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EFFECTS OF SUBOPTIMAL TEMPERATURE ON CELL DIVISION
IN HUMAN LYMPHOCYTES IN VITRO

by

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TABLE OF CONTENTS

	page
Introduction	1
Materials and Methods	13
Results and Conclusions	16
Discussion	19
Summary	27
Illustrations and Tables	29
Bibliography	42

INTRODUCTION

Studies were conducted on human lymphocytes in vitro aimed at ultimately defining the mechanism by which exposure to suboptimal temperature acts on cell division. Even though various effects of "cold" have been observed by several investigators, the mode of action of exposure to suboptimal temperature on the process of cell division is only vaguely understood.

Studies conducted with "cold" adapted mammalian cell lines of L (mouse subcutaneous fibroblasts), Detroit-6 (human bone marrow) and HeLa (human epidermoid carcinoma) lines have revealed that exposure to 4°C influences certain activities in the dividing cultures more than others. These cell lines were isolated from the survivors of wild cell populations exposed repeatedly to 4°C for increasing time periods, and have been maintained from two to four years. (Holeckova, et al, 1965) The three cold-adapted cell lines showed increased numbers of polyploid cells as compared to controls maintained at 37°C, which persisted for months after the last exposure. Immediately after 4°C exposure, increased endoreduplication (re-duplication occurring during interphase, producing diplochromosomes which may be observed as the cell enters mitosis) was evident. The cold-adapted cell lines showed increased synthetic efficiency and increased growth rate which was not seen in other polyploid lines investigated. (Cerny, et al, 1965; Holeckova et al, 1967)

Swedish investigators working with the human epithelial cell line Lu 106, and the fibroblast-like strain HEL, both derived from embryonic lung tissue, have described the effects of a single exposure to suboptimal temperatures of 3^o, 10^o, and 20^oC (most experiments at 3^oC) on the chromosomes. The frequency of polyploid chromosome numbers was increased in this short cold treatment. This increase was attributed by the investigators to a selective phenomenon since the short exposure would not likely have allowed time for the action of chromosome doubling mechanisms. A higher frequency of endoreduplications in Lu 106 was also noted and was compared to a similar tendency induced by a variety of treatments. Detailed karyotype analysis revealed a ruggedness in chromosome outlines, alternating segments of the chromosome showing strongly stained and unstained regions in irregular sequence along the chromosome length, the unstained regions appearing as constrictions. These constricted regions showed a tendency toward breakage, as a result of cold treatment. It was concluded that the constrictions were probably exaggerations of normal secondary constrictions, indicative of the chomomeric pattern. The pattern of breaks induced by cold treatment was different from the pattern produced by two DNA inhibitors studied, but the patterns produced by cold and DNA-inhibitors were more similar to each other than to patterns produced by either X-ray (random breakage) or measles. The observation that breaks induced by low temperature exposure produce definite patterns, while other types of treatment induce random breakage, was related to the fact that various treatments probably affect different stages of the chromosomal replication. It was

significant to the investigators that a non-specific influence such as suboptimal temperature might cause highly specific, localized chromosome breakage. Cold treatment produced a high incidence of dicentrics and acentrics, which were formed during the preceding G₁ or pre-DNA-synthesis stage of the cell cycle. (Hanpel, and Levan, 1964) Abnormal anaphases, with bridges and acentric fragments, were present in greater frequency after cold treatment.

The above studies indicate that temperatures of 3° and 40°C affect certain processes necessary for the normal functioning of the mitotic apparatus, as well as the morphology of the chromosomes.

Small temperature changes seem to produce less dramatic effects, but do affect the kinetics of certain parts of the division cycle differentially. Sisken and coworkers (1965), in studies with monolayer cultures of human amnion cells, found that temperatures of 34°-40.5°C affect all phases of the mitotic cycle; temperatures above and below the optimum prolonged all phases. (See Figure 1, and page 7) But the effects were most pronounced in some phases than others. They found G₁ and metaphase to be most sensitive to temperature change, G₂ and the period between completion of DNA synthesis and metaphase to be less sensitive, and anaphase essentially insensitive (with the qualification that higher temperature favors shorter anaphase in contrast to the U-shaped response of each of the other parts of the cycle). (Sisken, 1965; Sisken, et al, 1965)

Rao and Englebert (1966) studied the effects of temperatures ranging from 33°-40°C on HeLa cells in suspension culture. The immediate effect of a temperature shift was to increase the relative

duration of mitosis the longest, followed by G_1 , S, and G_2 in decreasing order. The durations of the phases increased during the transient period until they approached a steady-state value (in contrast to Sisken's studies in which such a progressive increase was not found). Determinations of the stage durations, after cells had reached the steady-state, revealed that at low temperatures the duration of mitosis and metaphase in particular was much prolonged. In the range from 25°C - 31°C , cells could not be grown in the steady state. During the transient from one temperature to another the rate of cell division approached zero, while the mitotic index increased with time. At these suboptimal temperatures, cells in metaphase had a normal appearance, with intact spindles and well-defined chromosomes. However, mitoses which accumulated in the cold did not divide in the normal manner when returned to 37°C . In shifting the cells from 37°C to 29°C , it was found that the duration of mitosis depends both on the temperature and the time the cells spend at this temperature before entering mitosis. When transferred to 29°C in G_2 and S, cells divided normally upon entering mitosis, while those transferred in G_1 showed abnormal anaphases; the chromosomes traveled to the poles individually or in small groups, and the sister chromatids often remained together (Rao, and Engleberg, 1966).

In summary, effects of subnormal temperatures upon cells cultured in vitro have been manifested on the mitotic process by producing polyploidy, on the chromosomal level in induced constrictions and breaks, and on the relative durations of G_1 , S, G_2 and mitosis in the cell cycle (G_1 and metaphase being prolonged at sub-normal temperature).

It would seem probable that these effects are interrelated--perhaps explicable in terms of a specific mechanism by which sub-normal temperature acts, or a site of action which would be common to chromosomal structure, and G_1 and metaphase activities, perhaps an action on certain types of proteins.

A clue to the mechanism might be found in studying polyploidy resulting from suboptimal temperature exposure. One might ask several questions, one of them being, at what point in the cell cycle does reduplication of the chromosomes occur?

Hsu and Moorhead (1956) describe five types of reduplication:

1. Inter-reduplication, or endoreduplication, occurs when chromosomes reduplicate during interphase. As the reduplicated chromosomes enter mitosis, diplochromosomes (paired homologs) may be observed.
2. Pro-reduplication, or endomitosis, occurs when the chromosomes are slightly condensed and within the nuclear membrane, as during prophase.
3. Meta-reduplication, or C-mitosis, is a result of metaphase without a normal spindle. (The spindle may be well-formed at first and then degenerate, or may never be formed normally.) The chromosomes are reconstituted into a nucleus without going through anaphase and without normal cytokinesis.
4. Ana-reduplication results when chromosomes move back to the equatorial area from the poles.
5. Telo-reduplication is a simple fusion of daughter cells after cytokinesis.

Since endoreduplication was noted in the above cold exposure studies immediately after exposure, the polyploidy persisting over several generations might be a result of normal division of endoreduplicated cells in the mitosis following a few endoreduplications in the first generation. Abnormal anaphases and prolonged metaphase (which means that the pole-to-pole movement during anaphase is

delayed) suggest a possible effect on the mitotic spindle apparatus, which could be an effect initiated during mitosis, or an effect initiated during interphase which shows up during mitosis.

Increased polyploidy is often cited as a means by which cells adapt to adverse, or other than normal, conditions. For instance, cells taken from normal mammalian organisms and grown in cell or tissue culture usually give rise, after years of subculturing, to cell lines, whose modal chromosome number is polyploid. This is a selective phenomenon, since the few polyploid cells in the first few generations of culture can survive conditions under which diploid cells would die out; consequently, the population after several generations would be predominantly polyploid.

The polyploidy resulting from below-normal temperature exposure might be explained in these terms, but the explanation falls short of explaining two observations made by the investigators working with cold-adapted cell lines (p. 1). It does not account for the initial endoreduplication observed, nor does it account for the increased growth rate which was not found in other polyploid lines studied.

The present research attempts were efforts to reproduce some of these effects of suboptimal temperature on short-term lymphocyte cultures, in particular, to induce polyploidy by subnormal temperature exposure. It would seem interesting to note whether or not cultures of relatively "de-differentiated" cells were effected in a fashion similar to the cell lines of epithelial and fibroblast-like cells; whether or not cells exposed only briefly in short-term culture to cold could be induced toward increased polyploidy. If this were the

case, this system might be studied in detail in an attempt to define the mode of action of suboptimal temperatures upon cell division, if indeed there is a specific effect.

