

FINAL REPORT

PROJECT NO. A-430

RESEARCH AND REPORTS ON
OZONE-INDUCED EFFECTS ON LIVING CELLS

By

ROBERT H. FETNER

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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

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APRIL 30, 1961



Engineering Experiment Station
Georgia Institute of Technology

Atlanta, Georgia

ENGINEERING EXPERIMENT STATION
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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-micro-photography. Attention was focused on cytoplasmic membrane mobility. Waves originate at the outermost periphery of the cell membrane and migrate toward the nuclear zone. These waves appear with a rhythmic 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.

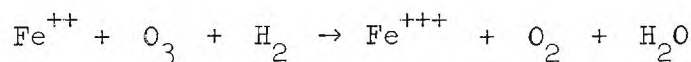
II. MATERIALS AND METHODS

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 investigations¹ 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.

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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:



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 investigators⁴ 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.

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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.)
<u>Interphase</u>	Chromatin granules are scattered in a homogeneous nuclear background. Nucleoli very irregular, appear as grape-like cluster of small spheres.	27
<u>Prophase</u>		
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
<u>Metaphase</u>	Nuclear membrane disappears; cell assumes a spherical shape.	13
<u>Anaphase</u>	Proximal ends of chromatids separate.	9
<u>Telophase</u>		
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.

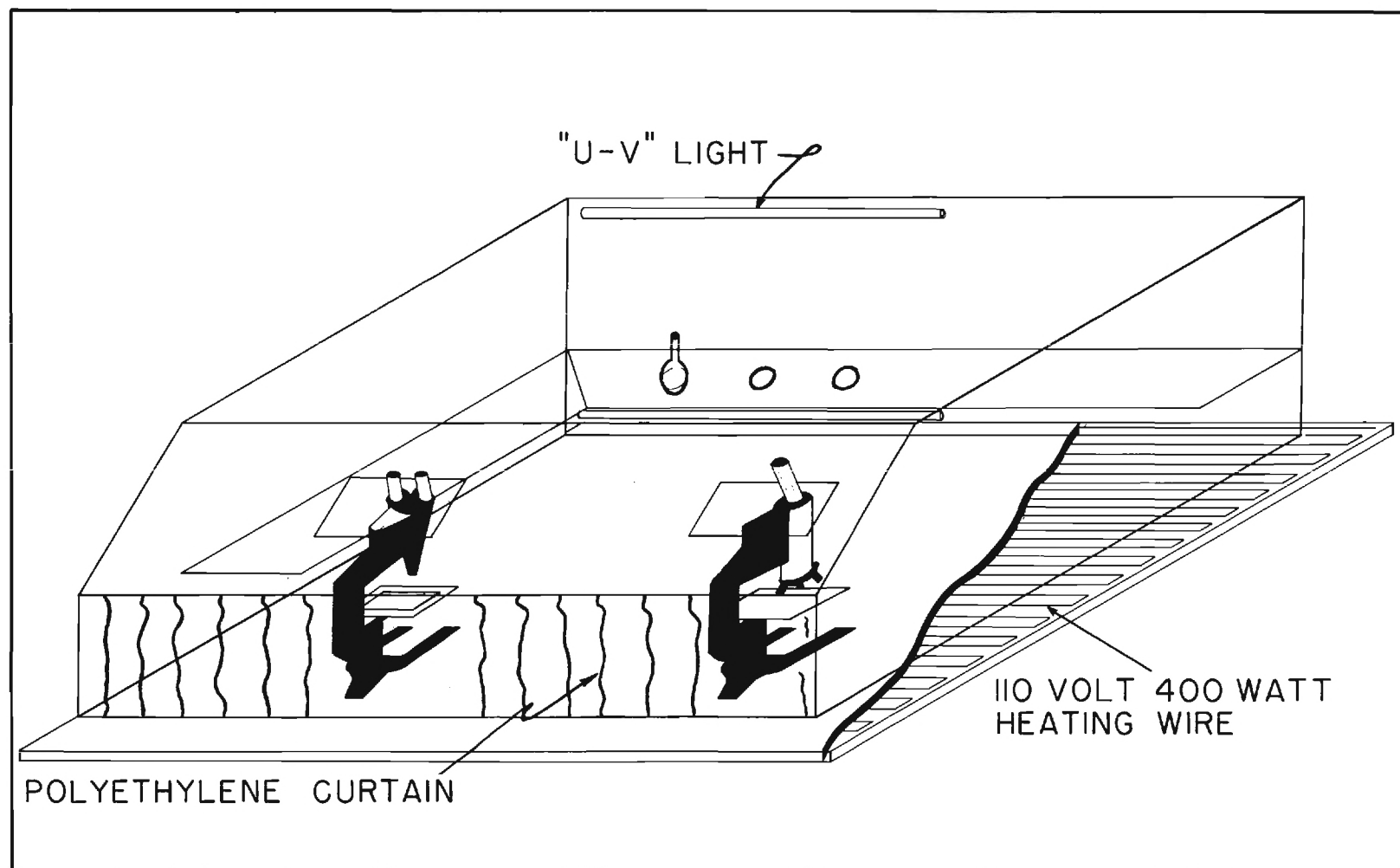
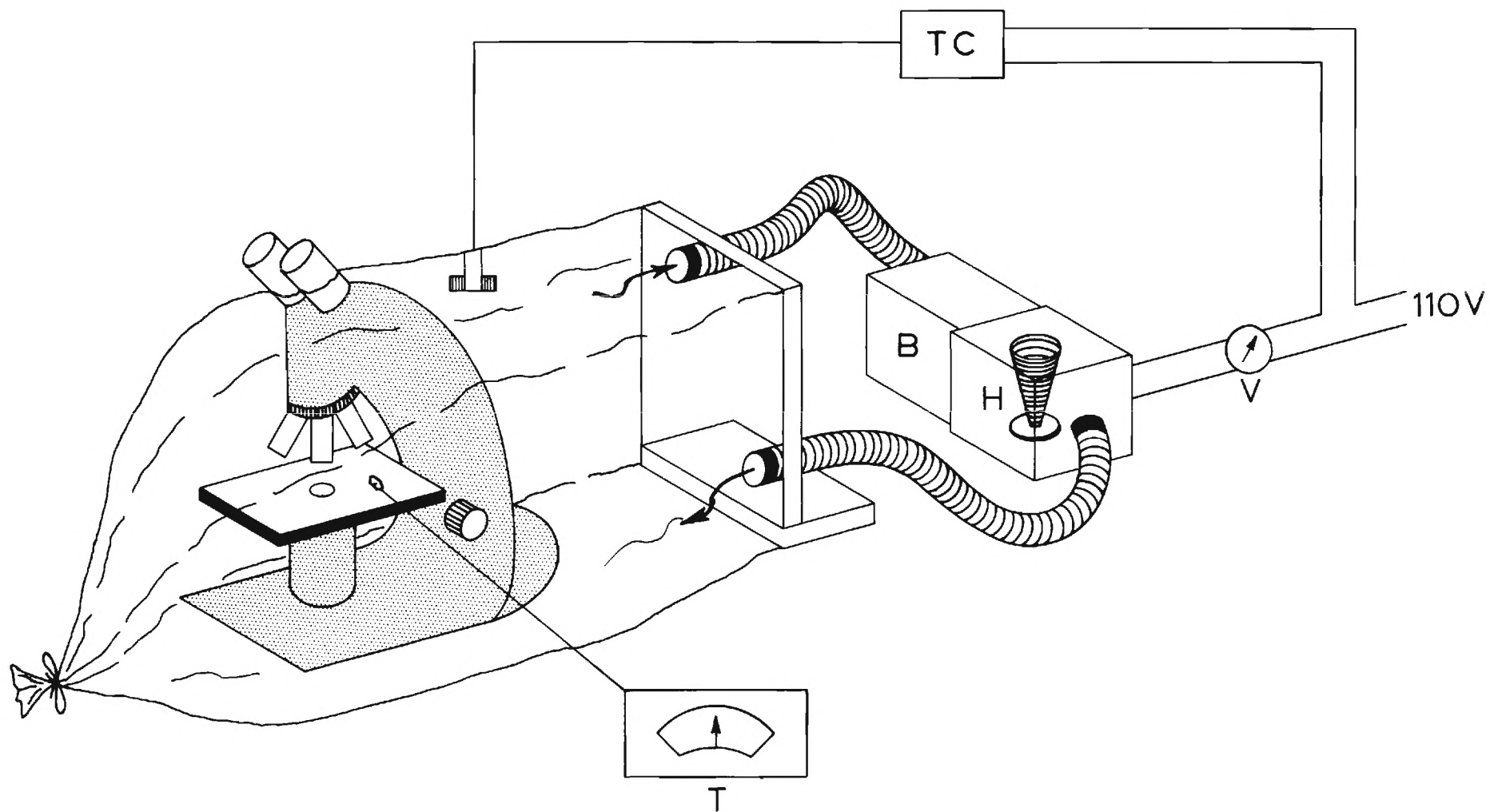


FIGURE 2. Microscope incubator.



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.

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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	<u>349</u>	<u>351</u>	2	2	<u>0.014</u>	<u>.90-.95</u>
Total	2713	2705			0.277	> .99

* Treatment was with physiological saline (control).

** $m = t \times n$; 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 line⁸ 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 solution⁹ 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
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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 rhythmic frequency in the flattened metabolic cells and are readily visible until they reach the nuclear zone (A in Figure 3).

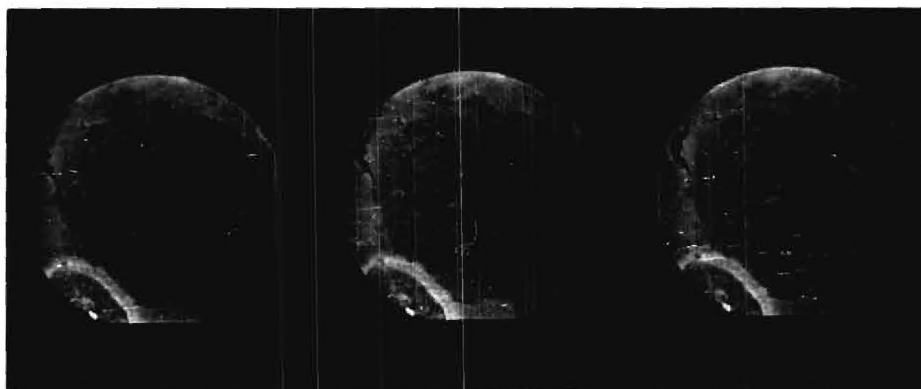
Ozone dosimetry was performed by rinsing the coverslip culture in saline and then exposing to ozone in the dosing bottle (see above). Fresh media was replaced and photographs commenced when the membrane waves reappeared. All preparations were maintained at $37.5 \pm 0.5^{\circ} \text{C}$.

