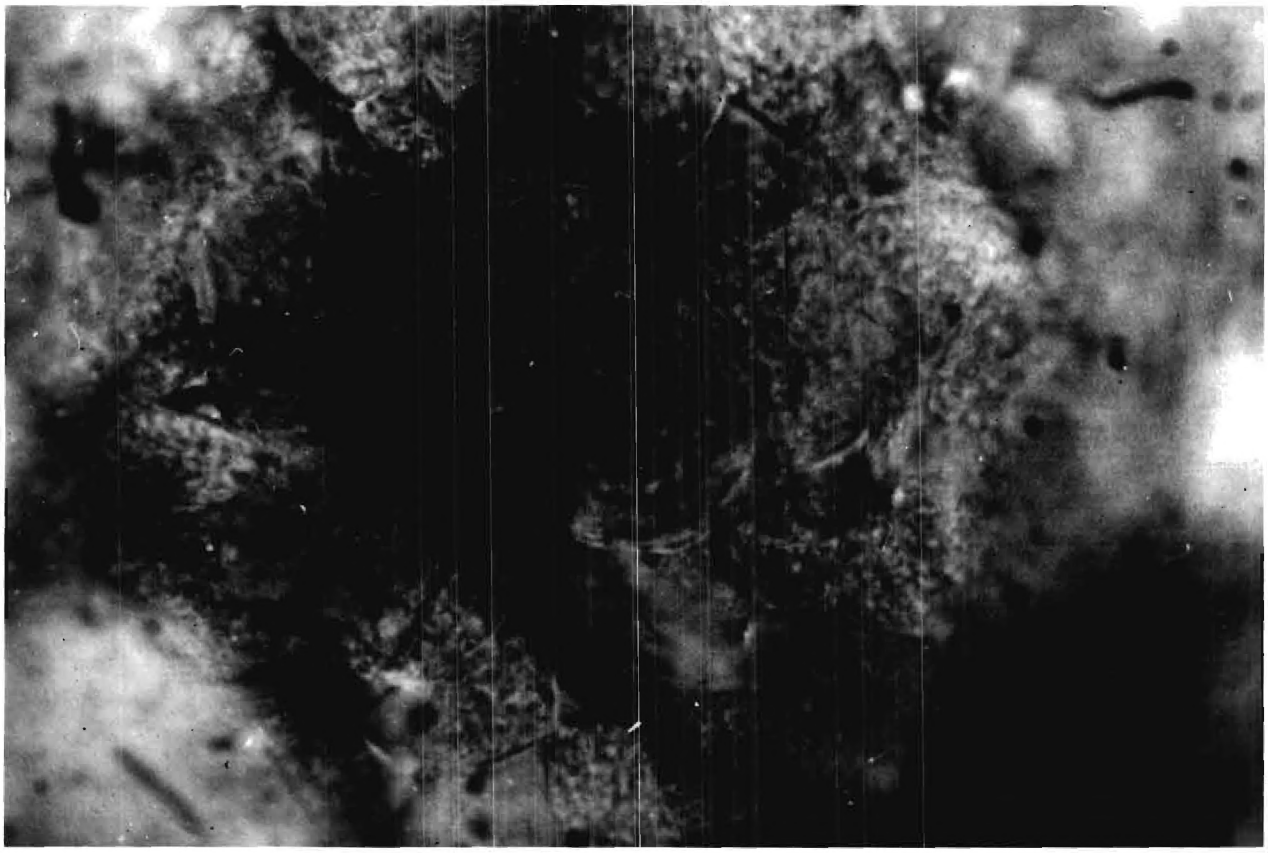


PLATE XV. Eight minutes later cells A and B in late telophase. Cell E in interphase.

PLATE XVI. Eleven minutes later cells A and B in late telophase. Cell C in early telophase.



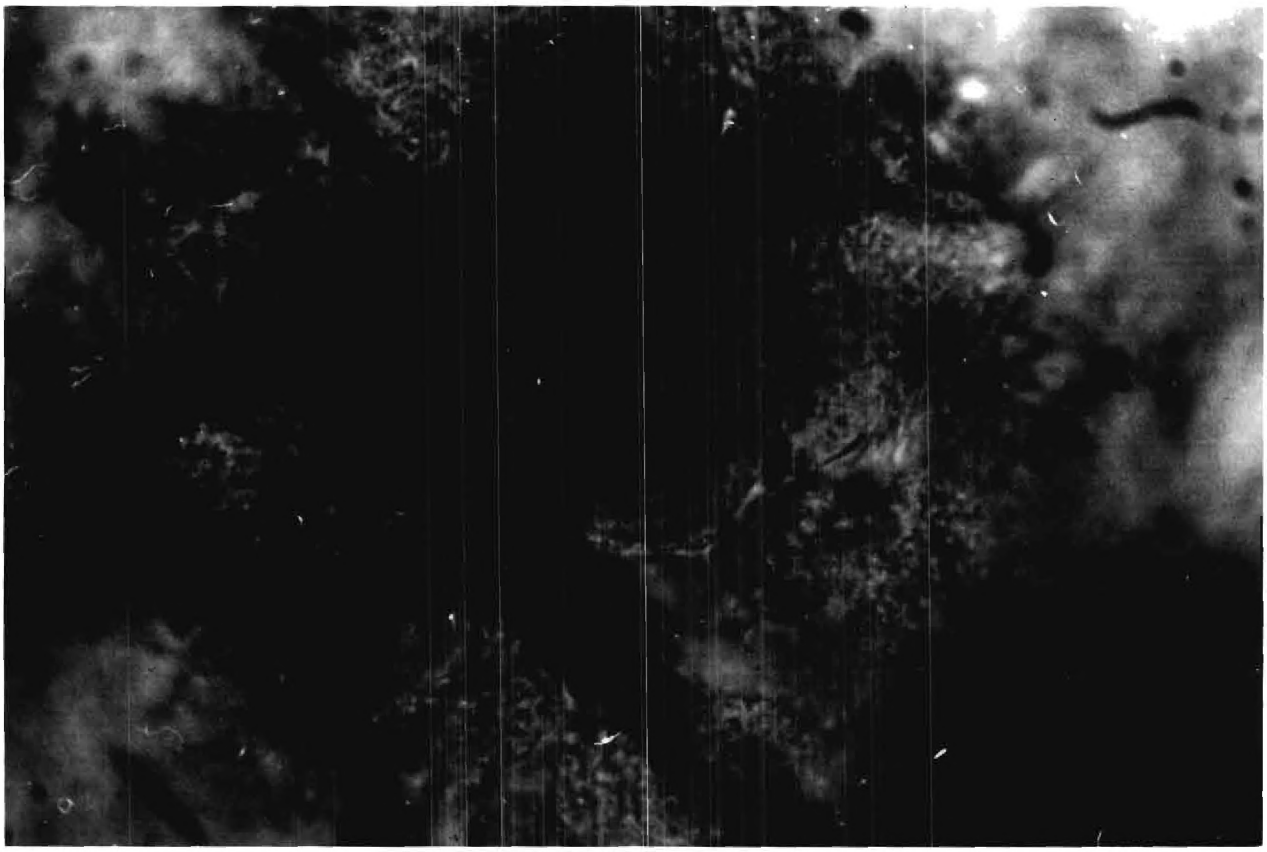


Plate XIII

After 4 minutes, the nucleoli have increased very sharply in size and are both spherical; this is more evident in cell B than in cell A, which is slightly out of focus for the nucleoli. Cell A indicates that the nucleus has increased sharply in size during the 4 minutes elapsing since the last photograph and also that the nuclear granules are becoming evident as the chromosome bodies themselves become more diffuse.

Plate XIV

In this photograph, cells A and B are still in mid telophase. The nucleoli have obtained their maximum spherical size. It appears that the nucleus is not a great deal larger than in the previous photograph. The mid telophase stage begins when the nucleoli become visible and ends when the nucleoli lose their spherical shape. The duration of this stage is 18 minutes.

Plate XV

Eight minutes later both cell A and cell B are in late telophase; the nucleoli have lost their spherical shape and have become irregular in outline. As the cells progress through late telophase the nucleoli will become progressively more irregular and will eventually be very difficult to identify. Late prophase begins with the loss of spherical form of the nucleoli and continues until the chromatin granules are scattered in a homogeneous nuclear background, at which time the chromosome bodies or threads are imperceptible.

PLATE XVII. Twelve minutes later cell A in late telophase.

PLATE XVIII. Twelve minutes later Cell A in interphase, linear arrangement of nuclear granules no longer evident.

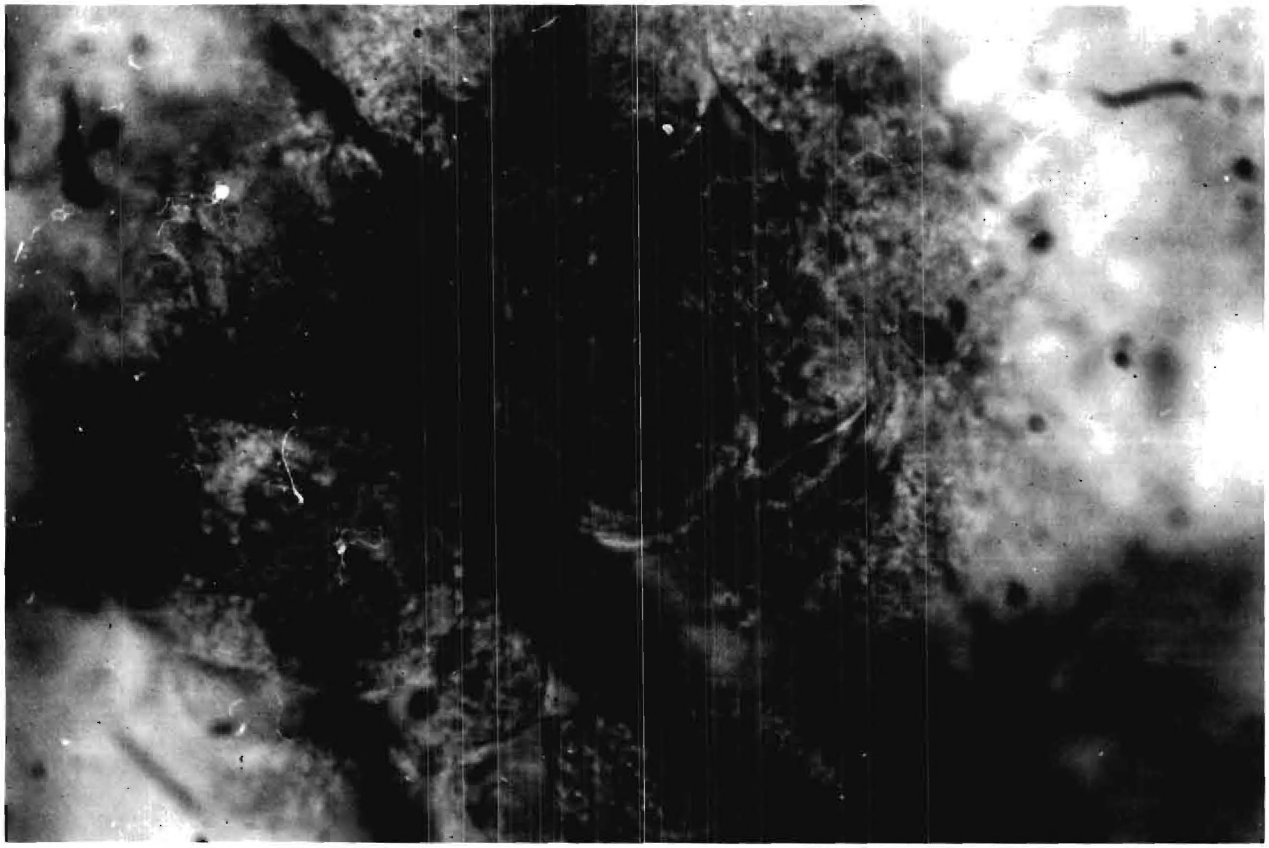
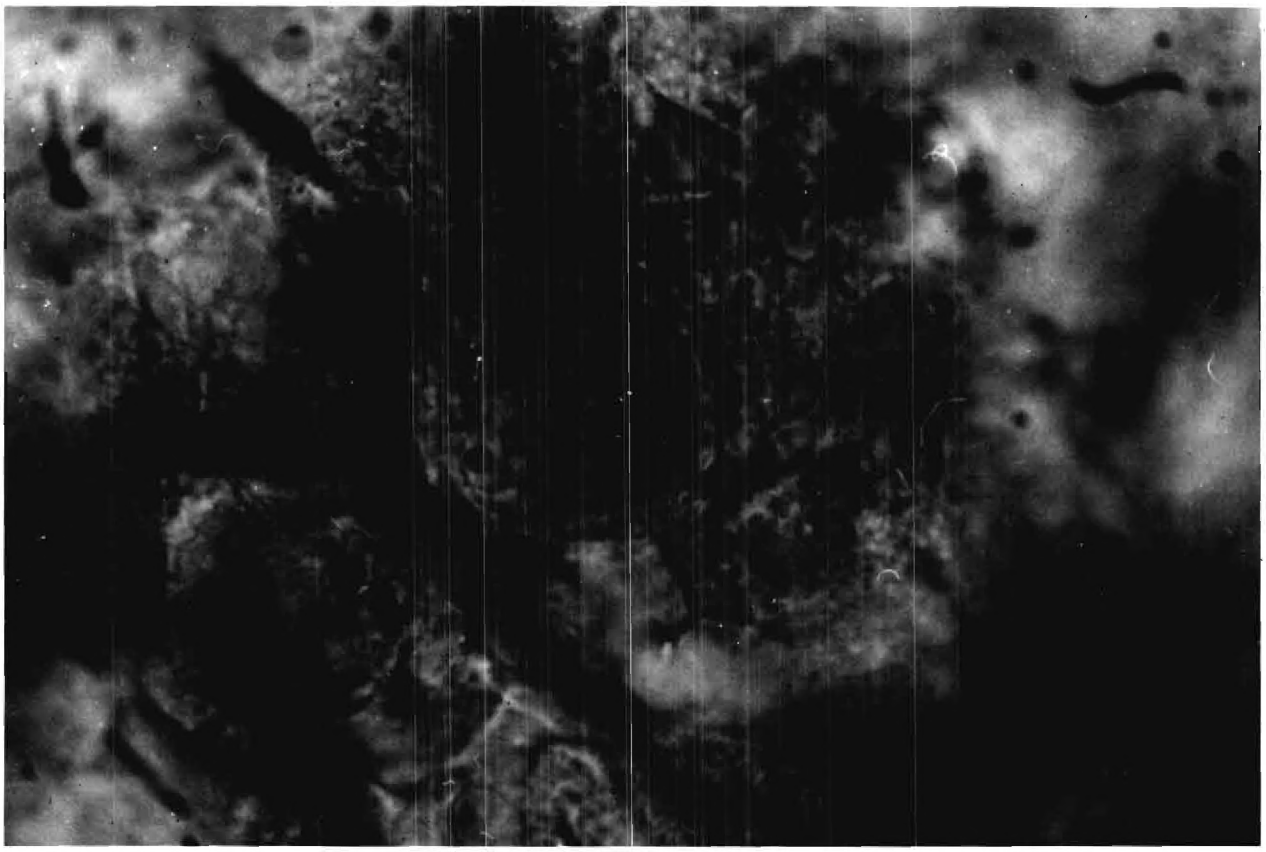


PLATE XIX. Twenty-five minutes later Cell A in very early prophase.

PLATE XX. Thirty-five minutes later Cell A in early prophase.



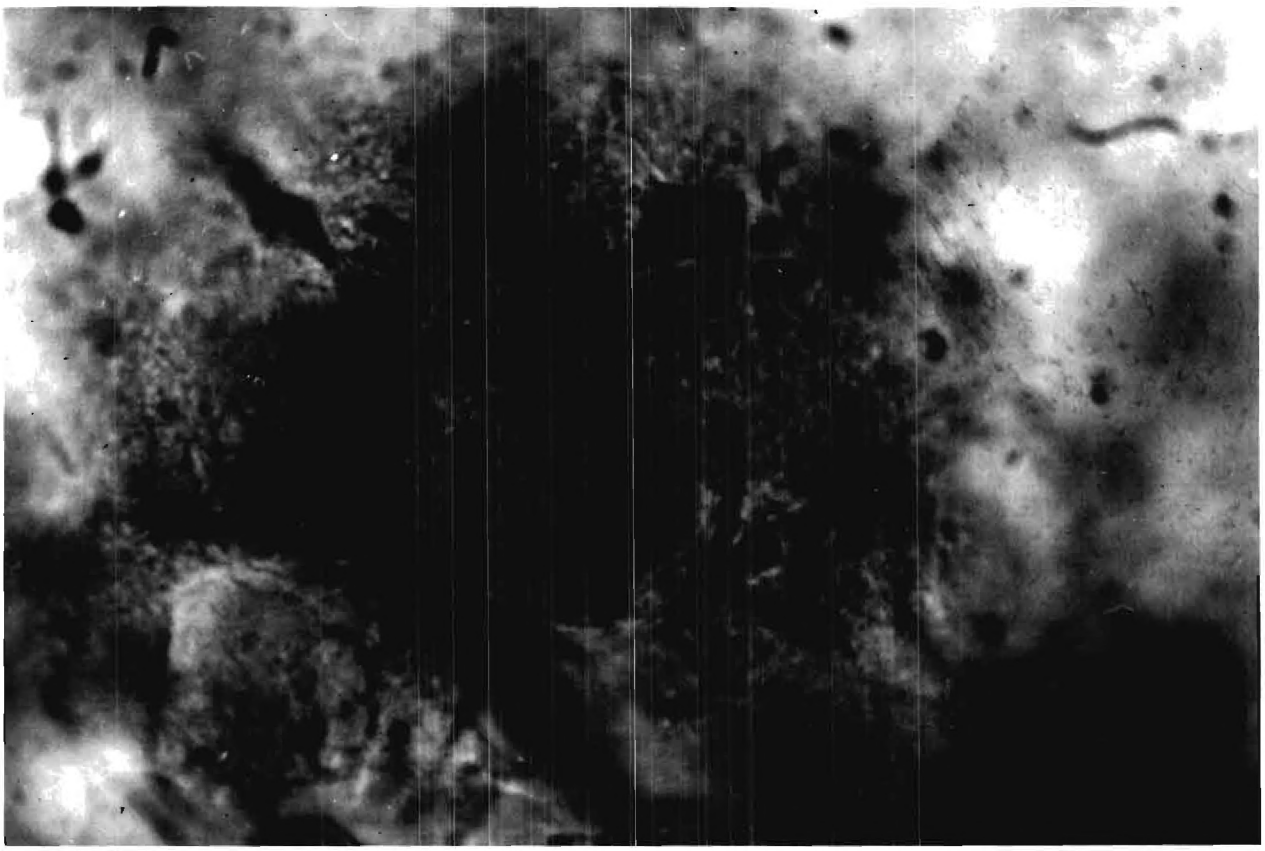


Plate XVI

In this photograph cell A is in late telophase, a stage of 33 minutes duration. Cell C is in early telophase; this particular cell was first identified 76 minutes ago when it was in early prophase.

Plate XVII

Cell A is still in late telophase and shows the progressive diffusion of the chromatin threads and the persistence of the nuclear granules.

Plate XVIII

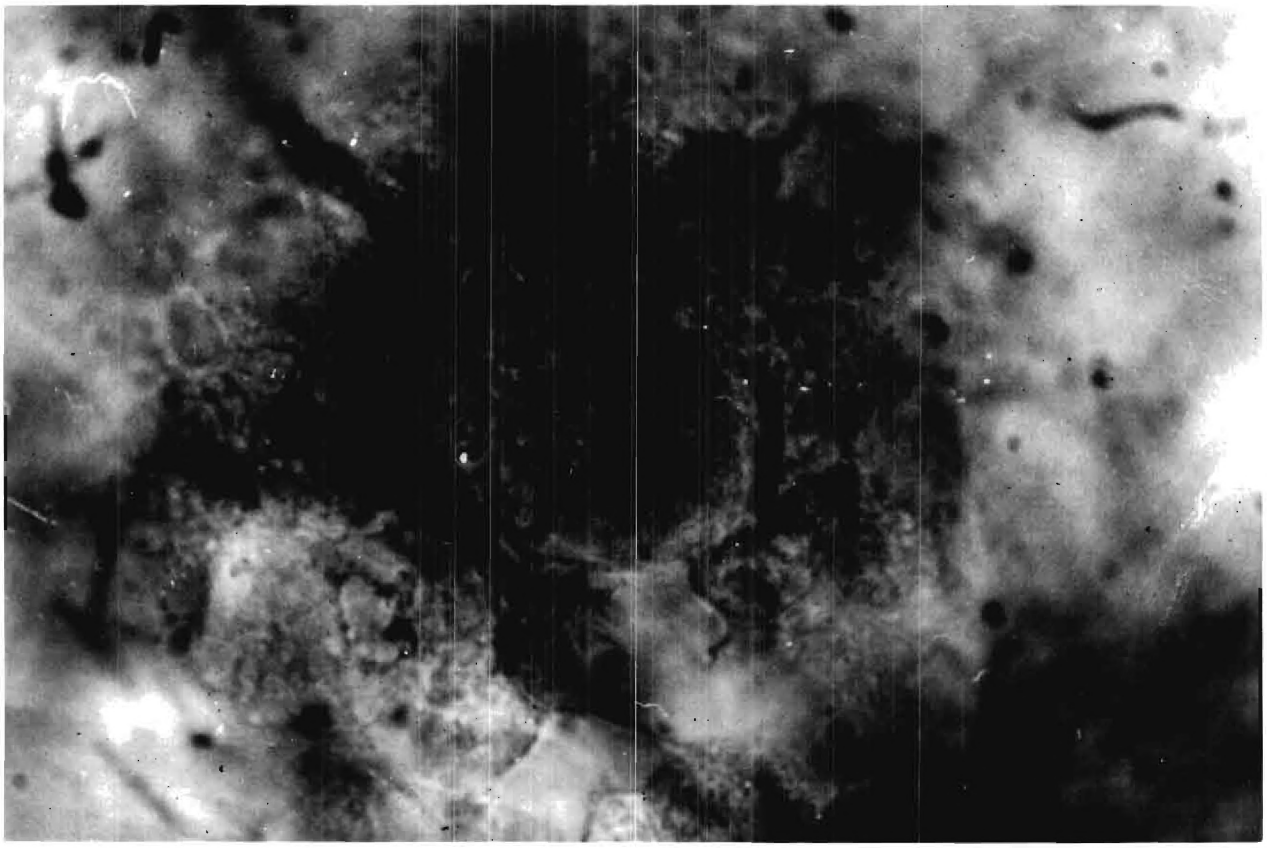
Cell A is now in interphase; the chromatin granules appear scattered throughout the homogeneous nucleus. Actually the nuclear granules lie immediately adjacent to the nuclear membrane and when the nucleus is in optical cross section, as in these photcgraphs, only a few of the nuclear granules are visible, mostly those around the periphery of the nucleus. However, if the preparation is focused up or down so as to be brought into optical alinement, the upper or lower part of the nearly spherical nucleus (as was seen in Cell E of Plate VI), then the granules become visible. The interphase stage begins with the above description and continues for 27 minutes, until barely visible threads appear among the nuclear granules.