In vitro cultures of human peripheral lymphocytes were used in these studies. Mature lymphocytes in the peripheral blood system in vivo do not normally divide, but are in the G_0 or G_1 stage. Medium and large lymphocytes in the lymph nodes and spleen divide and give rise to these peripheral blood lymphocytes. In vitro, small peripheral blood lymphocytes, with darkly staining nuclei, are transformed into large blast-like cells with palely staining large nuclei, showing abundant cytoplasm and prominent nucleoli. These blasts are capable of undergoing mitosis. Morphologically similar to undifferentiated, primitive blood cells, the blasts may be the result of de-differentiation. The transformation may occur spontaneously, (Sabesin, 1965), but the process is more effectively induced by the addition of phytohemagglutinin (PHA) to the cultures. PHA is an extract from the red kidney bean. Cultures containing PHA may contain 70% blastoid cells on the third day of culture, the diameters of the largest blasts being three times the size of small lymphocytes. Glycolysis is probably the main energy source for blastogenesis, which can occur in the absence of oxygen. Glucose is utilized, with the production of lactic acid. (Robbins, 1964) RNA synthesis takes place during the first 24 hours of culture (G_1), the initial response being the synthesis of non-ribosomal RNA, which is probably m-RNA. At some point in the 25-34th hour of culture, DNA synthesis begins. The first S period is estimated by Bender and Prescott (1962) to be approximately 12 hours, followed by a G_2

stage of six hours. Transformation, the progressive cell enlargement of the cells, is essentially completed in 24-48 hours. It seems that the initial cell cycle takes approximately 48 hours. In subsequent proliferation, the generation time is about 19-22 hours. Sasaki and Norman attribute the increase in DNA synthesis, which is evident from 48-72 hours, entirely to cell proliferation. After 60-72 hours of culture, cells are in their second cycle. A peak of mitosis is usually around the 72nd hour of culture.

The aspects of blastogenesis itself have been studied by several persons in the past four years. (Robbins, J., 1964; Sabesin, 1965) Interesting results were demonstrated by Epstein and Smith (1968) in recent studies in which mouse lymphocytes were induced by PHA to undergo transformation in vivo. The changes observed were similar to those seen in vitro. There was a marked shift in the mesenteric node and the peripheral blood, from small lymphocytes to medium and large ones, many resembling the blast-like cells seen in vitro.

During blastogenesis in cell culture, there are always some lymphocytes which do not undergo transformation, even in high concentrations of PHA. This is apparent in Figure 2, which shows small lymphocyte nuclei from cells which did not transform, along with large nuclei from blast cells, with their pale staining and prominent nucleoli. Figure 3 shows the nucleus of a blast-like large lymphocyte, actually 47μ in diameter. The cell nuclei in a typical field in our cultures ranged from $8-45 \mu$.

The generation time for human peripheral lymphocyte culture after the completion of blastogenesis has been reported from the

results of autoradiographic studies. The durations of the cycle stages as reported are as follow:

	Cave (1966)	Susaki and Norman (1966)	German (1964)
G ₁	4.6 hrs.	6 hrs.	
S	9.6	11	
G ₂	3.5	3	3-4 hrs.
M		<u>2</u>	
Total		22 hrs.	

The discrepancies in the reports of these investigators are probably a result of differences in method of culture. In our own laboratory it has been found that the number of blood cells per volume of culture medium is an important variable. A micro-technique, in which three drops of blood are cultured in five milliliters of medium, is employed in our laboratory.

The diagram in Figure 1 illustrates a generalized cycle, based on a total generation time of twenty hours duration. The cells transverse in a clockwise direction. G₁ occupies the part of the cycle from the end of telophase to the initiation of DNA synthesis. S is the period of DNA synthetic activity, and G₂ is the interlude between the cessation of DNA synthesis and mitosis, M. (A prolongation of G₁ would simply refer to the fact that DNA synthesis is delayed.)

Lymphocytes in tissue culture transverse the cell cycle asynchronously. At any one time in the cycle of 20 hours total, theoretically 1/2 of the cells would be synthesizing DNA, 1/4 in

the pre-DNA synthesis period of G_1 , 7/40 in G_2 , and 3/40 in mitotic division. In subjecting such a culture to suboptimal temperature, no cause and effect relationship between the time of exposure and a specific stage of the cell cycle can be postulated. In view of this, a synchronized culture would be desirable in which at any one point during the cycle, the majority of cells would be engaged in the same cyclic process. If this were accomplished, the sensitivity of G_1 and mitosis to temperature could be investigated more thoroughly. Indeed, such a synchronized culture would be valuable in future studies of any parameters during lymphocyte cell culture.

Several investigators have achieved partial synchronous cultures of mammalian cells by the application of an excess of thymidine, a 2'deoxyriboside of thymine (1- β -D-2-deoxyribofuranylthymine), which in normal physiological amounts is the precursor of DNA synthesis by the following scheme:

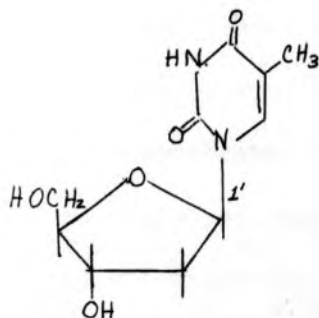
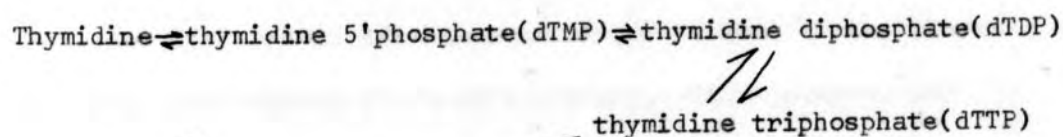


Figure 4. Thymidine

The thymidine is phosphorylated upon incorporation into the cell.

A large excess of thymidine (amounts of 2.0-25 mmoles have been used in previous studies) inhibits DNA synthesis. The mechanism is only vaguely understood;

investigators attempting explanations disagree, and the most recent persons using this technique do not speculate about it at all. Generally stated, the effect is probably a type of negative feedback inhibition, in which the excess thymidine inhibits one or more enzymes required for the conversion of immediate or distant precursors to their reaction products. (There is indication from recent studies, that excess thymidine blockage did not affect thymidine kinase activity. The investigators concluded that this enzyme forming system was apparently regulated by thymidylate and deoxycytidylate; Stubblefield and Murphie, 1967) If a regulation site were affected, the precise site of action would be difficult to define, since inhibition might occur at various points along the pathway which are not directly affected by the excess thymidine.

Xeros (1962) found that excess thymidine to a level of 2mM, applied for 24 hours to monolayers of genetically heterogeneous Chong-appendix cells effected partial synchrony. Accumulated C-mitoses in 6 1/4-12 1/2 hours after the release of the block were 44%, and between 6 3/4-12 3/4 hours, 40%. Peterson and Anderson (1964) achieved synchrony by applying two thymidine blocks to a suspension culture of Chinese hamster ovary (CHO) cells and in HeLa monolayers. Bootsma and co-workers (1964) synchronized a cell line of human kidney cells with a double block, obtaining 80-90% synchrony in the S phase after 1-3 hours, and 15-25% of cells in mitosis after 8-10 hours. Similar work was reported by Puck (1964), who employed the double block technique. He found that excess thymidine affects all stages of DNA synthesis, in that the first block stops cells at all points during the S phase. In HeLa

cells, synchronized by this method by Rao and Engelberg, 90% of the cells divided between 7-11 hours after the release of the second thymidine block. These workers emphasize that although as many as 98% of the cells accumulate at the beginning of S in a region about 5% of the total generation time, after release of the block and the ensuing mitosis, the cells are no longer in this synchrony due to intrinsic variability and the nonhomologous population.

The following series of studies was conducted in our laboratory in view of the overall plan to define the mechanism of action for the effects on cell division produced by below-normal temperature exposure. The studies to date are only preliminary steps in this direction. Since no previous work dealing with temperature effects had been initiated in our laboratory, the preliminary investigation dealt with the development of techniques for such a study and evaluation of these in regard to possibilities for future research.

Three series of experiments have been conducted:

- I. Lymphocyte culture under normal conditions at 37°C.
- II. Exposure of lymphocytes to 4°C and 15°C for varying lengths of time.
- III. Modification of the excess thymidine technique for inducing cultures to divide synchronously--as a possible aid in the temperature studies.

MATERIALS AND METHODS

Preparation of culture medium

Vials to contain media were autoclaved and the plastic tops were soaked in 70% ethanol, usually for several days. Using sterile technique, the following were mixed: Medium 199 (with sodium bicarbonate, Microbiological Associates), 800 ml.; Fetal bovine serum (Microbiological Associates), 200 ml.; Sodium heparin solution (1000 units/ml. solution, an anticoagulant), 20 ml.; phytohemagglutinin-M (Difco Laboratories, rehydrated with sterile distilled water), a mitogenic agent, 20 ml. Approximately five ml. of medium were poured into each culture vial. Vials containing medium were frozen until needed.

Method of culture and preparation of cells for cytological analysis

The method of lymphocyte culture was a modification of Moorhead's and Nowell's, developed for use in our laboratory by Dr. L. G.

Anderton and J. O. Hall. It involved the following:

- 1) Collection of blood--Three to four drops of blood obtained from the finger or arm of the subject by sterile technique were placed into prewarmed vials of sterile medium.
- 2) Incubation--The vials were stoppered tightly, gently swirled to mix, and incubated at 36.5°-37°C, for four days. Culture medium was observed daily for pH changes as indicated by the phenol red included in the medium and adjustment made as indicated.
- 3) Cells arrested at metaphase--On the morning of the fourth day (counting the day the culture is set up as day 0), 0.02 ml. of colchicine solution (100 g/ml., Nutritional Biochemicals Corporation) was added with a tuberculin syringe, and the culture was reincubated for 3-5 hours. Colchicine acts to arrest mitotic cells in metaphase by preventing spindle formation. (Tannock, 1967, and Kihlman, 1966, discuss various metaphase arrest agents).

