FACTORS AFFECTING GROWTH OF THE IMMATURE RAT OVARY IN VITRO WITH SPECIAL REFERENCE TO RIBONUCLEOTIDES

by

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INTRODUCTION

In a recent paper on the behavior of the nucleic acids in embryogeny Brachet (1947) advanced the view that the ribonucleotides may function as evocating agents. Considerable data support this concept. For example, in the developing amphibian embryo Brachet (1940, 1942, 1949) has found that the distribution of ribonucleic acid follows gradients which closely correspond to the morphogenetic gradients of Dalcq and Pasteels. Brachet further showed that explants lose their basophily during evocation, which suggests the diffusion of nucleic acid derivatives into the neighboring tissues. He observed that during the mitotic process the cell lost its cytoplasmic basophily due to ribonucleic acid, while desoxyribosenucleic acid was increased. Thus Brachet concluded that availability of a source of ribonucleic acid or its more diffusable derivatives may be a controlling factor also in mitoses.

Vincent and Dornfeld (1949) described the localization of the nucleic acids in the developing rat ovary. They found increased concentrations of ribonucleic acid in the region of the germinal epithelium showing the greatest proliferative activity. Preceding this proliferative

activity the tissues of the suspensory ligament and the ostium of the oviduct, which lie next to this active area, underwent an increase in ribonucleic acid content. It was thus suggested that a nucleic acid derivative might be involved in an evocatory role.

In the present study it was desired to demonstrate experimentally the effects of increased concentration of nucleotides on mitosis in the germinal epithelium. The method of *in vitro* culture was selected, with the ovary and its adnexa kept intact so as to retain normal tissue relations. Apart from its pertinence to the problem of ovarian development, the germinal epithelium when so used presents certain general advantages for the investigation of fundamental problems in cell reproduction. The rate of multiplication may be accurately measured and compared with a suitable control. Cell migration does not occur, thus the normal association with other tissues is maintained. The physical and chemical environment can be held within the tolerances found *in situ*. In addition, damage to the tissues by the usual methods of introduction of experimental substances can be avoided, and products that are released by cell injury can be washed away or diluted out.

MATERIALS AND METHODS

Culture Technique

Cultures of entire rat and mouse ovaries (Martinovitch 1938a, 1938b, 1939; Franke 1941) and fragments of rat and guinea pig ovaries (Diomidowa 1938) have been reported. In all of these investigations, however, fowl plasma coagula were used as a supporting and nutrient medium and chick embryo extract was used to promote growth. Since the use of a semisolid coagulum might well account for the migration of cells out of the explants as reported by these authors, and retard the exchange of metabolites between the explant and the medium, it was decided to use a basal fluid medium of serum and Tyrode solution in the present studies. Because of the highly effective and poorly characterized action of chick embryo extract in stimulating cell metabolism (Fischer 1946, Davidson 1947), the use of this substance was avoided.

Preparation of Serum

The serum used in the cultures was prepared from whole blood taken from female adult albino rats of the Sprague-Dawley strain. Since this is the same stock as the animals from which the ovaries were taken, the serum is in this respect homologous. The serum from several animals was pooled, and the amount prepared at any one

time depended on the number of ovaries available for culture. Each culture required 1/2 ml. of serum, and since the average yield of serum per donor animal was 2 ml., the number of animals to be bled could be roughly computed on this basis.

The animal to be bled was narcotized with Nembutal. An opening was made into the thoracic cavity through the ventrolateral body wall, exposing the heart and lungs. The aorta was cut through, allowing the blood to run into the thoracic cavity. It was quickly withdrawn in a 10 ml. syringe fitted with an 18 gauge needle, which had previously been flushed out with Tyrode solution and chilled in the refrigerator, then discharged into a 100 x 13 mm. Pyrex centrifuge tube. The blood was allowed to clot, and the mass was rotated with a glass rod in order to keep the coagulum free from the side of the tube. It was then centrifuged for 5 minutes at 4000 r.p.m. in an angle centrifuge. The clear serum was carefully pipetted off and discharged into the pool storage tube. By this method clear non-hemolytic serum could be prepared in a yield of about 40% of the whole blood.