Plate XIX

In this photograph very fine threads may be detected traversing through the body of the nucleus. The nuclear granules, which are not in good focus in this photograph, are considerably smaller than a few minutes previous. This is very early prophase and lasts 24 minutes. The nucleus has increased considerably in size during the interphase stage.

PLATE XXI. Twenty-two minutes later Cell A in early prophase.

PLATE XXII. Twenty minutes later Cell A in mid prophase. This completes entire mitotic cycle.



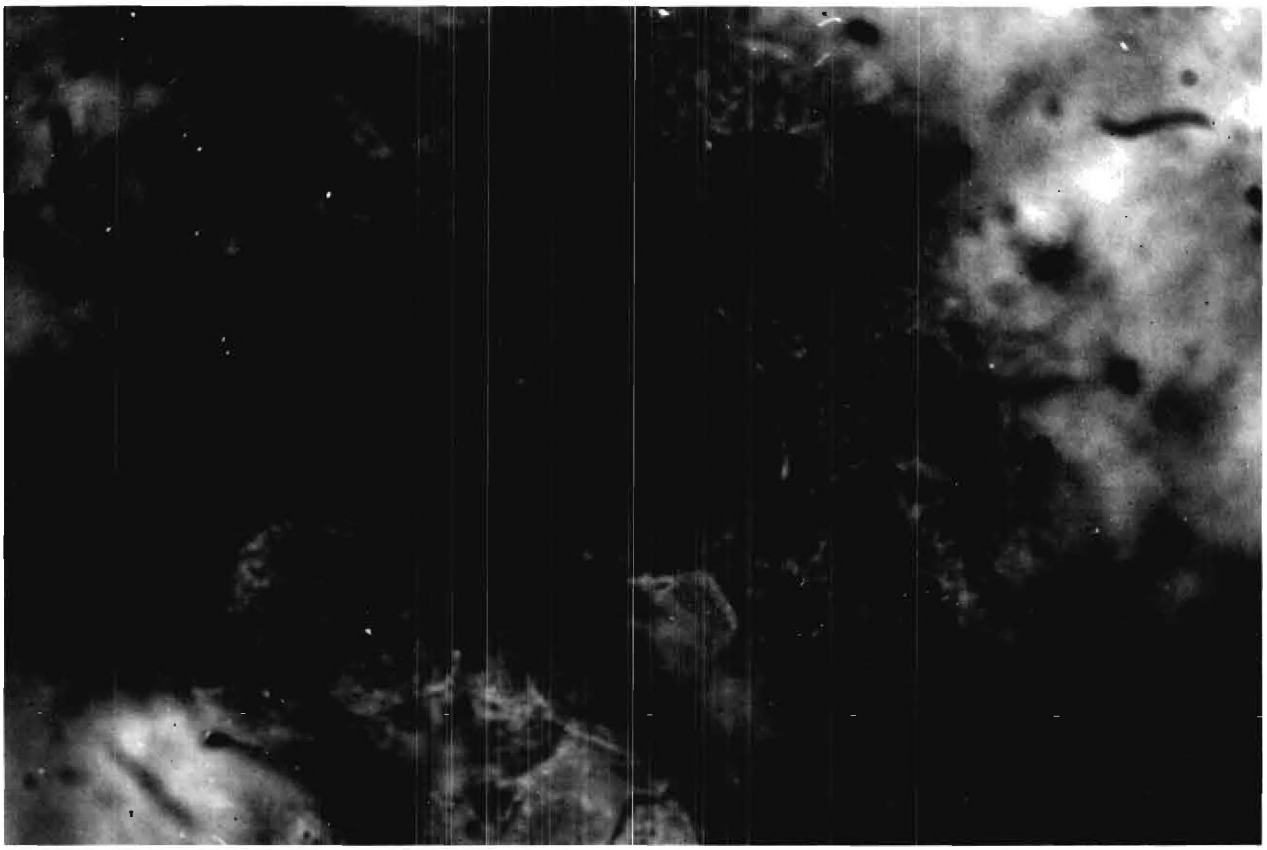


Plate XX

Cell A is in early prophase, the nuclear granules have disappeared, and the entire nucleus is filled with fine threads. It is not possible to follow an individual thread for any distance through the nucleus, but the nucleus gives the appearance of being uniformly filled with the fine chromatin threads. Early prophase begins when the nuclear granules disappear and ends when the chromosome threads become condensed enough so they may be followed from one part of the nucleus to another. Early prophase represents the mitotic stage of greatest duration, lasting 46 minutes.

Plate XXI

Cell A is still in early prophase; the chromatin threads have become more prominent.

Plate XXII

Cell A is now in mid prophase, the chromosomes are now distinct enough so that they may be followed from one part of the nucleus to another. During the early prophase there was a considerable increase in the size of the nucleus as viewed in optical cross section. This photograph terminates the sequence of plates and represents a time 208 minutes after the first picture. The cell represented in this plate is in the same stage as shown in Plate I, which was the beginning of this sequence.

Individual cells were studied after ozone exposure, and their progress was followed from one mitotic stage to another. The dosimetry procedure has been described in previous reports, and was followed in these experiments. In each experiment from one to two cells were followed per

preparation, depending upon the number of cells in the particular stage to be studied that could be found in one microscope field. Therefore, the total of six cells used in each experimental group represents from three to six replicate experiments. The cells were exposed to the ozone for 5 minutes and then the time recorded from the beginning of the next successive stage of mitosis and each succeeding stage until the cells entered anaphase. Table I presents the data from those cells exposed in anaphase. The duration of each succeeding stage, up to mid prophase, was not significantly different from that expected. Mid prophase and late prophase were significantly inhibited; however, once beyond this stage, the cells did proceed through metaphase to anaphase at the expected rate.

TABLE I

SIX CELLS TREATED IN ANAPHASE

Successive Stages	Total Duration (Minutes)	Expected* Duration (Minutes)	Difference (Minutes)	P**
Mid telophase	153	144	9	>.05
Late telophase	204	198	6	>.05
Interphase	154	162	8	>.05
Very early prophase	147	144	3	>.05
Early prophase	296	276	20	>.05
Mid prophase	192	96	96	<.01
Late prophase	288	96	192	<.01
Metaphase	80	78	2	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

In Table II, cells were treated in mid telophase. The cells progressed through successive stages at the expected rate until mid prophase and late prophase where there was a significant inhibition, and then proceeded through metaphase to anaphase as in the controls.

TABLE II

SIX CELLS TREATED IN MID TELOPHASE

Successive Stages	Total Duration (Minutes)	Expected* Duration (Minutes)	Difference (Minutes)	P**
Late telophase	212	198	14	>.05
Interphase	141	162	21	>.05
Very early prophase	149	144	5	>.05
Early prophase	273	276	3	>.05
Mid prophase	155	96	59	<.01
Late prophase	293	96	197	<.01
Metaphase	77	78	1	>.05

*Based on control = 208 minutes at 30° C.
**From Table for Goodness of Fit (Fisher).

Cells treated in late telophase (Table III) proceeded as the controls to mid prophase where there was a significant inhibition in mid prophase and late prophase, and then proceeded at the expected rate to anaphase. Cells treated in interphase (Table IV) also proceeded at the expected rate to mid prophase and late prophase where again there was a significant inhibition. Cells treated in very early prophase (Table V) were inhibited in

TABLE III

SIX CELLS TREATED IN LATE TELOPHASE

Successive Stages	Total Duration (Minutes)	Expected* Duration (Minutes)	Difference (Minutes)	P**
Interphase	149	162	13	>.05
Very early prophase	157	144	13	>.05
Early prophase	301	276	25	>.05
Mid prophase	183	96	87	<.01
Late prophase	307	96	211	<.01
Metaphase	82	78	4	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

TABLE IV

SIX CELLS TREATED IN INTERPHASE

Successive Stages	Total Duration (Minutes)	Expected* Duration (Minutes)	Difference (Minutes)	P**
Very early prophase	137	144	13	>.05
Early prophase	294	276	18	>.05
Mid prophase	127	96	31	<.01
Late prophase	307	96	211	<.01
Metaphase	82	78	4	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

mid prophase and late prophase and then proceeded at the expected rate to anaphase. Cells treated in early prophase (Table VI) proceeded to anaphase without inhibition as did cells treated in late prophase and metaphase (Tables VII and VIII).

TABLE V

SIX CELLS TREATED IN VERY EARLY PROPHASE

Successive Stages	Total Duration (Minutes)	Expected* Duration (Minutes)	Difference (Minutes)	P**
Early prophase	261	276	15	>.05
Mid prophase	133	96	37	<.01
Late prophase	176	96	80	<.01
Metaphase	75	78	3	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

TABLE VI

SIX CELLS IN EARLY PROPHASE

Successive Stages	Total Duration (Minutes)	Expected* Duration (Minutes)	Difference (Minutes)	P**
Mid prophase	107	96	11	>.05
Late prophase	112	96	16	>.05
Metaphase	72	78	6	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

TABLE VII

SIX CELLS TREATED IN MID PROPHASE

Successive Stages	Total Duration (Minutes)	Expected* Duration (Minutes)	Difference (Minutes)	P**
Late prophase	91	96	5	>.05
Metaphase	82	78	4	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

TABLE VIII

SIX CELLS TREATED IN LATE PROPHASE

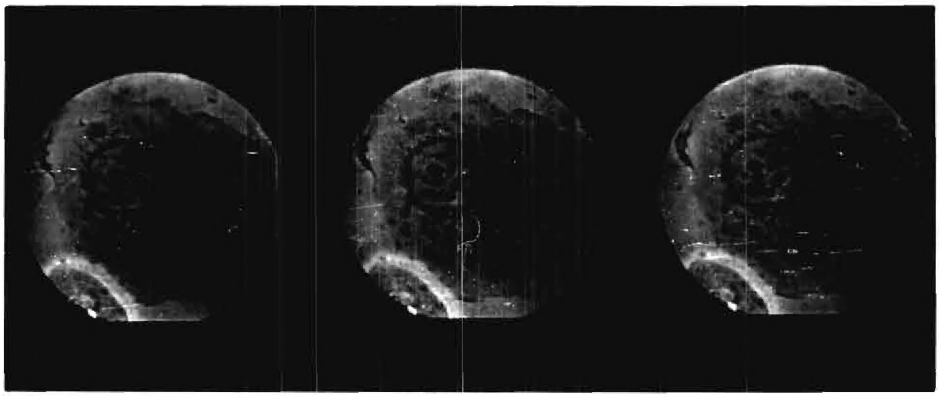
Successive Stages	Total Duration (Minutes)	Expected* Duration (Minutes)	Difference (Minutes)	P**
Metaphase	73	78	5	>.05

*Based on control = 208 minutes at 38° C.
**From Table for Goodness of Fit (Fisher).

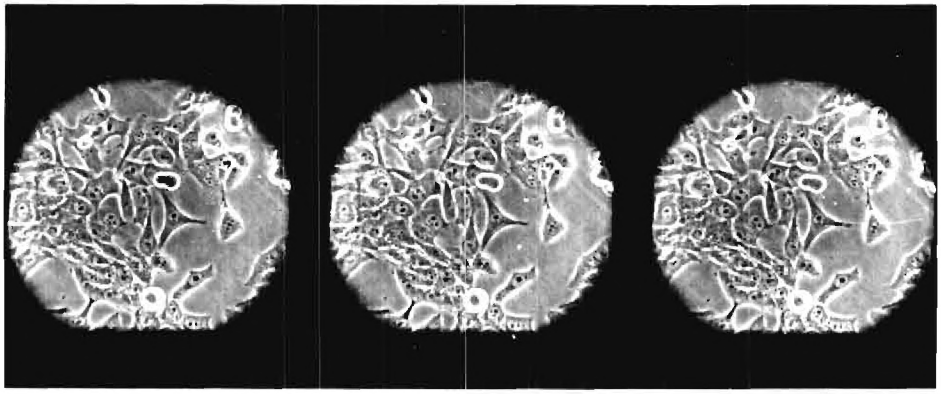
Cells exposed to ozone, at the threshold concentration, in the stages of anaphase, telophase, interphase, and very early prophase, will be significantly inhibited in passing through mid prophase and late prophase, and then will reach anaphase at the expected rate. The cells are sensitive during these times and this sensitivity is expressed by an inhibition during the late stages of prophase or conversely, the sensitivity is expressed by a delay in entering metaphase.

In the time lapse cinematography studies a technique was developed for cover slip culture of Hela cells which permits considerable manipulation without producing any obvious shock to the cells. Cover slips (22 x 44 mm) are coated with silicone grease (Dow-Corning) to cover one entire surface except for an area of approximately 0.5 centimeters diameter in the middle. A fresh preparation of trysinized Hela cells in suspension are placed as a drop in this clear zone and the preparation inverted over a depression slide so that contact is made between the top cover-slip containing the silicone grease and the other cover slip which comprises the bottom of the depression slide. The depression slides are made from ordinary microscope slides through which a hole has been etched by hydroflouric acid. The slides are inverted so that the cells become attached to the cover slip containing the silicone. The preparation is incubated at 37° C. overnight in a moist chamber composed of a petri dish lined with moist filler paper. After the cells have become attached to the glass and are actively growing, the cover slip may be detached from the slide and dipped into a saline solution; this does not detach the cells from the glass and permits the media to be changed by the addition of a fresh drop of culture fluid. The preparation may be inverted in a closed system over a bottle of ozone solution and the organisms in this way exposed to ozone after being freed of extraneous organic materials. If care is taken to maintain constant temperature conditions in the saline dip, the cells apparently suffer no noticeable effect in control preparations and may be maintained on the cover slips until they form a thick sheet of cells. The advantage of the silicone is that the "sitting drop" culture remains centrally orientated in the depression cavity and does not make

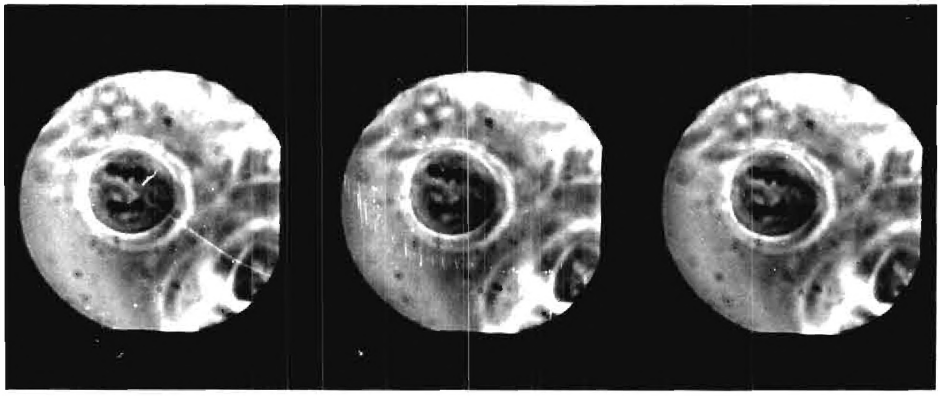
FIGURE 1. Reproductions from 16-mm. negatives of Hela cells in culture: A, a metabolic cell; the flaming of the cell membrane is visible at the upper left of the cell; B, low magnification of cell culture; C, dividing cell.



(A)



(B)



(C)

contact with the outside rim. Further, the silicone facilitates the process of rinsing and changing the media on the cultures since it repells liquids in all of the areas except the actual clear central zone where the cells are growing. This method has been used to obtain a catalogue of Hela cells photographed at various speeds; 1, 5, 15, and 60 frames per minute have been selected as the most probable times in which to study the various metabolic activities of the cells, and a time sequence of 15 frames per minute has been selected in the analysis of mitosis. In the preliminary analysis of these films attention is being concentrated on the motility of the cell membrane in the resting or metabolic cell (A in Figure 1). When the cell is in this highly flattened condition, periodic movements occur over the entire cell surface. These are most visible at the edges of the cell and, when viewed with time lapse photography, give a flaming appearance reminiscent of the flame cells in Platyhelminthes. This phenomenia is seen in all control preparations and should provide a good indicator of plasma membrane activity. This activity will be quantitated by measuring rate of movement with a frame-by-frame analysis.

Also in the metabolic cell the following is being studied: nuclear membrane activity, nuclear rotation and nucleoli activity.

In the mitotic cell the velocity of chromosome separation and the rate at which the cleavage furrow forms are being measured. All of these processes are under study and those which lend themselves to quantitation will be investigated in detail.

III. FUTURE PLANS

The work is proceeding as per proposal and no variations are anticipated.

IV. PERSONNEL

There has been no change in personnel.

V. FINANCIAL REPORT

For the period 31st December through 30th June, 1960, the following expenditures were made:

Personnel Services	$5,372.15
Material and Supplies	499.05
Freight and Express	4.92
Overhead	3,062.13
TOTAL	$8,938.25

A voucher in this amount has been submitted.

Respectfully submitted,

Robert H. Fetner
Project Director

Approved:

Wyatt C. Whitley, Chief
Chemical Sciences Divisic

FINAL REPORT

PROJECT NO. A-430

RESEARCH AND REPORTS ON OZONE-INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

- o - o - o - o -

CONTRACT NO. AF 41(657)-263

SCHOOL OF AVIATION MEDICINE

UNITED STATES AIR FORCE
RANDOLPH AIR FORCE BASE, TEXAS
R&D PROJECT NO. 7758-130

- o - o - o - o -

APRIL 30, 1961

Engineering Experiment Station
Georgia Institute of Technology
Atlanta, Georgia

ENGINEERING EXPERIMENT STATION
of the Georgia Institute of Technology
Atlanta, Georgia

FINAL REPORT

PROJECT NO. A-430

RESEARCH AND REPORTS ON OZONE-INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

- o - o - o - o -

CONTRACT NO. AF 41(657)-263

SCHOOL OF AVIATION MEDICINE

UNITED STATES AIR FORCE
RANDOLPH AIR FORCE BASE, TEXAS
R&D PROJECT NO. 7758-130

- o - o - o - o -

APRIL 30, 1961

TABLE OF CONTENTS

This report contains 45 pages.

LIST OF FIGURES

LIST OF TABLES

I. ABSTRACT

Living, unstained neuroblasts of the grasshopper Chortophaga viridifaciata were exposed in a closed system over solutions of known ozone concentration. Immediately after exposure the cells were observed continuously under the microscope. With a 5-minute exposure the threshold dose necessary to produce a significant delay in mitosis (progression to anaphase) was determined to be in the range 3.5 to 4.5 mg per liter of ozone. The inhibition was reversible and, although mitosis was delayed it went to completion. Cells dosed in stages preceding early prophase were inhibited and differed significantly from the controls in the increment of time taken to reach anaphase. At the time of ozone exposure cells more advanced than very early prophase proceeded to anaphase at a time not significantly different from the controls.

Once the threshold dose had been determined, data were obtained on the progression of neuroblast cells through successive stages of mitosis after ozone exposure in each of the eight stages of mitosis. It was found that cells exposed in anaphase, telophase, interphase and very early prophase were delayed significantly in passing through mid prophase and late prophase stages. Progression through preceding and succeeding stages was the same as control preparations. Thus exposure to ozone in any of the preprophase stages results in inhibition of mitosis in late prophase, and cells exposed later than the earlier stages of prophase proceed through the next cell division uninhibited. The sensitive stages to the threshold concentration of ozone are preprophase and the inhibited stages are mid and late prophase.