- 4) Sedimentation of cells--The contents of the vials were aspirated, and placed in a graduated conical centrifuge tube (pooling the three vials from each subject in one tube). Cells were centrifuged at 280-450 RCF for ten minutes, and the supernatant removed.
- 5) Washing of cells--8-10 ml. of Hanks balanced salt solution (Microbiological Associates) was added to the cells in the tube to wash them free of exogenous medium. Cells were resuspended by aspiration and centrifuged.
- 6) Hypotonic treatment--All but 0.5 ml. of Hanks was removed, and 2.0 ml. of glass distilled water was added, and allowed to stand 8-10 minutes. This hypotonic treatment caused hemolysis of the red blood cells and served to burst the nuclear membranes of the lymphocytes, dispersing the chromosomes.
- 7) Removal of red blood cells--Resuspension and centrifugation followed the hypotonic treatment, and all of the supernatant, including the "shadow cells" of the hemolyzed red blood cells were removed.
- 8) Fixation--Two ml. of freshly made fixative was added to each centrifuge tube, avoiding disruption of the pellet (glacial acetic acid: absolute methanol, 1:3). This was allowed to stand stoppered and undisturbed for 30 minutes. Upon resuspension, centrifugation was repeated. The supernatant was discarded and the cells were resuspended in approximately one milliliter of fresh fixative, a fine suspension being obtained by repeated aspiration with a fine-tipped pipette and continued change of fixative, if necessary.
- 9) Preparation of slides--One drop of cell suspension was allowed to run down the cold wet surface of each slide at a 45° angle (clean slides having been in a container of iced, distilled water). Each slide was immediately passed face down through a flame until it was just warm to the back of the hand. The slides were waved vigorously to complete drying as rapidly as possible. If metaphases were scarce or concentrated, adjustment was made by resuspension in less or more fixative.
- 10) Staining (on completion of drying)--
 - a) slides are hydrolyzed in 1 N HCl at 6°C for 10 minutes (to remove acid soluble protein not denatured previously)
 - b) rinsed in distilled water
 - c) placed in freshly made Giemsa for 8-15 minutes (distilled water: stock Giemsa:0.15M NH₄OH, 90:10:7)
 - d) dipped in Acetone I--4 dips, Acetone II--6 dips

- e) placed in acetone-xylol, 1:1 for 2 minutes,
- f) placed in Xylol I, 5-10 minutes, Xylol II, for 10 minutes,
- g) slides were mounted in permount and coverslips were placed on. The prepared slides were allowed to dry thoroughly and were subjected to microscopical analysis. Selected representative metaphases were photographed, and karyograms were prepared.

Preliminary studies under normal conditions of culture were conducted in order to become acquainted with the techniques involved. In the second series of experiments cultures were subjected to sub-normal temperatures of $4^{\circ} \pm 2^{\circ}\text{C}$ and $15^{\circ} \pm 2^{\circ}\text{C}$ for varying lengths of time and at different points during the culture period, from 48-72 hours. Each exposure was followed by incubation at 37°C , prior to harvesting the cells (with the exception of one experiment).

In the third series of experiments, application of excess thymidine was accomplished by a modification of the methods of Puck, Bootsma and co-workers, Peterson and Anderson, and Rao and Engelberg. After approximately 60 hours of culture, the medium was decanted from the vials containing the cells to be synchronized. Medium containing thymidine (3 mg/ 5 ml., 2.5mM) was filtered through a millipore filter, pore size 0.22 , in a Swinny Adapter, directly into the vials containing cells. Cells were resuspended and the vials incubated at 37°C . To remove excess thymidine, the medium was decanted, the cells washed in sterile Hanks balanced salt solution two or three times (with centrifugation at 130°CF), and the sterile media was added to the cells. The medium was changed in the control cultures when thymidine was added or removed from the experimentals. This procedure was repeated upon application and removal of the second block.

Upon the application of the first thymidine block, cells were incubated at 37°C for the duration of the total generation time, minus S, which allowed the cells to be stopped in the S stage. Upon release from the first block, cells were allowed to incubate for the duration of S, when a second thymidine block was applied--causing the accumulation of all the cells at the beginning of the S stage. After removal of this second block, the cells continued through S and G₂. Colchicine was added to arrest the cells in metaphase, and the cells are treated as indicated previously. Figure 5 illustrates the time sequence of events during induction of synchrony, based on arbitrary average values for the cell cycle time.

RESULTS AND CONCLUSIONS

I. Culture under normal conditions

In the lymphocyte cultures of four days duration at 37°C, the modal chromosome complement was diploid, with a chromosome number of 46. Figure 6 shows a typical chromosome spread (of a female) from a 4-day old culture, and Figure 7 is a karyogram of this spread.

II. Exposure of cultures to 4°C and 15°C

The results of these experiments are compiled in Table I. In these preliminary studies on subnormal temperature exposure, cells exposed to 4°C seemed to divide normally upon reincubation at 37°C. In those subjected to 15°C, there was evidence of a premature separation of sister chromatids and of the type of heterogeneous

staining reported by Hanpel and Levan. (See pp. 1 and 2) Figures 8 and 9 show these effects in their most exaggerated forms. The incidence of polyploidy was insignificant. Figure 10 shows a polyploid spread with otherwise normal chromosomes. The polyploid cell in Figure 8 shows the effects mentioned above. (Both of these spreads are from Exp. C.)

It would be difficult to make any generalizations concerning the results of these experiments. The time at which cold was applied varied unsystematically from one experiment to another as can be seen in Table I, and time did not permit repetition of each of these. More detailed cytological examination of the prepared slides might reveal other minor effects, but such study probably could not be evaluated in view of the lack of any rigorous design in the original planning of these studies.

III. Synchrony with excess thymidine

Results are indicated in Table II. Exp. A was based on a generation time of G_1 -9 hours, S-6 hours, G_2 -10 hours, M-1 hour. Experimentals showed a mitotic index of 0.247 as compared with 0.03 in the controls. The metaphases examined appeared normal, as in Figure 11. The subsequent experiments B, C, and D were based on the generation time-- G_1 -5 hours, S-10 hours, G_2 -3.5 hours, M-1.5 hours, which corresponded with that reported by Cave, and Susaki and Norman. In B, the experimentals and controls showed the same mitotic index. Results were inconclusive in C and D, due to the bacterial contamination found. The count of cells in D revealed a mitotic index of only 0.083 in experimentals, and 0.023 in controls.

The relative lack of success of the most recent synchrony experiment may be due in part to the generation time used as a basis for the thymidine schedule. It is possible that Cave's and Susaki and Norman's values are not applicable under the conditions in our lab. Furthermore, the durations of the various stages in the cycle may be shifted as a result of the thymidine inhibition. Rao and Engelberg in their studies found that the duration of S plus G₂ was 7 hours in the synchronized cells in contrast to 10.5 hours in unsynchronized cells at 37°C. The various stage durations could be delineated by autoradiographic studies, but at present this cannot be accomplished in our lab.

If this synchrony technique were perfected for use in our lymphocyte culture, it could be used in connection with suboptimal temperature exposure, as stated elsewhere. Before this could be done effectively however, a more rigorous means of maintaining constant temperatures would need to be devised, as well as a more efficient means for gradually shifting the temperature of the cultures to avoid "temperature shock" in some of the studies.

Biochemical studies of the sort accomplished with cell lines, might be valuable in conjunction with a synchronous lymphocyte culture, in efforts to compare the characteristics of short-term and long-term culture on the molecular level--and in relation to differentiated and undifferentiated mammalian tissue. It would be interesting to extend these effects to a longer-term lymphocyte culture, by subculturing the cells in fresh medium 72 hours after the initiation of incubation, and thereafter every 40 or so hours. McCarty and co-workers (1965) were able to isolate and propagate a

continuous culture of human lymphoblasts (CCRF-CEM) from the peripheral blood of a patient with acute lymphoblastic leukemia. Chromosome counts revealed modal numbers of 46 and 47 chromosomes.

DISCUSSION

The attempts to induce polyploidy in short-term lymphocyte culture and the use of excess thymidine to induce synchrony in these cultures have been relatively unsuccessful to date. But these experiments, even though not producing the desired results, are valuable, as any such preliminary steps would be, in evaluating experimental design in relation to possibilities for future studies. It is evident that subsequent experiments should be undertaken only on the basis of very rigorous experimental design. Culture techniques and temperature exposure parameters should be strictly uniform. Prior to further use of synchrony techniques, the durations of the cell-cycle stages would need to be determined for the conditions in our laboratory. Autoradiography could be used to accomplish this. If the equipment were available, time-lapse cinemicrography would be valuable in determining the durations of the various mitotic stages, as well as allowing one to view the mitotic events as they happen. Polarization optics could be employed to view the highly organized spindle structures. Autoradiographic studies with isotopically labeled protein precursors, such as leucine and valine, and arginine and lysine (which are the main two amino acids in histones) might be designed in connection with tritiated thymidine and/or suboptimal temperature exposure, in order to determine the effects of the low temperatures on DNA and protein synthesis.

Research such as that suggested briefly above might aid in the attempt to elucidate cell division activities during and after exposure to suboptimal temperatures. (But since such studies would require use of unfamiliar techniques and require various time-consuming experimentation, it would be unrealistic to attempt this in our cytogenetics laboratory at the present time.)

