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FINAL TECHNICAL REPORT

The initial goal at the outset of these studies was to construct a clinostat which could support mammalian cell culture. The clinostat was selected as a means by which to simulate microgravity conditions within the laboratory, by constant re-orientation of cells with respect to the gravity vector. The experimental objective of the first series of studies was to examine the effects of this simulated microgravity on in vitro meiotic maturation of oocytes, using mouse as the model system. Follicular oocytes were recovered and matured in vitro at various rates of clinostat rotation. Specific endpoints included assessing the timing and frequency of germinal vesicle breakdown and polar body extrusion, as well as the structural and numerical properties of meiotic chromosomes at Metaphase I and Metaphase II of meiosis.

The studies were then directed toward examining the effects of clinostat rotation on fertilization in vitro. Specific endpoints included examining the timely appearance of male and female pronuclei (indicating fertilization) and the efficiency of extrusion of the second polar body. Particular attention was paid to detecting anomalies of fertilization, including parthenogenetic activation and multiple pronuclei. Finally, for our preliminary studies on mouse embryogenesis, we modified a key feature of our clinostat--that of the position of the cells during rotation. We found a means to immobilize the cells during the clinostat rotation, permitting the cells to remain at the axis of rotation yet not interfering with cellular development.

A. Clinostat for Use in Mammalian Oocyte and Embryo Culture in Vitro

The clinostat we initially constructed and subsequently modified is based upon the design originally developed by Tremor and Souza (1) for studies of the effects of simulated microgravity on amphibian development. The first modifications were necessary to accommodate the in vitro culture requirements of mammalian oocytes and embryos, including a 37°C temperature, continuous gas exchange in a constant atmosphere of 5% CO₂ in air, and tissue culture-grade chambers. Under clinostat rotation conditions, and with these requirements, this means that gas exchange must be effected without spilling culture media from the non-airtight vessel. A major modification of our original design involved the inclusion of a rotating shaft in the vertical (or control) position. This shaft is driven by the same motor as that which drives the horizontal (or experimental) shaft. This design permits simultaneous experimental and control rotation and provides an excellent internal control.

1. The Clinostat-Incubator: The assembled instrument is depicted schematically in Figure 1. There are several aspects of the assembly which should be noted. First, the attachment of the motors to the rotating shaft was designed so that the motors can be interchanged outside the incubator with minimal effort. Second, beveled gears were installed so that the main rotating shaft drives two secondary shafts, oriented perpendicular to one another. This permits concomitant vertical and horizontal rotations and serves as an internal control for experimental parameters (such as

room temperature. The oocytes were subjected to the following hypotonic-fixation protocol: hypotonic--4 changes, 3 min each; hypotonic--5th change, 20 min; distilled H₂O--2 changes, 5 min each; removal of excess H₂O by blotting; fixation with 1 drop of freshly prepared 3:1 ethanol:glacial acetic acid. The slides were air dried and stained with 2% toluidine blue in dimethylhydantoin formaldehyde resin and were visualized and photographed at 100X with oil immersion optics. Selected specimens were destained and restained with 3% Giemsa in 0.001 N NH₄OH solution for higher resolution of chromosome morphology.

Experiments in the initial year involved first, a rigorous assessment of the extent to which the rate of meiotic maturation of our static controls--using the culture system modified for the clinostat--was comparable to the rate of meiotic maturation under standard laboratory conditions; and second, a preliminary analysis of meiotic maturation of oocytes rotated on the clinostat at low RPM. In the second year of these studies, we conducted an extensive series of meiotic maturation experiments over a range of clinostat rotation speeds (1/4 RPM to 100 RPM). This analysis required more experiments than originally anticipated. First, it became clear that it would be of interest to extend the rotation speeds into the fast-clinostat range (100 RPM). Second, it was necessary to perform a sufficient number of experiments for meaningful statistical analysis of the subtle nature of the effects we were observing.

Endpoints for analysis included rate of germinal vesicle breakdown and progression through Metaphase I or II. Oocytes were also examined under a dissecting microscope immediately upon removal from rotated or static culture for any obvious cellular abnormalities such as a granular or necrotic appearance of the cytoplasm, fragmentation, rupture of the zona pellucida, clumping of cumulus cells, etc. In addition, limited cytogenetic examination of the chromosomes was obtained, with emphasis on numerical (disjunctive) abnormalities.

Qualitative evaluation of the condition of the cells was made immediately following rotation in all experimental and control cultures. There was no obvious production of fragmented or otherwise damaged cells at any rotation rates. In some of the experiments, some granularization of the oocyte cytoplasm was noted. However, no impairment of germinal vesicle breakdown or meiotic progression could be correlated with the granular-appearing cytoplasm.

The results of 65 experiments (total n = 3089 oocytes) which were suitable for analysis of the rate of meiotic maturation and 52 experiments (total n = 1930 oocytes) used to analyze progression to Metaphase II are summarized in Fig. 2 and 3. The following criteria for inclusion in this analysis were imposed: a) greater than 70% meiotic maturation (germinal vesicle breakdown) and progression to Metaphase II in the static control culture; and b) a sample size or number of oocytes (n) equal to or greater than 5 per culture. The former criterion was important to ensure that the culture system was capable of supporting meiotic maturation. The latter was important for statistical analysis since percentages were being compared.

Several conclusions regarding the effects of clinostat-rotation on mouse meiotic maturation can be drawn. The rate of resumption of meiosis was similar under static and all horizontal-rotation (experimental) and vertical-rotation (mechanical control) conditions (Fig. 2). This

observation is not surprising, and indeed, would be anticipated in view of the rapidity of the oocyte's response to the stimulus to resume meiosis. Changes in the germinal vesicle (oocyte nucleus) membrane can be observed ultrastructurally within 30 minutes following release from the ovarian follicle (2). To inhibit germinal vesicle breakdown, cellular changes due to the rotation conditions would have had to elicit an effect quite rapidly.

In contrast, the observations on the rate of progression to Metaphase II of meiosis involve cellular processes over a 16 hour culture period. As noted in Fig. 3, oocytes matured under clinostat rotation conditions at 1/4, 1, 10, and 30 RPM proceed to Metaphase II at rates comparable to the static and vertical-rotation controls. At 100 RPM, however, there was a slight inhibition of entry into Metaphase II. This inhibition in achieving normal chromosome disjunction was statistically significant (Chi square analysis, $p = 0.05$). Of particular importance in these experiments was the inclusion of a simultaneous vertical-rotation control to ensure that any effects observed were not simply due to features of rotation such as vibration.