III. RESULTS

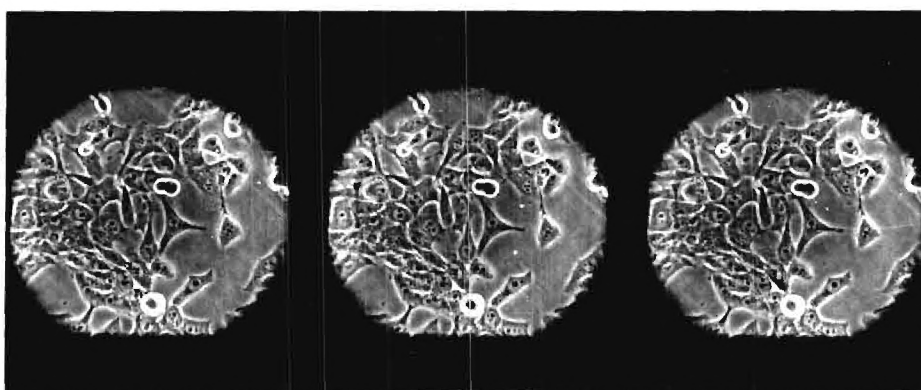
A. Description of Mitosis in Neuroblast Cells

A complete sequence of mitosis in the neuroblast cells of Chortophaga 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

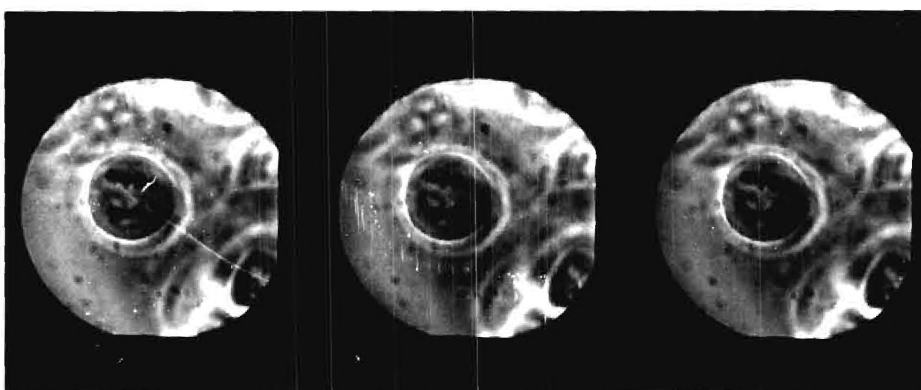
FIGURE 3. Reproductions from 16-mm. negatives of HeLa cells in culture: A, a metabolic cell; the flaring of the cell membrane is visible at the upper left of the cell; B, low magnification of cell culture; C, dividing cell.



(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

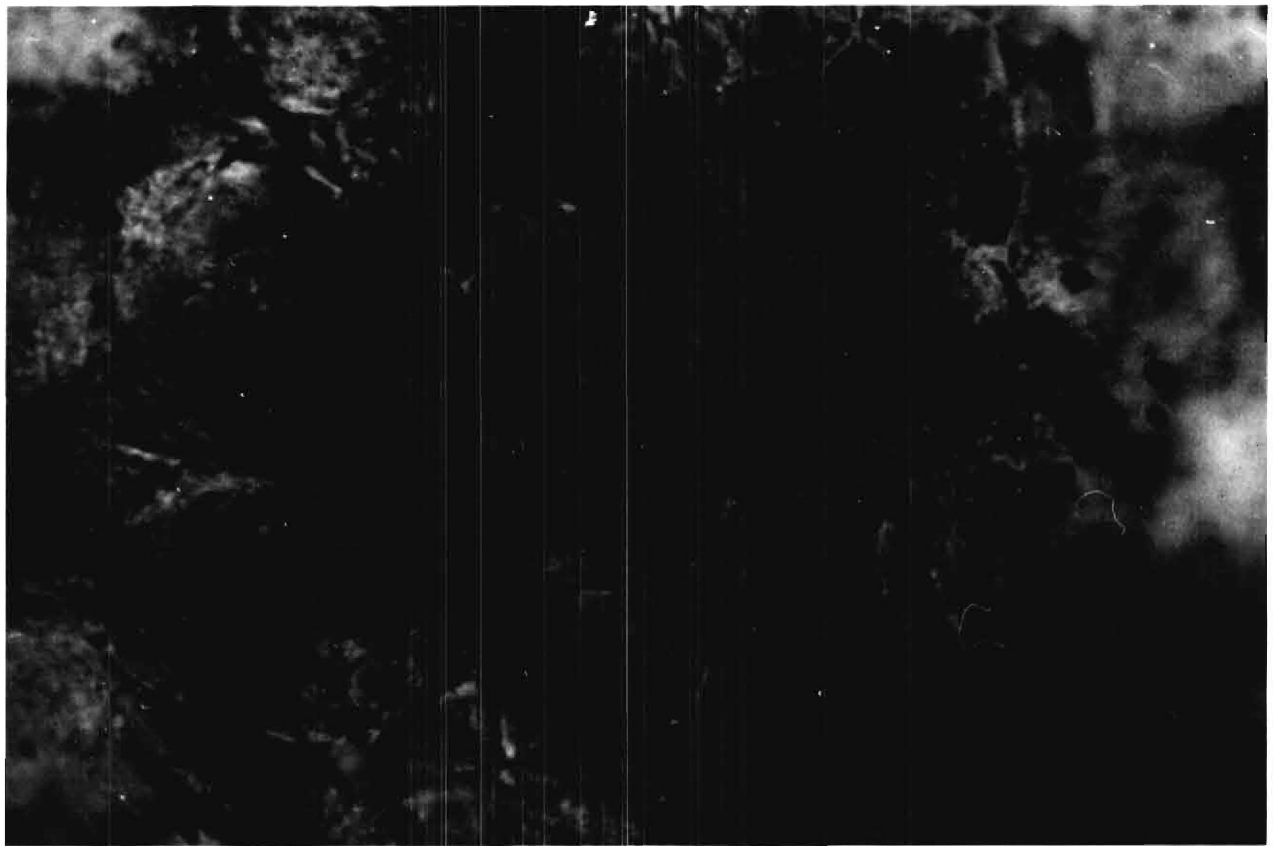
Cell A is in the terminal phase of late prophase and the nuclear membrane appears to be in the process of breaking down or disappearing in the lower

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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 mitochondria 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.

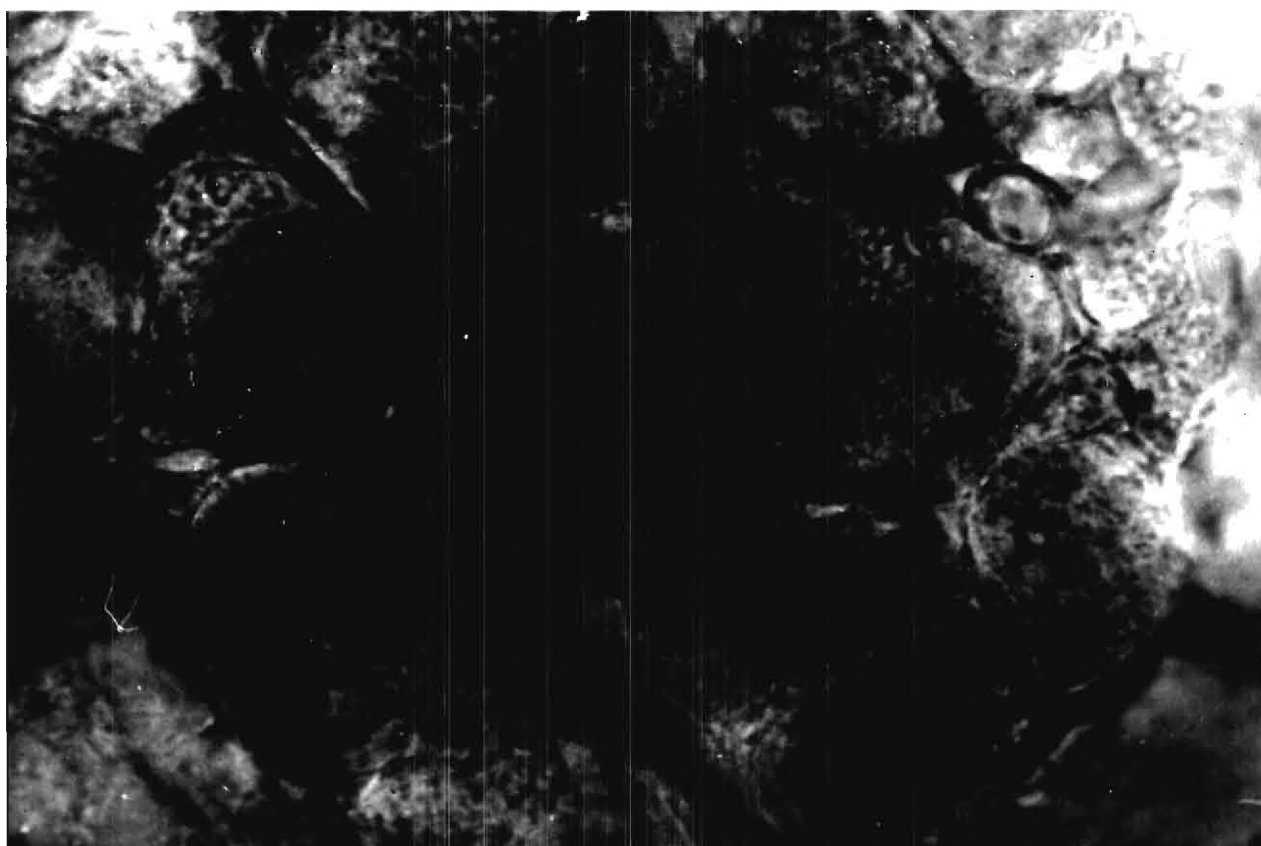
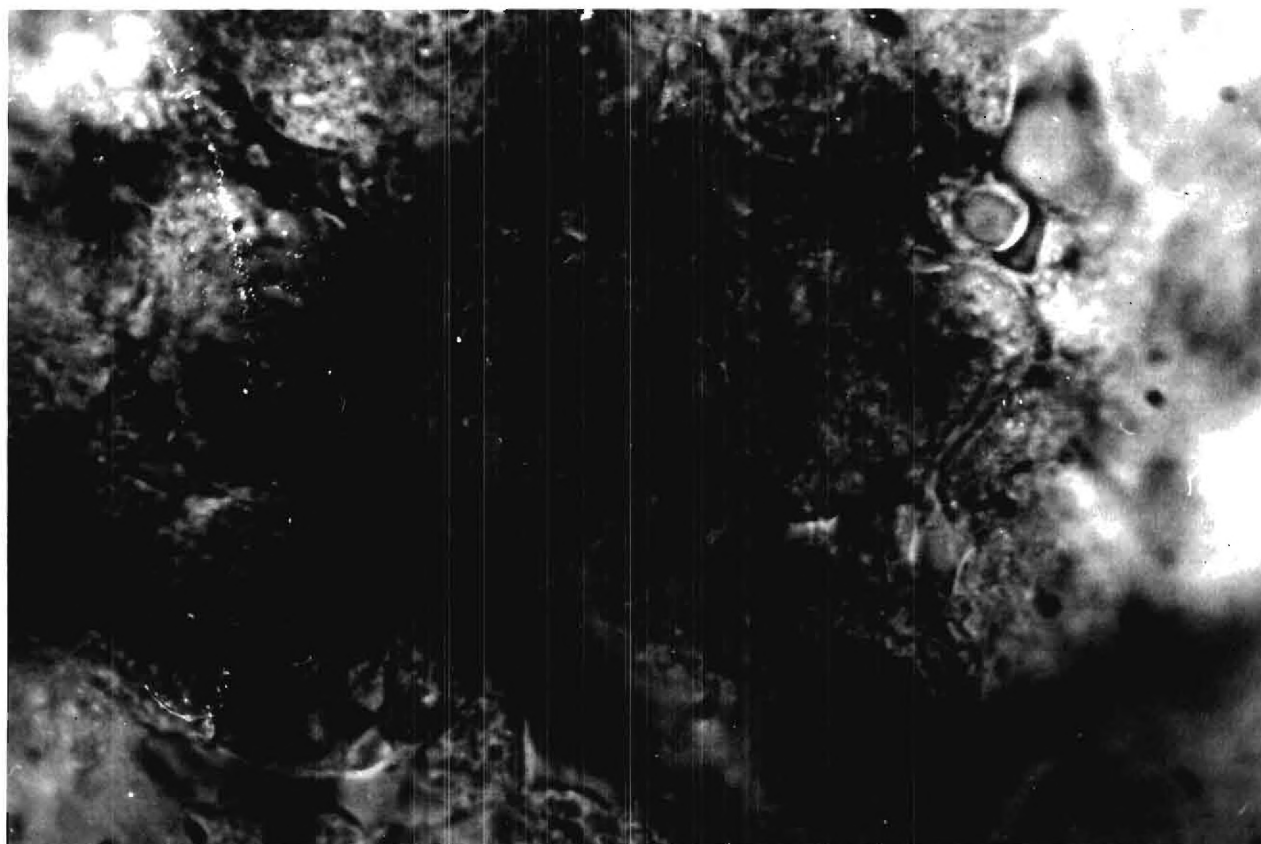