Various cellular phenomena were investigated by time lapse cine-microphotography. Attention was focused on cytoplasmic membrane mobility. Waves originate at the outermost perifera of the cell membrane and migrate toward the nuclear zone. These waves appear with a rythmic frequency in metabolic or flattened cells. Replicate measurements indicate that five such waves will appear in 159 ± 6 seconds. After ozone exposure the frequency of waving was found not to be significantly different from control preparations although dosed cells would round up and fail to exhibit any activity for some time.

II. MATERIALS AND METHODS

A. Ozone Generation and Analysis

Ozone was generated at room temperature in a Welsbach Model 23 laboratory ozonator from cylinder oxygen and scrubbed successively through 0.8-normal sulfuric acid and a 2-per-cent potassium iodide trap. The ozone used for dosimetry was that present as dissolved ozone in the 0.8-normal sulfuric acid solution. It has been shown in previous investigations[1] that ozone is reasonably stable under such conditions--at least the rate of decomposition will be minimized and will be less than 10 per cent over the period of the experiment. The concentration of ozone present in such a solution is a function of the distribution coefficient between the ozone and the gas immediately above it. This in turn is determined by the temperature. At, or close to, room temperature (23° C) the distribution coefficient for ozone is about 0.24.[2] This means that at saturation the ozone concentration in the solution will be about one-quarter of that present in the gas being bubbled through it.

- - - - -

1. R. S. Ingols, R. H. Fetner and W. H. Eberhardt, Adv. in Chemistry 21, 102 (1959).

2. F. Kawamura, J. Chem. Soc. (Japan) 53, 783 (1932)

The Welsbach ozonator is capable of producing ozone from pure oxygen in concentrations from about 1 to 10 per cent by weight--depending on the voltage operation, rate of gas flow, and certain other variables. We have found that scrubbing ozone through 400 ml of 0.8-normal sulfuric acid for 20 to 30 minutes produces a near-saturated solution and, as a matter of convenience, we allow this period of time for scrubbing. The final concentration of ozone was determined by varying the voltage on the ozonator and/or by diluting the resultant solution. After the ozone solution had been generated it was poured into a glass-stoppered bottle. This particular bottle has a very narrow mouth and the top has been ground smooth so that a tight fit will be obtained when a coverslip is inverted over the open neck. The bottle has a ground glass stopper and special care is necessary to insure that no contaminating or reducing substances are present as these would effectively reduce the ozone concentration. Several methods for ozone analysis have been evaluated in previous investigations.[3] The ferrous-ferric system was selected as the best method for these experimental procedures. This analytical procedure involves the oxidation of the ferrous ion to the ferric ion by ozone:

$$Fe^{++} + O_3 + H_2 \rightarrow Fe^{+++} + O_2 + H_2O$$

Enough ferrous ammonium sulfate in 0.8-normal sulfuric acid was added to the test solution to make a final concentration of approximately 0.5-normal ferrous ammonium sulfate solution. This was back-titrated with potassium permanganate standardized against sodium oxalate. Insofar as this analysis is ordinarily conducted in dilute sulfuric acid solutions, it lends itself particularly well to our experimental procedure and eliminates the necessity of changing the

3. Ingols, loc cit.

pH during the analytical procedure as would be necessary, for example, with potassium iodide. Another advantage of this analytical method is the sharp end point evidenced in the permanganate titration.

B. Neuroblast Culture and Ozone Dosimetry

Six-day Chortophaga viridifasciata embryos incubated at 37° C (equivalent of 12-day embryos of Carlson at room temperature) were used in the experimental work. This material has several advantages: the work of previous investigators[4] has established the precision and noncyclic mitotic rate of these particular cells, and the large size and exceptional visibility make refined in vitro observations possible. Cultural procedures employed are essentially those developed by Carlson and his co-workers. The thickness of such preparations precludes the effective use of phase contrast optics, so all observations were made with ordinary microscopy. Details of cell division are clearly visible and a photographic sequence has been made of a single neuroblast cell through the entire mitotic cycle. The criteria used to determine the beginning of each mitotic stage are given in Table I.

- - - - -

4. J. G. Carlson, J. Morphol. 66, 71 (1942); J. G. Carlson, Cold Spring Harbor Symp. Quant. Biol. 9, 104 (1941); J. G. Carlson, J. Morphol. 71, 449 (1942); J. G. Carlson, J. Cellular Comp. Physiol. 26, 165 (1945); J. G. Carlson, Biol. Bull. 90, 109 (1946); J. G. Carlson, A. Hollaender and M. E. Gaulden, Science 105, 187 (1947); J. G. Carlson, A. Hollaender and M. E. Gaulden, J. Cellular Comp. Physiol. 31, 149 (1948).

TABLE I

CRITERIA USED TO DETERMINE THE VARIOUS MITOTIC STAGES

Stage	Description of initiation of stage	Duration (Min.)
Interphase	Chromatin granules are scattered in a homogeneous nuclear background. Nucleoli very irregular, appear as grape-like cluster of small spheres.	27
Prophase		
very early	Chromosome threads become visible among the nuclear granules.	24
early	Nuclear granules disappear; the nucleus is filled with fine chromosome threads.	46
middle	Threads increase in thickness and may be followed from one place in nucleus to another.	16
late	About seven chromosomes are near the nuclear membrane in one-fourth optical cross section.	16
Metaphase	Nuclear membrane disappears; cell assumes a spherical shape.	13
Anaphase	Proximal ends of chromatids separate.	9
Telophase		
mid	Cleavage furrow appears to be complete.	24
late	Nucleoli lose their spherical shape; chromatin granules arranged in linear sequence.	33

To eliminate the temperature shock-effect present in the experimental results of other investigators using this material, it was decided to conduct all operations as close to the incubation temperature (38° C) as would be feasible. To this end, a constant-temperature work space was constructed in which all of the preparatory steps and dosimetry could be accomplished (Figure 1). Temperature control was maintained in this inoculation box through thermostatic control of the radiant-heating bottom surface. Heat is supplied through a 400-watt radiant-heating wire located beneath a 1/4-inch plate glass surface. This plate glass bottom acts as a continuous heat-radiating surface, and the high heat loss and continuous heat input of this system results in a small amplitude of temperature change while permitting the front of the box to remain open and considerable manipulation to take place through the open front. In operation this box automatically maintains a temperature between 35° and 37° C. By preparing samples under such conditions and then transferring them to the microscope incubator, the temperature shock-effect was eliminated.

A new type microscope incubator which offers several unique advantages was designed and put into operation (Figure 2). This incubator is an inflated polyvinyl bag through which hot air is forced in a closed circulating system. Forced-draft incubating systems offer good temperature control if there is a large enough temperature differential between the desired incubator setting and room temperature. The flexible polyvinyl bag offers an additional advantage over rigid systems--namely, the microscope controls may be manipulated from the exterior of the bag without breaking into the closed forced-draft system. This is accomplished by pressing in on the bag and manipulating the controls directly through the polyvinyl

FIGURE 1. Controlled temperature work space.

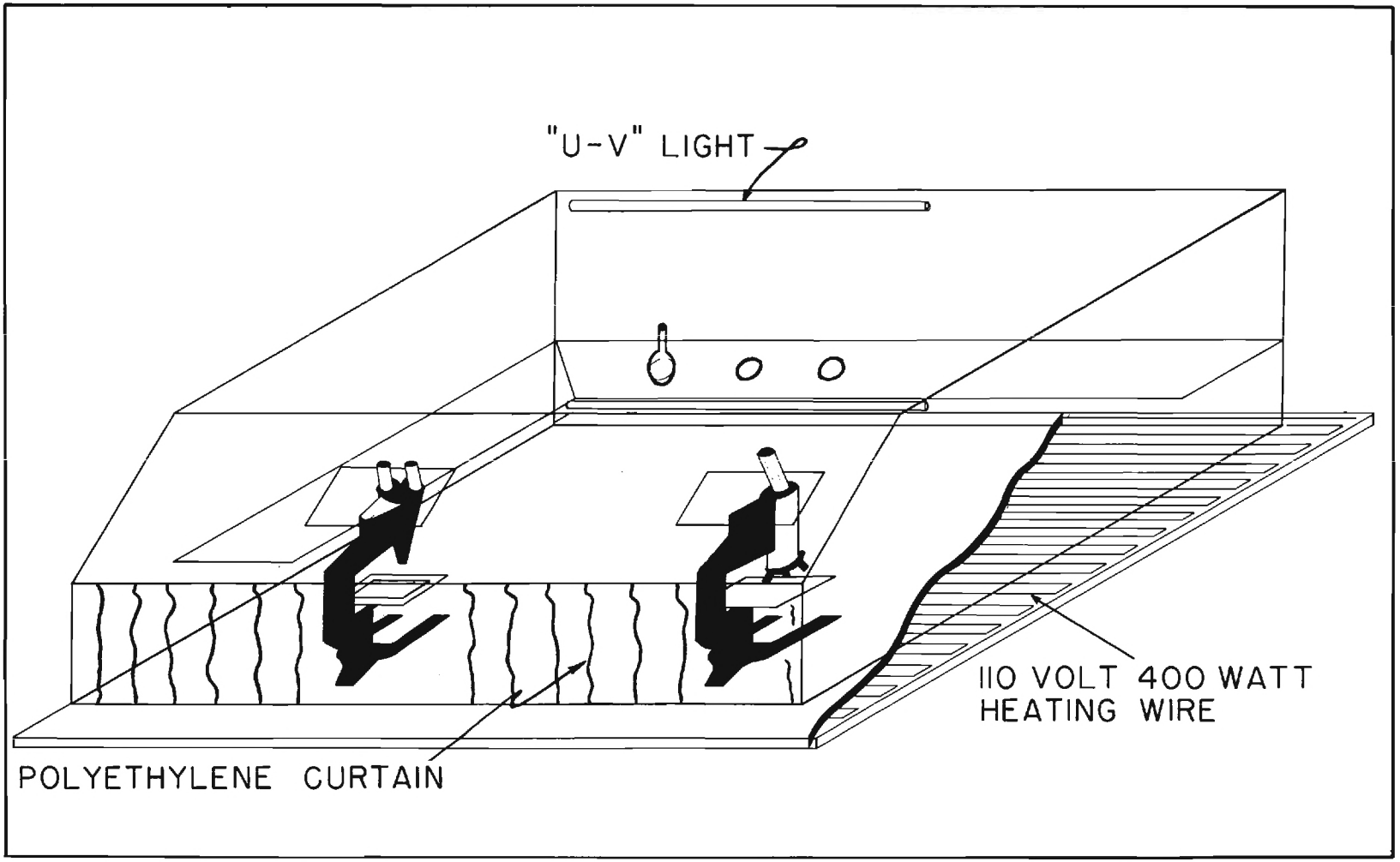
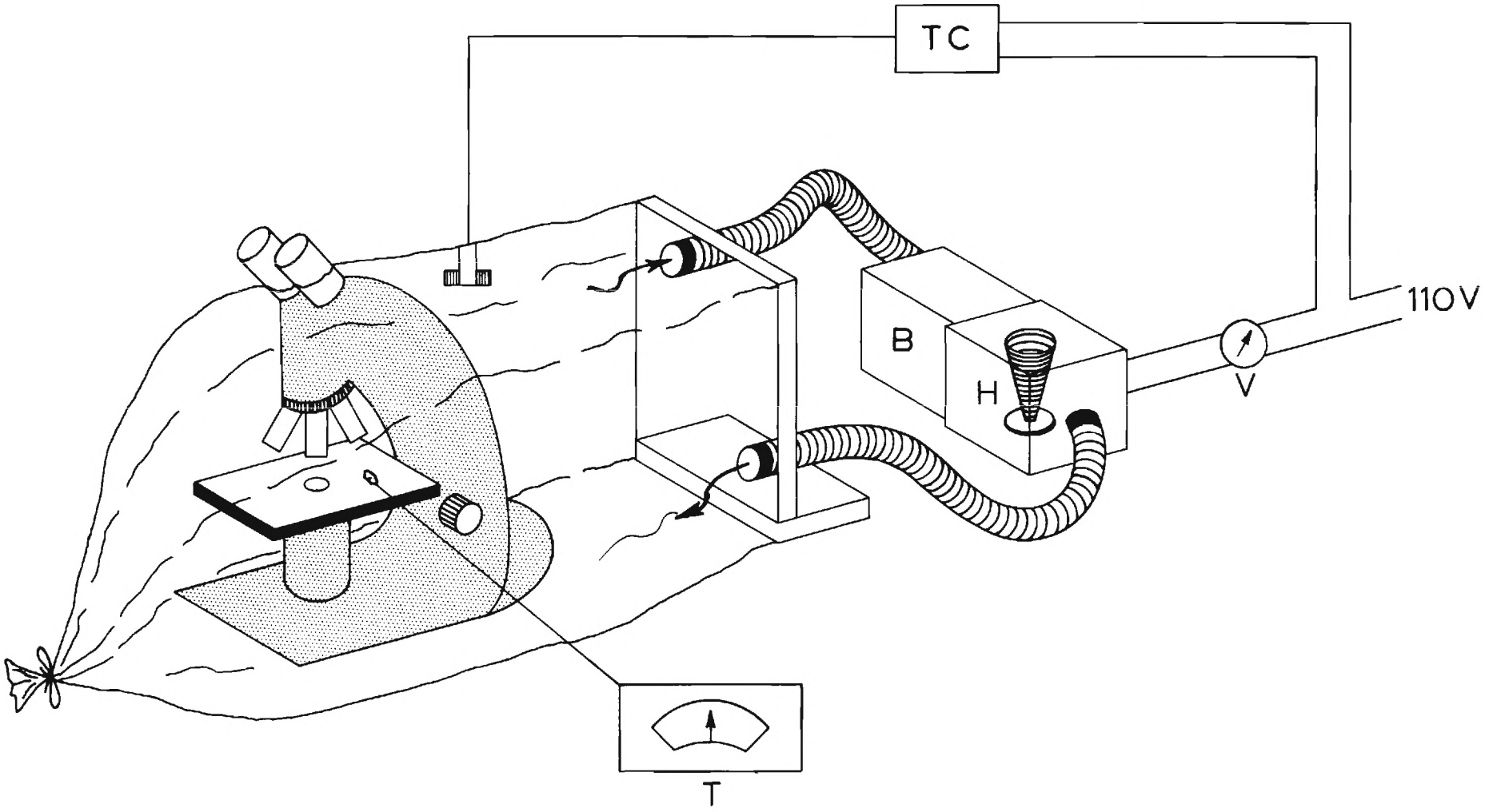

"U-V" LIGHT
110 VOLT 400 WATT
HEATING WIRE
POLYETHYLENE CURTAIN

FIGURE 2. Microscope incubator.


TC
110V
B
H
V
T

material. This overcomes one of the more serious drawbacks to a forced-draft system--that of having to break into and disturb the continuous air flow.

In the dosimetry of the hanging-drop preparations, the embryos were exposed to ozone by inverting the preparations on the cover slip and placing them on the top of the sample bottle; pains were taken to insure a complete seal between the cover slip and the ground lip of the bottle. Under these conditions, the concentration of ozone in the gas above the liquid and in contact with the drop containing the embryo preparation may be estimated from the distribution coefficient of ozone at this temperature.[5] After a specified contact time, the solution was replaced in the preparation, yolk material added, and observation begun immediately. This particular method of dosimetry seemed to be dictated by several considerations: (1) the requirements of the embryo for a balanced physiological solution, (2) the advantage of a short contact time, and (3) the necessity of eliminating reducing materials from the embryo preparations during ozone contact. It has been found in other investigations that ozone is not effective biologically until all reducing materials in solution have been eliminated.[6] The presence of even small amounts of reducing agents provides marked protection from the ozone effect. Controls were run by exposing preparations in the same manner except that oxygen alone had been bubbled through the solution. The controls were not significantly different from theoretical; Table II shows the result of a typical control run.

- - - - -

5. Kawamura, loc cit.

6. R. H. Fetner and R. S. Ingols, J. Gen. Microbiol. 15, 381 (1956).

TABLE II

NEUROBLAST CELLS TREATED* IN INTERPHASE

THIRTEEN OF THE 100 CELLS TREATED WERE IN THIS STAGE

Mitotic Stage	Total Duration	Theoretical Duration** (m)	Difference (d)	d^2	$\frac{d^2}{m}$	P
Prophase						
Very early	314	312	2	4	0.012	.90-.95
Early	601	597	4	16	0.027	.80-.90
Middle	200	204	4	16	0.078	.70-.80
Late	203	204	1	1	0.005	.90-.95
Metaphase	172	169	3	9	0.053	.80-.90
Anaphase	119	117	2	4	0.034	.80-.90
Telophase						
Early	311	312	1	1	0.003	.95-.98
Late	444	439	5	25	0.052	.80-.90
Interphase	349	351	2	2	0.014	.90-.95
Total	2713	2705			0.277	> .99

*Treatment was with physiological saline (control).

**m = t x n; where t = no. of cells, n = duration of each stage from Carlson and Hollaender.[7]

Occasionally the embryo in a preparation would die, usually by drying up. This, however, did not confuse interpretation of the data because in such instances the preparation became highly refractive and all of the cells died. Any deviation from iso-tonicity is immediately detectible by changes in the optical characteristics of the preparations.

- - - - -

7. Carlson and Hollaender, loc cit.

A 5-minute dose time was used in all of the experiments; this was selected as a compromise between a time adequate for diffusion of ozone into the cell and yet short enough to permit accurate determination of the stage treated.

C. Human Cell Culture and Dosimetry

In the human cell culture studies, two types of cell strains were used in preliminary work: Hela and KB strains. As the work progressed the KB strain was selected as the primary study material. The KB cell line[8] was originally obtained from Dr. W. E. McLimans of the Communicable Disease Center, Atlanta. It was grown in a media composed of Eagles' amino acid and vitamin solution[9] in Hank's salt solution to which 10 per cent pooled human serum had been added. Routine incubation was at 35° C.

In the time lapse cine-microphotography studies, a technique was developed for cover slip culture of cells which permits considerable manipulation without producing any obvious shock to the cells. Cover slips (22 x 44 mm) are coated with silicone grease (Dow-Corning) to cover one entire surface except for an area of approximately 0.5 centimeter diameter in the middle. A fresh preparation of trysinized cells in suspension are placed as a drop in this clear zone and the preparation inverted over a depression slide so that contact is made between the top cover-slip containing the silicone grease and the other cover slip which acts as the bottom of the depression slide. The depression slides are made from ordinary microscope slides through which a hole has been etched by hydrofluoric acid. The slides are inverted so that the cells become attached to the cover slip containing the silicone. The

8. H. Eagle, Proc. Exp. Biol. Med. 89, 362 (1955).

9. H. Eagle, Science 130, 432 (1959).

preparation is incubated at 37° C overnight in a moist chamber composed of a petri dish lined with moist filler paper. After the cells have become attached to the glass and are actively growing, the cover slip may be detached from the slide and dipped into a saline solution; this does not detach the cells from the glass and permits the media to be changed by the addition of a fresh drop of culture fluid. The preparation may be inverted in a closed system over a bottle of ozone solution and the organisms in this way exposed to ozone after being freed of extraneous organic materials. If care is taken to maintain constant temperature conditions in the saline dip, the cells apparently suffer no noticeable effect in control preparations and may be maintained on the cover slips until a thick sheet of cells is formed. The advantage of the silicone is that the "sitting drop" culture remains centrally orientated in the depression cavity and does not make contact with the outside rim. Further, the silicone facilitates the process of rinsing and changing the media on the cultures since it repels liquids in all of the areas except the actual clear central zone where the cells are growing.

D. Time Lapse Cine-microphotography of Cell Cultures

All motion studies were recorded with a Sieman camera on Plus X 16 mm film. Film was developed in Kodak D-19 or Microdol developers. In preliminary investigations individual cells were photographed at 1, 5, 15 and 60 frames per minute and were analyzed using a time-motion study projector (Bell and Howell Co.) which permits the film to be run forward or in reverse at various rates of speed or frame by frame.

After a preliminary survey of the films it was decided to concentrate our attention on membrane mobility effects produced by ozone. This decision

was dictated in the belief that this might be a cellular phenomenon which would be sufficiently reproducible for quantitative studies. To this end a number of control cells were photographed at 1 frame per second and were analyzed with a projection speed of 14 frames per second. The time required for five successive waves to appear at the interface of the cell membrane was recorded and was used as the quantitative criteria of membrane mobility. When viewed at the proper rate of motion study, waves appear at the outermost attachment of the cytoplasmic membrane to the glass surface and move toward the center of the cell in a well defined wave front. These waves appear with a rythmic frequency in the flattened metabolic cells and are readily visible until they reach the nuclear zone (A in Figure 3).

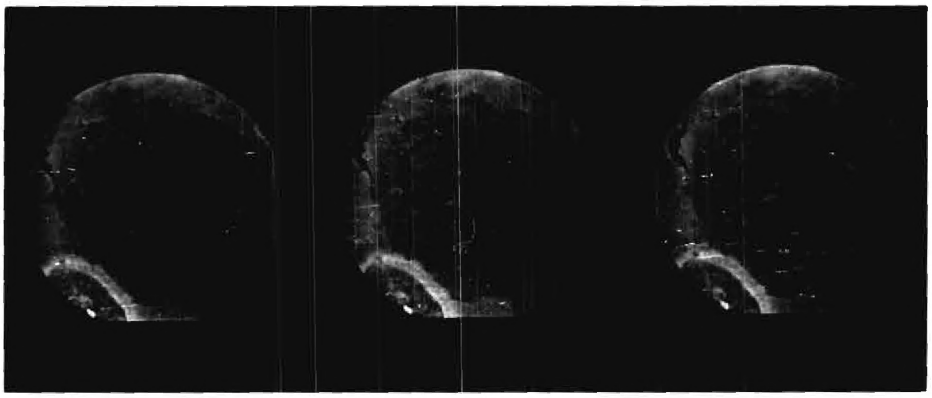
Ozone dosimetry was performed by rinsing the coverslip culture in saline and then exposing to ozone in the dosing bottle (see above). Fresh media was replaced and photographs commenced when the membrane waves reappeared. All preparations were maintained at 37.5 ± 0.5° C.