Several correlations can be postulated and questions can be explored in relation to the temperature effect on cell division. Since the most obvious correlation of temperature changes would appear to be on proteins, a consideration of protein synthesis during the cycle would be relevant (although temperature shifts could effect existing proteins as well as newly synthesized ones). Work by Donnelly and Sisken (1967) with puromycin and actinomycin D suggests that in order for division to occur, new protein molecules must be synthesized within a 30-60 minute period prior to metaphase. Are these perhaps the spindle proteins? They found that inhibition of nucleolar RNA synthesis did not effect the entry of cells into mitosis for periods up to three hours, inferring that cells in the latter part of the cell cycle contain a 1-3 hour supply of ribosomes utilizable for the synthesis involved in preparation for division. Cells, in which ribosomal RNA was inhibited in G_1 , required more time to get to division; cells in which ribosomal RNA was inhibited during late S or G_2 were not delayed in entering division, but the succeeding cycle was prolonged. This infers that ribosomal RNA synthesis in G_2 is necessary for the ensuing G_1 and/or S activities.

Shapira and Levina (1967) have also been concerned with protein synthesis during the cycle. Their studies in particular on human lymphocyte cell culture, indicate that nuclear protein synthesis

takes place before DNA synthesis, but after a preceding nine hours of RNA synthesis. Their experiments with pulse and prolonged labeling with tritiated arginine and lysine permitted them to conclude that synthesis of proteins and in particular chromosomal protein, proceeds during all of the mitotic cycle, taking place in both prophase and metaphase. The rate in G_1 is 2-3 times less than in S and G_2 .

Subnormal temperature appears to prolong the duration of metaphase in several studies, and seems to maintain an intact, normal spindle apparatus, yet subnormal temperature has little if any effect on the duration of anaphase. This would indicate that any abnormal anaphases resulting from subnormal temperature exposure would be a result of activities prior to the "initiation" of anaphase. Since cells transferred to suboptimal temperatures during G_1 , showed abnormal anaphases, perhaps proteins are synthesized during G_1 which are specifically used in anaphase activities.

Protein synthesis is usually preceded by RNA synthesis. Robbins and Scharff (in Cell Synchrony, 1966) give evidence that the rate of RNA synthesis increases from G_1 to G_2 , remaining steady through S, but decreases to a very low level in metaphase. In relation to a possible effect of temperature on RNA, Byfield (Whitson, et al, 1967) has suggested that the effect of temperature shock in Tetra-hymena might be an effect of drastically reducing the half-life of RNA. It might be possible in the future to relate temperature effects specifically to some of the synthetic activities mentioned above. A generalized summary of these activities and temperature effects is diagrammed in Figure 12.

Also of interest is a consideration of the labilities of structural proteins involved in cell cycle processes. Mazia (1961) mentions in addition to the relation between temperature and rate, the relation between temperature and the physical state of gels and polymers. In this category would fall the spindle apparatus, composed of proteins existing in the spindle configurations for only a fraction of the cell cycle. Mazia's investigation and isolation of the spindle apparatus in sea urchin eggs has allowed extensive biochemical analysis of the apparatus. Much emphasis has been placed on the interconversion of thiol and sulfhydryl linkages in the formation and activation of the spindle. Mazia hypothesizes that other types of weak intermolecular links may be important and warrant consideration. He found evidence in early studies of secondary bonds, probably hydrogen bonds, by solution of the apparatus with urea. It is interesting that the conditions favorable for maintaining the stability of the isolated spindle were favorable to more hydrogen bonding and less hydrophobic bonding (cold temperature, phenol, pH lower than normal). (Hydrophobic bonds are formed when two or more non-polar groups come into contact.) During anaphase the main spindle fibers elongate and eventually "dissolve." Mazia cited hydration of the gel as a possible explanation. Also feasible would be a change in the nature of weak intermolecular bonds, resulting in an "unfolding" or "refolding" of tertiary or quaternary protein structure. M. Joly, in considering the denaturation of proteins, found that the ratio of hydrogen to hydrophobic bond as they vary with temperature, is

important to the stability of protein structure in aqueous systems. In general, both hydrogen bonding and hydrophobic bonding stabilize protein structure, but temperature changes affect these two types of bonding differently. The strength of hydrophobic bonds decreases with decreasing temperature, below 60°C, while the strength of hydrogen bonds increases with decreasing temperature. Joly found that below a maximum temperature (about 60°C), the effect of hydrophobic bonds predominates; the helix stability of the protein decreases with decreasing temperature. Thus, at low temperatures, increased hydrogen bonding and decreased hydrophobic bonding would stabilize the helix. A similar effect might possibly be applied to fibrous proteins and the transitions between crystalline and amorphous forms. For these reasons, the effect of low temperature on mitosis may be one of stabilizing the spindle proteins configuration to such an extent that anaphase, with dissolution of the spindle, is not initiated normally, preventing normal cytokinesis. The effect might also be on the level of chromosomal proteins. Hydrogen bonds bind the chromosomal nucleic acid-chromosome protein complex to histones. (The ratios of histone to DNA are identical for metaphase and interphase chromosomes. Metaphase chromosomes, in an amount equal to histone, contain a mixture of non-histone proteins soluble in HCl--most probably the acid-soluble ribosomal protein, according to Bonner and his co-workers, 1968.)

In considering possible mechanisms of subnormal temperature action on cell division, one is reminded that the basic mechanism is a simple one of thermodynamics. The rates of biochemical reactions are a function of temperature and concentration of reactants.

The rate limiting step in a biochemical pathway at 37°C is probably not the rate-limiting step at a different temperature. For this reason, cells maintained at a subnormal temperature would be expected to contain different relative amounts of macromolecular constituents. On the molecular level, the effect would be quite specific for each enzyme-protein, or particular reaction, but in the complex intercellular environment, many reactions are interrelated in a web of biochemical pathways. Changes in temperature would be expected to alter protein structure, alteration in tertiary and quaternary structure in turn altering micro-hydrophillic and hydrophobic surroundings. Such effects would encompass enzyme activity and subsequent macromolecular synthesis, structural proteins, membrane permeability and transport, energy relations, etc. Therefore, the attempt to delineate a specific mechanism for the effect of subnormal temperature exposure is perhaps at best, unrealistic. Such an attempt must remain on the level of correlation between overall surface evidences of the effects of exposure. If the effect of subnormal temperature was to decrease the rates of a few processes, its overall effect might be to increase the rate of another process which would not be morphologically or biochemically "visible" under normal conditions. This could result simply from a shift in relative rates, or result from activation or inhibition of certain macromolecules at the new temperature, both of which would produce the same overall effect. Even so, the localization of suboptimal temperature influence to specific types of activities (like metaphase and G_1) may help to elucidate the process of cell division, if one remembers that these correlations are arbitrary from a thermodynamic standpoint.

Studies on in vitro culture cannot be directly extrapolated to explain conditions in vivo, for very obvious reasons. Yet such speculation is nevertheless attractive to the newly initiated biologist! Malignant tissue is generally composed of undifferentiated, or "de-differentiated" cells, undergoing active mitoses, similarly to lymphocyte culture. Does "blastogenesis" perhaps occur in the genesis of malignancy? Cancerous cells tend to be more frequently polyploid than non-malignant tissues. Is this a cause, or effect relationship?

In events of low temperature surgery, one might wonder if localized cold application would affect surrounding cells undergoing mitotic activity. Holeckova and his co-workers note that their cold-adapted cell line gains increased resistance to cold without the neurohumoral regulation common in a mammalian organism. These adapted cells are able to live in medium depleted of glucose, which could be caused by increased utilization of lipid substrate, and show increased oxygen consumption, similarly to cold-adapted mammals. Le Blanc has studied the aspects of peripheral tissue adaptation to cold in the rat. He observed in rats exposed to 6°C, that if cold exposure blocked mitosis only for a short time, and if the number of layers in the stratum corneum of the skin increased as well as the number of capillaries, then cold injury was not observed and adaptation took place. In the skin of the abdomen of rats (6°C) there was an increase in the number of mast cells after 1/2—2 months, with a decrease after 4 months of exposure. Similarly, in the skin of the fingers of fishermen adapted to cold water, more

mast cells were found than in control subjects. (Proceedings--Temp. Acclimation, 1963) One might wonder whether or not these mitotically dividing skin cells might be polyploid.

The consideration of possible sites of differential temperature influence on the cell cycle, and the consideration of correlations between synthetic and structural activities having, at best, only vague delineations, is relatively speculative in nature. This is dictated by the lack of abundant research in this area, and also by the inherent nature of an influence as general as a shift to suboptimal temperatures. Temperature shifts would be expected to affect all metabolic processes to different extents. The specificity of action of subnormal temperature would depend upon the level of organization from which one views "specificity." If regarded from the standpoint of overall metabolism, the influence is a nonspecific one. If regarded from the level of particular biochemical processes occurring in an isolated duration of time, the influence could be thought of as specific; yet the boundary lines set up to define such a grouping of processes would be limits arbitrarily imposed. The mechanism for suboptimal temperature influence may of necessity remain undefined in view of the complexity of a cell's milieu intérieur.

SUMMARY

Studies were conducted on human lymphocytes in vitro aimed at ultimately defining the mechanism by which exposure to suboptimal temperature acts on cell division. Investigators working with cell lines have reported that subnormal temperature affects 1) the mitotic process by inducing polyploidy, 2) the chromosomes by causing constrictions and breaks, and 3) the relative durations of G_1 , S, G_2 and mitosis in the cell cycle (G_1 and metaphase being prolonged).

As a preliminary step to relating these effects in a mechanism of action, attempts were made to reproduce some of these effects in human peripheral lymphocytes cultured in vitro by a modification of the method of Moorhead and Nowell (1964). Three series of experiments were conducted: 1) pilot lymphocyte culture under normal conditions at 37°C; 2) exposure of cultures to 4° and 15°C for varying lengths of time; 3) application of excess thymidine (to level of 2.5 mM) by a modification of the methods of Puck, Bootsma and co-workers, Peterson and Anderson, and Rao and Engelberg, to induce synchrony in the dividing cells as a possible aid in the temperature studies.