Figure 8

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.

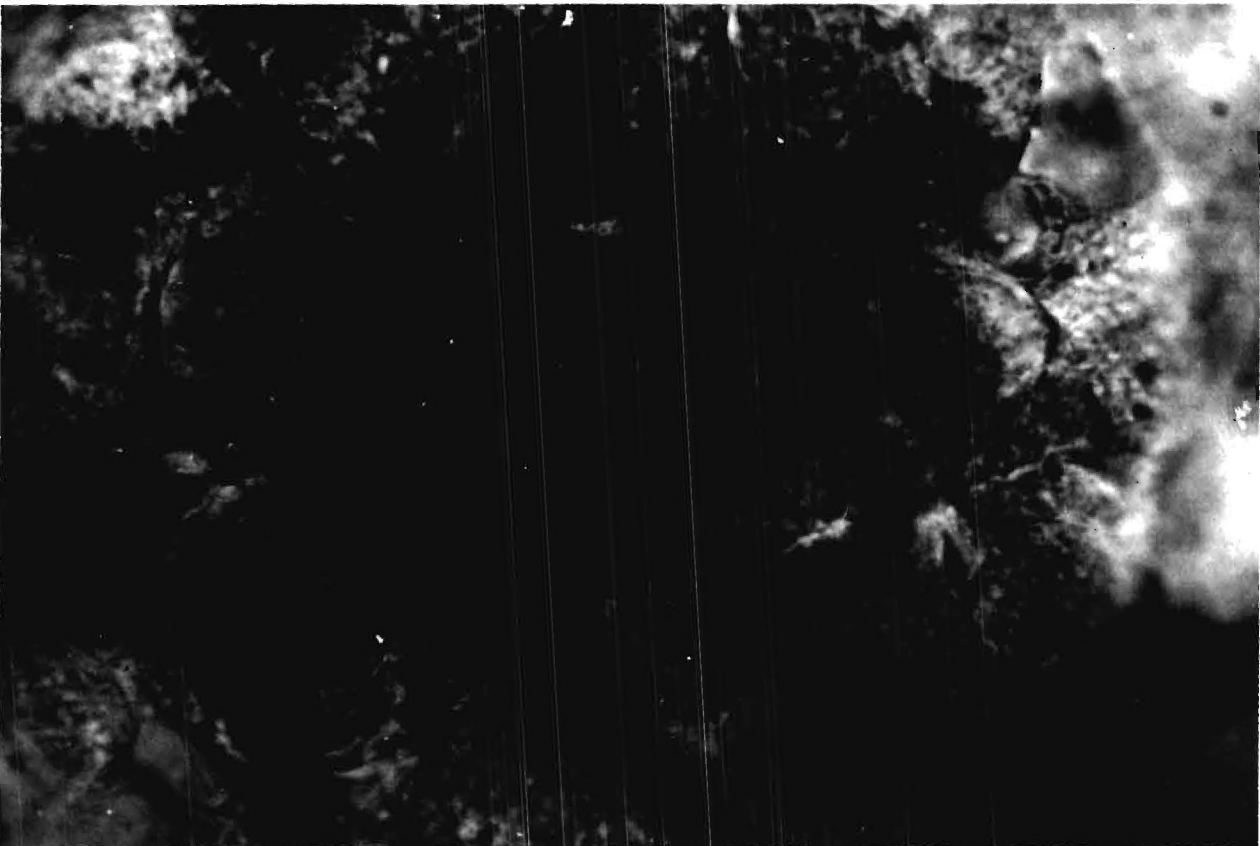
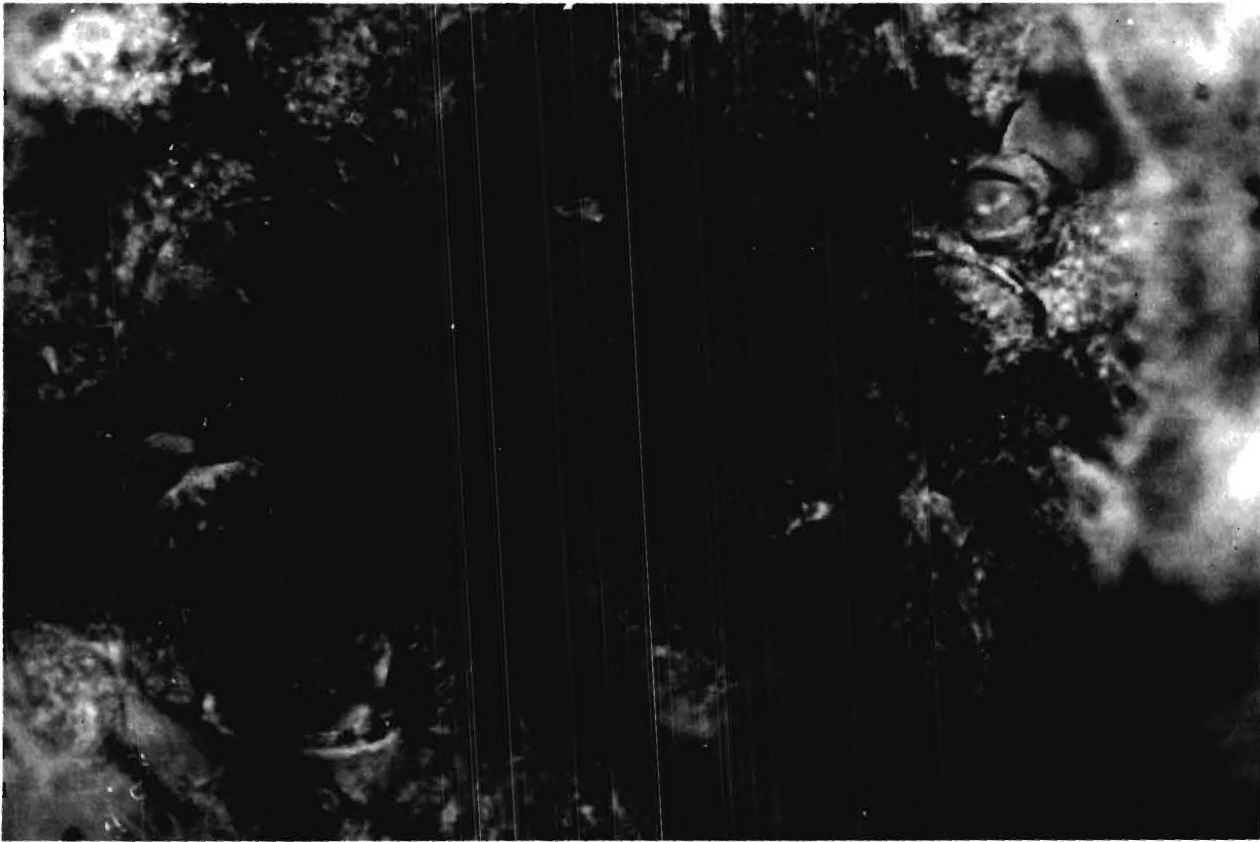
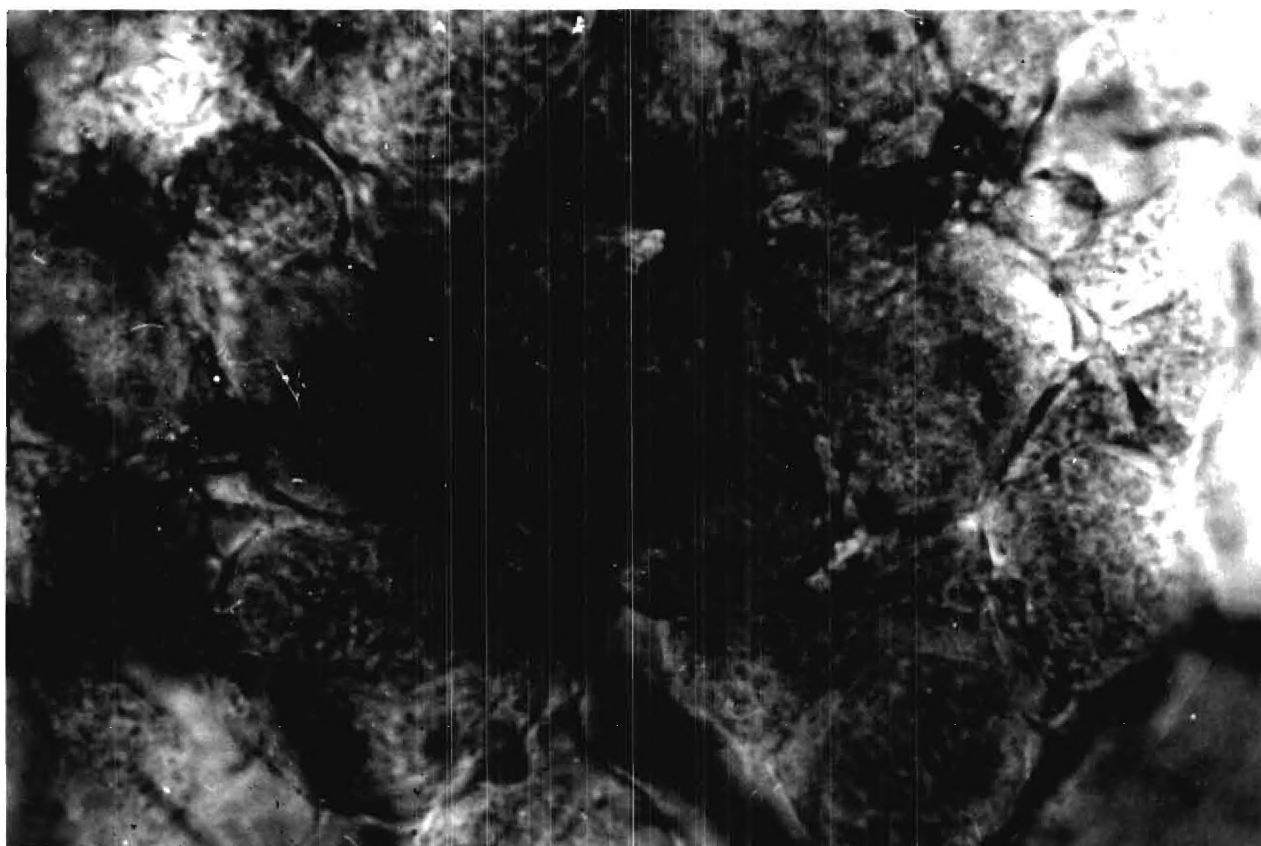
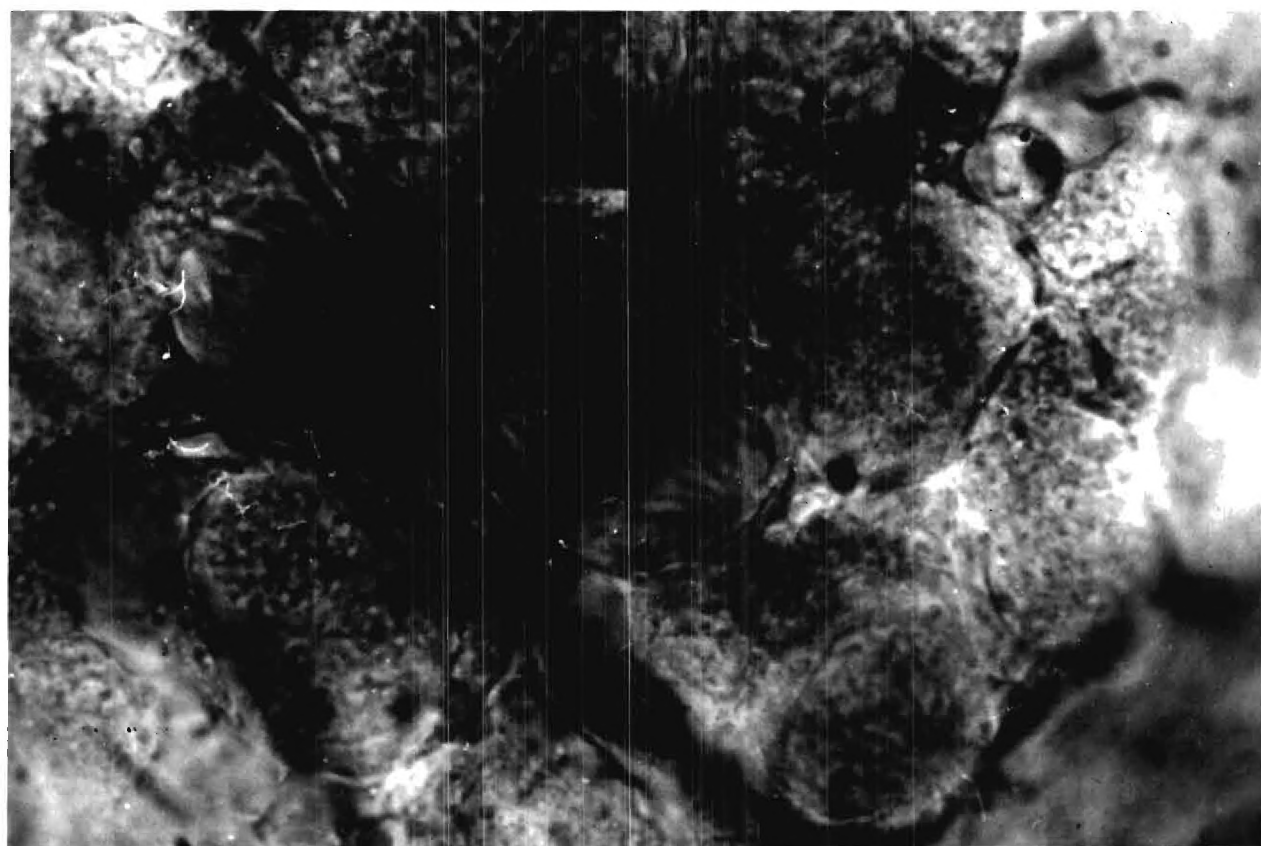