Preparation of Nucleotide Solutions

The pentose nucleotides, adenylic acid, guanylic acid, cytidylic acid, and uridylic acid dissociate as moderately strong acids in aqueous solution. In order to

incorporate these substances in the culture medium at the hydrogen ion concentration of normal serum (pH 7.2-7.6), it is necessary to bring about neutralization and conversion of the ionic equilibrium to that of a soluble salt. It was decided to test the activity of the nucleotides at a concentration of .0025 M in the culture medium. Since the medium in all cases consisted of 50% Tyrode solution and 50% serum, the nucleotides were dissolved in the Tyrode fraction at twice the desired concentration (.005 M), which when mixed with an equal volume of serum resulted in a final concentration of .0025 M. In practice, 10 ml. of a nucleotide solution was prepared at one time, o.o5 millimoles of nucleotide being first dissolved in 5 ml. of Tyrode solution. The pH was then adjusted to 7.4 by addition of Tyrode solution containing 2 mg./ml. of added sodium carbonate. Sufficient further Tyrode was then added to bring the volume to 10 ml. The isotonicity of the Tyrode solution is not appreciably altered by the addition of the nucleotide and the neutralization.

The solutions prepared in the manner just described, the sources of the nucleotides used, can be summarized as follows:

Solution A. 17.4 mg. yeast adenylic acid (adenosine-3-phosphoric acid) (Schwarz, lot HA-4815C, m.w. 347.24) was added to 10 ml. Tyrode solution and neutralized with sodium carbonate.

Solution B. 18.4 mg. yeast guanylic acid (Schwarz, lot HC-4605, m.w. 363.24) was added to 10 ml. Tyrode solution and neutralized with sodium carbonate.

Solution C. 16.2 mg. yeast cytidylic acid (Schwarz, lot HC-4901A, m.w. 323.21) was added to 10 ml. Tyrode solution and neutralized with sodium carbonate.

Solution D. 16.2 mg. yeast uridylic acid (Nutritional Biochem. Corp. lot 6060, m.w. 324.19) was added to 10 ml. Tyrode solution and neutralized with sodium carbonate.

Preparation of Aseptic Tyrode Solution

The Tyrode solution used has the following composition (Parker 1938):

Sodium chloride	8.00 gm.
Potassium chloride	0.20 gm.
Calcium chloride ($CaCl_2$)	0.20 gm.
Magnesium chloride ($MgCl_2.6H_2O$)	0,10 gm.
Sodium acid phosphate ($NaH_2PO_4.H_2O$)	0.05 gm.
Sodium bicarbonate ($NaHCO_3$)	1.00 gm.
Glucose	1.00 gm.
Water (double glass distilled) to make	1000 ml.

As an aid in maintaining asepsis, 2 mg. of Potassium Penicillin G Abbott were added to each liter of the above solution. No unfavorable effects could be traced to its use in this concentration.

Preparation of Cultures

Animals. Female animals were selected from litters of Sprague-Dawley strain albino rats. The litters ranged in age from 6 hours to 2 days post partum. The animals were isolated in finger bowls prior to the culture preparation.

Culture Tubes. The culture vessels were 100 x 13 mm. rimless Pyrex tubes. The empty tubes were arranged in a rack in pairs, fitted with rubber stoppers, and labeled according to the conditions of the experiment.

Solutions. The Tyrode solution, nucleotide solutions, and rat serum were prepared well in advance of the culturing and were stored in the refrigerator. One ml. pipettes fitted with rubber bulbs were used to dispense the solutions into the culture tubes. First, one-half ml. of serum was pipetted into each of the culture tubes. Next, one-half ml. of Tyrode solution was pipetted into each of the tubes which was to receive a control ovary (one ovary of each pair). One-half ml. of Tyrode containing the nucleotide was then pipetted into the remainder of the tubes. Extra tubes were prepared containing serum and each of the nucleotide solutions being used. These received a few drops of a phenol red solution, and were used to follow visually the pH changes during the addition of gas (explained below).