III. RESULTS

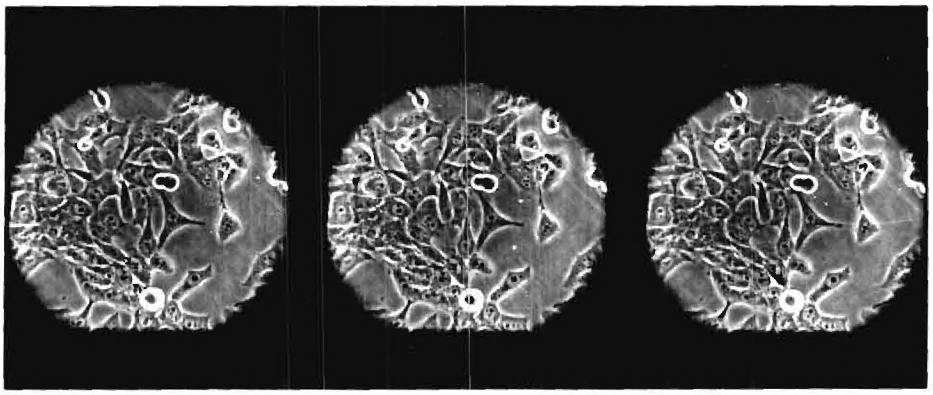
A. Description of Mitosis in Neuroblast Cells

A complete sequence of mitosis in the neuroblast cells of Chorotophaga embryos is presented on the following pages. To the best of our knowledge this is the only such sequence which has been made available. Because the thickness of the specimen precludes effective use of phase contrast optics, these pictures were made using bright field microscopy. The sequence is of the same field in a Chortophaga preparation and covers a period of 208 minutes at 38° C. In the description of the stages which follows, cell A (indicated in the legends which accompany each photograph) is the cell which

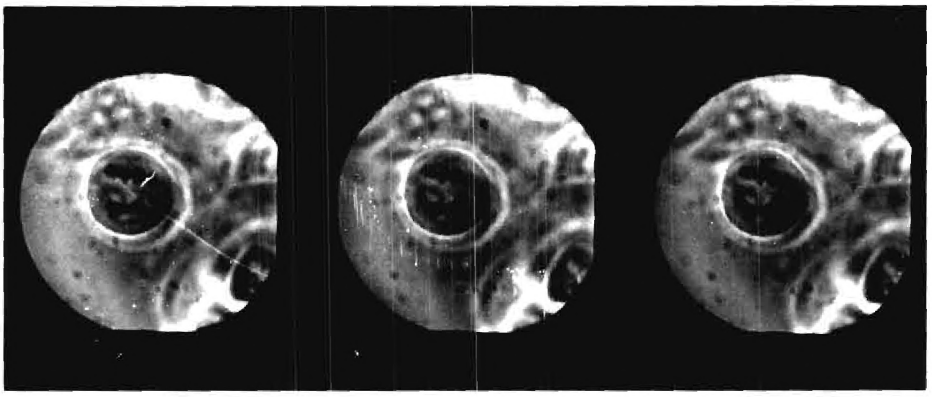
FIGURE 3. Reproductions from 16-mm. negatives of Hela cells in culture: A, a metabolic cell; the flaming of the cell membrane is visible at the upper left of the cell; B, low magnification of cell culture; C, dividing cell.



(A)



(B)



(C)

was maintained in focus. Other cells, however, are described as they appear in focus to demonstrate various other stages of division. A description of each figure follows:

Figure 4

At the initiation of this sequence Cell A is in mid prophase. This stage is described as beginning "when the chromosome threads can be followed from one part of the nucleus to another."[10] In all stages of prophase the nucleus has a homogeneous core, approximating a somewhat doughnut shape although it is not visible in this photograph because the focus of the microscope is above the core area. This stage of mitosis lasts 16 minutes and ends when the chromosomes are considerably more condensed. Cell B is in late prophase, the next stage of division.

Figure 5

Twelve minutes later Cell A is now in late prophase. This stage is described as beginning "when about seven chromosomes are seen near the nuclear membrane in one-fourth of the optical section of the nucleus." Also in this stage the nuclear membrane or the outline of the nucleus becomes irregular as it conforms to the shape of the increased size of the chromosomes. Cell B shown in this plate is slightly more advanced than Cell A and the entire cell has begun to round up and has lost its hemispherical shape. This indicates the end of very late prophase. The duration of late prophase (including very late prophase) is 16 minutes. Cell C is in early prophase.

Figure 6

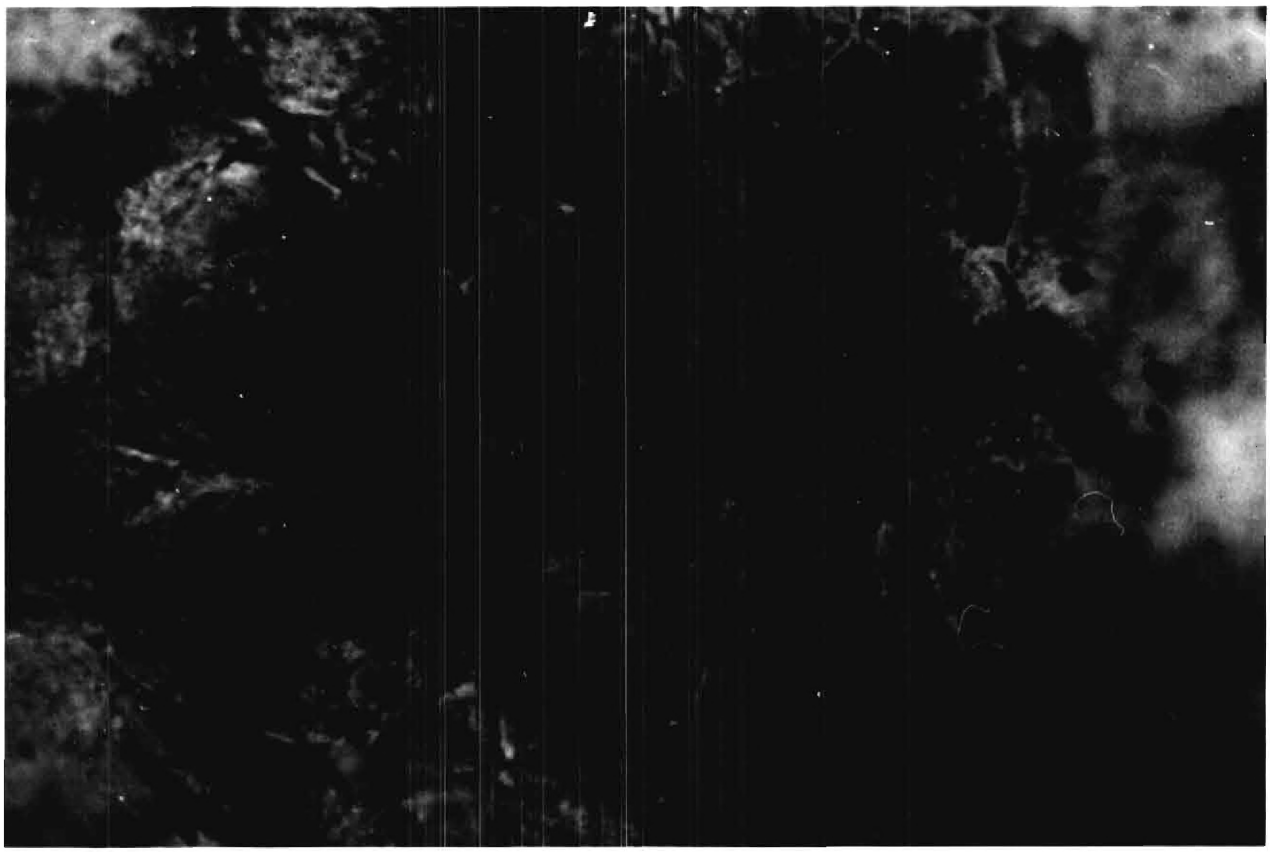
Cell A is in the terminal phase of late prophase and the nuclear membrane appears to be in the process of breaking down or disappearing in the lower

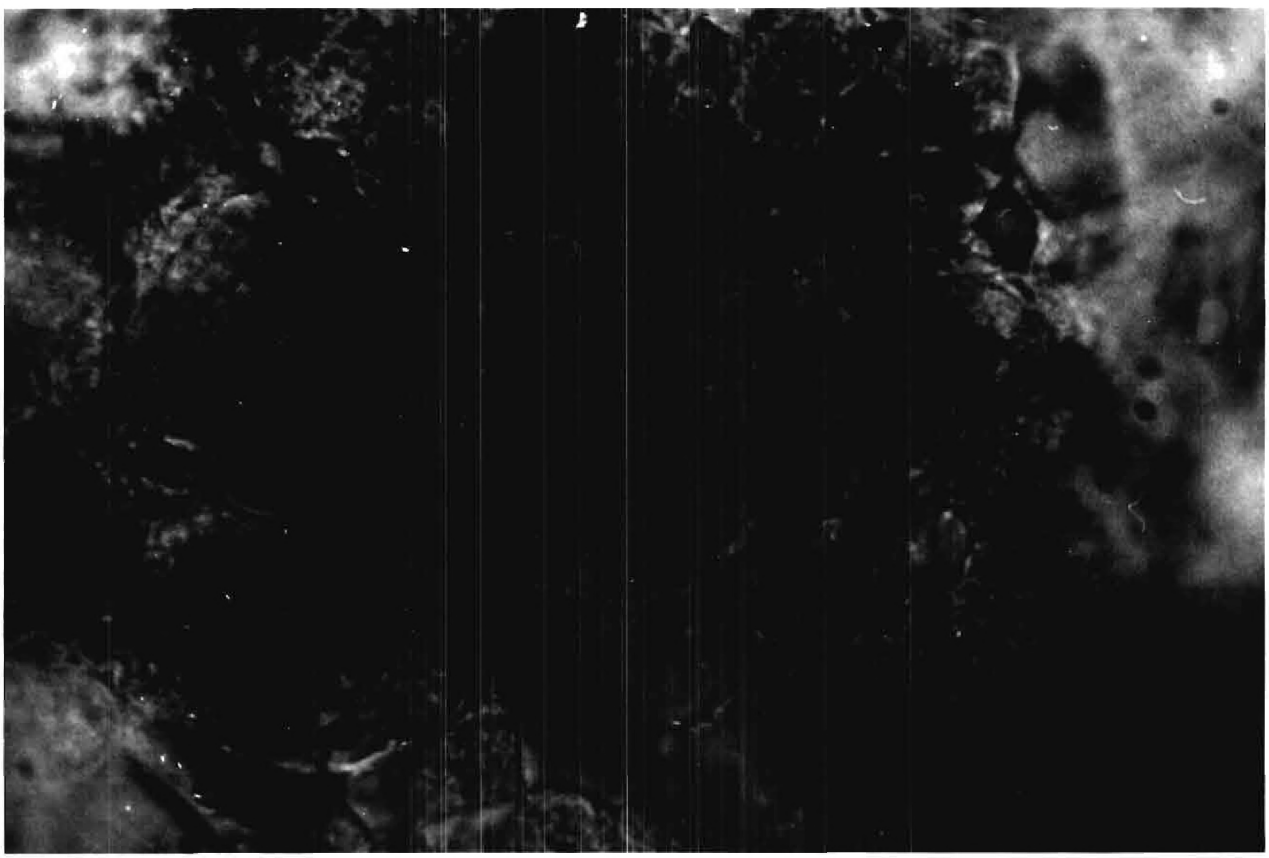
- - - - -

10. W. St. Amand, Rad. Res. 5, 65 (1956).

FIGURE 4. At beginning of sequence, Cell A in mid prophase Cell B in late prophase.

FIGURE 5. Twelve minutes later, Cell A is in late prophase nuclear outline becoming irregular due to the protrusion of the chromosomes. Cell B still in late prophase. Cell C in early prophase.





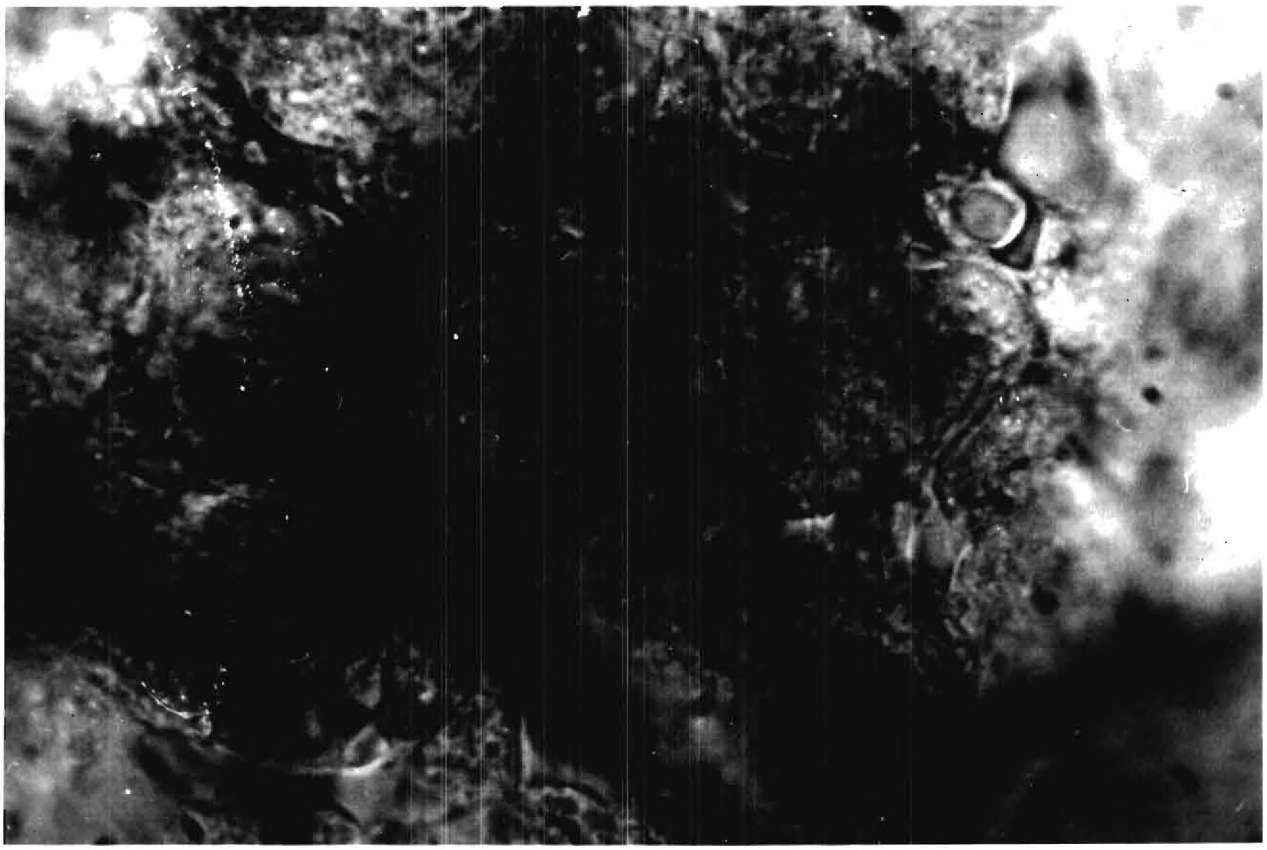
right hand quadrant of the nucleus. The late prophase stage ends with the disappearance of the nuclear membrane. Cell B, while not in good focus, is in the prometaphase stage--the nuclear membrane having disappeared entirely and the chromosomes not yet having become orientated on the equatorial plate.

Figure 7

Cell A, having passed through prometaphase or the period from the disappearance of the nuclear membrane to the orientation of the chromosomes on the equatorial plate, is now in the metaphase stage. The prometaphase condition lasts only 4 minutes and has taken place between the photograph of Figure 6 and Figure 7. In the metaphase condition the spindle mechanism has reached maximum development and the centric ends of the chromosomes lie on the equatorial plate. A clear zone is visible in Cell A on both sides of the equatorial plate. This zone remains free of the mitochondria which are visible in the cytoplasm at this stage because of the gelation of the protoplasmic mass. They are obscured in other preparations because of the blurring effect produced by Brownian movement; however, during metaphase the mitochondira are immobilized in the gelated cytoplasm. Cell A represents a side view of the metaphase figure and Cell B a slightly tilted view. A polar view of the metaphase plate presents a typical "wagon wheel" type of metaphase with the central zone free of chromosomes. Cell C clearly shows the central core through a prophase nucleus which is evident because the plane of focus intersects the core area. Also, in this figure, Cell B shows the concave-convex shape of the prophase cell. This is typical of these cells and enables one to anticipate the direction of the next mitosis because the daughter cells are budded alternately from each lobe of the concave surface.

FIGURE 6. Thirteen minutes later Cell A still in late prophase, Cell B in prometaphase; the nuclear membrane having disappeared and the cell assuming a spherical shape.

FIGURE 7. Five minutes later, Cell A has passed through prometaphase and is in the early stages of metaphase. The chromosomes are becoming orientated on the equatorial plate and are almost at their maximum constriction (minimum length). The cell is spherical. Cell B is slightly more advanced in metaphase. Cell C in mid prophase; this nucleus is doughnut shaped with the focus intersecting the nuclear core. Cell D is in late prophase and demonstrates the concave-convex cell shape.



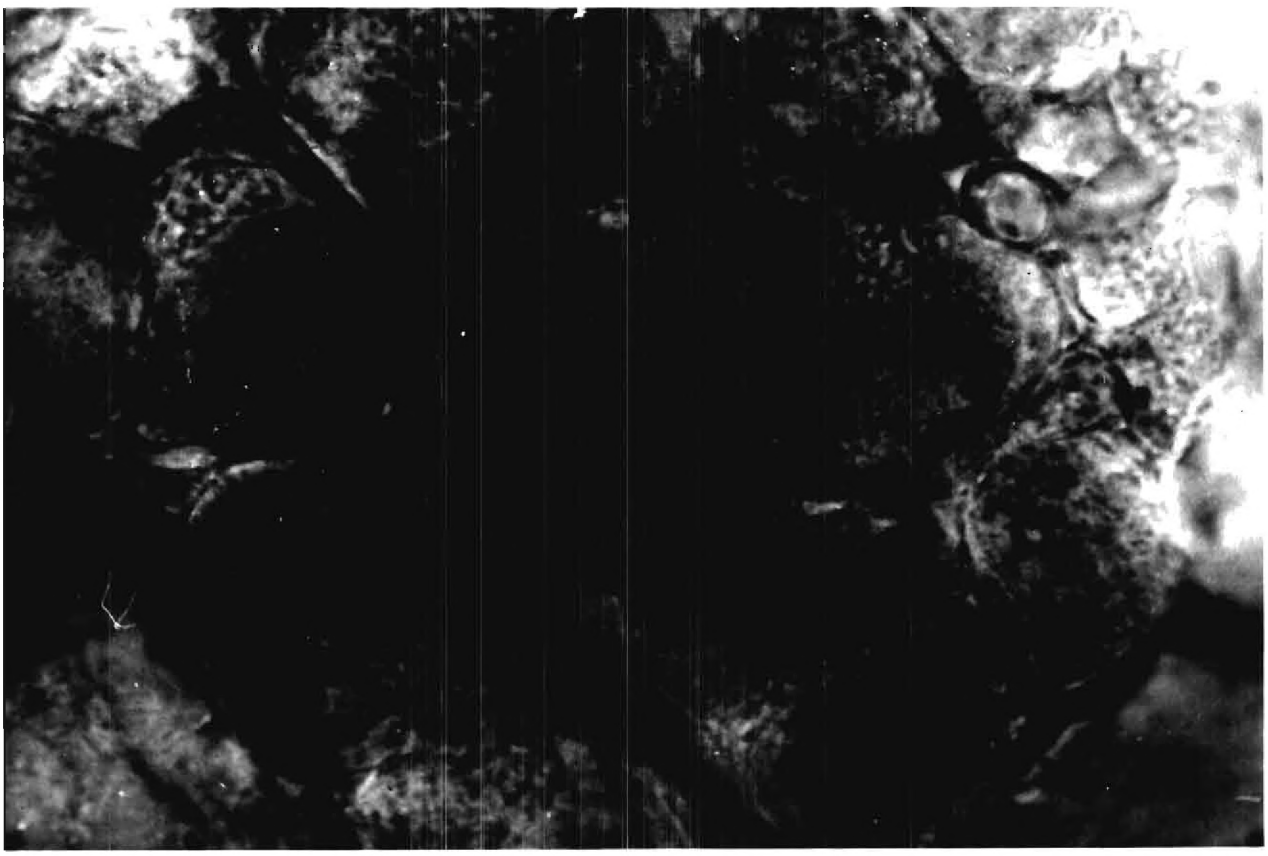


Figure 8

Cell A is still in metaphase; the cleared ozone or centrosome is clearly evident in the lower right hand corner of the cell. The mitochondria are still visible as discrete bodies. Cell B, in which the focus is at the distal ends of the chromosomes, demonstrates the double nature of the chromosome bodies. This indicates the initiation of anaphase. The metaphase stage has a duration of 9 minutes. Cell C has progressed to mid prophase.