FIGURE 10. Two minutes later both Cells A and B in anaphase; in both cells daughter chromosomes are pressed against cell membrane at point where daughter will bud, while maternal chromosomes are orientated more centrally in what will become mother cell. Cell C in late prophase.

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.

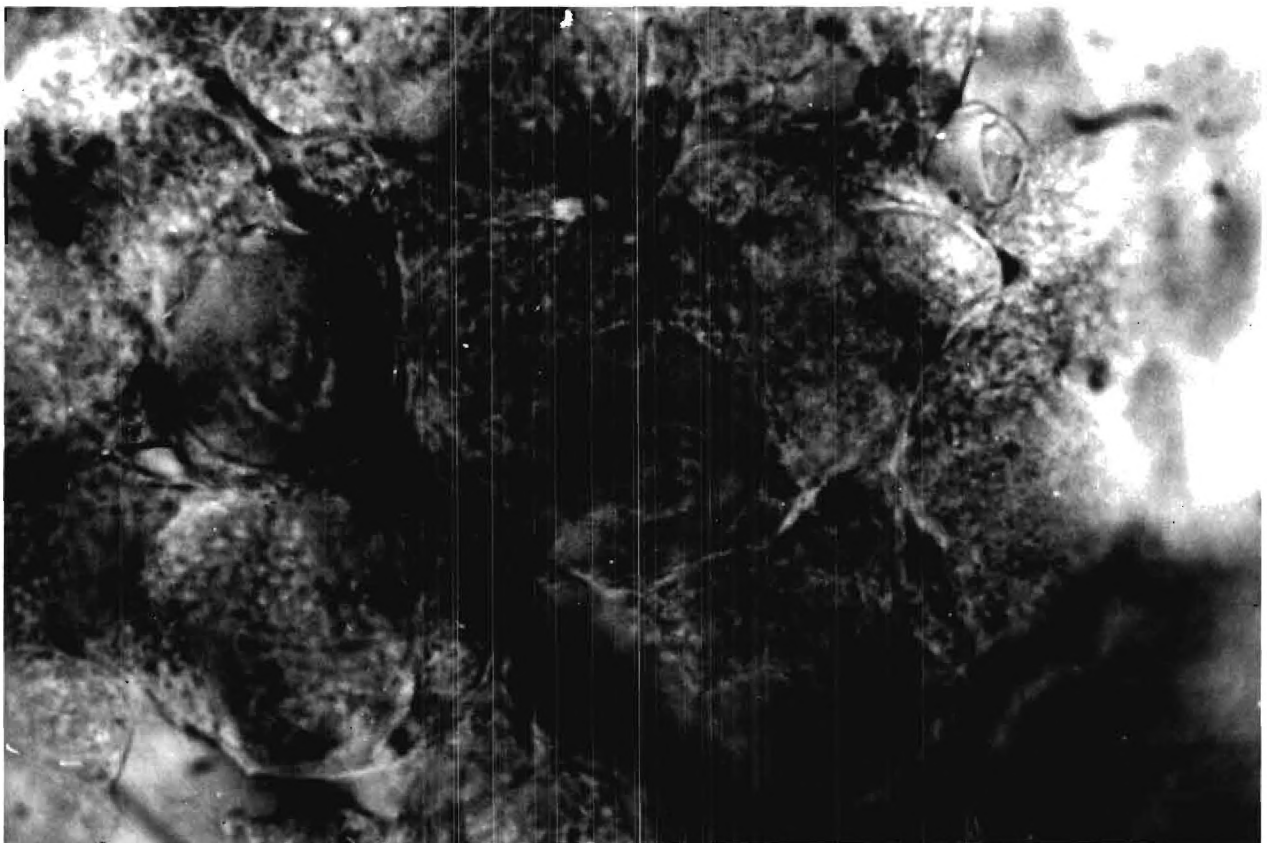
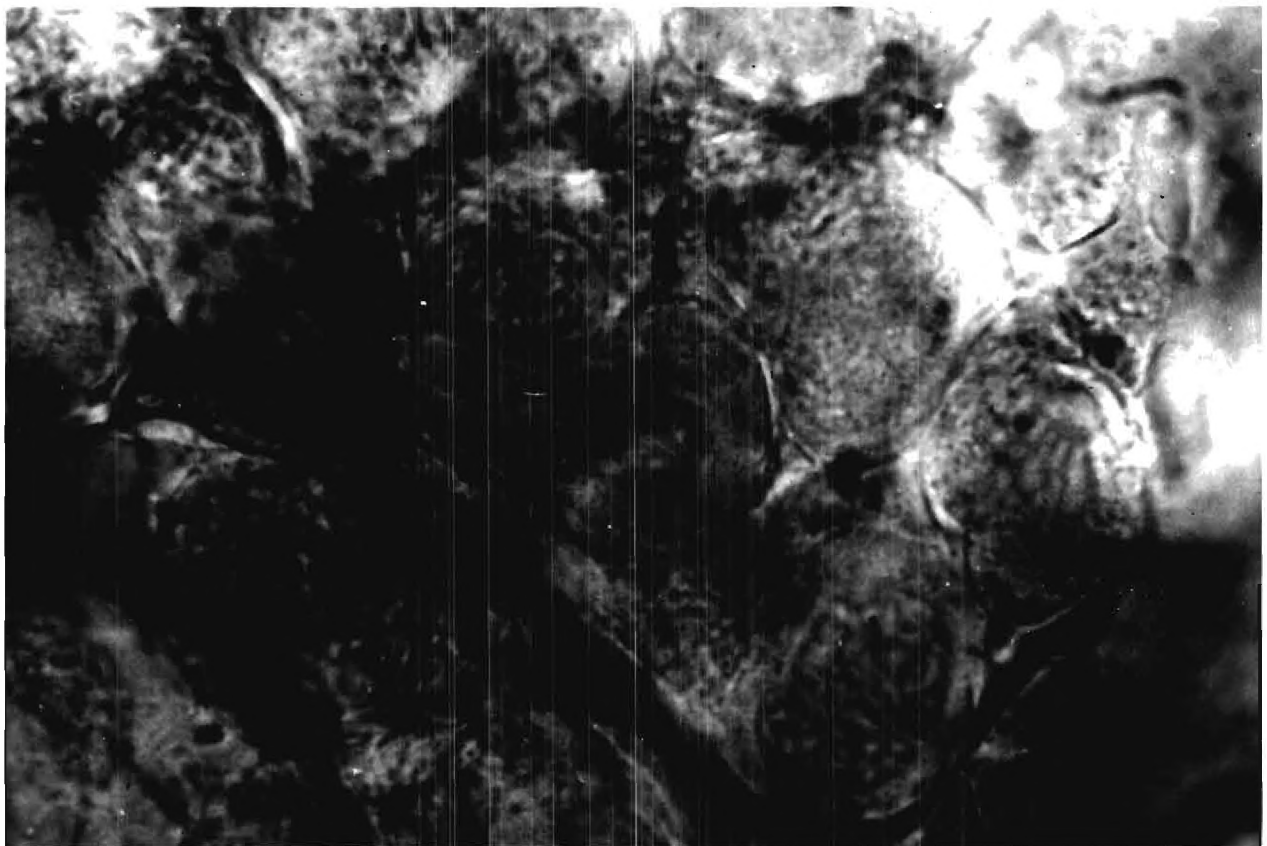