The results were inconclusive. Exposure to 15°C induced a high incidence of exaggerated constrictions, and early separation of chromatids in one experiment. Partial synchrony was achieved

in one attempt, but subsequent experiments aimed at improving the technique did not collaborate this, although they did indicate that further experimentation was warranted.

In considering the possible mechanism of subnormal temperature action on cell division, one is reminded that the basic mechanism is thermodynamic; temperature changes affect the rates of individual biochemical reactions differentially. On the level of cell cycle activities these effects would be evident from an overall, nonspecific standpoint, as affecting some activities more than others. This permits correlation between subnormal temperature and nucleic acid and protein synthesis, and subnormal temperature and the integrity of structural proteins participating in mitosis. These correlations may elucidate the process of cell division; however, they must remain arbitrary in view of the complexity of the biochemical interrelationships in the cell.

ILLUSTRATIONS
AND
TABLES

<p>Fig. 1. <i>...</i></p>	<p>...</p>	<p>...</p>	<p>...</p>
<p>...</p>	<p>...</p>	<p>...</p>	<p>...</p>
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...

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EXPERIMENT	A		B		C		D	
	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control
Date	11/29/67-12/3/67		12/8/67-12/12/67		1/9/68-1/13/68		2/5/68-2/9/68	
Sex of subject	female - FAC		female - MFB		female - MFB		female - MFB	
Age	22 yrs		22 yrs		22 yrs		22 yrs	
Method for obtaining blood	venepuncture		venepuncture		finger prick		finger prick	
Temperature exposure	4-5°C for 6 hr applied after 51 hr of culture at 37°	37°C	3°C for two 3½ hr periods (after 54 and 74 hr)	37°C	14-15°C for 2 hr after 53 hr of culture, 3½ hr after 66 hr, 2½ hr after 75 hr	37°C	15-20°C (mainly 16°C) for 27 hr, followed immediately by harvesting of cells - omitting colchicine and hypotonic treatment, to view intact cells	37°C, cells harvested without colchicine or hypotonic treatment
Age of culture when cells harvested	98 hr	98 hr	96 hr	96 hr	96 hr	96 hr	95 hr	
Results	no. cells counted	2294	†(1035)	(extremely faint and irregular staining prevented evaluation)	2416	7808	no significant differences in experimentals and controls. Cells were clumped and irregularly shaped. No mitotic figures were observed in the experimentals.	
	no. mitoses	108	(52)		151 (11 in prophase)	210 (6 in prophase)		
	*mitotic index (MI)	0.047	0.050		0.061	0.025		
	no. polyploid	1 triplod			2			
	appearance of chromosomes	normal	normal		many of chromosomes appeared "knobby-like" due to heavy and light stained regions in sequence along chromosome arms. 31 out of 40 metaphases had one or more chromosomes with separated chromatids. See Figures 8 and 9 for most exaggerated cases.	staining of chromosomes relatively uniform. 9 out of 40 metaphases had one chromosome with separated chromatids, and one had 4 chromosomes with separated chromatids		

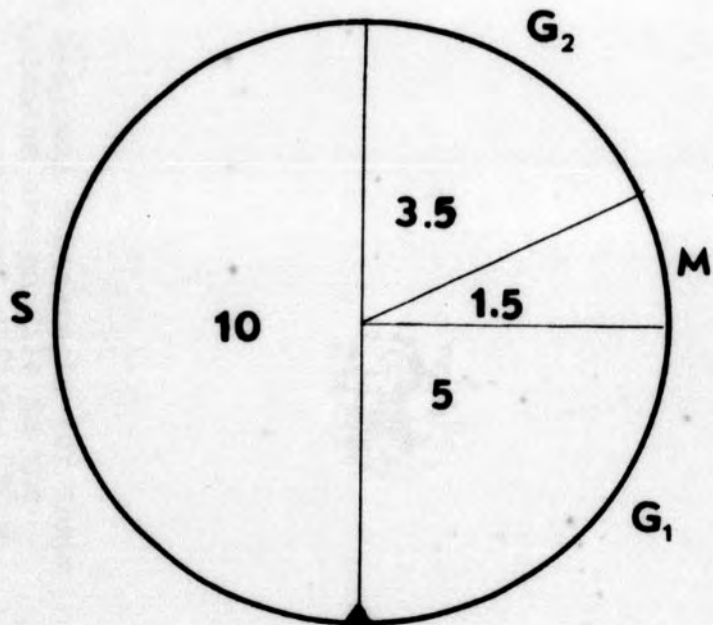
Table I

* MI is the fraction of cells in mitosis; no. mitoses/total cell count.

† () indicate that staining of the cells was faint and/or irregular and difficult to count.

EXPERIMENT	A		B		C		D		
	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	
Date	2/26/68 - 3/2/68		3/14/68 - 3/19/68		3/30/68 - 4/5/68		4/24/68 - 4/29/68		
Sex of subject	female - MFB		female - MFB		male - LHK		female - MAL		
Age	22 yrs		22 yrs		22 yrs		21 yrs		
Method for obtaining blood	venepuncture		venepuncture		venepuncture		venepuncture		
Treatment	Application of excess thymidine	yes	no	yes (6 vials)	no (6 vials)	yes	no	yes	no
	Thymidine sterilized by millipore filter	not completely		not completely		no		yes	
	Subjection to 15°C	no		3 vials treated with thymidine, and 3 controls, at 15°C for 14 hr, harvested without colchicine.		no		no	
Results	no. cells counted	6650	3097	37°C - 8571	(2031)	complete	5238	1443	
	no. mitoses	1645 (52 in prophase)	97	626	(149)	bacterial	353	33	
	mitotic index	0.247	0.031	0.073	0.073	contamination	0.093	0.023	
	no. polyploid	4	0	2 (1 endoreduplicated)	2				
	appearance of the stained preparations	normal (see figure 11)		37°C - very faintly stained 15°C - cells clumped, looked very much like those in Exp. D, Table I.			Partial bacterial contamination in both experimentals and controls, of 2 types: 1) spore-former, g-pos. rods in long chains, acid producing (similar in gross morphology to Lactobacillus); 2) g-pos. irregularly shaped rods, non-acid producing (similar to Corynebacterium).		

Table II



Human lymphocytes at 37° C

Total: 20 hrs.

Fig. 1

2nd thymidine block stops cells prior to DNA-synthesis (S-phase)

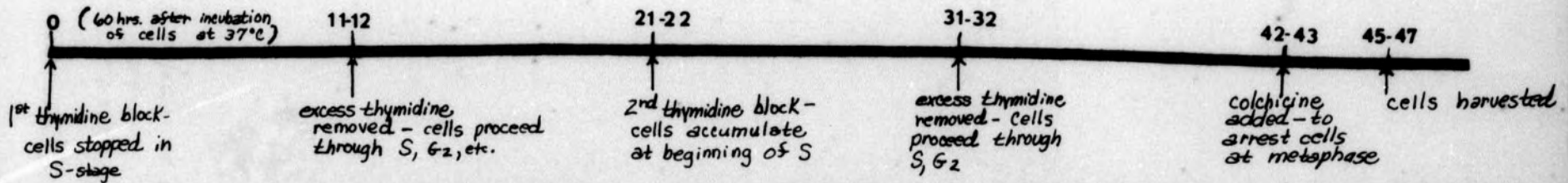
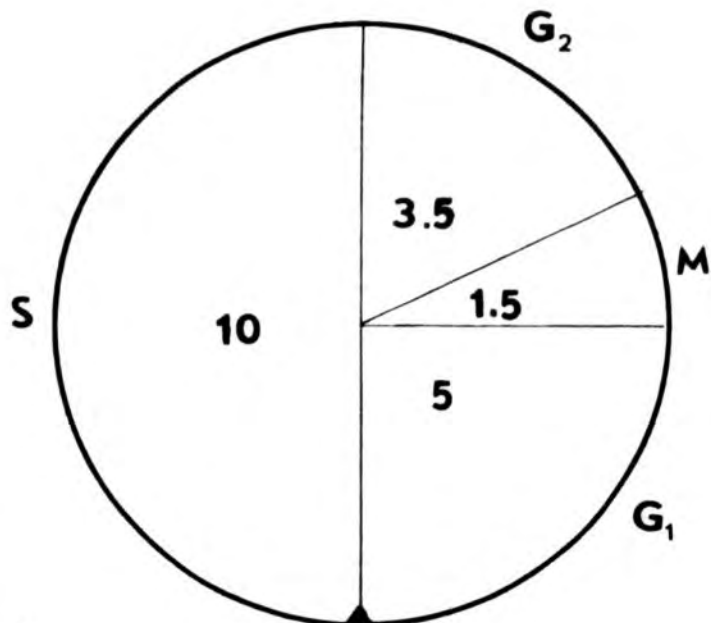


Fig. 5

THYMIDINE SCHEDULE



Human lymphocytes at 37°C

Total: 20 hrs.