Finally, the experiments were also analyzed for structural or numerical abnormalities in the Metaphase I and Metaphase II chromosome complements. No abnormalities have been noted to date (examples in Fig. 4) and it is not anticipated that striking abnormalities will be observed in the remaining experiments to be analyzed.

C. In Vitro Fertilization Under Clinostat-Rotation

To conduct the fertilization experiments proposed as our second specific objective, it was necessary to use different culture media, as noted below. An improvement in the tissue culture dishes was also developed (diagrammed in Fig. 5). Essentially, a plate with 5 wells (LUX 5250), each approximately 9mm in diameter and 5mm in height, was used as the tissue culture chamber. Media and cells were placed in the center well. A strip of sterilized Petriperm membrane (Tekmar, Inc.) was placed over the well, hydrophilic side toward the culture, and secured in place with a rubber O-ring. The chambers were mounted onto the rotating plates in the experimental (horizontal rotation) and control (vertical rotation) orientations.

To accomplish fertilization *in vitro*, our protocol essentially followed those of Hoppe and Pitts (3) and Jagiello and Lin (4). Young (25 to 45 days old) virgin females (F_1 offspring of C57Bl/6 females and DBA/2 males, purchased from Jackson Laboratories, Bar Harbor, Maine) were superovulated by intraperitoneal injection of 10 I.U. of PMSG (Organon) followed 48 hours later by 2.5 I.U. of hCG (Organon). Animals were sacrificed 14-16 hours later.

To recover ovulated oocytes, the ovary, oviduct, and a short segment of the uterus were removed to a small culture dish containing fertilization medium. Under the dissecting microscope, the egg mass can be visualized in the ampulla of the oviduct and removed by gentle teasing with a needle. Spermatozoa were obtained by removing the cauda epididymides and vasa deferentia of mature, fertile males, (also F_1 offspring of the C57 Bl/6 x DBA/2 cross), pricking the epididymides with a needle and transferring the released spermatozoa into capacitation medium. The capacitation medium is a modification of that developed by Hoppe and Pitts (3) with NaCl adjusted to

6.4 g/l and Na-lactate omitted. Spermatozoa were capacitated for 2 hours in static conditions prior to being placed with ova. Sperm were diluted to $8-10 \times 10^5$ motile sperm/ml. For insemination, 7 ul of this suspension was added to 140 ul of the fertilization media containing 20 to 40 ova.

The chambers were mounted immediately onto the clinostat. After 3-4 hrs, the rotation was interrupted briefly (<15 min) to recover ova from the fertilization medium. The ova were observed briefly, under the dissecting microscope, for any obvious abnormalities such as fragmentation or complete absence of adhering cumulus cells. The ova were transferred to 350 ul of standard culture medium (3) in the clinostat chambers and subjected to static or rotated conditions for an additional 4 hours.

By about 6 hours, most of the cumulus cells had dispersed. Remaining cumulus cells were removed by brief treatment with 0.1% hyaluronidase in Hank's Basic Salt Solution at room temperature. Dislodged cells were separated from the ovum by drawing the ovum into a large bore-micropipet and the cumulus-free ovum was transferred to culture medium for examination. Fertilized ova were processed for morphological examination using our slight modification (5) of the whole-mount procedure described by Chang and Hunt (6). This involves fixation for 30 min in 1% glutaraldehyde, followed by fixation in 10% formalin for 24-48 hours. Staining was accomplished with 0.1% toluidine blue in dimethylhydantoin formaldehyde resin. The advantages of the whole-mount procedure include the ease and rapidity of processing and the retention of the spatial orientation of the polar bodies and pronuclei.

Rotation speeds were selected using our results from the meiotic maturation experiments as a guide. Therefore, we focused our attention on rotation at 100 RPM, with a few experiments being conducted at 1/4 RPM. The results of 45 experiments (total n=990 ova) are summarized in Figures 6 - 8. We have detected no abnormalities in the appearance of the fertilized ova (note Fig. 6) or differences in the efficiency of achieving normal fertilization between experimentally rotated and control rotated cultures (Fig. 7). In addition, we did not see any increase in the proportion of ova which underwent parthenogenetic activation (Fig. 8). This observation was of considerable interest since mammalian ova can be easily activated by stimuli (mechanical, temperature, ETOH, etc., reviewed in ref. 7) other than a fertilizing sperm. We were careful to note, therefore, that our culture system on the clinostat did not produce this situation.

To date, we conclude that the re-orientation of the cells did not result in impairment of fertilization per se. However, as is noted briefly below and discussed in greater detail in the experiments outlined in Specific Objectives, there are questions that remain to be answered regarding the developmental potential of such embryos.

D. Modification of the Culture System to Immobilize Cells at the Axis of Rotation

We were aware of the theoretical limitations in using the clinostat (e.g. 8,9), especially as regards free-falling bodies (10). However, for the meiotic maturation and first stage of the fertilization studies, technical aspects of the biological system prevented alternative experimental design. For the embryogenesis and second-stage

fertilization experiments proposed in our new specific objectives, we have been able to modify the culture system in order to immobilize the cells at the axis of rotation. This was accomplished by embedding the cells in low melting point agarose, a modification of protocols described by Willadsen (11). A sterile 1.2% solution of agarose (BRL No. SS17UB) in 0.9% (w/v) NaCl in distilled, deionized water was prepared. The agarose solution was heated above its melting point of 65°C, 3 ml were aliquoted into a 35mm petri dish, allowed to cool to 37°C and to equilibrate in the tissue culture incubator for one hour before use.

To conduct embryo culture, 45 to 70 day old virgin female B6D2F1/J mice (F₁ offspring of C57 Bl/6 females and DBA/2 males; purchased from Jackson Labs., Bar Harbor, Maine) were mated with mature (60 - 120 day old) male B6D2F1/J mice. Mice were checked for the presence of vaginal plugs within 9 hours after mating. To recover 2-cell embryos, animals were sacrificed 35-37 hours after mating. The oviduct and a very short segment of the uterus were removed to an empty petri dish. A sterile 2 ml Hamilton syringe with a blunt, 30 gauge needle was filled with culture medium. Under the dissecting microscope, the needle was inserted into the end of the oviduct, the oviduct held snugly to the needle by a pair of watchmaker forceps, and each oviduct flushed with ~0.25 ml of culture medium.