Figure 9

Cell A has begun anaphase, initiated by the separation of the proximal ends of the chromatids. The double nature of the chromosomes may be seen by viewing their distal ends.

Cell B is in a more advanced stage of anaphase, the proximal ends having achieved a considerable separation.

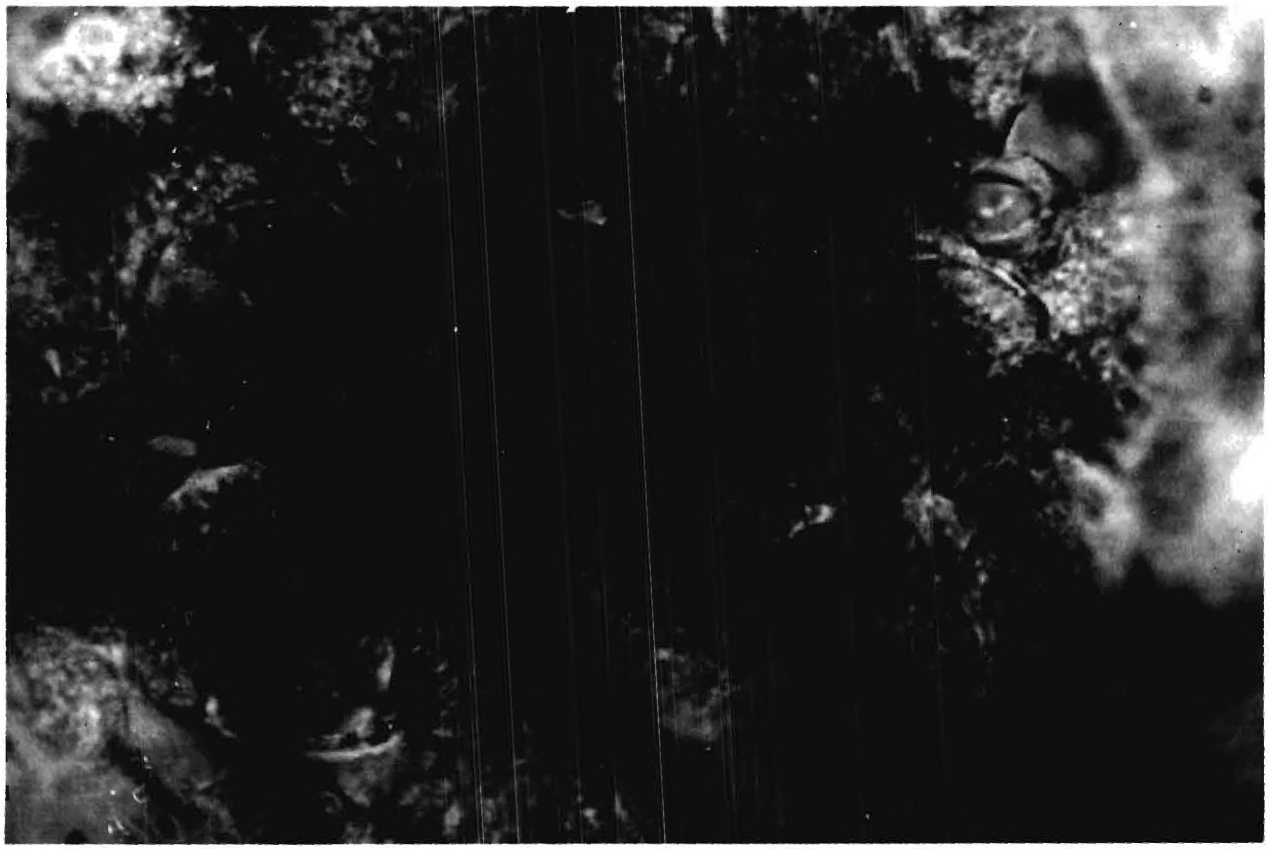
Cell E is of particular interest because it demonstrates very clearly the nuclear granules visible during the late telophase stage. The smaller size of the nucleus is also evident.

Figure 10

Two minutes later the anaphase figures have considerably advanced, the chromosomes being well separated in both Cell A and in Cell B, which is more advanced than Cell A; the left hand set of chromosomes have migrated to the cell membrane while the right hand group of chromosomes has become more centrally orientated in the cell. In both of these cells the set of chromosomes to the right will remain in the mother cell, and the left set will push against the cell membrane and bud out into the daughter ganglion cell. In Cell A, most of the chromosomes have completely separated; however, there is one, a long

FIGURE 8. Five minutes later Cell A in metaphase; Cell B at end of metaphase; end view of chromosomes demonstrates double nature. Cell C in mid prophase. Cell E in late telophase.

FIGURE 9. Five minutes later both Cells A and B in early anaphase. Cell C in late prophase. Cell E shows late telophase nuclear granules, focus at periphery of nuclear membrane.



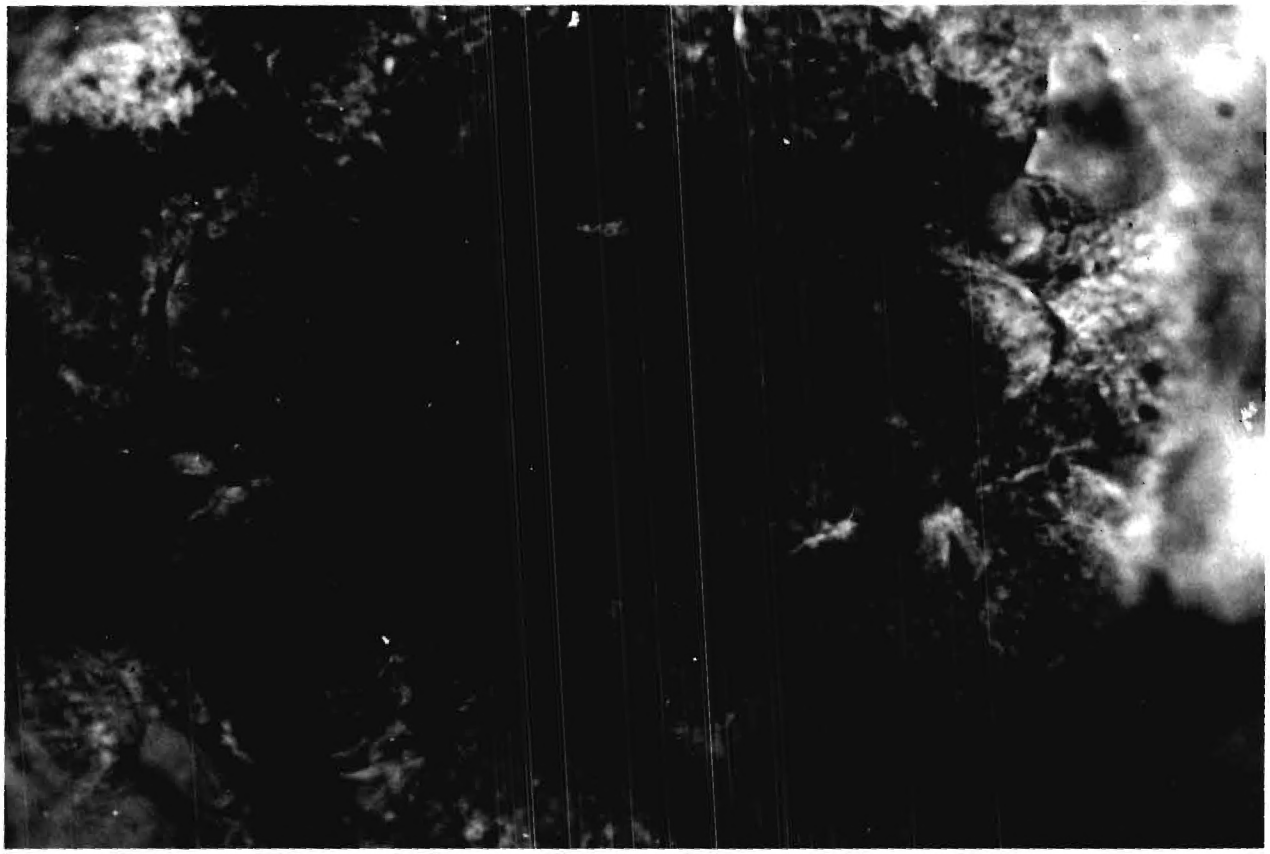
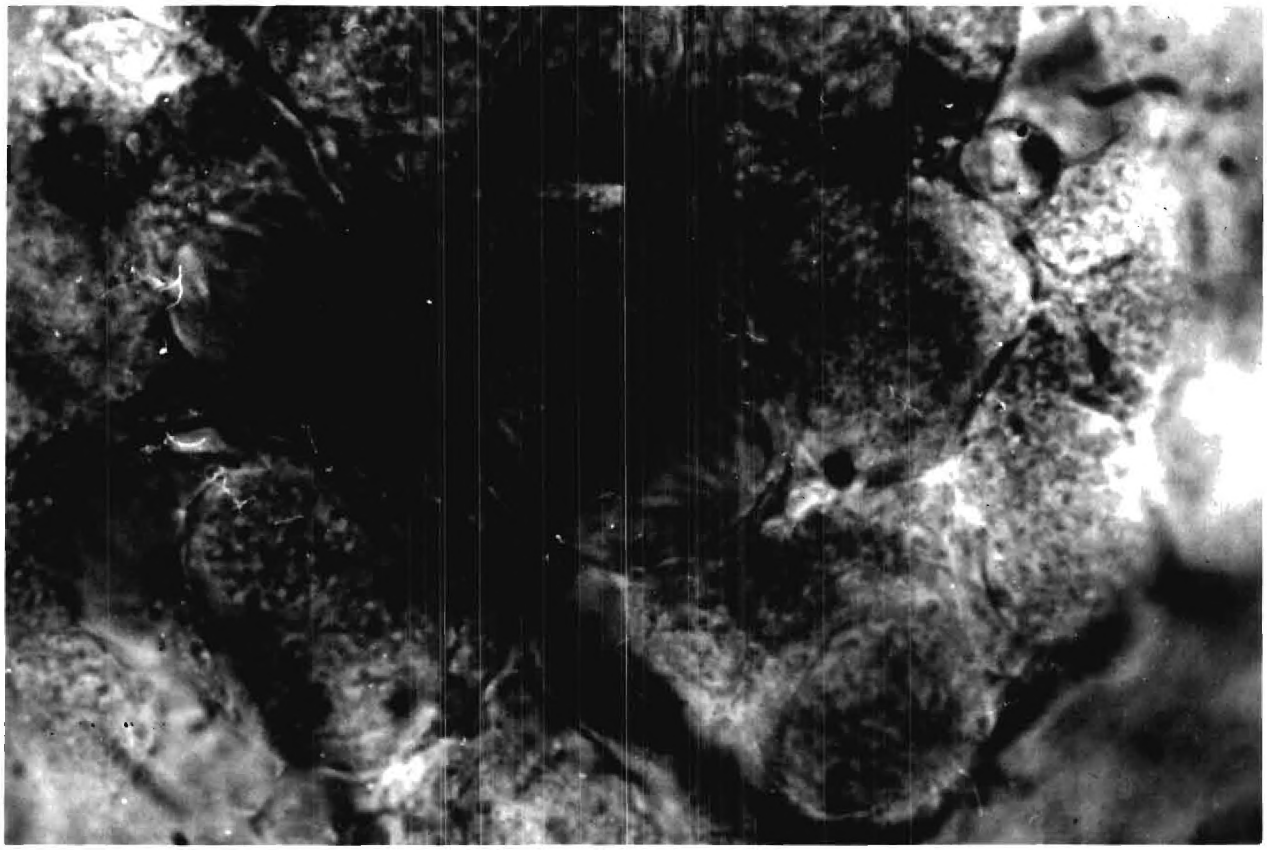
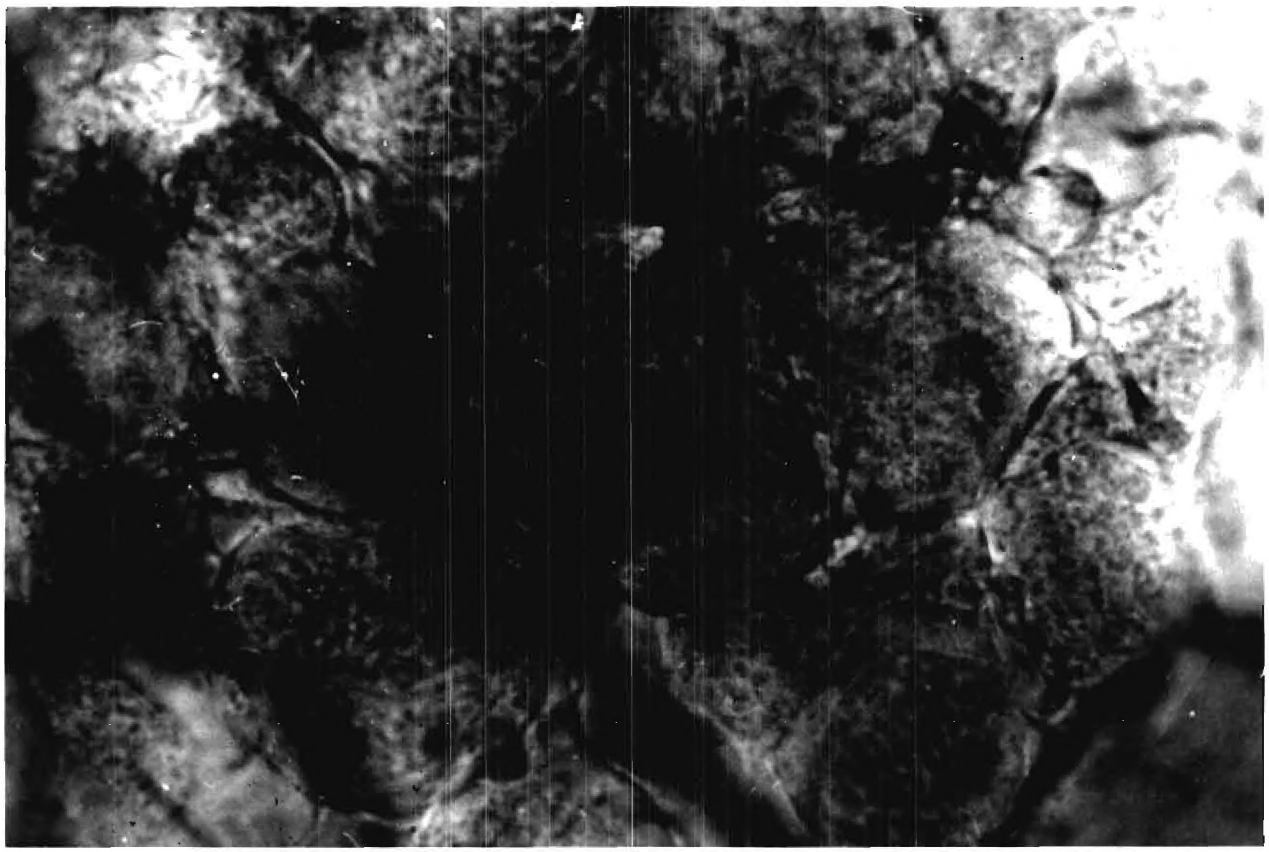


FIGURE 10. Two minutes later both Cells A and B in anaphase; in both cells daughter chromosomes are pressed against cell membrane at point where daughter will bud, while maternal chromosomes are orientated more centrally in what will become mother cell. Cell C in late prophase.

FIGURE 11. One minute later Cells A and B in late anaphase. In Cell B the daughter chromosomes are beginning to extrude into a cell. Cell C in late prophase. Cell D in late prophase.





pair, in the upper right of the cell in which two sister chromosomes are apparently still in conjunction.

Figure 11

This photograph taken one minute later demonstrates the rapidity with which the anaphase stage is accomplished. The separation of the chromosomes during anaphase is very rapid, being completed when the cleavage furrow appears to be complete. This entire stage of mitosis requires 9 minutes. Cell A demonstrates the budding out of that portion of the cell which will become the daughter ganglion cell, and Cell B demonstrates the uneven division of the cell. In Cell B, as in Cell A, the central orientation of the chromosomes in the mother cell is plainly shown in contrast to the orientation of the daughter chromosomes in the budding ganglion cell.

Figure 12

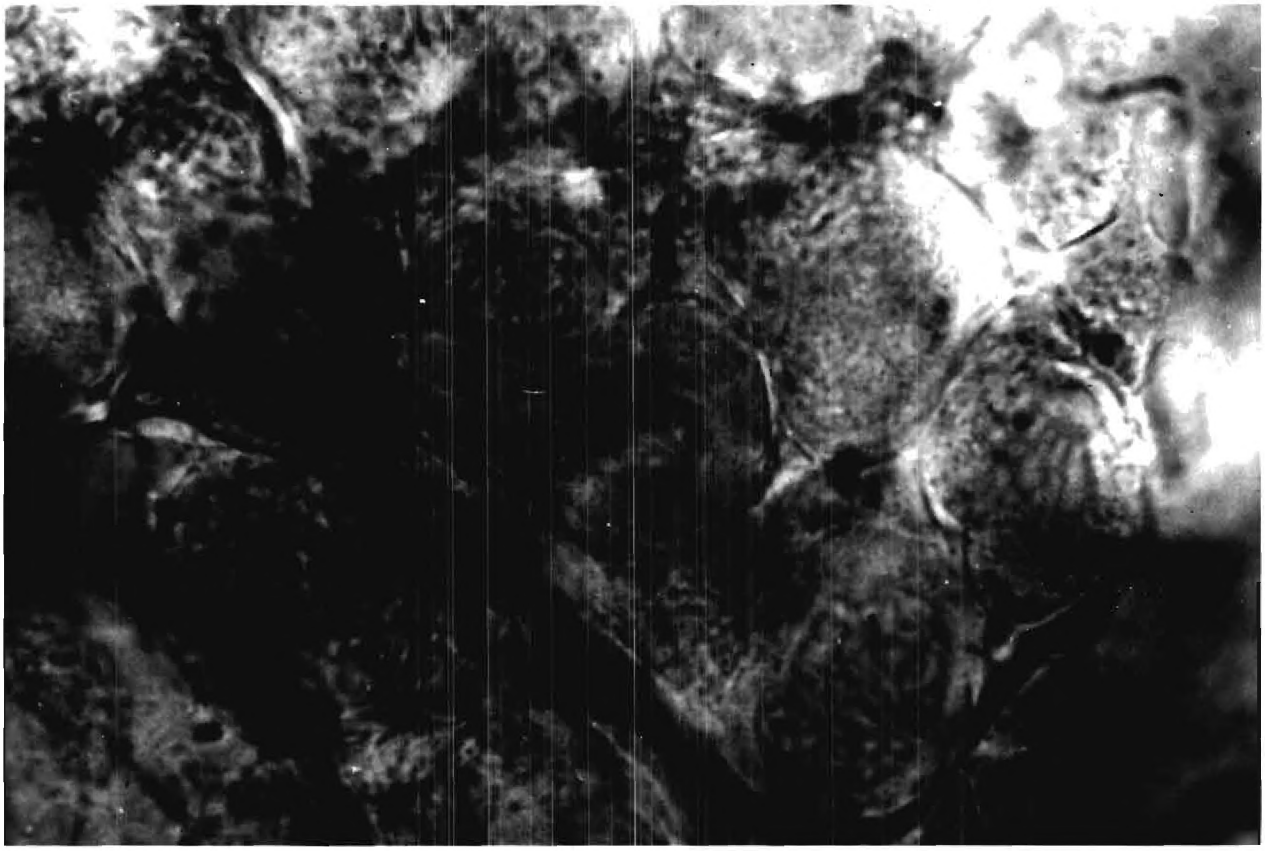
Cell A is in late anaphase, the daughter cell having reached its maximum extrusion and the cleavage furrow having begun to form between the two cells. After the cleavage furrow is complete, the daughter cell comes back and rests tightly against the mother cell. Cell B, which is slightly advanced of Cell A, has completed anaphase, the cleavage furrow having completely separated the two cells and the daughter ganglion cell having returned to a position adjacent to the mother cell.

Figure 13

Both Cell A and Cell B are in early telophase. This stage commences when the cleavage furrow appears complete and terminates when the nucleoli become visible. It is a very short stage, of 6 minutes duration.

FIGURE 12. Two minutes later Cell A in late anaphase, Cell B in early telophase. Cells C and D in late prophase.

FIGURE 13. Two minutes later both Cells A and B in early telophase. Cell E in late telophase.