Figure 14

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.

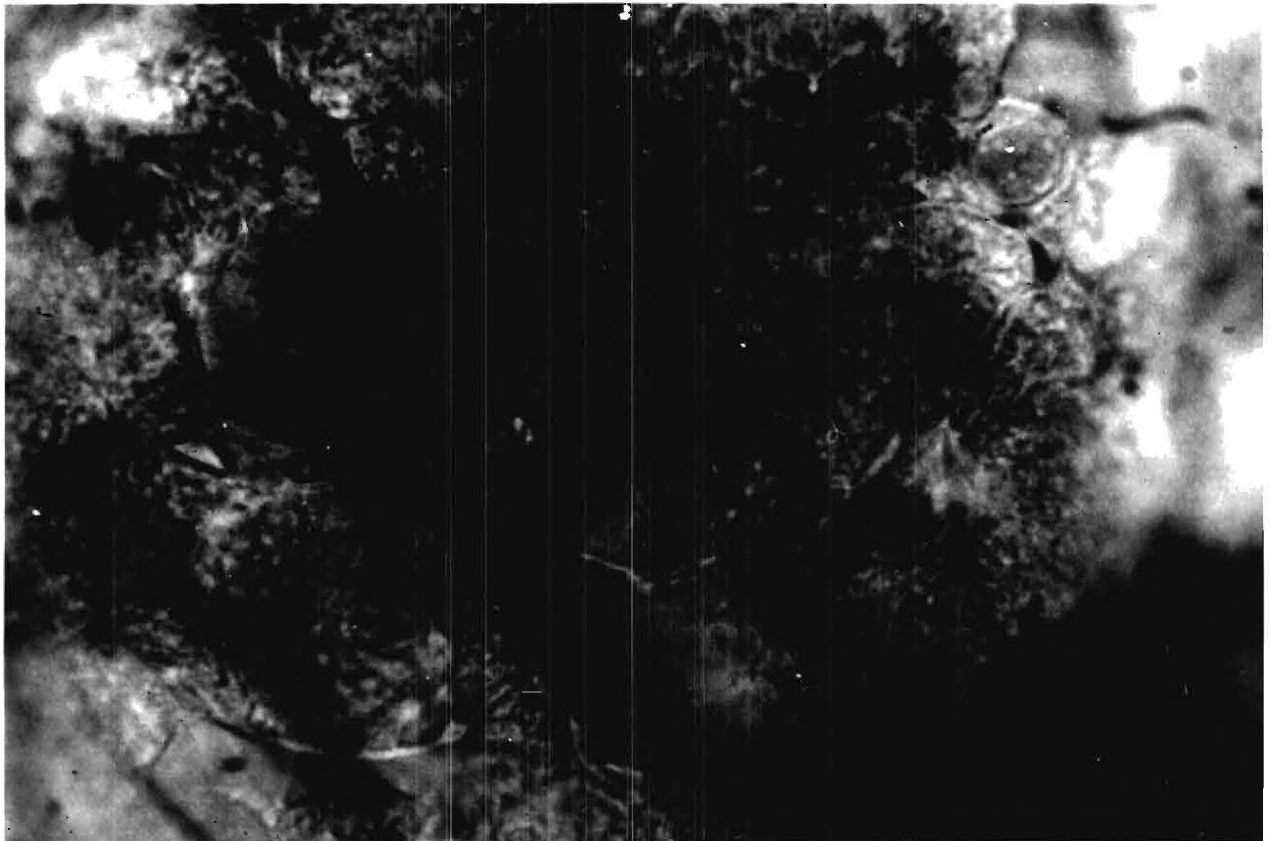
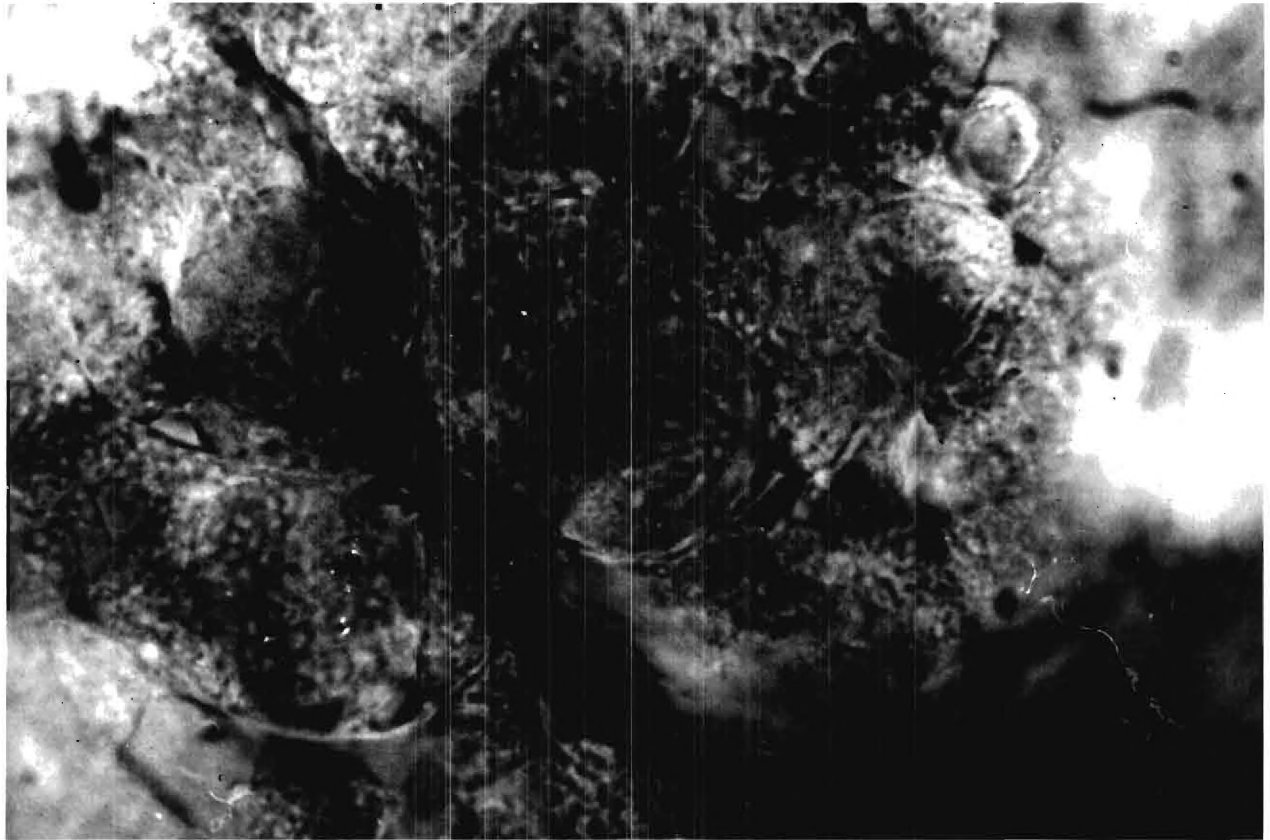


FIGURE 16. Four minutes later Cells A and B in mid telophase, nucleoli increasing in size.