Embryos were pooled and observed briefly for number of cells and any obvious abnormalities, such as the lack of a zona pellucida. Normal 2-cell embryos were immediately transferred with a pulled micropipette to the gas equilibrated, 37°C agar solution. The embryos were picked up a few seconds later in a small amount of agar solution. The agar containing the embryos was then expelled from the micropipette into the clinostat cell culture chamber (Fig. 5), placed in a gas pack unit (BBL 60622), gassed with 5% CO₂ 5% O₂ 90% N₂, and allowed to solidify at room temperature for 5-10 min. The number and appearance of the embryos in the agar was scored, the agar was overlaid with 180 ul of culture medium (Appendix, Table 1), and the chambers were assembled as described in the *in vitro* fertilization experiments. The chambers were immediately mounted onto the rotating plates in the experimental (horizontal rotation) and control (vertical rotation) orientations. A static control was also placed in the incubator.

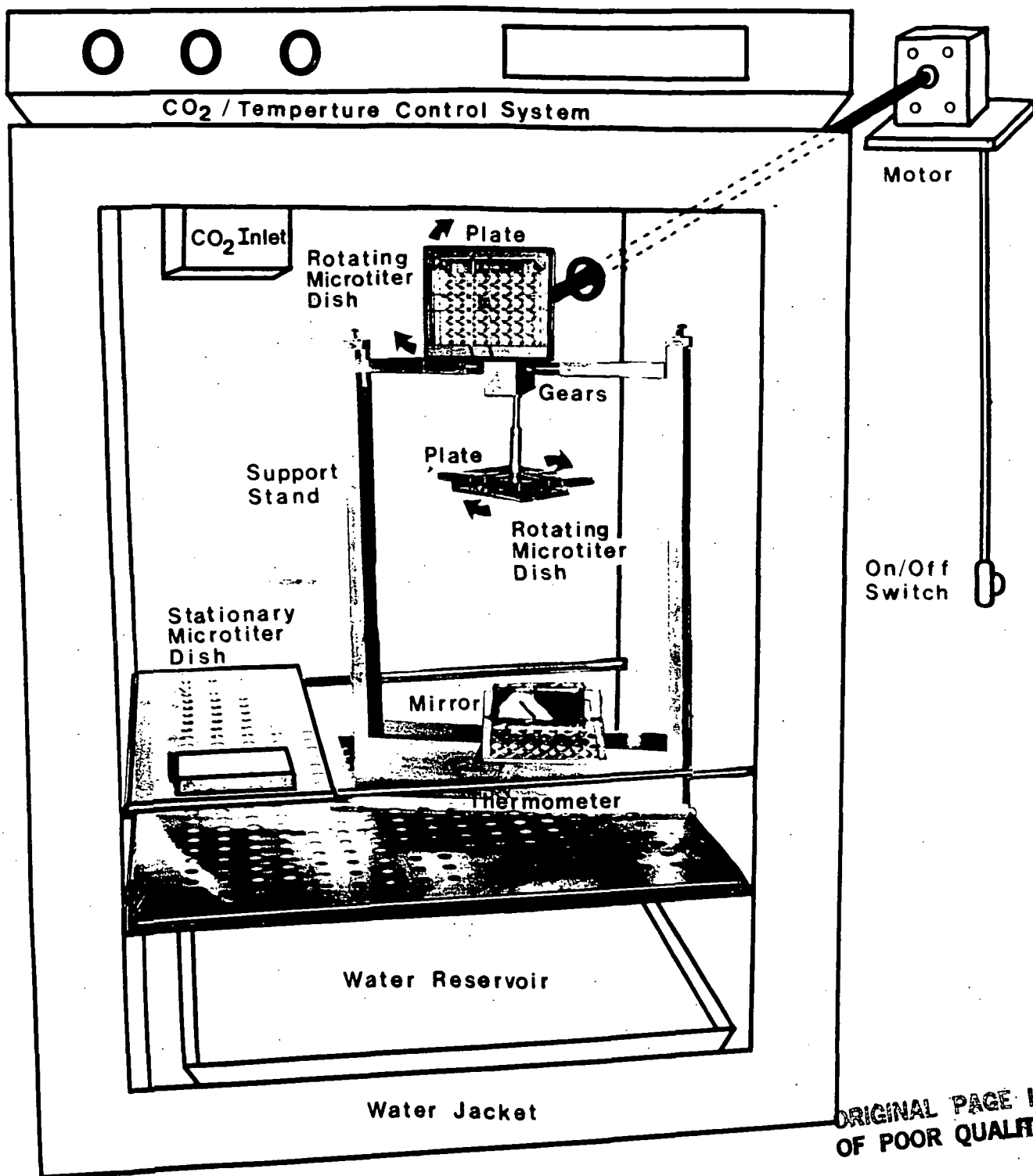
The clinostat was stopped after 24 hours of rotation. The embryos in the agar were scored under the dissecting microscope for progression to the 8-cell stage and for any obvious abnormalities such as necrosis or fragmentation (Fig. 9). Embryos in agar were processed for morphological examination using a paraffin embedding procedure (Fig. 10). This involved aspirating the media from the clinostat cell culture chamber, washing 3x with a phosphate buffered saline solution, and fixing the embryos in agar for 24 hours in Bouin's fixative. Fixation was followed by 3 washes with distilled water and then by alcohol dehydration: 50% EtOH - 30 min; 70% EtOH - 60+ min; 80% EtOH - 30 min; 90% EtOH - 30 min; 2x 100% EtOH - 30 min each. Dehydration was followed by a 10 min xylene wash. The agar with embryos was then embedded in paraffin and sliced on a microtome into 5 um sections. Temporary staining was accomplished with 0.2% toluidine blue in dimethylhydantoin formaldehyde resin, diluted 1:1 with water. Sections were examined under a Zeiss-14 light microscope and photographed. Sections with embryos were destained from toluidine blue and then stained with hematoxylin and eosin.

Our preliminary observations, illustrated in Figs. 9 and 10, show that this culture system can support a timely progression from the 2-cell to 8-cell embryo. At the level of examination under the dissecting microscope, there were no obvious differences in the experimental and control embryos. Clearly, however, the questions of both the cellular and molecular criteria of normal development as well as the developmental potential of such embryos remain to be studied.

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CLINOSTAT FOR MAMMALIAN CELL CULTURE



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Figure 1. Schematic diagram of the clinostat used for mammalian tissue culture studies.