Excision of Ovaries. The animals were rendered unconscious by cervical dislocation and pinned out ventral side up on a large rubber stopper. The umbilicus was seized with a forceps and an incision made with a fine scissors through the skin on one side commencing at the angle of the ribs and crossing over to the thigh of the leg on the opposite side. This was repeated in the opposite direction, and the skin was pulled back exposing the abdominal musculature. The same incision was made through the musculature exposing the viscera. The umbilicus was then pulled posteriorly and pinned between the hind legs. The musculature on each side was pinned back. The ovaries lie just laterally and posterior to the kidneys. The oviduct lies against the dorsal body wall and continues posteriorly as the horn of the uterus. The oviduct was grasped with a fine forceps as close as possible to the ovary and cut with the scissors on the distal side close to the forceps. A second cut was made with the ovary lifted out from the body wall, completing the excision. The ovary and adnexa were placed in a covered dish of Tyrode solution for rinsing. The opposite ovary was removed in the same manner. After rinsing for a few minutes in Tyrode solution, each ovary was placed in its designated tube. Extreme care was exercised in handling the explant so as to avoid tissue injury.

Addition of Gaseous Medium. The presence of a small percentage (2-5%) carbon dioxide is required in each culture tube to adjust and maintain the pH of the medium. It was found necessary to replace the remainder of the atmosphere with oxygen in order to maintain the ovary in good condition *in vitro*. The apparatus used in these experiments consisted of a tank of anesthetic oxygen-carbon dioxide mixture, containing 90% oxygen and 10% carbon dioxide, to which was attached a "T" tube. One branch of this conducted part of the mixture through a column of Ascarite, which removed the carbon dioxide. This branch which contains 100% oxygen after leaving the Ascarite column, and the other branch containing the original mixture, were then mixed in the proportions necessary to give the final desired concentration of carbon dioxide. This was done by flow meters on each branch, and an adjustable valve in one of the branches. The two branches were brought together through a "Y" tube, and the resulting mixture conducted through a length of rubber tubing, and into the culture tubes. With this apparatus any desired concentration of carbon dioxide between zero and ten percent could be easily obtained by suitable adjustment.

In practice, extra tubes containing culture medium, but without ovaries, were prepared. Phenol red indicator was added to these. The carbon dioxide content of the

gaseous medium was adjusted to the proportion necessary to bring the pH of these tubes to 7.4. Then the stopper was removed from each culture tube, the gas allowed to flow into the tube for 30 seconds, and the stopper replaced.

Incubation. After addition of the gaseous medium, the culture tubes were placed in an incubator, which was equipped with a rotating apparatus. In this device the tubes were held in a slant position, making an angle of 10° with the horizontal, and were rotated at 2 r.p.m. This served to keep the explant from adhering to the wall and increased the rate of exchange between the gaseous medium and the culture fluid. Fig.2 demonstrates necrosis caused by adherence to the wall of the tube and incomplete circulation of the medium.

Colchicine Addition. After 16 hours of incubation the cultures were opened and one drop of Tyrode solution containing 0.2 mg./ml. of colchicine was added. This gives a colchicine concentration of .01mg./ml. of culture fluid, which has been found ample to halt mitosis. The cultures were then regassed and the pH checked, after which they were returned to the incubator. Eight hours later the ovaries were removed from the tubes and placed in fixative.

Processing of Cultured Ovaries

The ovaries were fixed in Zenker-formol for 6 hours, washed for at least 10 hours, and run through 5 changes of dioxan for dehydration. After rinsing for 1 min. in xylol, they were run through 5 changes of paraffin and embedded. Sectioning followed at 10 micra and the ovaries were mounted serially. The sections were run through xylol and graded alcohols to water, mordanted for 5 minutes in a 2.5% ferric ammonium sulfate solution, stained for 5 minutes in a 0.5% solution of Heidenhain's hematoxylin, and destained 5 minutes in a saturated solution of picric acid. Washing in alkaline tap water for 30 minutes removed the picric acid, and brought the tissues to a light blue color. The sections were run through graded alcohols, cleared in xylol, and mounted in Canada balsam.

Method of Measurement to Determine Mitotic Rate

The mitoses occurring during the last 8 hours of the 24 hour culture period are stopped at metaphase by the action of the colchicine. Since the entire ovary was mounted in 10 micra sections these mitoses are easily visible, and a total count was made of the number accumulated in the germinal epithelium. This number was divided by 5 to give the average number of mitoses occurring in one-fifth of the area of the germinal epithelium. Then a projection tracing was made of the perimeter of every

5th section through the ovary, and its length in centimeters determined by the use of a map reader. These lengths were totaled and divided by the linear magnification of the projection, thus giving for one-fifth of the ovarian surface, the length in centimeters of areas of germinal epithelium 10 micra in width. Multiplying by this width provided the area, which is reduced for convenience to square millimeters. The number of mitoses per square millimeter can then be easily determined.