Fig. 1

2nd thymidine block stops cells prior to DNA-synthesis (S-phase)

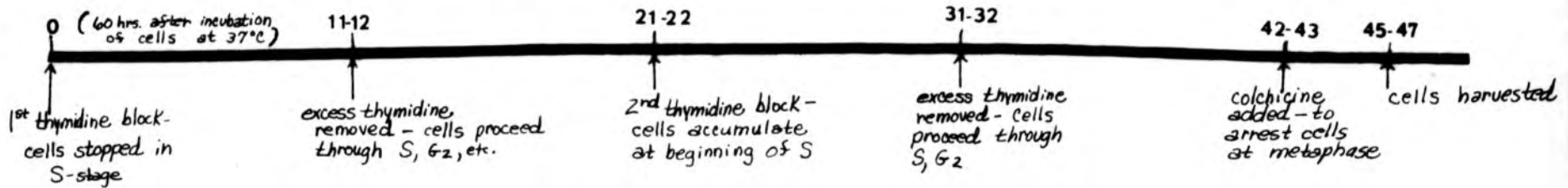



Fig. 5

THYMIDINE SCHEDULE

Figure 2

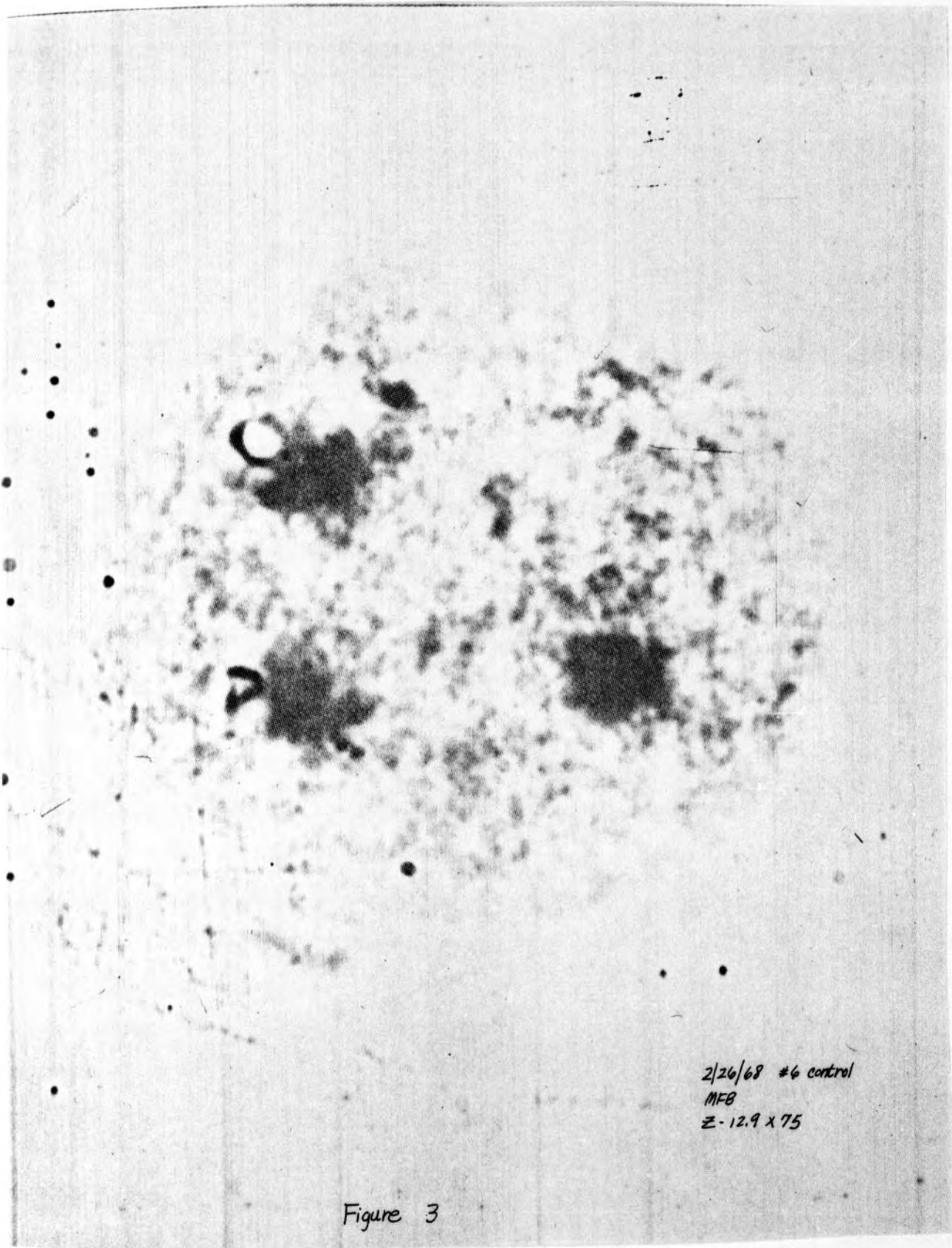


The original photographs for these
photocopies are on file in the
Biology Department office, UNC-G.

2/26/68 #4 Control

MF8

Z 6.3x99.5



2/26/68 #6 control
MFB
Z-12.9 x 75

Figure 3

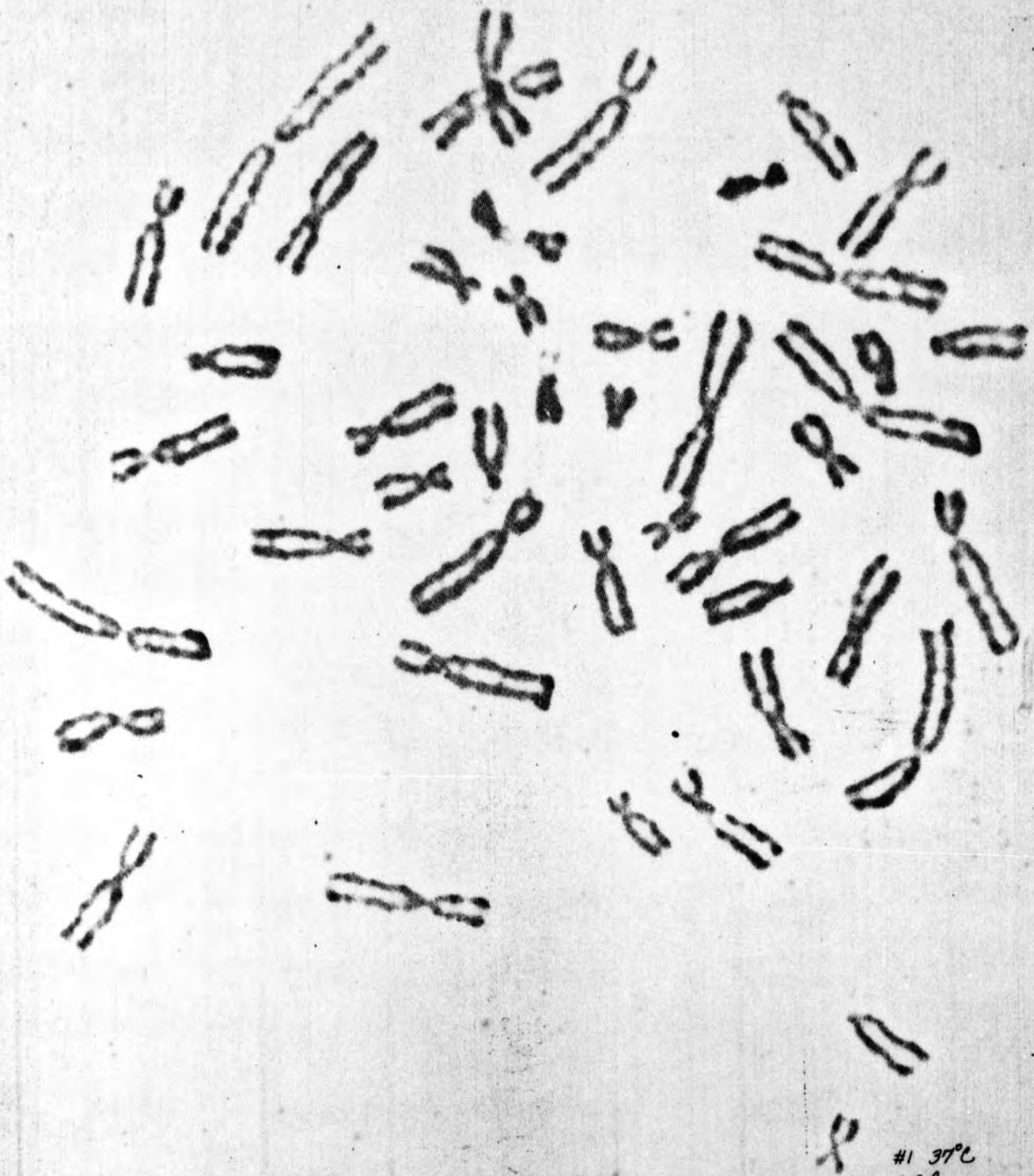
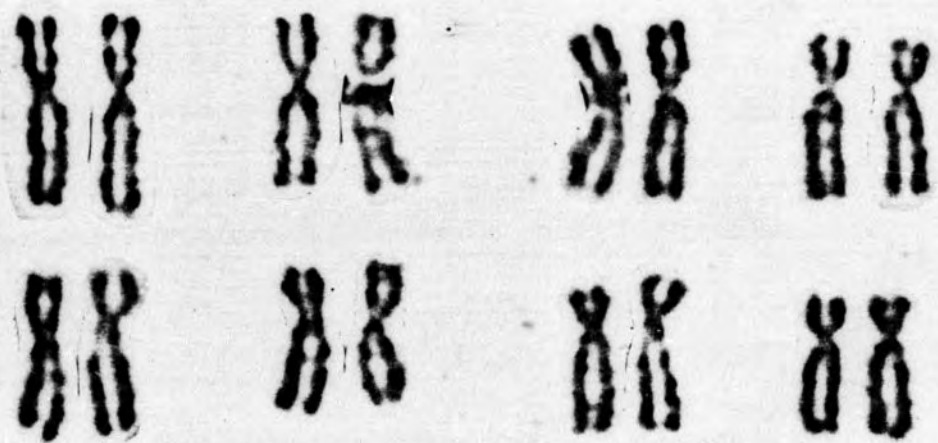