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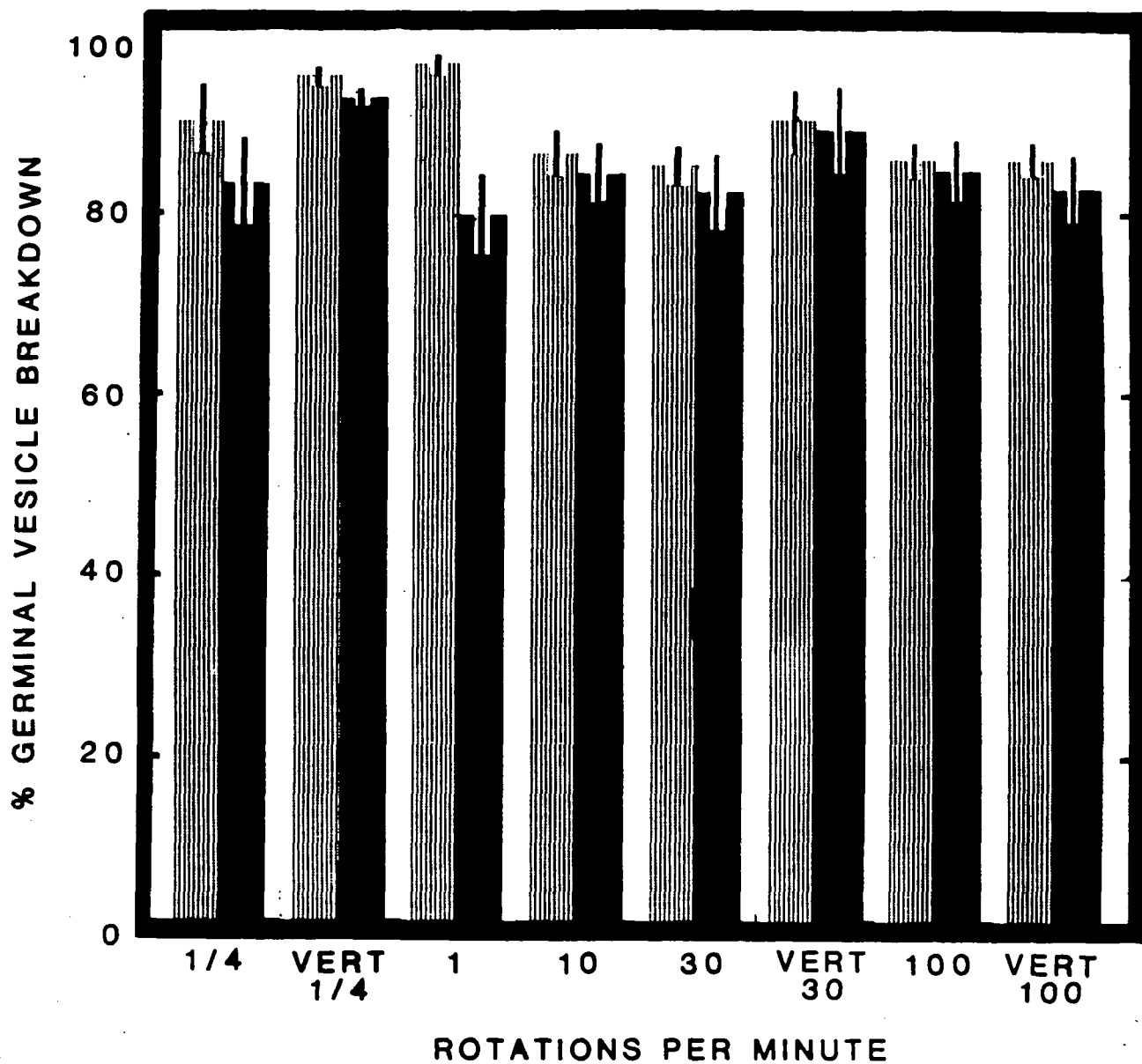


Figure 2. Bar graph of the results of experiments evaluating the percentage of oocyte germinal vesicle breakdown at various rates of rotation on the clinostat (solid bars) as compared to static controls (striped bars). Average percentages are shown + standard error. "Vert" refers to rotation in the vertical (control) axis.