OBSERVATIONS AND DATA

Effect of Variations in Culture Technique.

In the development of the culture technique and the subsequent testing of the nucleotides for their effect on mitosis in the germinal epithelium, 140 ovaries from 70 albino rats were used. Of this number 46 ovaries were expended in the development of the culture method. The technique described in the previous section represents the method in its final stage of development. Initially two sets of cultures (A1-A2) were prepared to which neither oxygen nor carbon dioxide were added. These gases were present only in their atmospheric concentrations. Consequently the pH of these early cultures was variable, and areas of cellular necrosis predominated over normal areas in the explants (Fig.1). It was found, however, that mitosis in some tissues continued despite the low oxygen

tension and pH variations (Fig. 1). In fact, mitoses were found in explants at pH's as extreme as 8.2 in the alkaline range and 6.9 in the acid range, with the gaseous medium that of the atmosphere. Addition of the oxygen-carbon dioxide mixture resulted in greatly improved cultures (A3-A7). The pH was stabilized in the range 7.2-7.6 by the low porportion of carbon dioxide (Ca.2%) and the high oxygen tension reduced the extent of necrosis to a small area in the center of the mass or at the point where the mass rested against the culture tube wall in the incubator (Fig. 2). Finally, the B series of cultures were made, using the roller device which gently agitated the ovaries and the fluid medium during incubation. This represented the final improvement on the technique. All of the ovaries of the B series were of such histological condition, that it was difficult, if not impossible, to distinguish them from uncultured normal ovaries (compare Figs. 3 and 4). However, a thorough cytological study of the material was not attempted, but left for future investigation. The present study was limited to the quantitative estimation of the effect of the nucleotides on mitosis in only one of the tissues present, the germinal epithelium.

Effect of Ribonucleotide Addition.

The mitotic rate per square millimeter of germinal epithelium was determined by the method previously described for each ovary of the 47 pairs used in this study. Of this total 44 pairs were used to test the action of the ribonucleotides, as follows: cytidylic acid, 10 pairs; uridylic acid, 12 pairs; adenylic acid 11 pairs; guanylic acid, 11 pairs. Of each pair one member served as control, the other was exposed to the nucleotide. The control ovary was either the right or left one, the order being successively alternated. The remaining 3 pairs were taken from animals injected with colchicine for an 8 hour period. The total number of mitoses counted in one-fifth of each ovary, and the surface area for each ovary are tabulated in Table 1. The mitotic rate of each ovary calculated from the data in Table 1, is given in Table 2, The letter number combination is used to assign the following information. The first letter and the number following it designate the serum lot used. The entire combination designates the animal from which the pair of ovaries was taken.

For ease in visual comparison the results are presented in Graphs 1,2,3, and 4. The intersect of the mitotic rate of the control ovary, measured along the horizontal axis, with that of the corresponding

experimental ovary, measured along the vertical axis, fixes the position of the plot. It is seen that if the rates of right and left ovaries were the same, these points would lie along a 45° line bisecting the quadrant. A plot below the line indicates inhibition, the vertical distance to the 45° bisect being a measure of this inhibition. The best straight line in the least square sense is drawn through these plots.

Analysis of variance at the 5% significance level allows the following conclusions to be drawn:

1. There is no significant difference in the response of right and left ovaries to the action of the nucleotides. (Smallest value of critical region = 4.96; highest value of F = 1.266).

2. Inhibition of mitosis occurs in ovaries treated with adenylic acid, guanylic acid, and cytidylic acid. (Largest value of critical region = 4.96; smallest value of F = 5.34).

3. There is no significant difference between the mitotic rates of the ovaries treated with uridylic acid, and those of the controls. (Critical region = above 4.96; F was less than 1.)