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

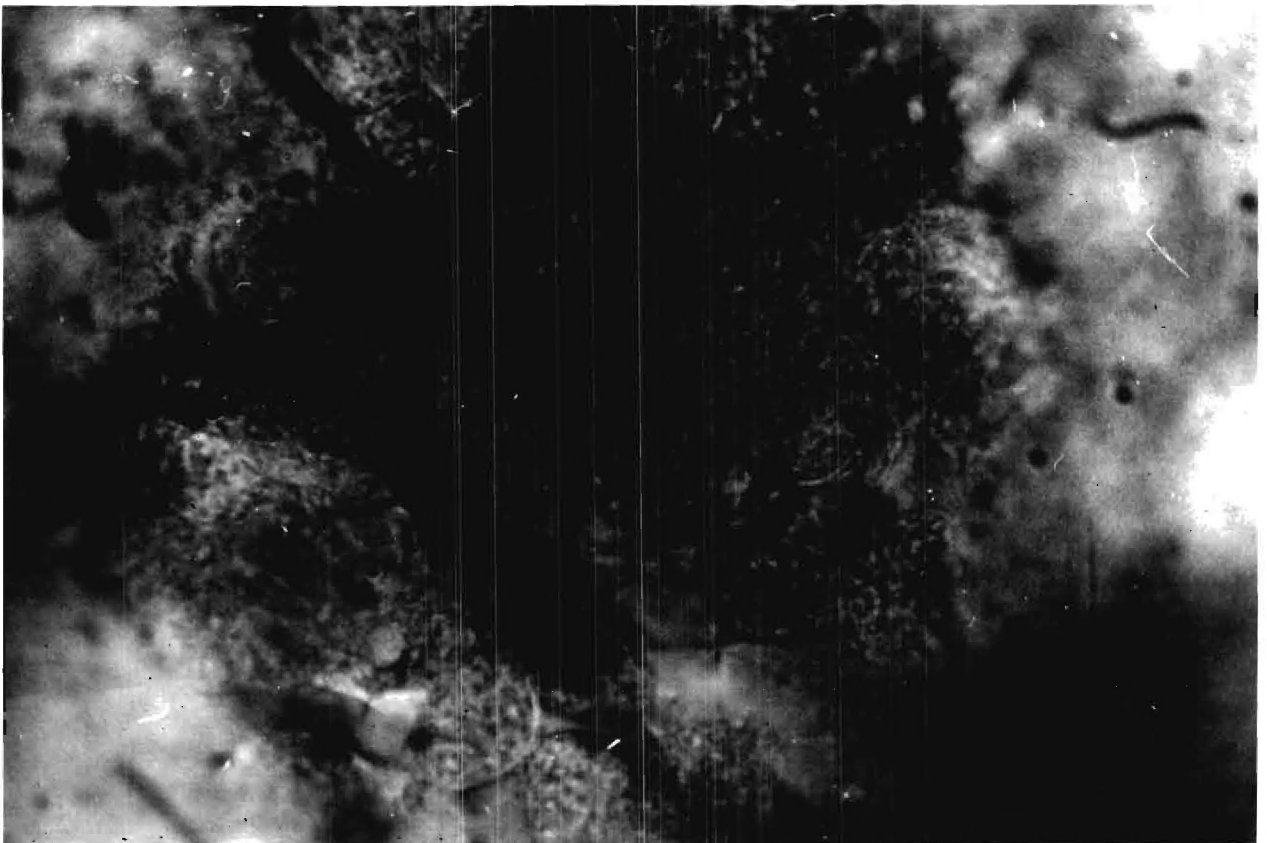
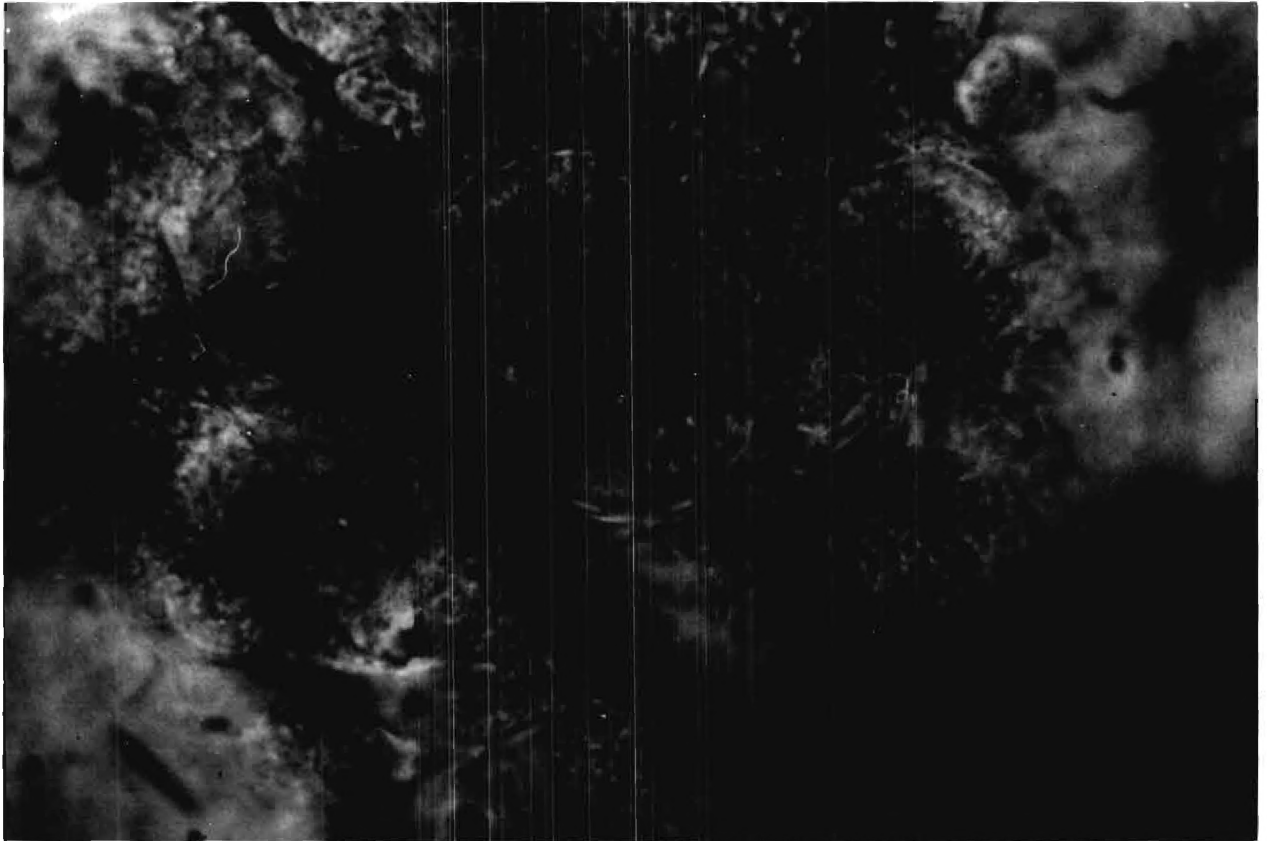
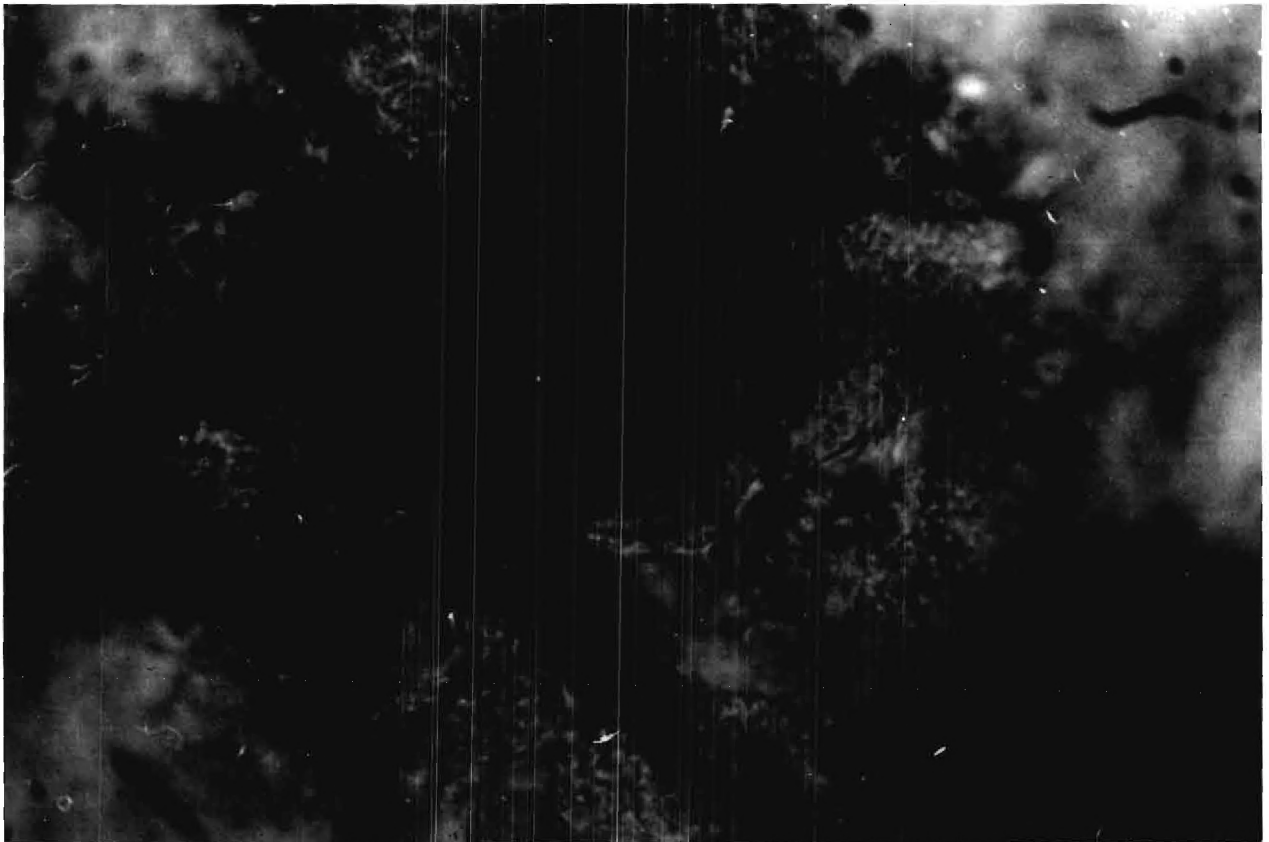
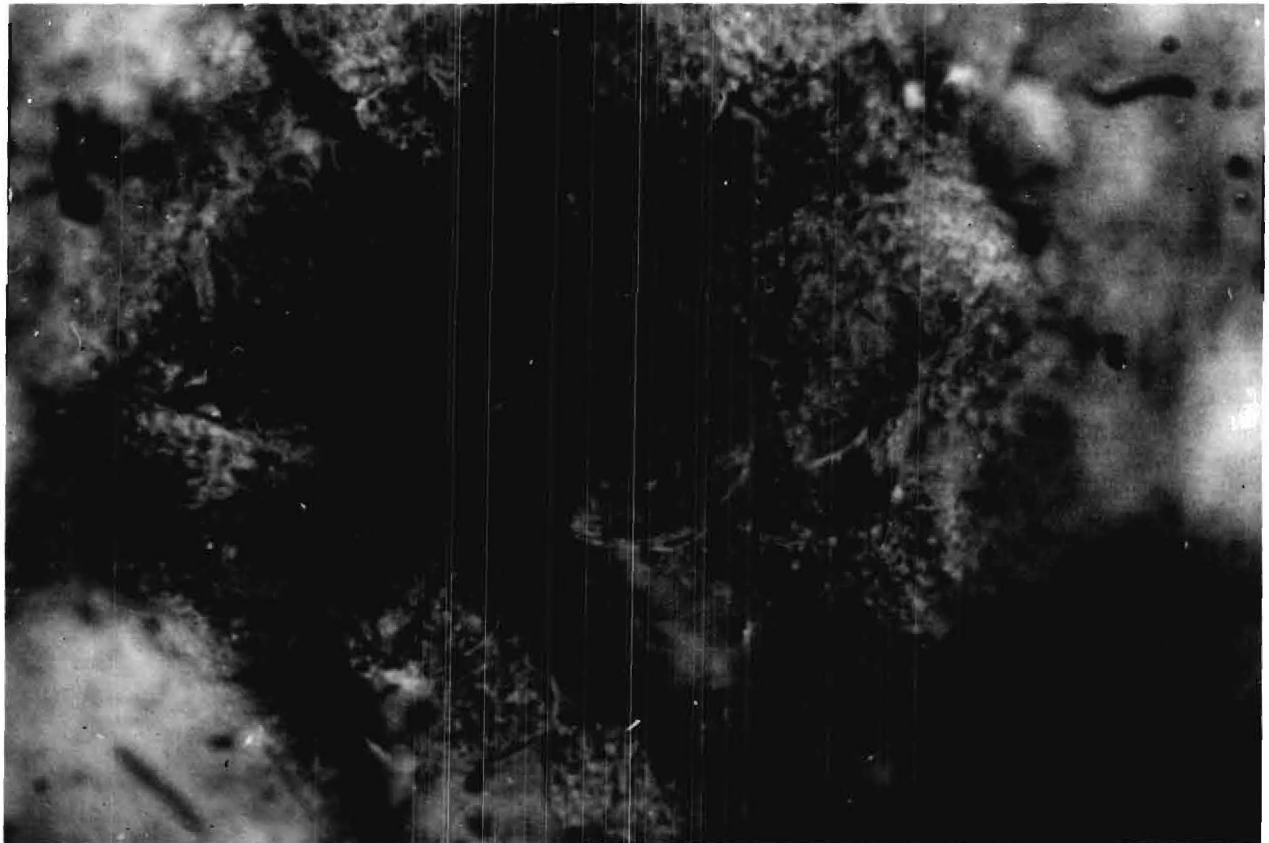