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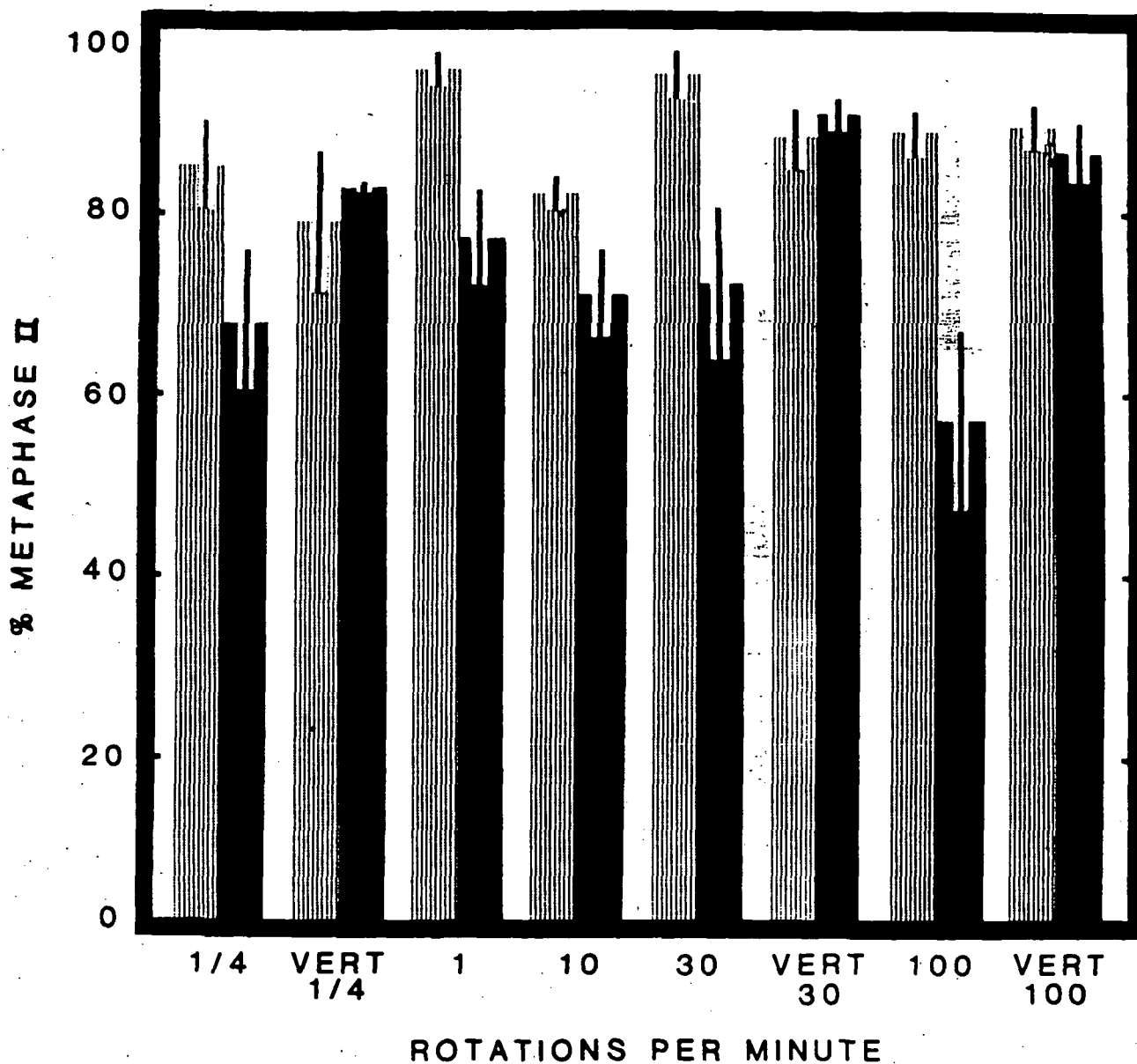
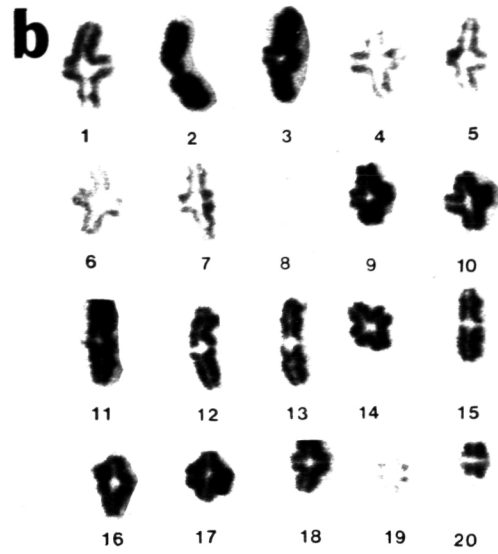
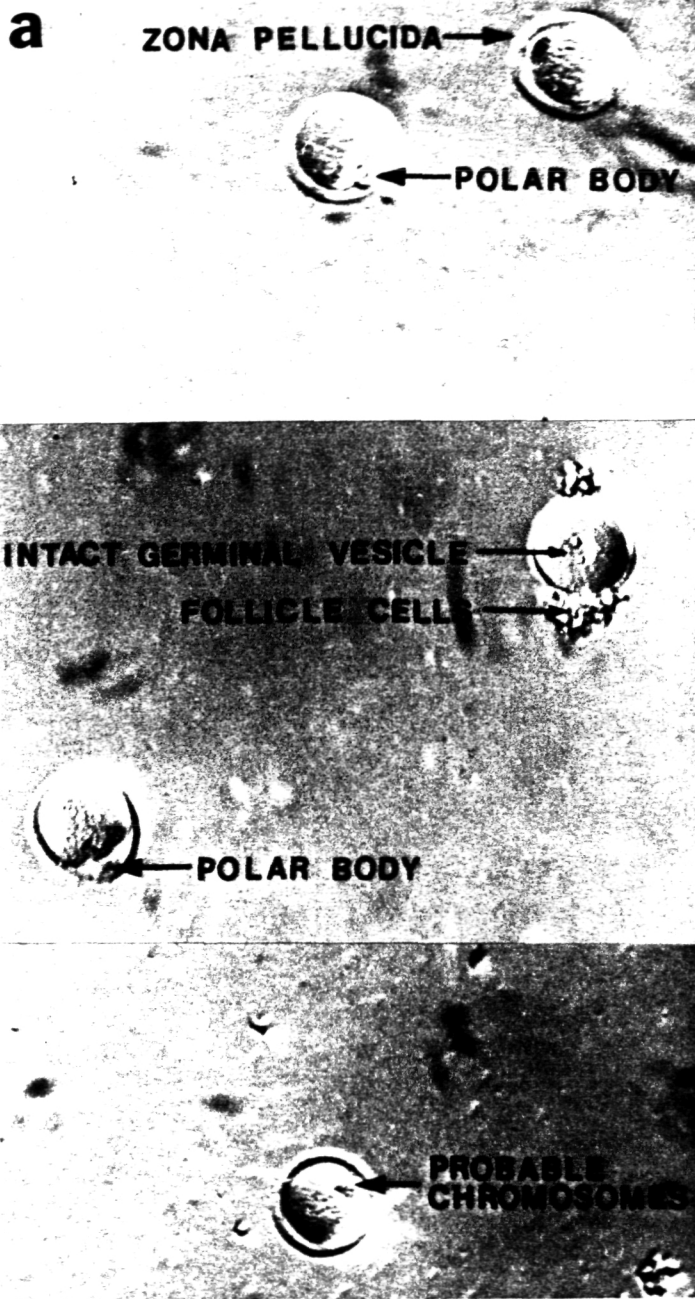
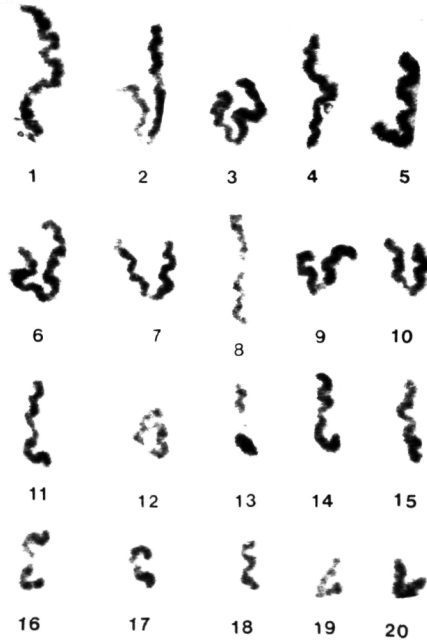


Figure 3. Bar graph of the results of experiments evaluating the percentages of oocytes reaching Metaphase II of meiosis at various rotation rates on the clinostat (solid bars) as compared to static controls (striped bars). Average percentages are shown + standard error. "Vert" refers to rotation in the vertical (control) axis.