TABLE ONE

Number of Mitoses and Surface Area of Germinal Epithelium

Treatment	Culture Pair	Total No. Mitoses in Exp. Ovary	Area of G.E.(1/5 Total)	Total No. Mitoses in Cont.Ovary	Area of G.E.(1/5 Total)
Cytidylic Acid	A2B	21	.154mm^2	32	.145mm^2
	A4B	22	.112	40	.116
	A5A	100	.136	385	.134
	A5C	60	.171	135	.112
	A5E	65	.134	280	.153
	A5G	60	.136	225	.112
	A6A	7	.156	51	.167
	A6B	23	.156	69	.161
	A7A	24	.159	51	.144
	A7B	22	.155	44	.138
Uridylic Acid	B1-7	29	.109	13	.110
	B1-9	29	.113	24	.116
	B1-11	113	.132	106	.128
	B2-1	8	.127	12	.110
	B2-3	19	.130	38	.132
	B2-5	68	.124	100	.113
	B2-7	46	.115	59	.107
	B2-9	3	.092	14	.112
	B2-11	9	.144	8	.054
	B3-13	17	.127	51	.140
	B3-15	62	.120	53	.122
	B3-17	18	.124	10	.118
Adenylic Acid	A4E	0	---	20	.100
	A4F	0	---	76	.108
	A4G	21	.113	42	.111
	A7F	27	.170	35	.149
	A7G	55	.143	62	.146
	A7H	36	.135	64	.141
	B4-7	6	.139	36	.116
	B4-9	16	.109	78	.118
	B4-11	5	.123	16	.111
	B4-13	0	---	0	---
	B4-15	5	.114	7	.127

TABLE ONE (Cont.)

Treatment	Culture Pair	Total No. Mitoses in Exp. Ovary	Area of G.E.(1/5 Total)	Total No. Mitoses in Cont.Ovary	Area of G.E.(1/5 Total)
Guanylic Acid	B1-1	13	.108mm^2	21	.110mm^2
	B1-3	12	.128	254	.126
	B1-5	96	.149	213	.152
	B3-1	0	---	74	.113
	B3-3	4	.124	17	.122
	B3-5	20	.129	57	.125
	B3-7	2	.112	48	.133
	B3-9	10	.143	26	.133
	B4-1	9	.125	6	.107
	B4-3	5	.122	50	.117
	B4-5	8	.128	90	.119
		Right Ovary		Left Ovary	
In Situ Colchicine 8 hours	B1-A	4	.150	5	.142
	B1-B	62	.167	61	.156
	B1-C	33	.155	22	.139

TABLE TWO

Mitotic Rates of Cultured Ovaries

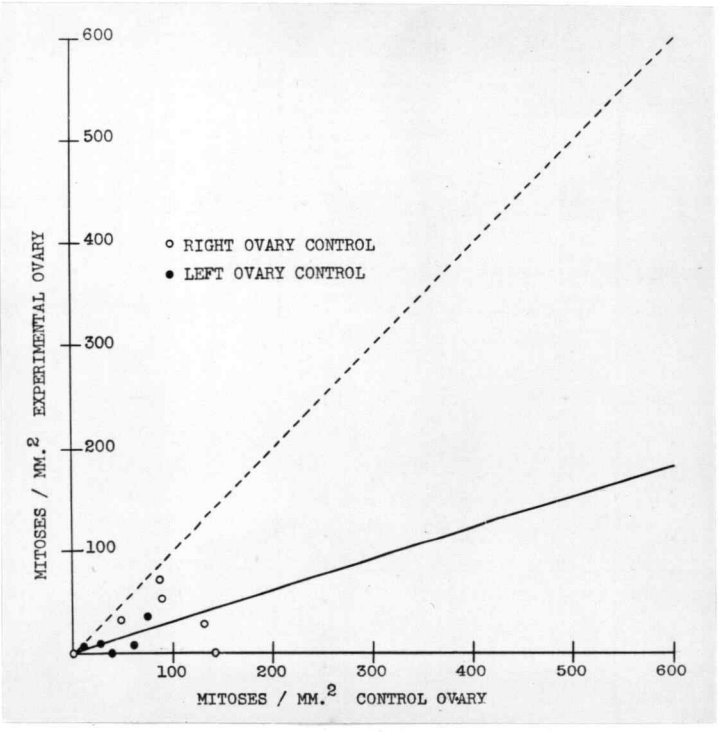
Treatment Remarks	Culture Pair	Rate Experimental Ovary and (Side) Mitoses/mm^2		Rate Control Ovary and (Side) Mitoses/mm^2	
Cytidylic Acid	A2B	27.0	(R)	44.1	(L)
	A4B	39.3	(L)	69.0	(R)
	A5A	147.0	(R)	575.0	(L)
	A5C	70.0	(L)	241.0	(R)
	A5E	97.0	(R)	366.0	(L)
	A5G	89.0	(L)	402.0	(R)
	A6A	8.97	(L)	61.0	(R)
	A6B	29.4	(L)	85.7	(R)
	A7A	30.2	(R)	70.8	(L)
	A7B	28.0	(R)	64.0	(L)
Uridylic Acid	B1-7	53.2	(R)	23.6	(L)
	B1-9	51.3	(L)	41.4	(R)
	B1-11	171.0	(R)	165.0	(L)
	B2-1	12.6	(L)	22.0	(R)
	B2-3	29.0	(R)	54.0	(L)
	B2-5	111.0	(L)	177.0	(R)
	B2-7	80.0	(R)	110.0	(L)
	B2-9	6.52	(L)	28.0	(R)
	B2-11	12.5	(R)	29.4	(L)
	B3-13	26.7	(L)	73.0	(R)
	B3-15	103.0	(R)	87.0	(L)
	B3-17	29.0	(L)	17.0	(R)
Adenylic Acid	A4E	0	(R)	40.0	(L)
	A4F	0	(L)	140.0	(R)
	A4G	37.2	(R)	76.3	(L)
	A7F	32.3	(R)	47.0	(L)
	A7G	76.2	(L)	84.9	(R)
	A7H	53.3	(L)	90.8	(R)
	B4-7	8.63	(R)	62.0	(L)
	B4-9	29.3	(L)	132.0	(R)
	B4-11	8.13	(R)	29.0	(L)
	B4-13	0	(L)	0	(R)
	B4-15	8.77	(R)	11.0	(L)