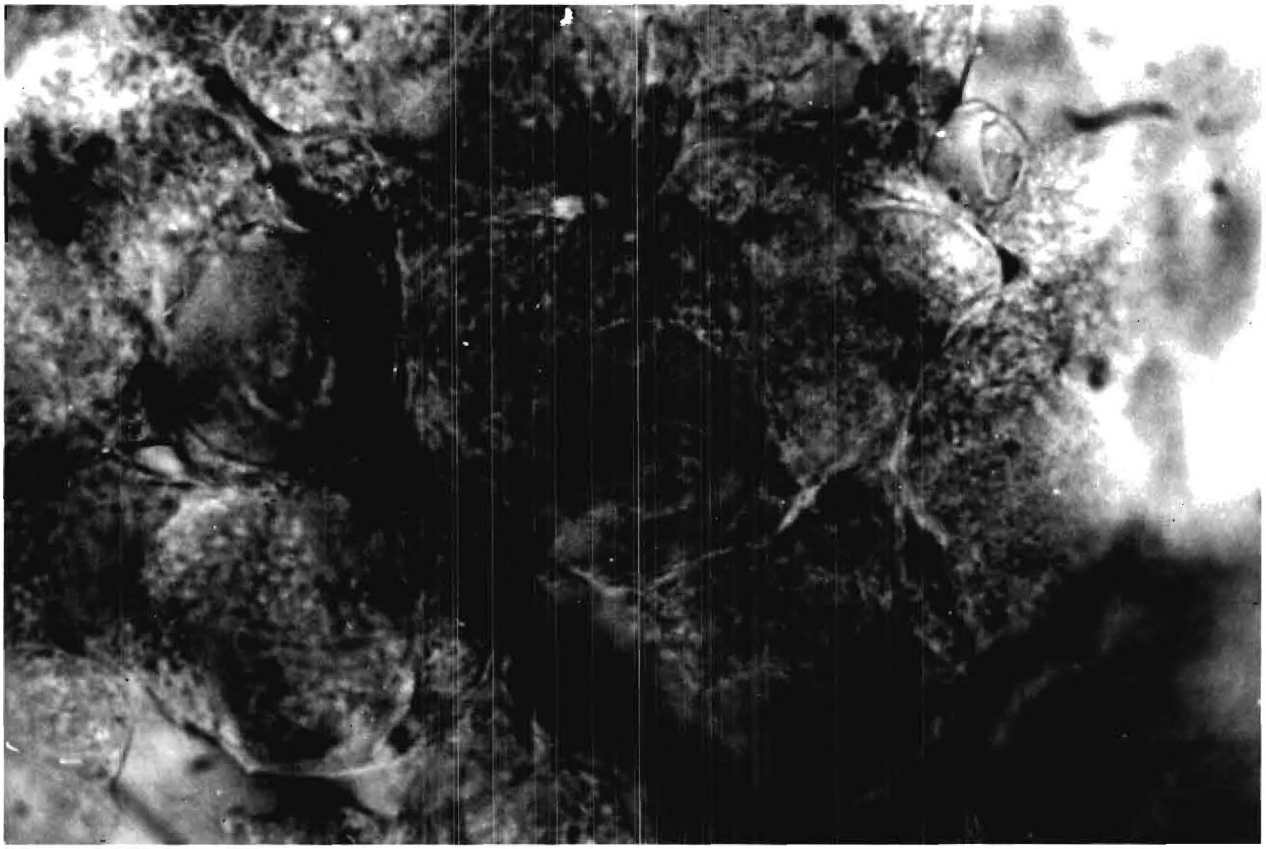


Figure 14

Three minutes later than the previous picture Cell A is still in early telophase. The nucleus, however, has rounded up considerably into a definitely formed body. Cell E again is in late telophase.

Figure 15

Cell A is in mid telophase as is Cell B. The nucleoli have become visible in the nucleus as small refractive spheres; both cells are still nearly spherical.

Figure 16

After 4 minutes, the nucleoli have increased very sharply in size and are both spherical; this is more evident in Cell B than in Cell A, which is slightly out of focus for the nucleoli. Cell A indicates that the nucleus has increased sharply in size during the 4 minutes elapsing since the last photograph and also that the nuclear granules are becoming evident as the chromosome bodies themselves become more diffuse.

Figure 17

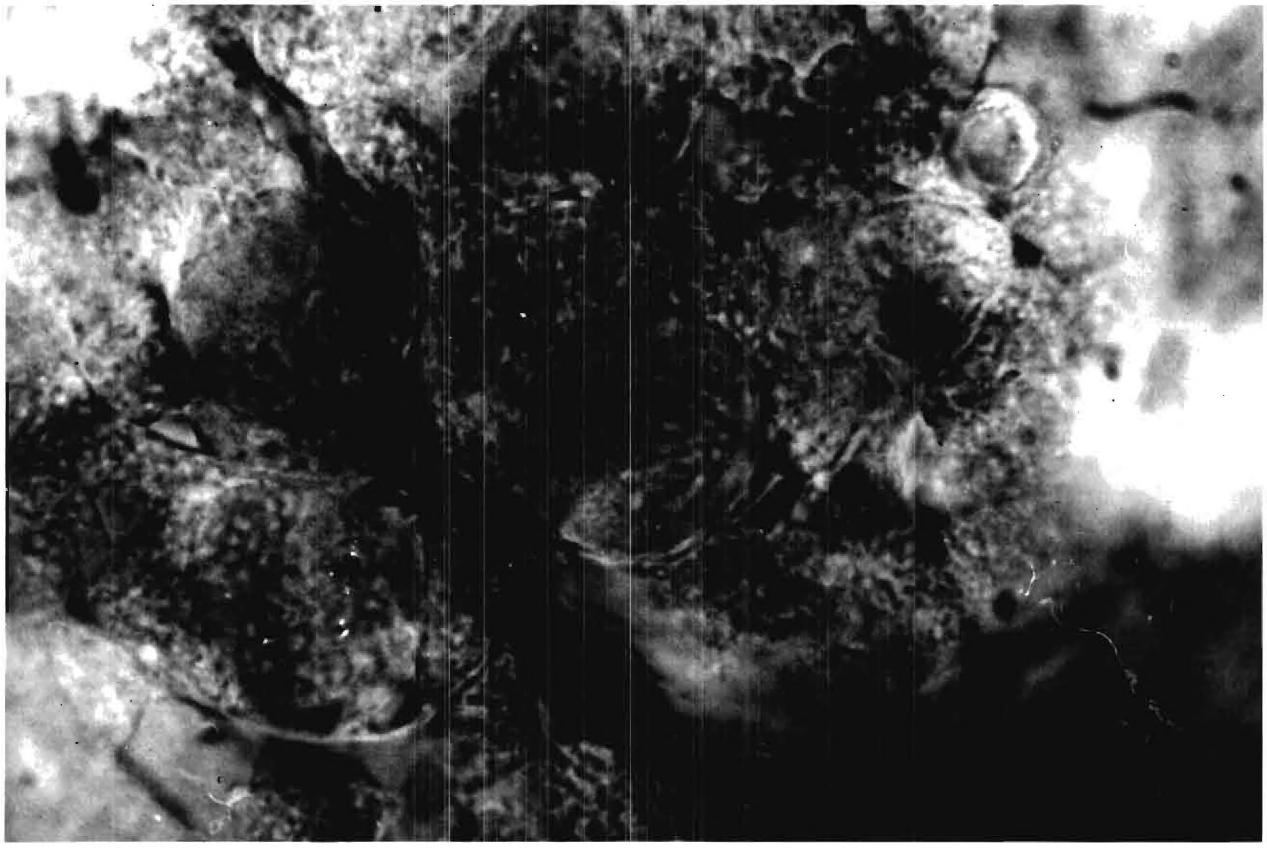
In this photograph, Cells A and B are still in mid telophase. The nucleoli have obtained their maximum spherical size. It appears that the nucleus is not a great deal larger than in the previous photograph. The mid telophase stage begins when the nucleoli become visible and ends when the nucleoli lose their spherical shape. The duration of this stage is 18 minutes.

Figure 18

Eight minutes later both Cell A and Cell B are in late telophase; the nucleoli have lost their spherical shape and have become irregular in outline. As the cells progress through late telophase the nucleoli will become

FIGURE 14. After 3 minutes Cells A and B still in early telophase. Cell E in late telophase.

FIGURE 15. Three minutes later Cells A and B in mid telophase, nucleoli have become visible as small refractive spheres. Cell E in late telophase.



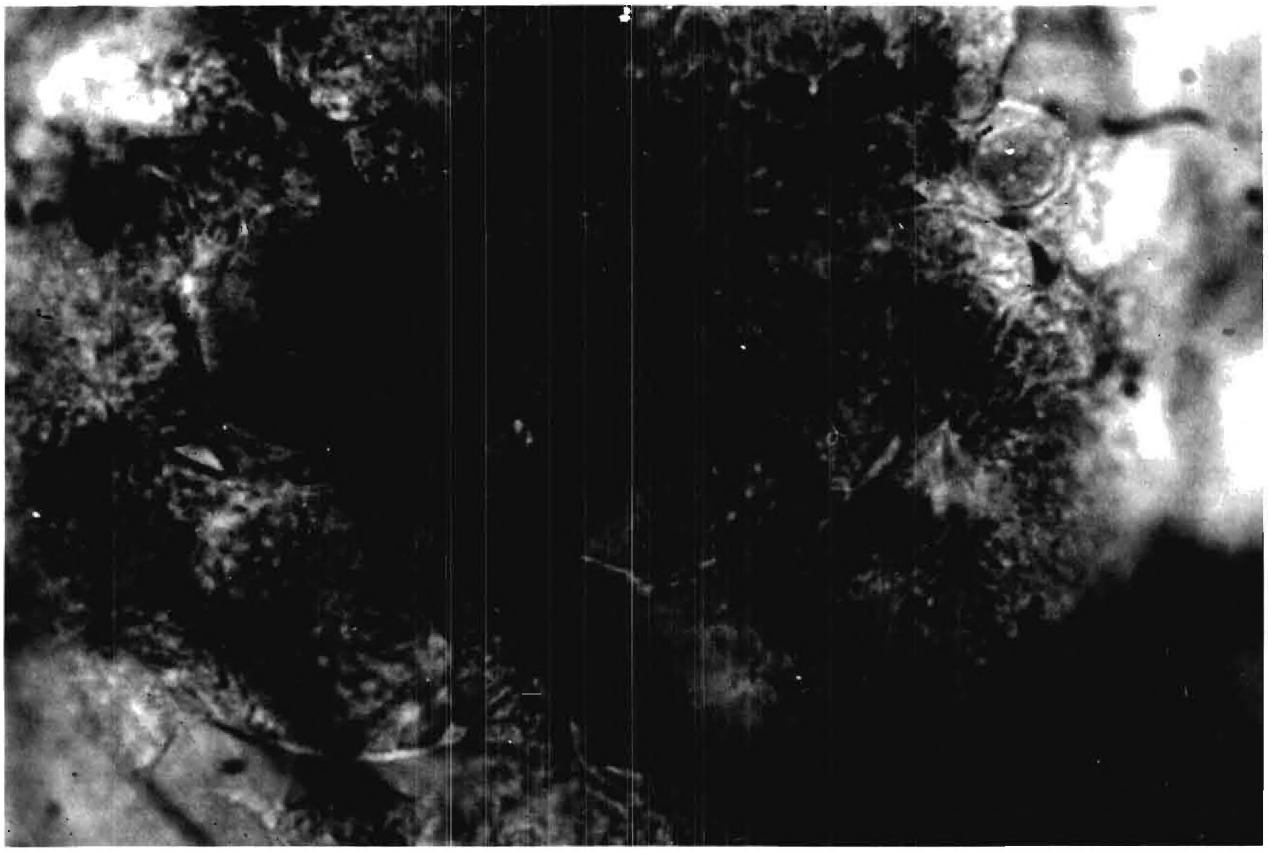
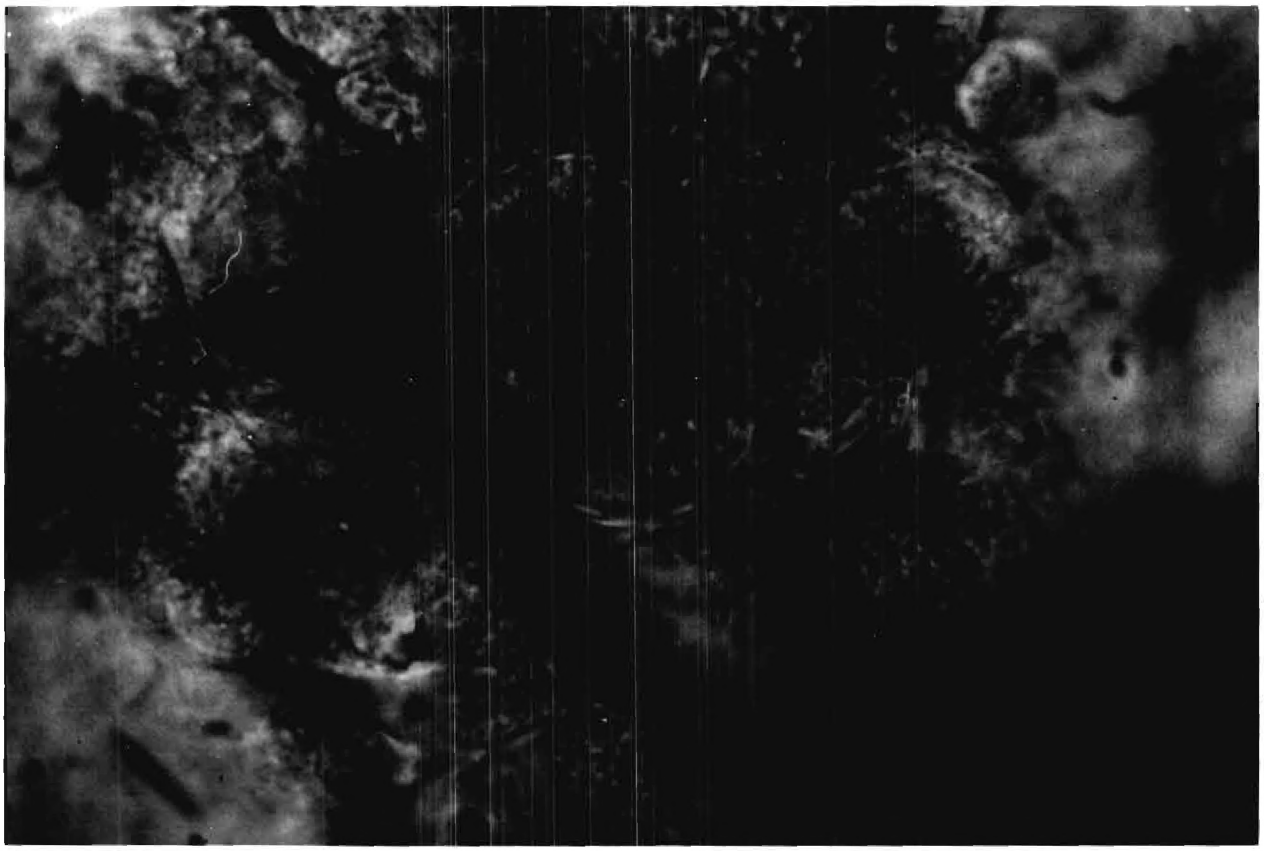


FIGURE 16. Four minutes later Cells A and B in mid telophase, nucleoli increasing in size.

FIGURE 17. Eight minutes later Cells A and B in mid telophase, nucleoli have attained maximum spherical size.



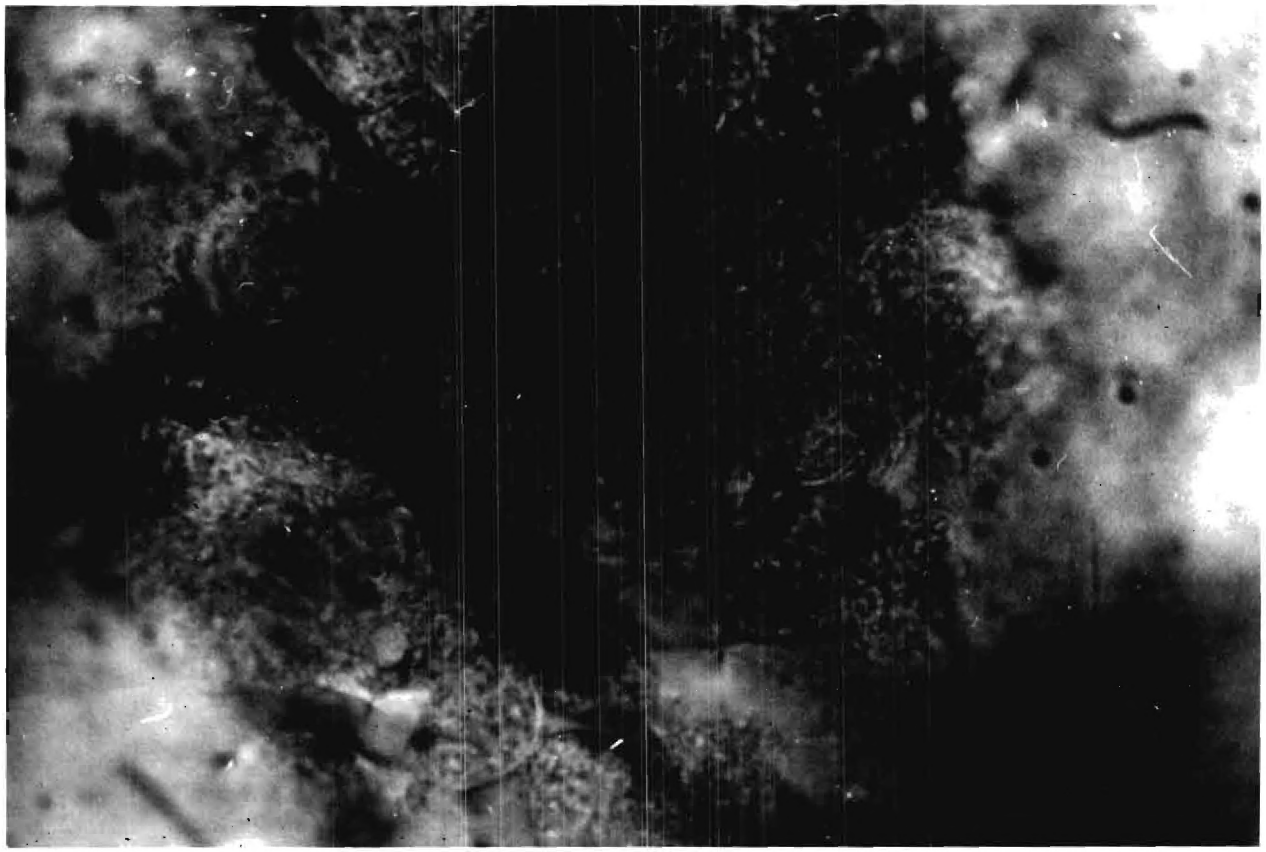
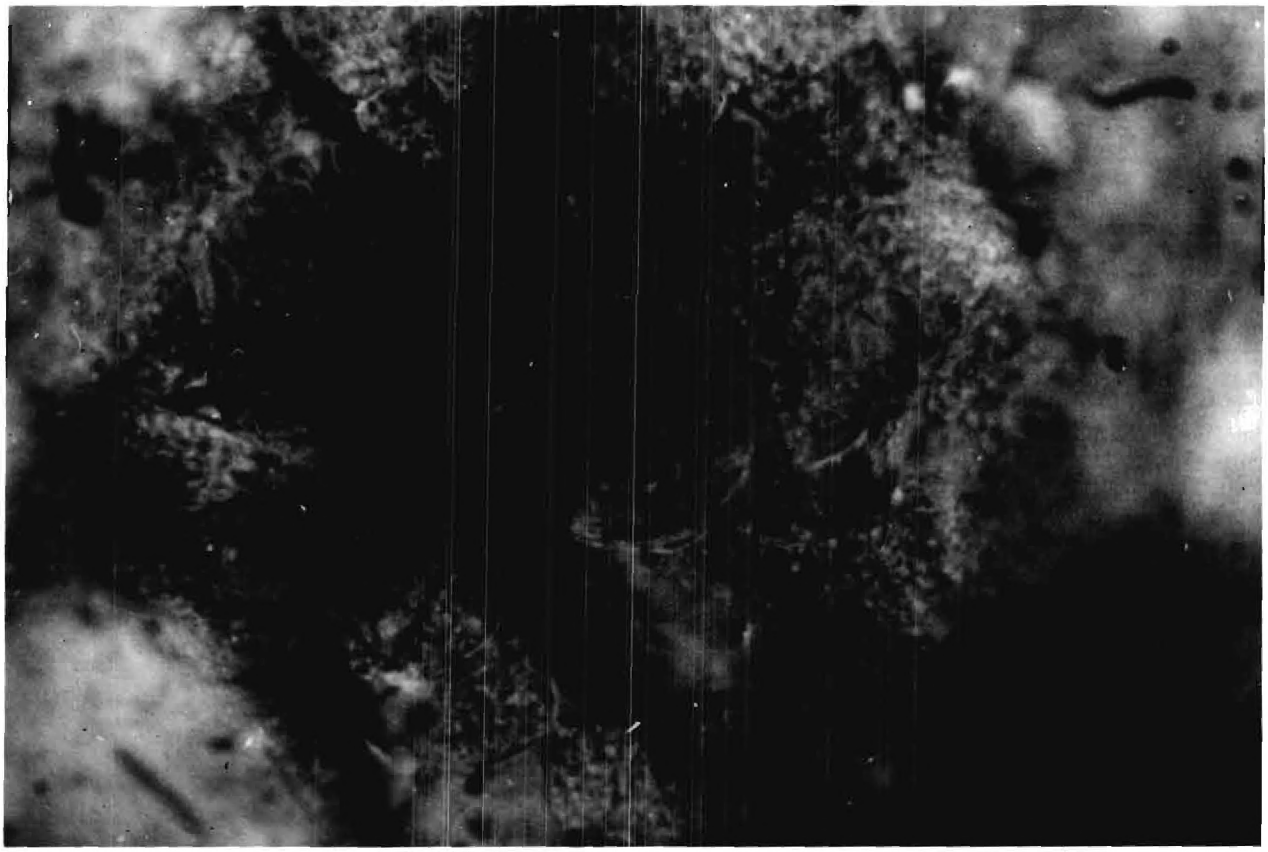
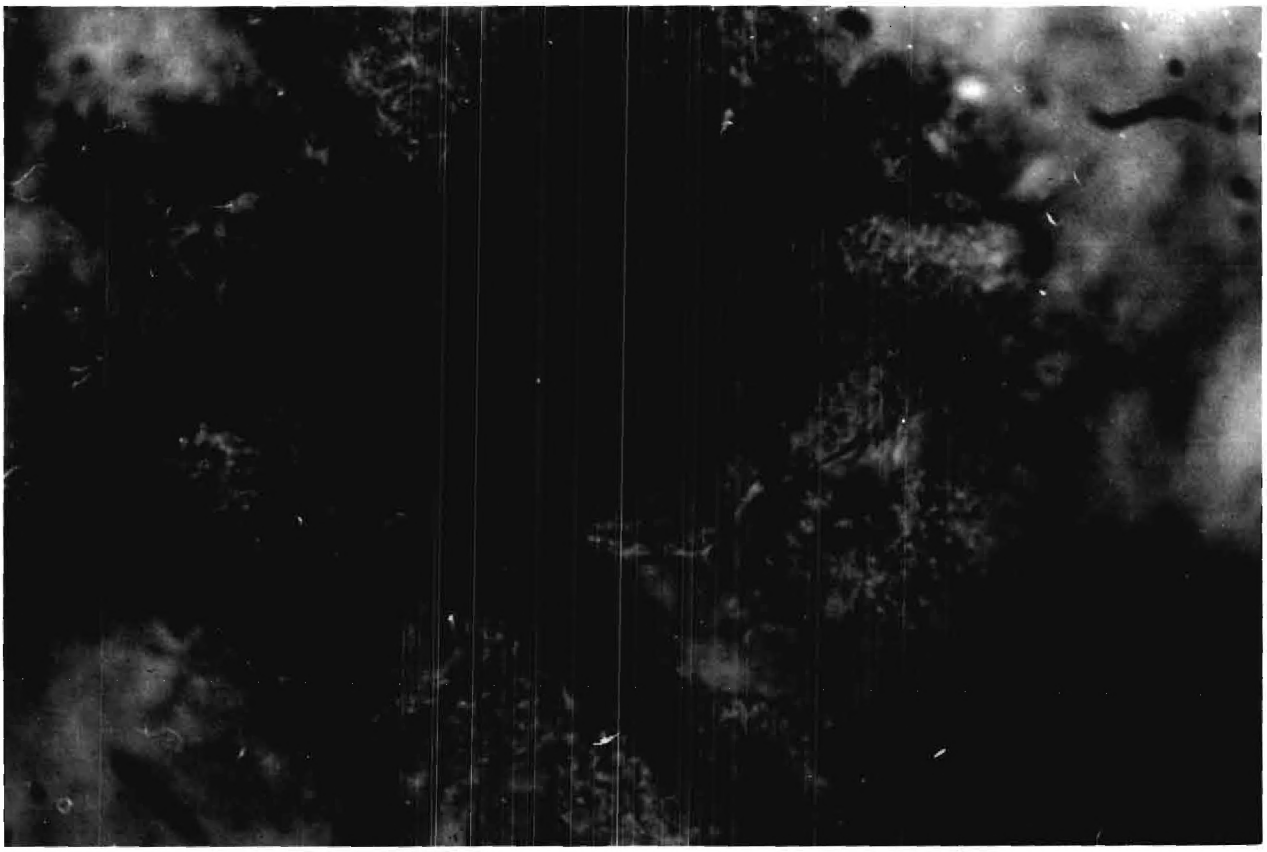