M I



M II

Figure 4

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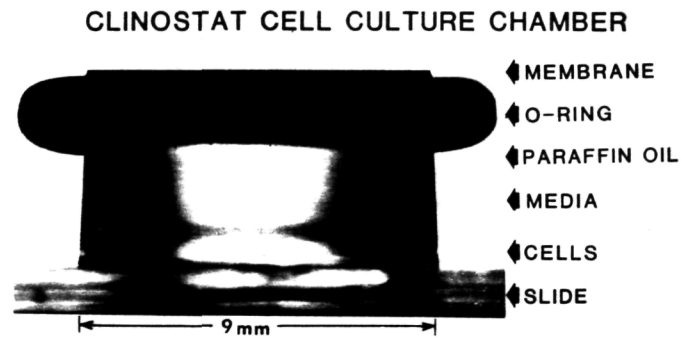


Figure 5. Diagram of an individual well chamber of the modified clinostat cell culture system using gas-permeable membranes.

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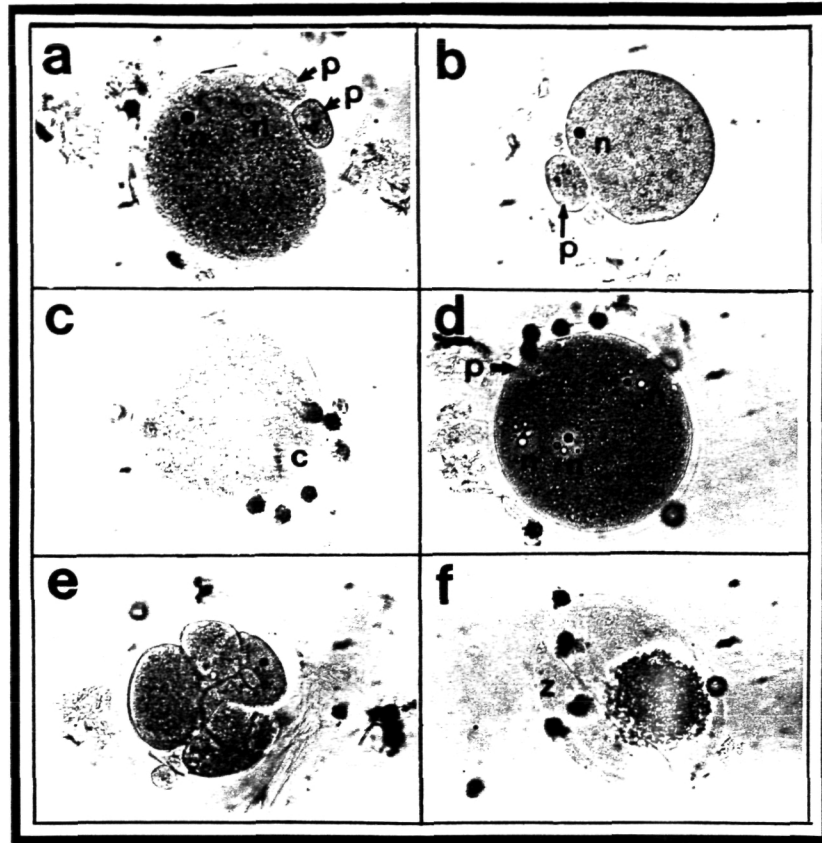


Figure 6. Photomicrographs of in vitro fertilized ova, clinostat rotated at 1/4 RPM, demonstrating normal and abnormal endpoints. (Mag = 212x)
n = pronucleus; p = polar body; c = chromosomes;
z = zona pellucida.

- (a): normal fertilization, with one male and one female pronucleus and two polar bodies.
- (b): parthenogenetically activated ovum (only one pronucleus).
- (c): unfertilized ovum with metaphase II chromosomes.
- (d): polyspermic fertilization, with three pronuclei. Polar body is out of plane of focus in upper left.
- (f): necrotic ovum.
- (e): fragmented ovum

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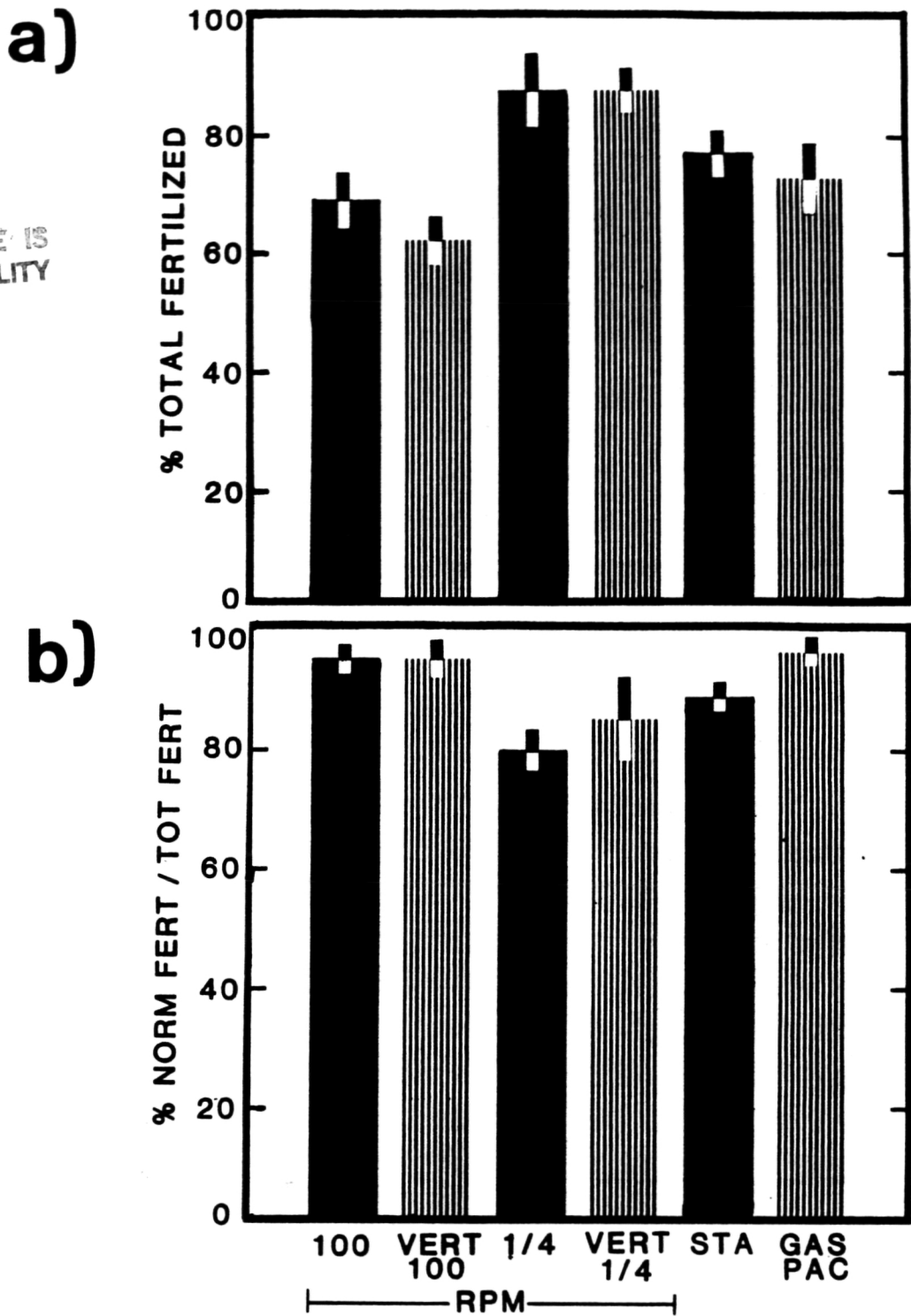