Figure 6

#1 37°C
LGA
Z 16.9 x 113.5

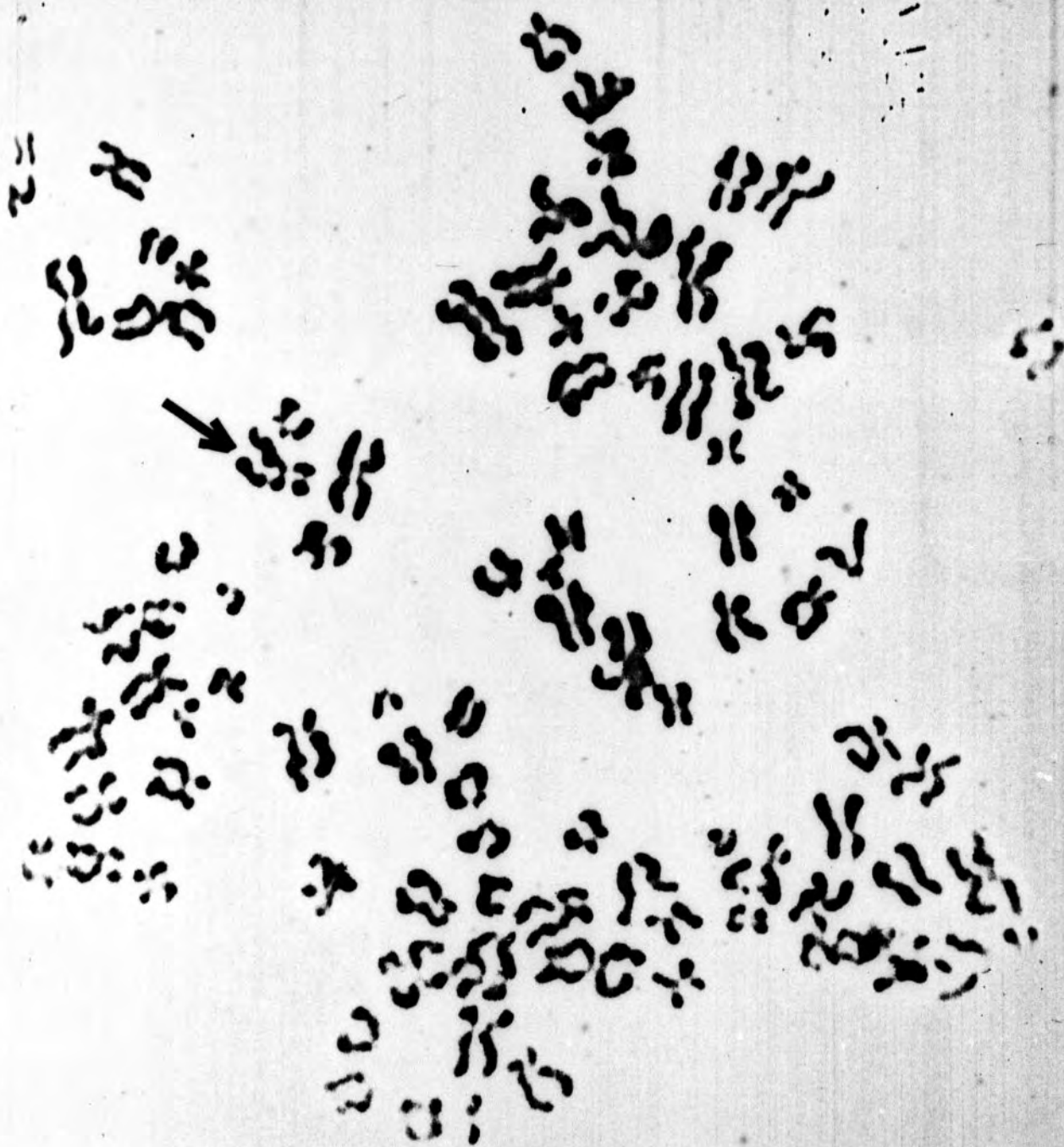


C (x, 6-12)



UNC-G LGA
S Lide 1
16.9 x 113.5 Z
37°C

Figure 7



1/9/68 H6 15°C
MFB
Z 10.2 x 95.4

Figure 8

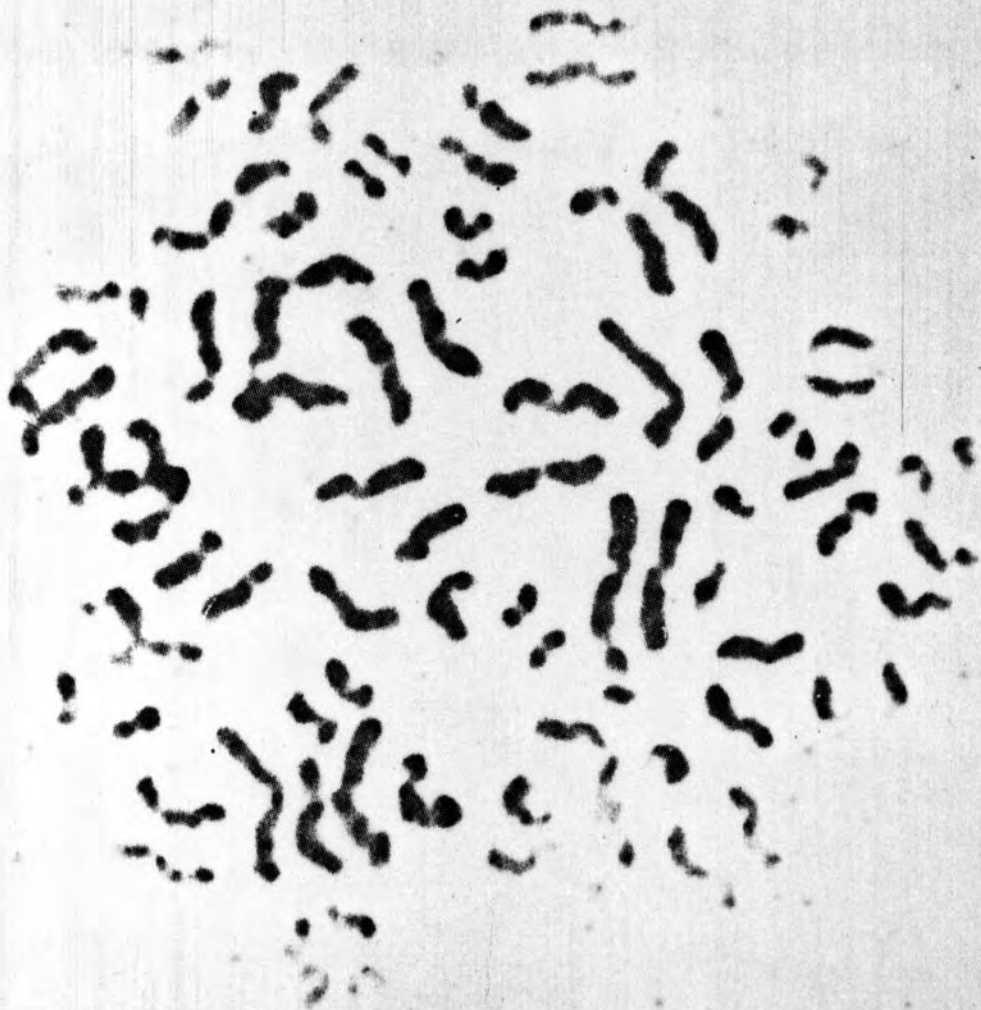
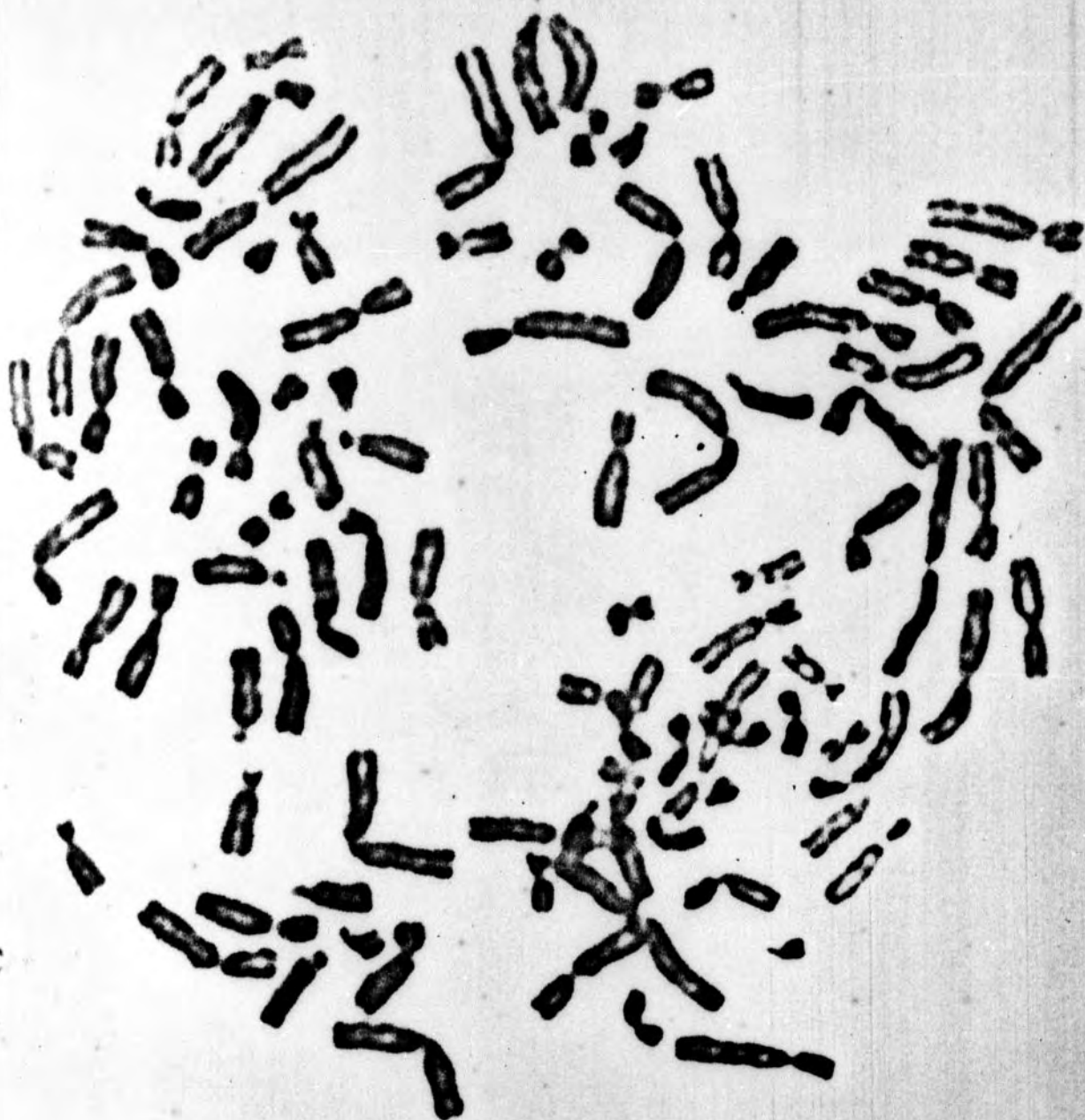