FIGURE 18. Eight minutes later Cells A and B in late telophase. Cell E in interphase.

FIGURE 19. Eleven minutes later Cells A and B in late telophase. Cell C in early telophase.





progressively more irregular and will eventually be very difficult to identify. Late prophase begins with the loss of spherical form of the nucleoli and continues until the chromatin granules are scattered in a homogeneous nuclear background, at which time the chromosome bodies or threads are imperceptible.

Figure 19

In this photograph Cell A is in late telophase, a stage of 33 minutes duration. Cell C is in early telophase; this particular cell was first identified 76 minutes ago when it was in early prophase.

Figure 20

Cell A is still in late telophase and shows the progressive diffusion of the chromatin threads and the persistence of the nuclear granules.

Figure 21

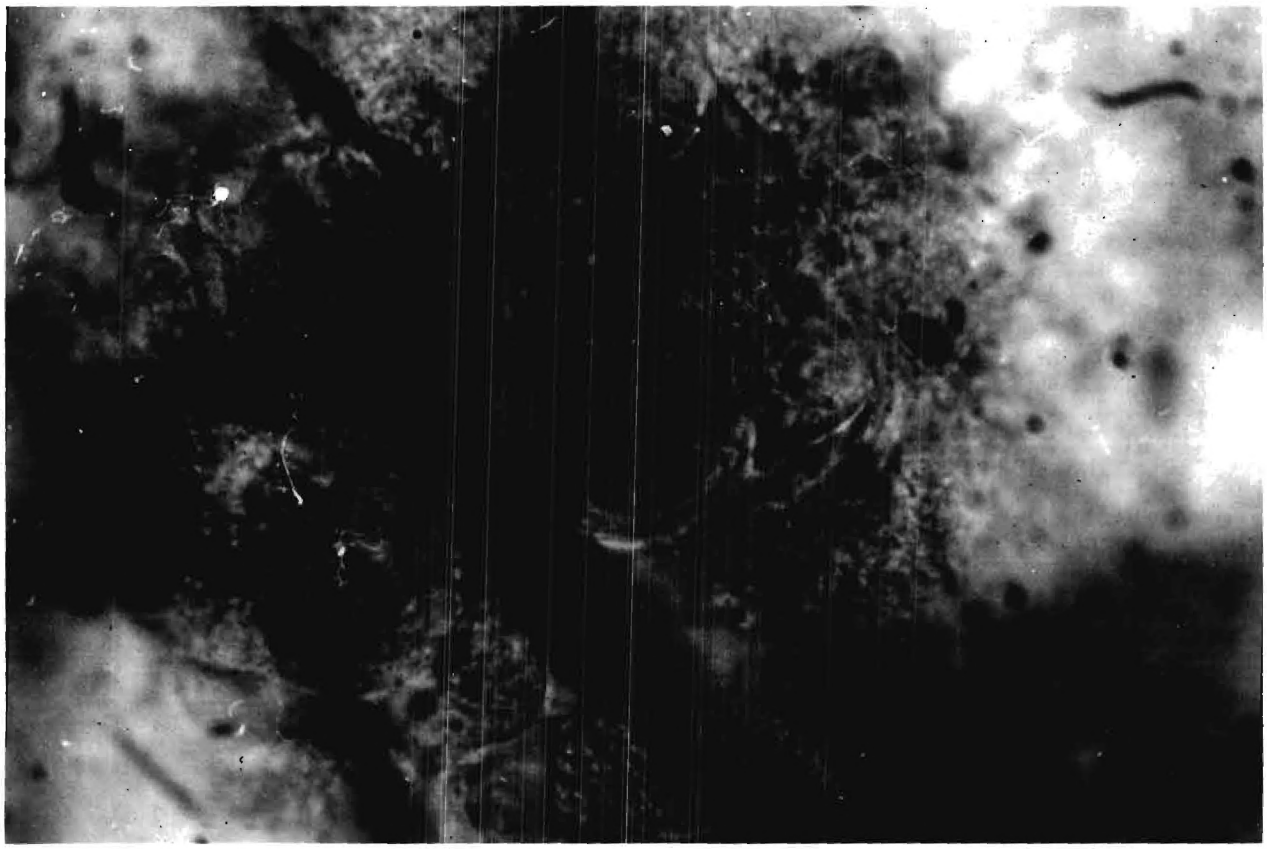
Cell A is now in interphase; the chromatin granules appear scattered throughout the homogeneous nucleus. Actually the nuclear granules lie immediately adjacent to the nuclear membrane and when the nucleus is in optical cross section, as in these photographs, only a few of the nuclear granules are visible, mostly those around the periphery of the nucleus. However, if the preparation is focused up or down so as to be brought into optical alinement, the upper or lower part of the nearly spherical nucleus (as was seen in Cell E of Figure 9), then the granules become visible. The interphase stage begins with the above description and continues for 27 minutes, until barely visible threads appear among the nuclear granules.

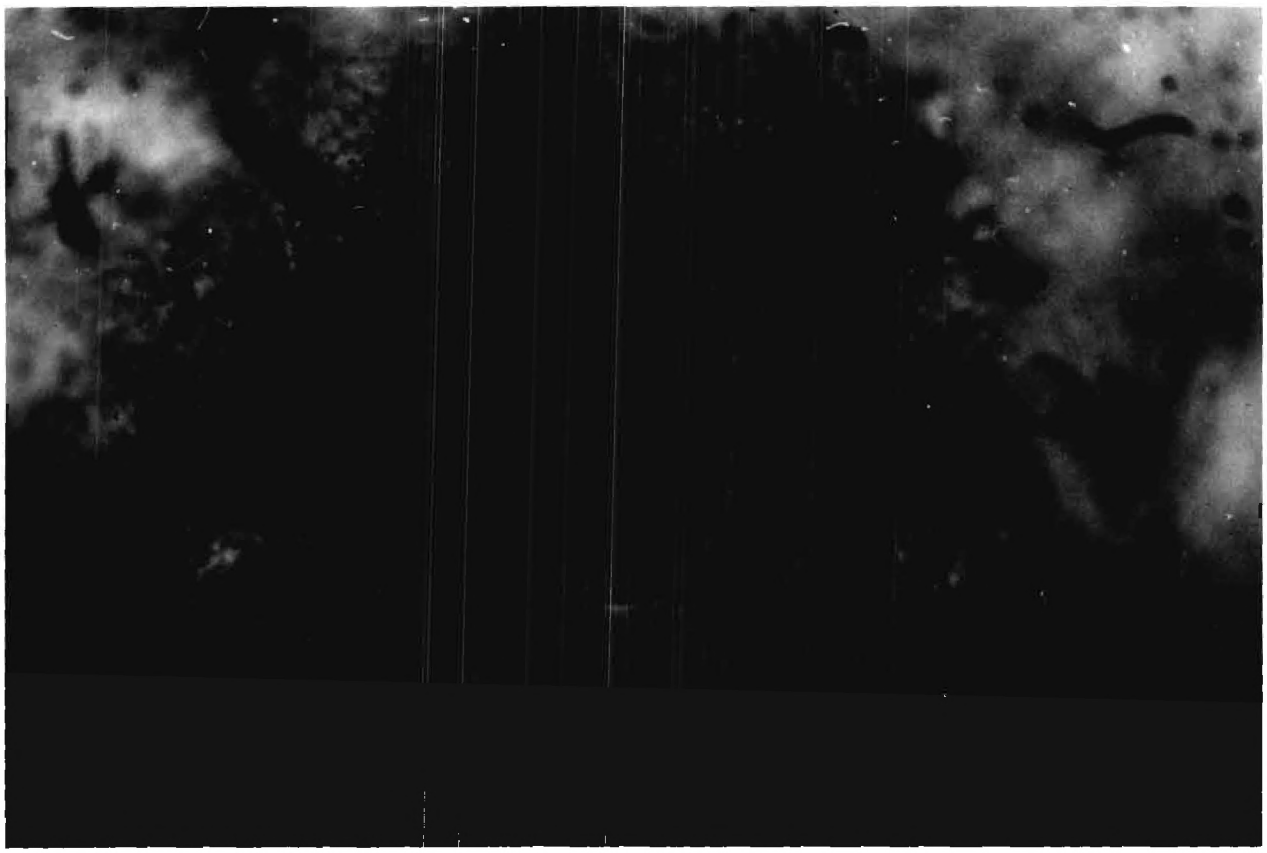
Figure 22

In this photograph very fine threads may be detected traversing through

FIGURE 20. Twelve minutes later Cell A in late telophase.

FIGURE 21. Twelve minutes later Cell A in interphase, linear arrangement of nuclear granules no longer evident.





the body of the nucleus. The nuclear granules, which are not in good focus in this photograph, are considerably smaller than a few minutes previous. This is very early prophase and lasts 24 minutes. The nucleus has increased considerably in size during the interphase stage.

Figure 23

Cell A is in early prophase, the nuclear granules have disappeared, and the entire nucleus is filled with fine threads. It is not possible to follow an individual thread for any distance through the nucleus, but the nucleus gives the appearance of being uniformly filled with the fine chromatin threads. Early prophase begins when the nuclear granules disappear and ends when the chromosome threads become condensed enough so they may be followed from one part of the nucleus to another. Early prophase represents the mitotic stage of greatest duration, lasting 46 minutes.

Figure 24

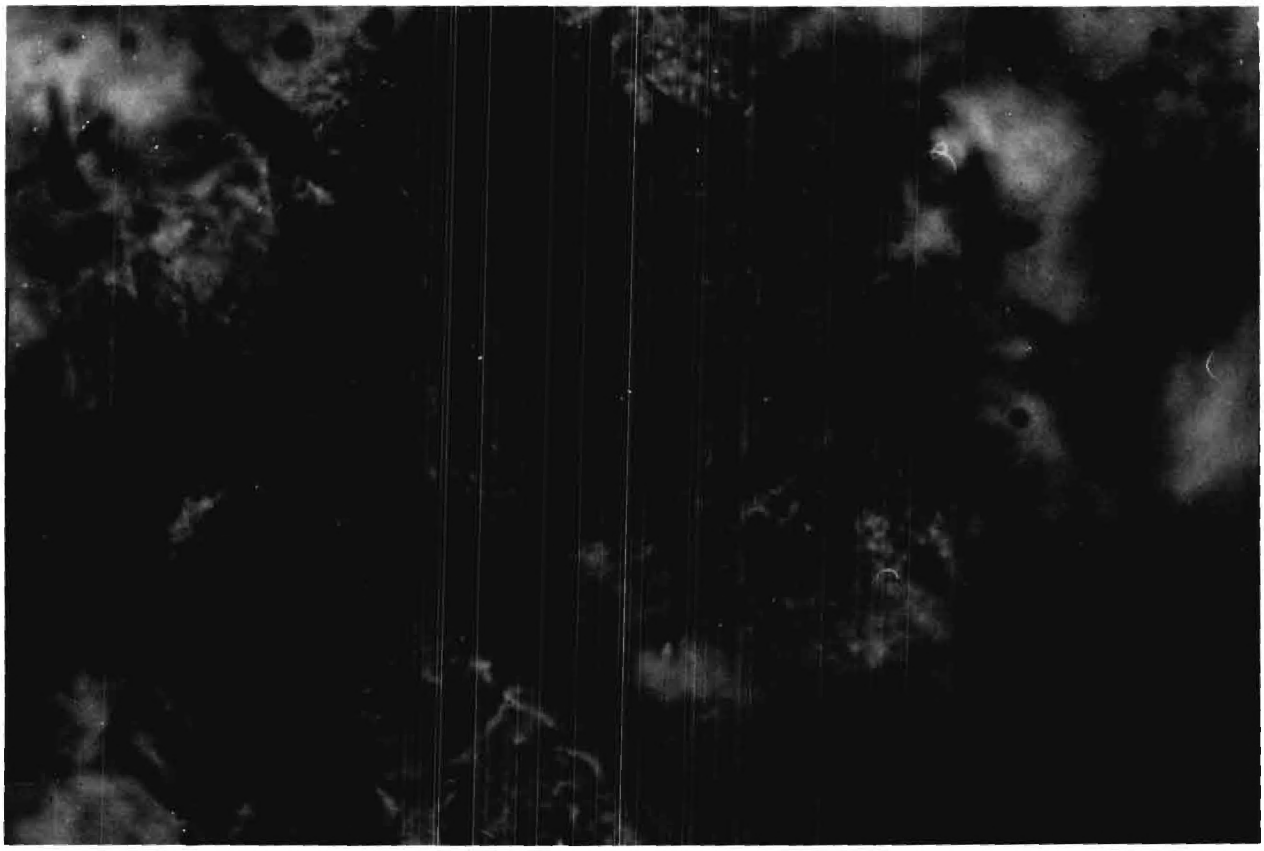
Cell A is still in early prophase; the chromatin threads have become more prominent.

Figure 25

Cell A is now in mid prophase, the chromosomes are now distinct enough so that they may be followed from one part of the nucleus to another. During the early prophase there was a considerable increase in the size of the nucleus as viewed in optical cross section. This photograph terminates the sequence of figures and represents a time 208 minutes after the first picture. The cell represented in this figure is in the same stage as shown in figure 4, which was the beginning of this sequence.

FIGURE 22. Twenty-five minutes later Cell A in very early prophase.

FIGURE 23. Thirty-five minutes later Cell A in early prophase.