Figure 7. Bar graphs of the results of rotating ova and sperm on the clinostat during fertilization (% \pm standard error).

- A. Percentages of the fertilized ova out of the total number of ova placed in culture are compared between experimental (100, 1/4 RPM) and control (Vert 100, Vert 1/4, STA, GASPAC) conditions. "Vert" refers to rotation in the vertical (control) axis; Gaspac refers to a method control for the static culture system (STA).
- B. Percentages of the total number of fertilized ova that showed normal fertilization under experimental and control conditions.

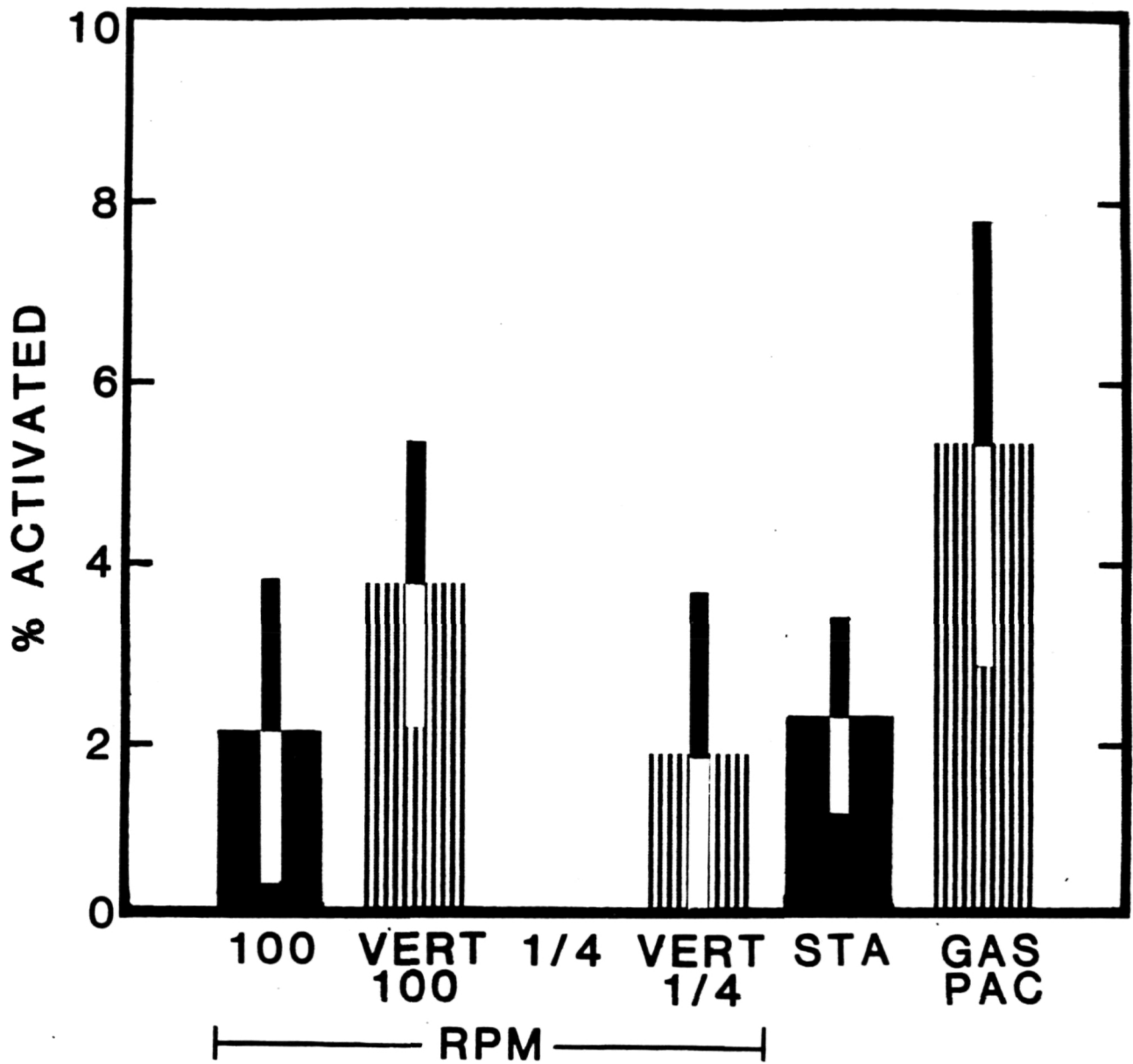


Figure 8. Percentages out of the total number of ova placed in culture of the total number of ova that underwent parthenogenetic activation.