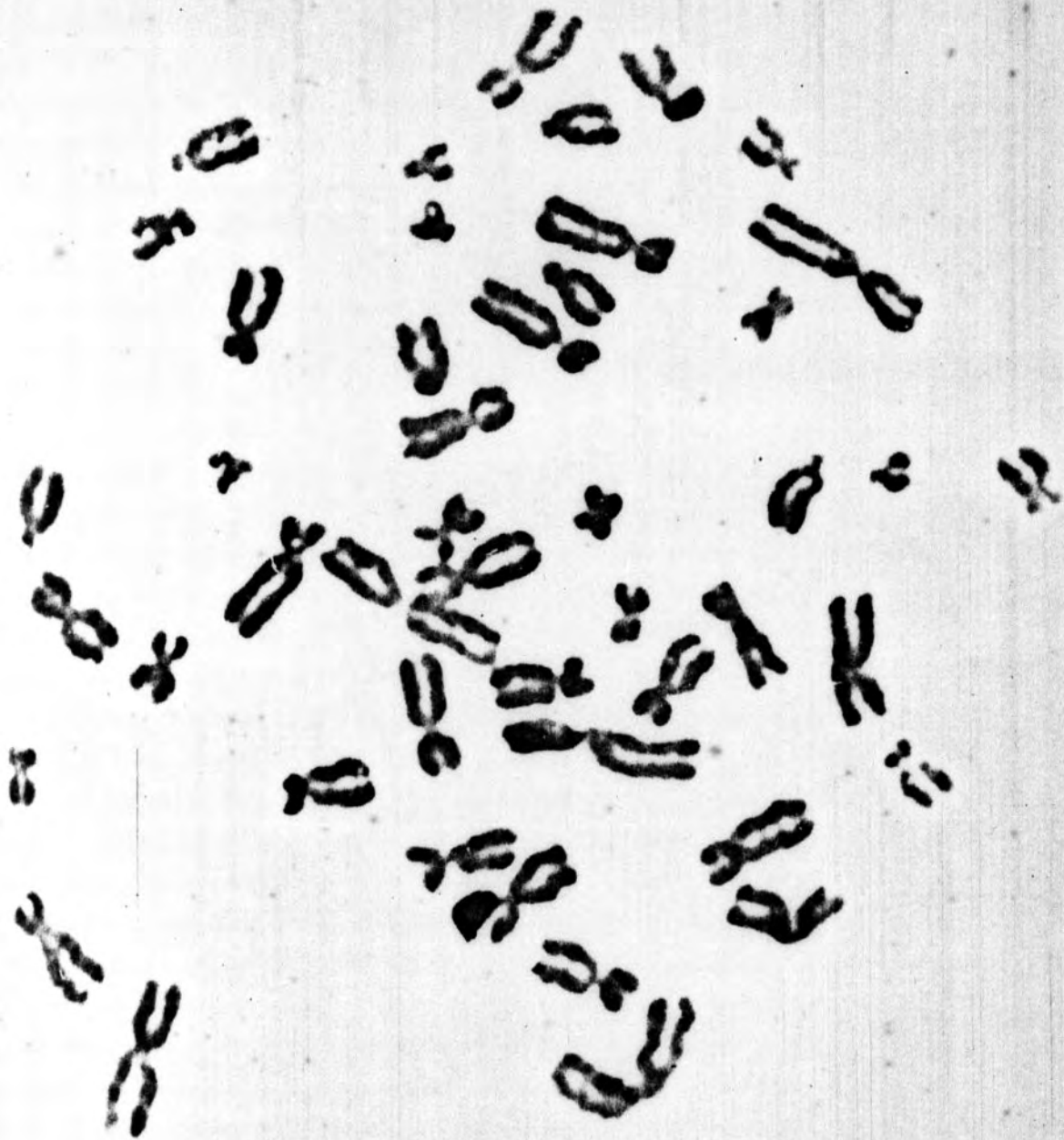
Figure 9

1/11/68 #6 15C
MFB
Z 10.2 x 95.4



1/9/68 #6 15°C
MFB
Z 14.5 x 85.6

Figure 10



2/26/68 #8 Synchrony
MFB
Z-10.25 x 76

Figure 11

Suboptimal temperature

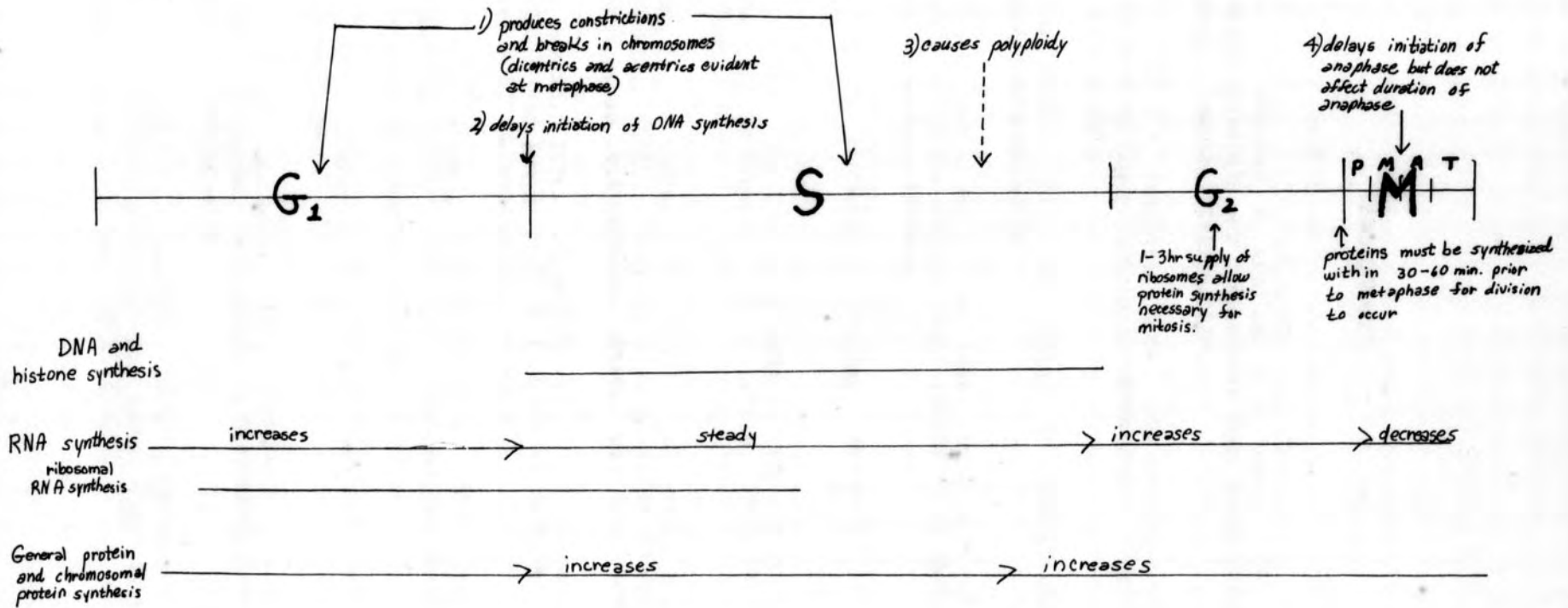


Figure 12

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