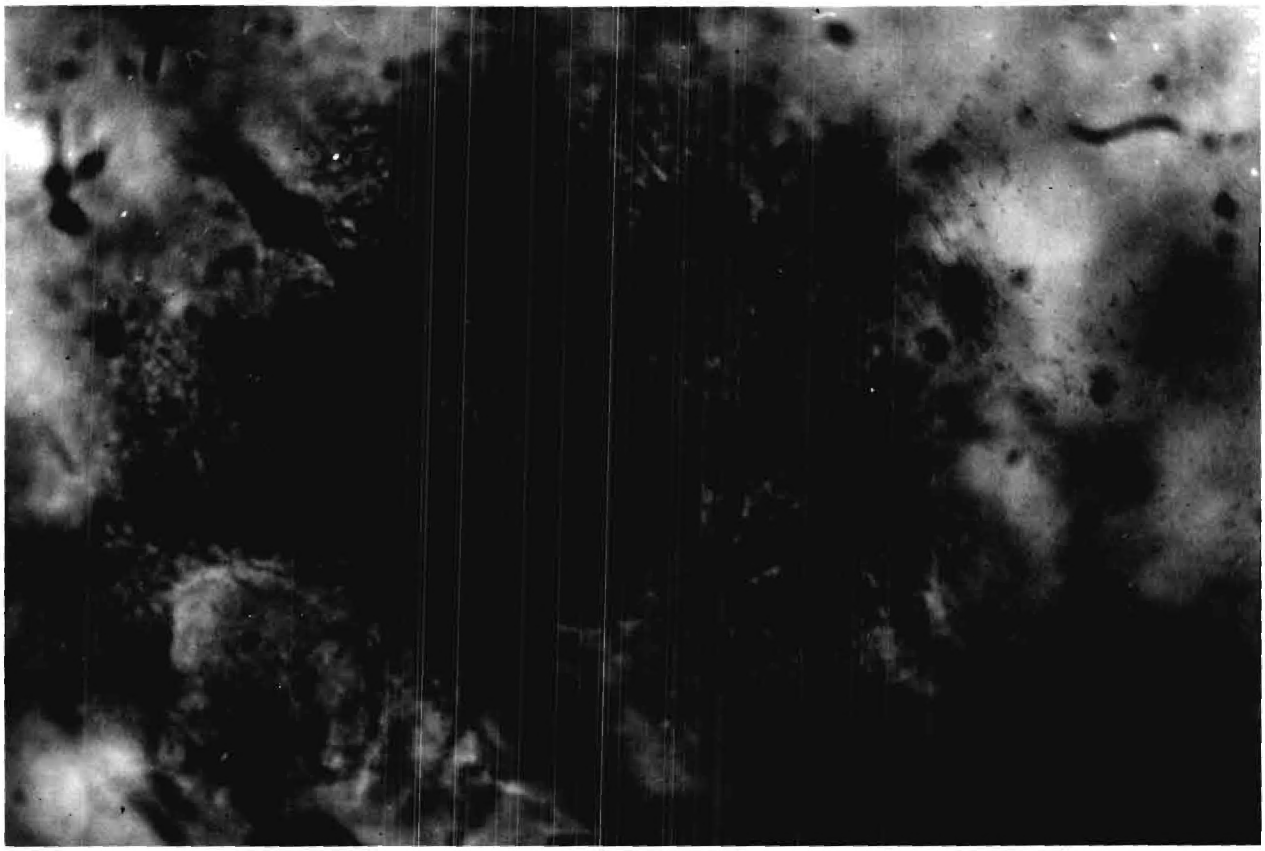
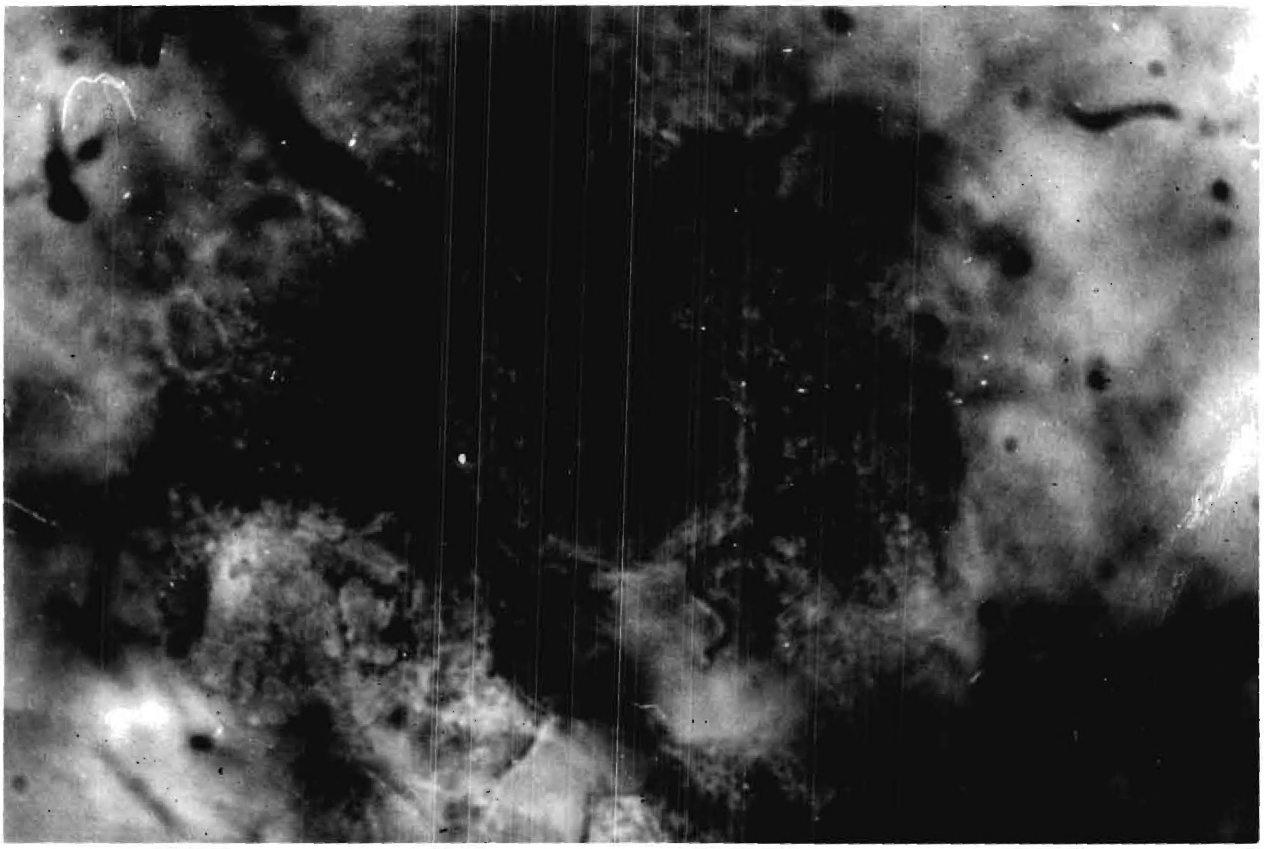
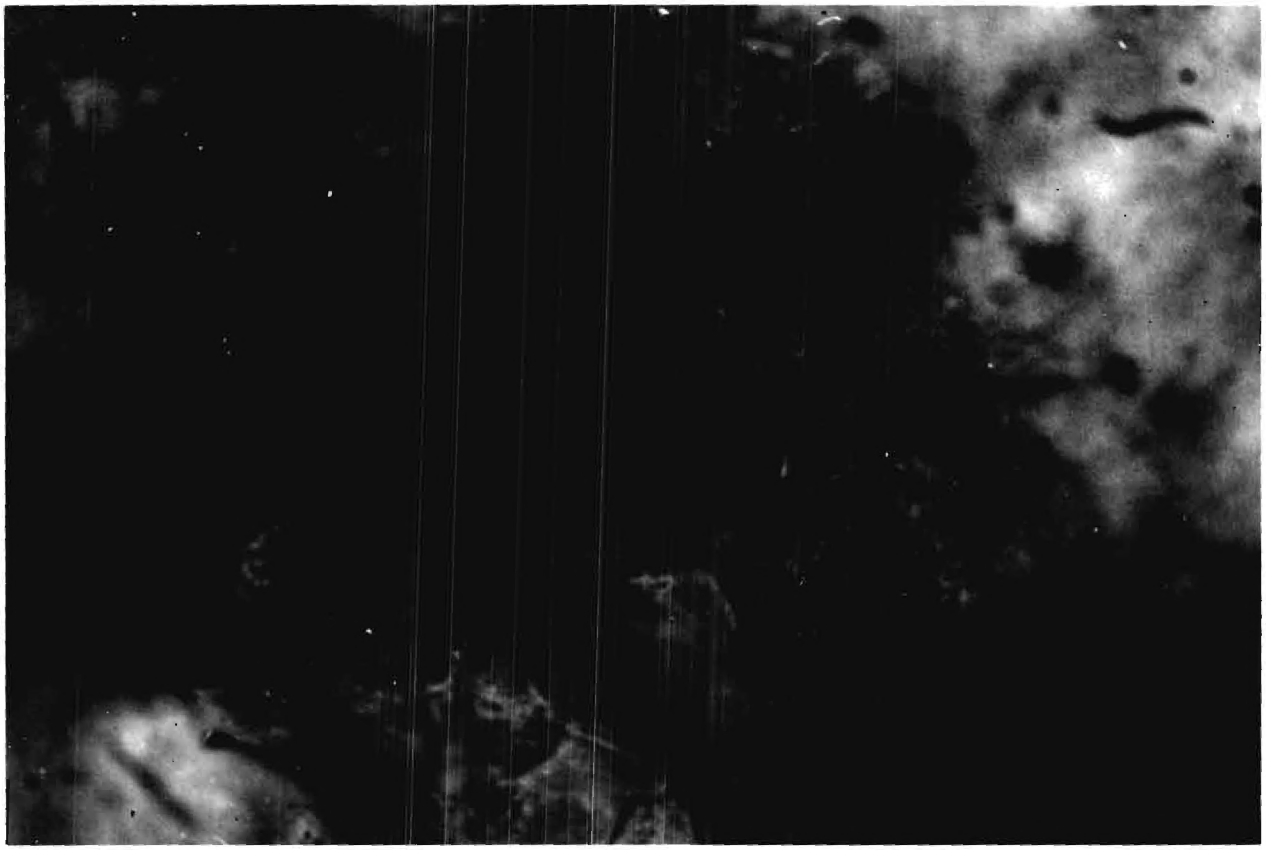


FIGURE 24. Twenty-two minutes later Cell A in early prophase.

FIGURE 25. Twenty minutes later Cell A in mid prophase. This completes entire mitotic cycle.





B. Threshold Ozone Exposure Necessary to Inhibit Mitosis

A series of experiments was run to determine the concentration of ozone necessary to produce a significant inhibition of mitosis. The results are shown in Table III.

TABLE III

DURATION OF MITOSIS AFTER EXPOSURE TO

VARIOUS CONCENTRATIONS OF OZONE FOR 5 MINUTES

Ozone Solution (Mg/L)	No. of Cells	Total Mitotic Time (Min.)	Theoretical Time* (Min.)	Difference	P**
0.0 to 1.5	18	3764	3744	20	> .5
1.5 to 2.5	22	4530	4576	46	> .5
2.5 to 3.5	18	3845	3744	101	0.10
3.5 to 4.5	16	5216	3328	1888	< .01

* Based on control = 208 minutes at 38° C.
** From Table for Goodness of Fit. (Fisher)

Under these experimental conditions no effect was detected until the ozone concentration in the dosing bottle was from 3.5 to 4.5 mg per liter of ozone. In this concentration range there was a significant inhibition of mitosis; however, the effect was reversible and mitosis would go to completion, although delayed. This range, 3.5 to 4.5 mg per liter of ozone, was the concentration used in all of the dosimetry experiments. The lower concentrations of ozone undoubtedly represent the "ozone demand" of this particular solution. It was not feasible to work with higher concentrations of ozone because of the excessive duration of mitotic inhibition.

C. Mitotic Stages Sensitive to Threshold Ozone Exposure

Quantitative information on the effects of ozone on each of the specific stages of mitosis was obtained by replication. To this end preparations were dosed and placed under the microscope, the preparations were mapped, and each cell was followed until the time of next anaphase. The total time involved from the end of dosing to the commencing of observation was 5 minutes; therefore, it is possible to predict the stage dosed because all of the stages are of at least twice this duration.

TABLE IV

TIME TO REACH NEXT ANAPHASE OF CELLS

TREATED WITH 3.5 TO 4.5 MG PER LITER OF OZONE

State Treated	No. Cells	Total Time To Next Anaphase (Min.)	Theoretical* Time (Min.)	Difference	P**
Interphase	9	1841	1154	687	< .01
Very early prophase	8	924	824	120	< .01
Early prophase	8	578	544	34	.15
Mid-prophase	7	263	259	4	> .5
Late prophase	10	225	230	5	> .5
Metaphase	5	27	22.5	4.5	> .5
Anaphase	6	1881	1221	660	< .01
Mid-telophase	6	1365	1104	261	< .01
Late telophase	4	937	634	303	< .01

*Based on control = 208 minutes at 38° C.
**From table for Goodness of Fit. (Fisher)

The data in Table IV indicate that cells dosed earlier than early prophase (very early prophase, interphase, late telophase, mid-telophase and early telophase) are inhibited by this dose of ozone and differ significantly from controls in the increment of time taken to reach the next anaphase. Cells more advanced at the time of ozone exposure than very early prophase proceed to anaphase at a time not significantly different from the controls.

D. Effect of Ozone Exposure on Progression of Mitosis

Individual cells were studied after ozone exposure, and their progress was followed from one mitotic stage to another. In each experiment from one to two cells were followed per preparation, the number to be followed depending upon the number of cells in the particular stage to be studied that could be found in one microscope field. Therefore, the six cells used in each experimental group represent from three to six replicate experiments. The cells were exposed to the ozone for 5 minutes and then the time recorded from the beginning of the next successive stage of mitosis and each succeeding stage until the cells entered anaphase. Table V presents the data from those cells exposed in anaphase.

TABLE V

SIX CELLS TREATED IN ANAPHASE

Successive Stages	Total Duration (Min.)	Expected* Duration (Min.)	Difference (Min.)	P**
Mid telophase	153	144	9	> .05
Late telophase	204	198	6	> .05
Interphase	154	162	8	> .05
Very early prophase	147	144	3	> .05
Early prophase	296	276	20	> .05
Mid prophase	192	96	96	< .01
Late prophase	288	96	192	< .01
Metaphase	80	78	2	> .05

*Based on control = 208 minutes at 38° C.

**From table for Goodness of Fit. (Fisher)

The duration of each succeeding stage, up to mid prophase, was not significantly inhibited; once beyond this stage, the cells did proceed through metaphase to anaphase at the expected rate. Table VI presents the results when cells were treated in mid telophase. The cells progressed through successive stages at the expected rate until mid prophase and late prophase, where there was a significant inhibition, and then proceeded through metaphase to anaphase as in the controls.

TABLE VI

SIX CELLS TREATED IN MID TELOPHASE

Successive Stages	Total Duration (Min.)	Expected* Duration (Min.)	Difference (Min.)	P**	
Late telophase	212	198	14	>	.05
Interphase	141	162	21	>	.05
Very early prophase	149	144	5	>	.05
Early prophase	273	276	3	>	.05
Mid prophase	155	96	59	<	.01
Late prophase	293	96	197	<	.01
Metaphase	77	78	1	>	.05

*Based on control = 208 minutes at 30° C.
**From table for Goodness of Fit. (Fisher)

Cells treated in late telophase (Table VII) proceeded as the controls to mid prophase where there was a significant inhibition in mid prophase and late prophase, and then proceeded at the expected rate to anaphase.

TABLE VII

SIX CELLS TREATED IN LATE TELOPHASE

Successive Stages	Total Duration (Min.)	Expected* Duration (Min.)	Difference (Min.)	P**
Interphase	149	162	13	> .05
Very early prophase	157	144	13	> .05
Early prophase	301	276	25	> .05
Mid prophase	183	96	87	< .01
Late prophase	307	96	211	< .01
Metaphase	82	78	4	> .05

*Based on control = 208 minutes at 38° C.
**From table for Goodness of Fit. (Fisher)

Cells treated in interphase (Table VIII) also proceeded at the expected rate to mid prophase and late prophase where again there was a significant inhibition.

TABLE VIII

SIX CELLS TREATED IN INTERPHASE

Successive Stages	Total Duration (Min.)	Expected* Duration (Min.)	Difference (Min.)	P**
Very early prophase	137	144	13	> .05
Early prophase	294	276	18	> .05
Mid prophase	127	96	31	< .01
Late prophase	307	96	211	< .01
Metaphase	82	78	4	> .05

*Based on control = 208 minutes at 38° C.
**From table for Goodness of Fit. (Fisher)

Cells treated in very early prophase (Table IX) were inhibited in mid prophase and late prophase and then proceeded at the expected rate to anaphase.

TABLE IX

SIX CELLS TREATED IN VERY EARLY PROPHASE

Successive Stages	Total Duration (Min.)	Expected* Duration (Min.)	Difference (Min.)	P**
Early prophase	261	276	15	> .05
Mid prophase	133	96	37	< .01
Late prophase	176	96	80	< .01
Metaphase	75	78	3	> .05

*Based on control = 208 minutes at 38° C.
**From table for Goodness of Fit. (Fisher)

Cells treated in early prophase (Table X) proceeded to anaphase without inhibition as did cells treated in late prophase and metaphase (Tables X and XI).

TABLE X

SIX CELLS IN EARLY PROPHASE

Successive Stages	Total Duration (Min.)	Expected* Duration (Min.)	Difference (Min.)	P**
Mid prophase	107	96	11	> .05
Late prophase	112	96	16	> .05
Metaphase	72	78	6	> .05

*Based on control = 208 minutes at 38° C.
**From table for Goodness of Fit. (Fisher)

TABLE XI

SIX CELLS TREATED IN MID PROPHASE

Successive Stages	Total Duration (Min.)	Expected* Duration (Min.)	Difference (Min.)	P**
Late prophase	91	96	5	> .05
Metaphase	82	78	4	> .05

*Based on control = 208 minutes at 38° C.
**From table for Goodness of Fit. (Fisher)

TABLE XII

SIX CELLS TREATED IN LATE PROPHASE

Successive Stages	Total Duration (Min.)	Expected* Duration (Min.)	Difference (Min.)	P**
Metaphase	73	78	5	> .05

*Based on control = 208 minutes at 38° C.
**From table for Goodness of Fit. (Fisher)

E. Membrane Mobility in Control KB Cell Cultures

The results of measurements made on eight control metabolic cells from different preparations are given in Table XI.

TABLE XIII

FREQUENCY WITH WHICH WAVES APPEAR AT THE PERIFERA OF CELL MEMBRANE IN METABOLIC KB CELLS. ALL OBSERVATIONS AT 37.5 ± 0.5° C.

Cell No.	Duration of 5 Waves (Sec.)	Time+ (Sec.)	Average Time Per Cell (Sec.)
1	11	154	
	9	126	150
	12	168	
2	9	126	
	12	168	158
	12	168	
3	15	210	
	10	140	177
	13	182	
4	9	126	
	10	140	140
	11	154	
5	10	140	
	12	168	163
	13	182	
6	11	154	
	12	168	163
	12	168	
7	14	196	
	13	182	182
	12	168	
8	9	126	
	12	168	140
	9	126	
Total	272	3808	159

(+) Duration of 5 waves (Sec.) x No. Frames Sec. on projection (14) x photographs per Sec. (1).

$\bar{x}$ = 159, σ = 16, n = 8, 159 ± 6 Sec./5 waves.

The average of three measurements of five waves on eight different metabolic cells was 159 seconds with a standard deviation of 16. This would represent about 32 seconds between waves.

F. Membrane Mobility in KB Cells after Ozone Exposure

After cells were exposed to from 3 to 4 mg/L of ozone solution for 5 minutes there was a considerable delay before the metabolic cells commenced cytoplasmic waving. If the duration of ozone exposure exceeded much over 5 minutes the cells would round up and dislodge from the glass surface.

Table XIV shows the results from three different cells when the cytoplasmic waving commenced after ozone exposure.

TABLE XIV

FREQUENCY WITH WHICH WAVES APPEAR AT THE PERIFERA OF CELL MEMBRANE IN METABOLIC KB CELLS AFTER OZONE EXPOSURE. CELL OBSERVATION AT 37.5 ± 0.5° C.

Cell No.	Duration of 5 Waves (Sec.)	Time (Sec.)	Average (Sec.)
1	15	210	
	10	140	163
	10	140	
2	9	126	
	12	168	154
	13	182	
3	11	154	
	11	154	144
	9	129	
$\bar{x}$ = 153, σ = 16, n = 3			

Once the wave mobility of the cell began after ozone exposure the frequency with which the waves appeared was not significantly different (less than twice the standard error of the difference of the means) from the controls.

IV. DISCUSSION

Mitosis is a physiological process. The progression of a cell through division is a function of physio-chemical processes acting within genetically determined parameters; each of the various mitotic stages are dependent for their predetermined completion on events in previous stages as well as events transpiring in the specific stage. Mitosis provides an exquisitely sensitive as well as fundamentally significant test process.

Quantitation in such studies is rendered difficult by the limited number of biological systems, of adequate visibility, where noncyclic mitosis occurs. Marine and amphibian eggs have been studied extensively, although intercellular visibility is poor; mammalian cell suspensions in the exponential growth phase is one recently developed system and the neuroblast mitosis used in this study is another.

The inherent difficulty of studying a dynamic system with static techniques has imposed additional limitations on such investigations and continuous observation of the living cell appears to be a necessary requirement. Evidence of mitotic stage reversal after irradiation[11] demonstrates the fallacy of assumptions based on data obtained from slide preparations.

A. Mitotic Inhibition in Neuroblast Cells

In these investigations it was found that ozone produced a specified effect upon mitosis. At the threshold concentration cells exposed in anaphase,

11. J. G. Carlson, J. Morphol. 71, 449 (1942).

telophase, interphase, and very early prophase were delayed significantly in passing through the next mid and late prophase stages. Cells more advanced than very early prophase proceeded to anaphase at a time not significantly different from the controls. It thus appears that the stages sensitive to ozone under these conditions are preprophase and the inhibited stages are mid and late prophase. These data suggest that the mitotic activity of ozone is similar in some respects to the responses to high energy irradiation, but there are significant differences. Exposure to ozone does not result in a reversion of prophase stages which has been described for irradiation with low irradiation doses reversion is limited to cells in late prophase and there is an accumulation of mid prophases; with higher doses all prophase cells revert to an interphase condition. Thus with irradiation we have a critical stage, late prophase, and a reversion process which is dose dependent. The higher the dose, the greater the reversion. This reversion phenomenon was absent with ozone exposure; however, late prophase was the stage in which the mitotic inhibition was expressed.

Ozone is of particular interest in irradiation biology because the decomposition of ozone in solution produces the same active radicals formed in water by high energy irradiation. The chemical activity of free radicals produced by the catalytic decomposition of hydrogen with ferric ions has recently been discussed[12] and Phillips[13] describes the production of chromosome aberrations in barley by these hydrogen peroxide precursors. From studies of the kinetics and mechanisms of ozone decomposition Alder and Hill[14]

- - - - -

12. R. F. Kimball and N. Gaither, Proc. Soc. Exp. Med. Biol. 80, 525 (1952); G. Scholes and J. Weiss, Exp. Cell Res. (Supp. 2), 219 (1952).

13. L. L. Phillips, Science 124, 889 (1956).

14. M. C. Alder and R. H. Hill, J. Amer. Chem. Soc. 72, 1884 (1950).

assumed that in solution the reactions

$$HO_3^+ + OH^- \rightleftarrows 2HO_2 \qquad (1)$$

$$O_2 + HO_2 \rightarrow OH + 2O_2 \qquad (2)$$

are responsible for disappearance of ozone and are relatively slow and determine the rate of reaction. Also, these authors indicate that reaction (1) represents an equilibrium which is maintained so long as ozone is present. The decomposition of ozone in solution is catalyzed by hydroxyl ions and is dependent upon temperature. The evidence presented in this study suggests that only a part of the irradiation effect on mitosis can be explained in terms of active radical formation in water. Such products produced external to the cell do not cause a reversion of the prophase stages of mitosis.

B. Membrane Mobility Effects

After exposure to ozone there was a considerable delay before the metabolic cells commenced cytoplasmic waving. When the waving began, however, the frequency with which the waves appeared was not significantly different from controls. There does not appear to be a lasting effect on this cellular process after ozone exposure. Narcotizing agents are capable of affecting membrane activity (unpublished data) and it might be concluded that ozone does not have a narcotizing effect, as would be expressed by decreasing the mobility of the cell membrane.

The demonstrated reproducibility of this cellular process may provide a parameter of cellular function which will prove useful for quantitative studies. Predictable indications of cellular activity which may be used to

characterize cell cultures are urgently needed if *in vitro* experimentation is to become a quantitative discipline.

V. FINANCIAL REPORT

For the period 31st December 1960 through 30th April 1961, the following expenditures were made:

Personnel Services	$24,037.51
Material and Supplies	2,104.19
Travel	196.99
Freight and Express	23.73
Equipment	2,828.16
Overhead	14,613.12
TOTAL	$43,803.70

Respectfully submitted:

Robert H. Fetner
Project Director

Approved:

Wyatt C. Whitley, Chief
Chemical Sciences Division

J. E. Boyd, Director
Engineering Experiment Station