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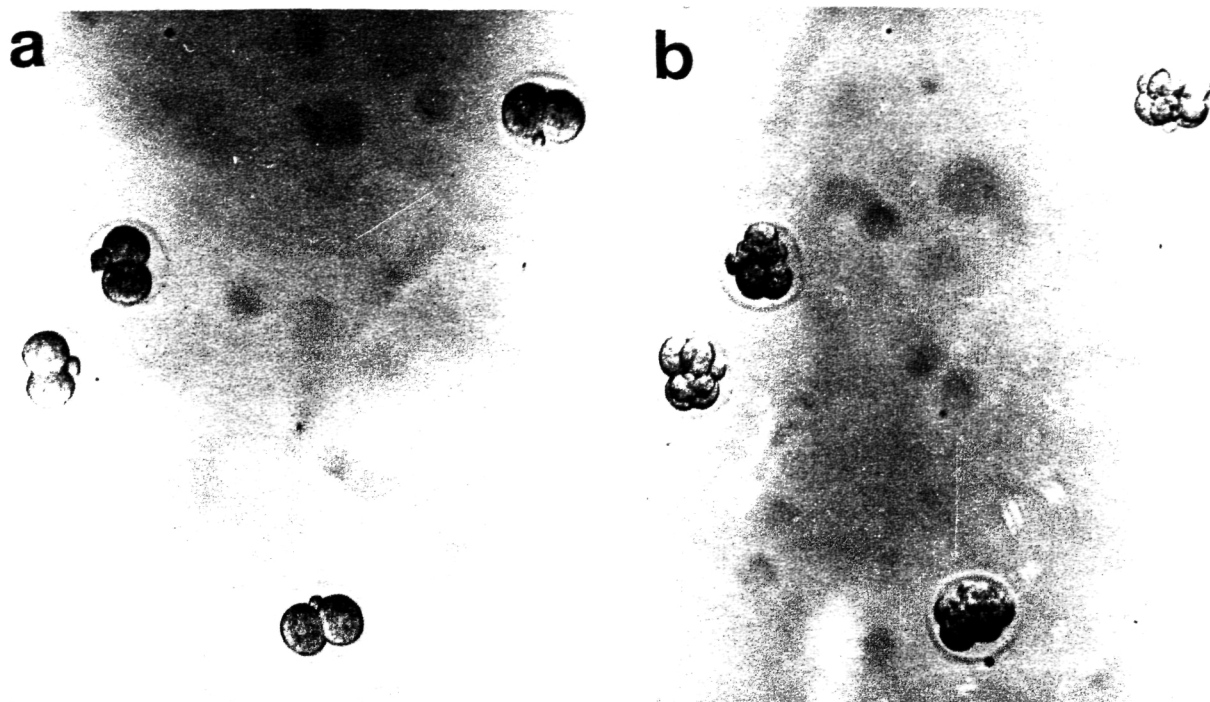


Figure 9. Photomicrographs of the same mouse embryos embedded in agar before (a) and after (b) clinostat rotation for $19\frac{1}{4}$ hours. Each two-cell embryo can be scored, rotated, and then re-located and examined for progression to the 8-cell stage. Photographed under a Wild Dissecting microscope (Mag = 120x).

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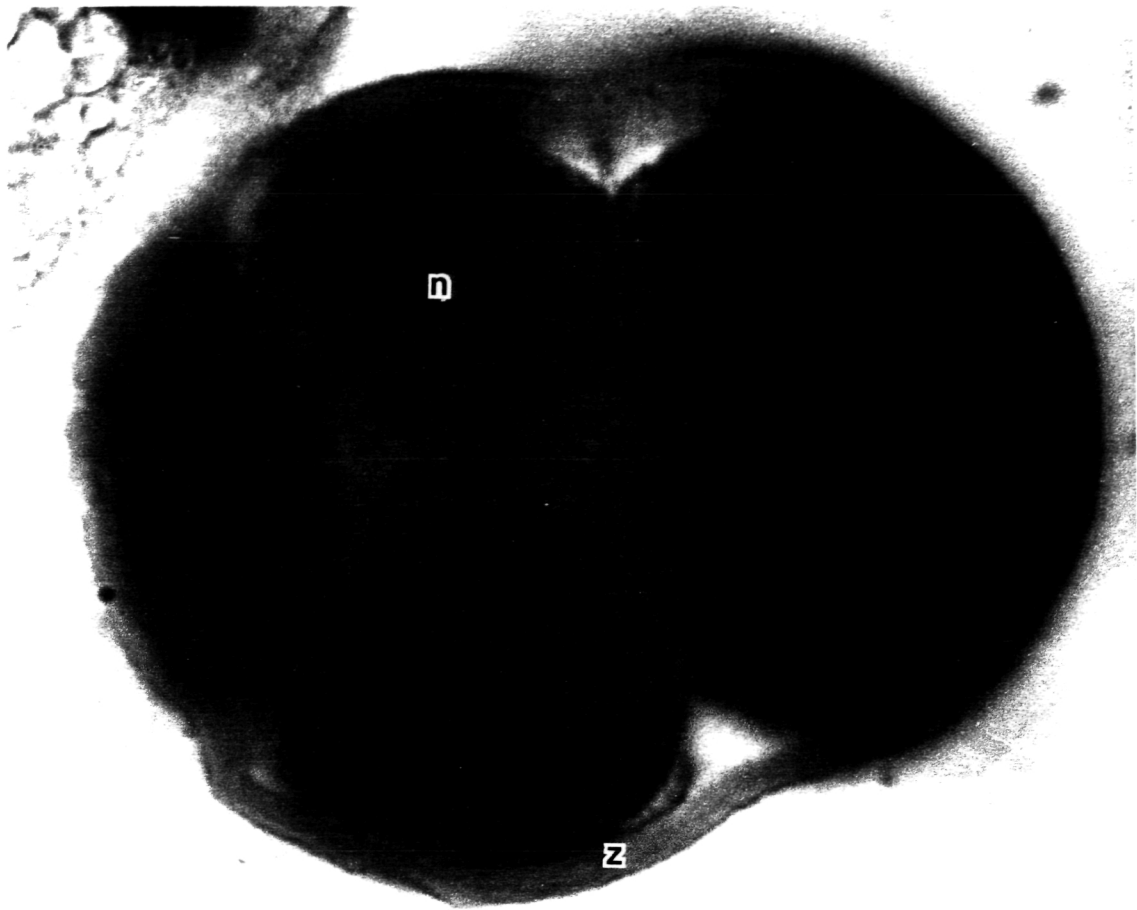


Figure 10. Photomicrograph of a histological section of
an embryo embedded in agar, fixed with Bouin's
and processed through paraffin
n = nucleus; z = zona
pellucida. (mag 1280x)

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