FIGURE 18. Eight minutes later Cells A and B in late telophase. Cell E in interphase.

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

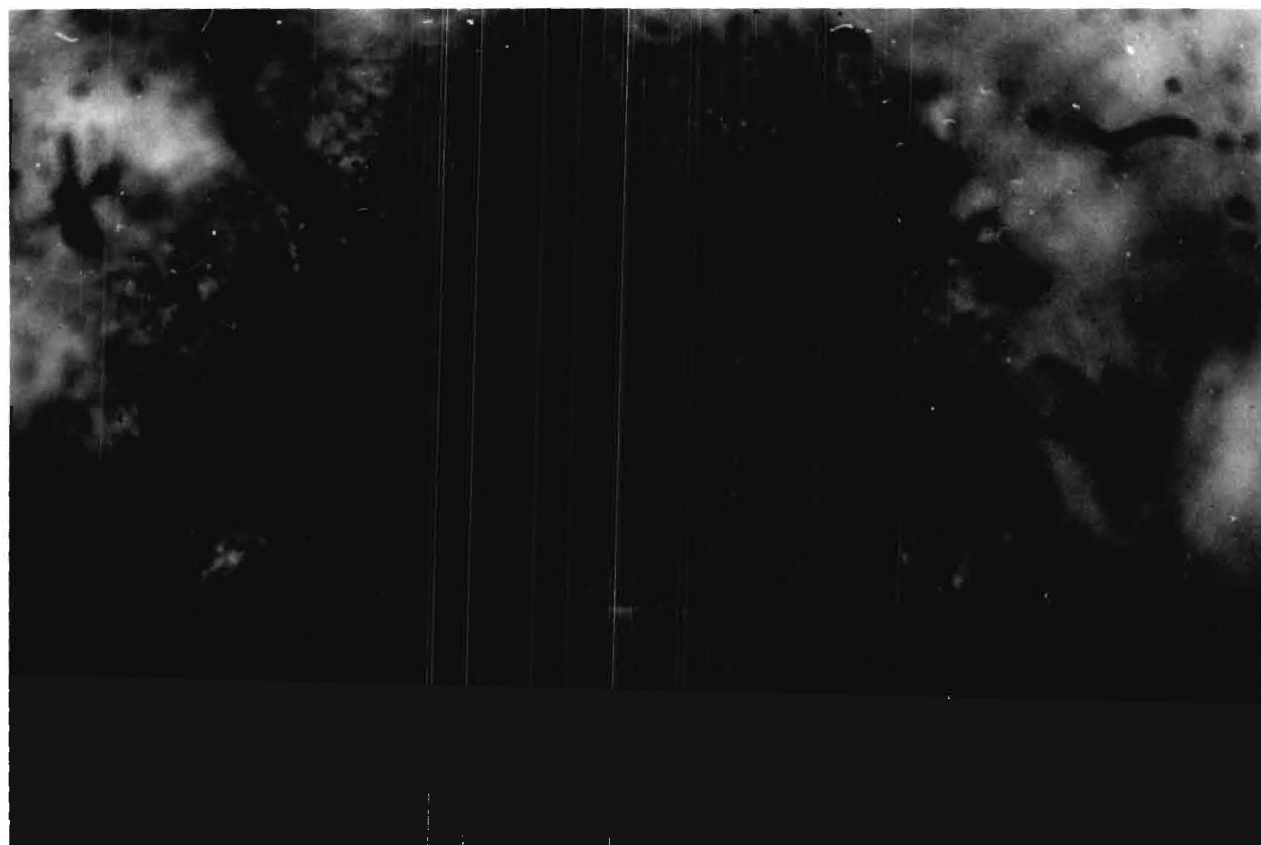
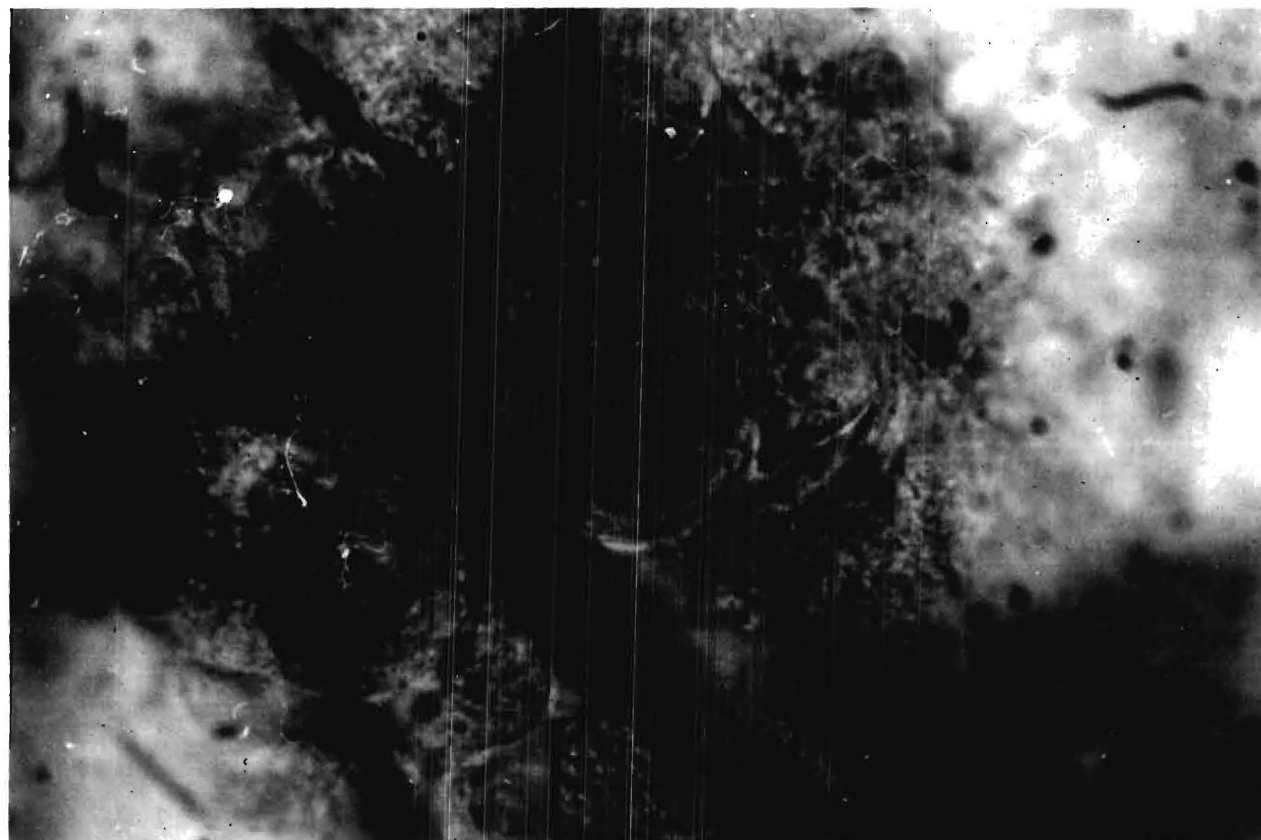
Cell A is now in interphase; the chromatin granules appear scattered throughout the homogeneous nucleus. Actually the nuclear granules lie immediately adjacent to the nuclear membrane and when the nucleus is in optical cross section, as in these photographs, only a few of the nuclear granules are visible, 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.

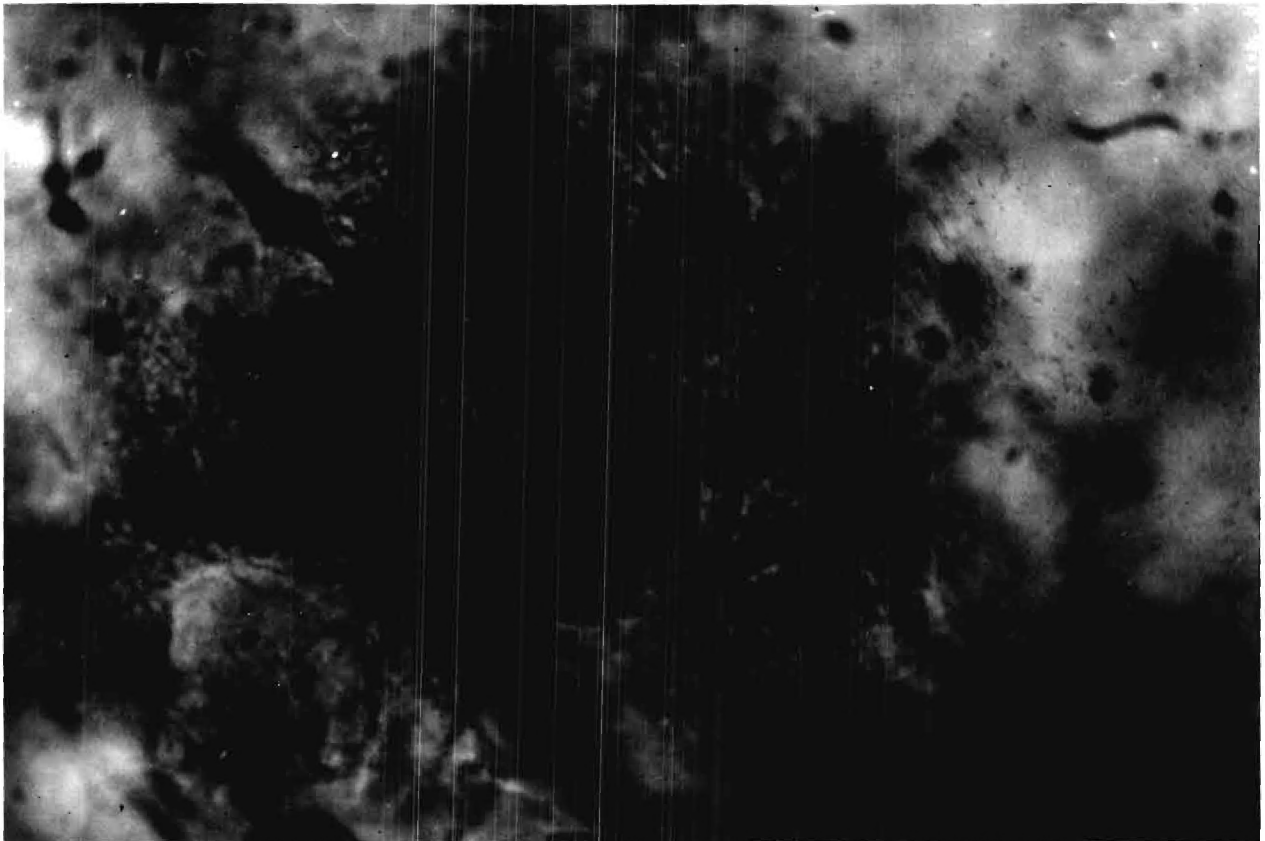
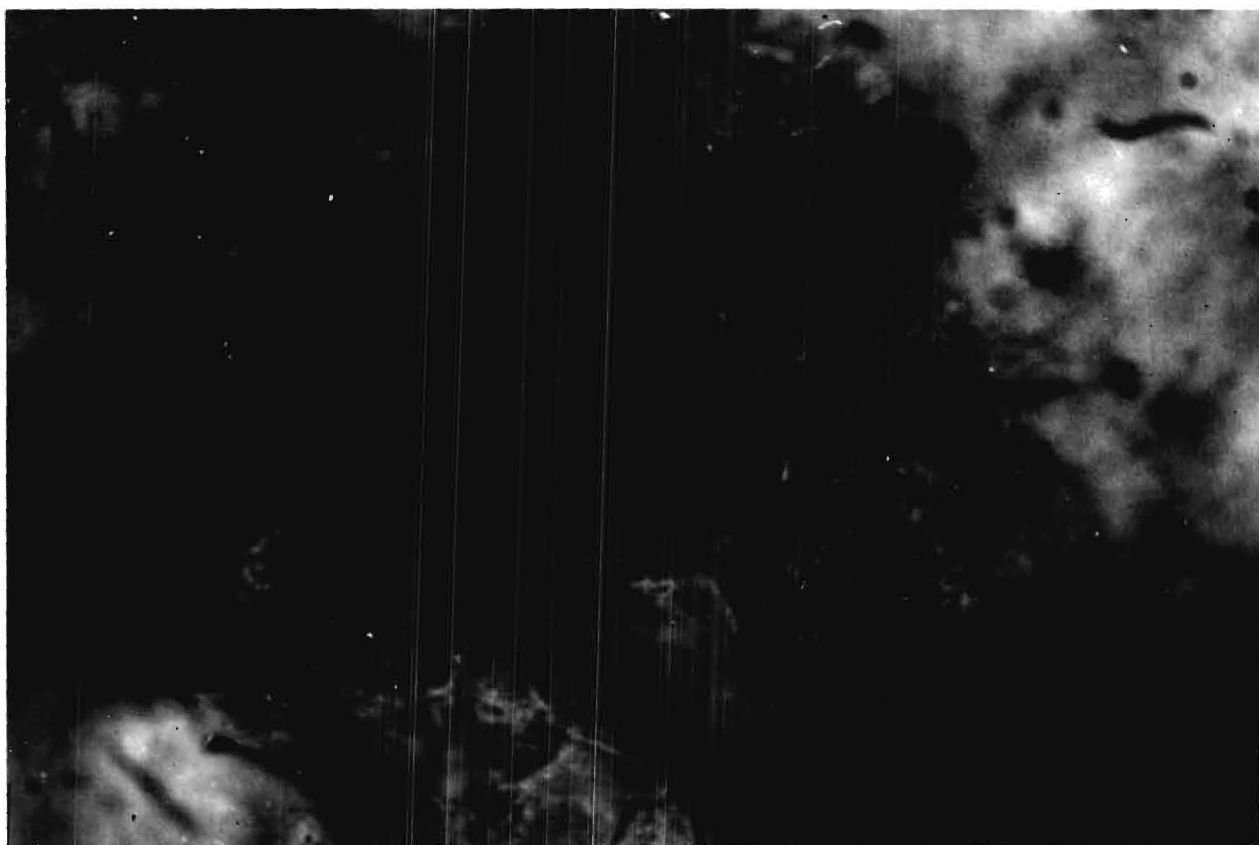
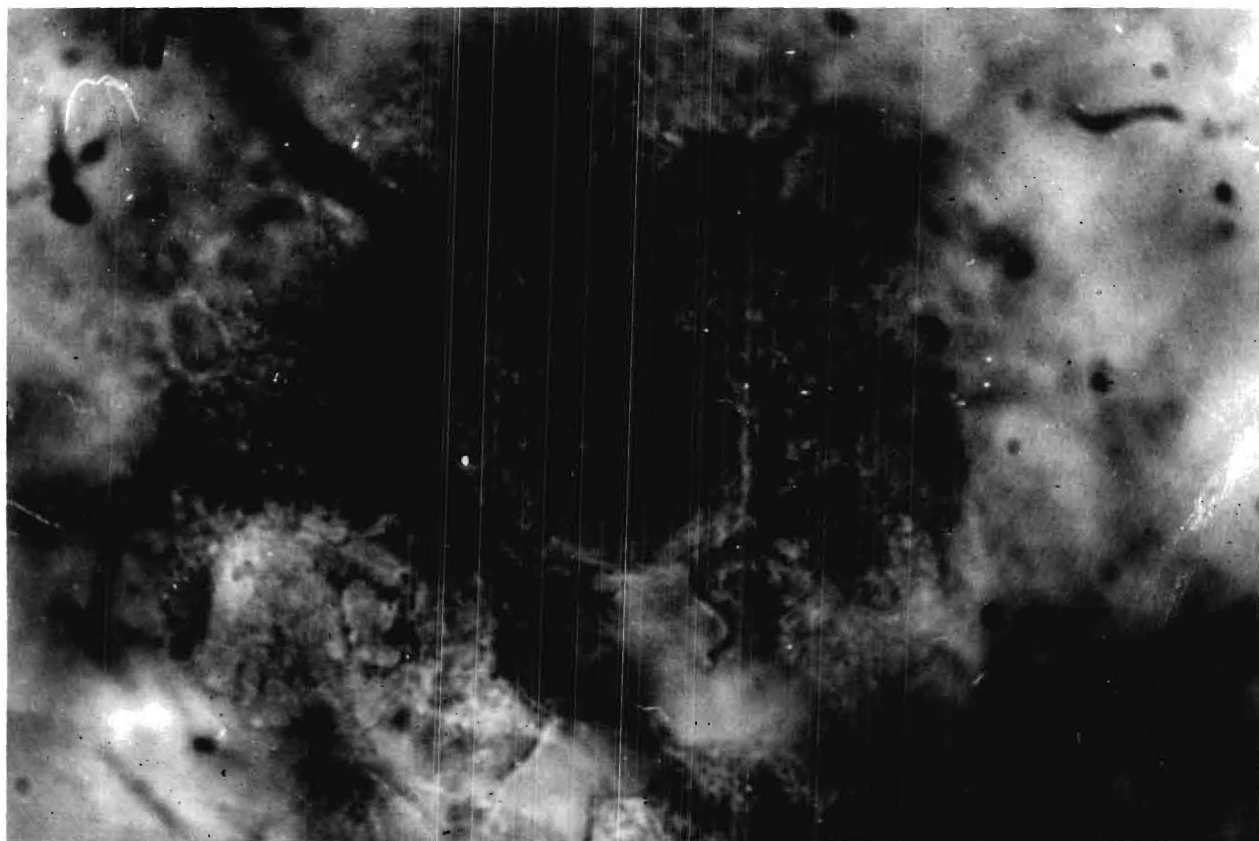


FIGURE 24. Twenty-two minutes later Cell A in early prophase.

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

<u>State Treated</u>	<u>No. Cells</u>	<u>Total Time To Next Anaphase (Min.)</u>	<u>Theoretical Time (Min.)</u>	<u>Difference</u>	<u>P**</u>
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

<u>Successive Stages</u>	<u>Total Duration (Min.)</u>	<u>Expected* Duration (Min.)</u>	<u>Difference (Min.)</u>	<u>P**</u>
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

<u>Successive Stages</u>	<u>Total Duration (Min.)</u>	<u>Expected* Duration (Min.)</u>	<u>Difference (Min.)</u>	<u>P**</u>
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 $37.5 \pm 0.5^{\circ} \text{C}$.

Cell No.	Duration of 5 Waves (Sec.)	Time ⁺ (Sec.)	Average Time Per Cell (Sec.)
1	11	154	150
	9	126	
	12	168	
2	9	126	158
	12	168	
	12	168	
3	15	210	177
	10	140	
	13	182	
4	9	126	140
	10	140	
	11	154	
5	10	140	163
	12	168	
	13	182	
6	11	154	163
	12	168	
	12	168	
7	14	196	182
	13	182	
	12	168	
8	9	126	140
	12	168	
	9	126	
Total	272	3808	159
(+) Duration of 5 waves (Sec.) x No. Frames Sec. on projection (14) x photographs per Sec. (1). $\bar{x} = 159, \sigma = 16, n = 8, 159 \pm 6 \text{ Sec./5 waves.}$			

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 $37.5 \pm 0.5^{\circ}$ C.

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

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 irradiation¹¹ 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 discussed¹² and Phillips¹³ 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 Hill¹⁴

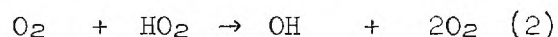
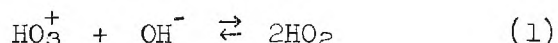
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12. R. F. Kimball and N. Gaither, Proc. Soc. Exp. Med. Biol. 80, 525 (1952); G. Scholes and J. Weiss, Exp. Cell Res. (Supp. 2), 219 (1952).

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



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	<u>14,613.12</u>
TOTAL	\$43,803.70

Respectfully submitted:

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