TABLE TWO (Cont.)

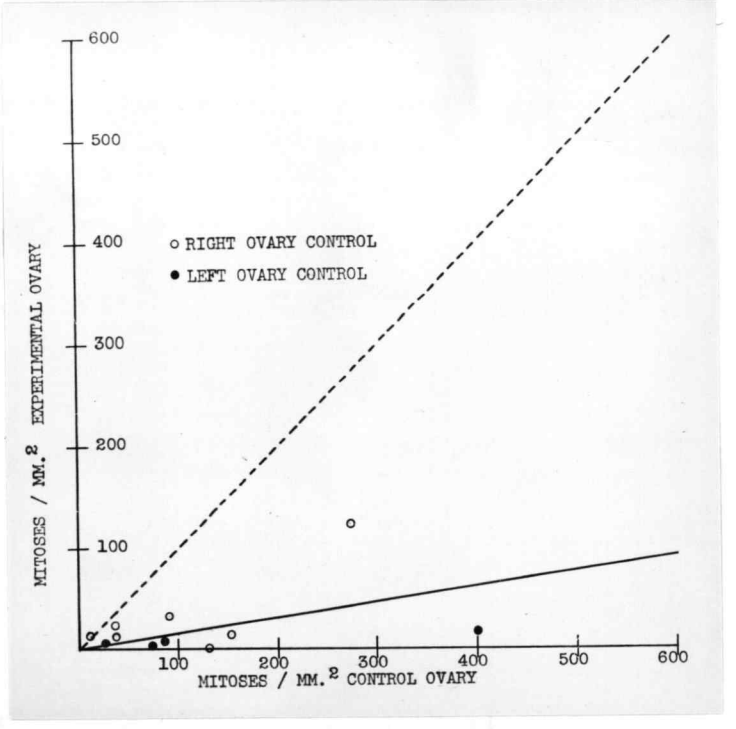
Treatment Remarks	Culture Pair	Rate Experimental Ovary and (Side) Mitoses/mm^2	Rate Control Ovary and (Side) Mitoses/mm^2
Guanylic Acid	B1-1	24.0 (L)	38.2 (R)
	B1-3	18.7 (R)	403.0 (L)
	B1-5	122.0 (L)	273.0 (R)
	B3-1	0 (L)	131.0 (R)
	B3-3	6.45 (R)	27.8 (L)
	B3-5	31.0 (L)	91.2 (R)
	B3-7	3.57 (R)	73.1 (L)
	B3-9	13.9 (L)	39.0 (R)
	B4-1	14.4 (L)	11.2 (R)
	B4-3	8.10 (R)	85.4 (L)
	B4-5	12.5 (L)	151.0 (R)

Mitotic Rates of Ovaries in Situ

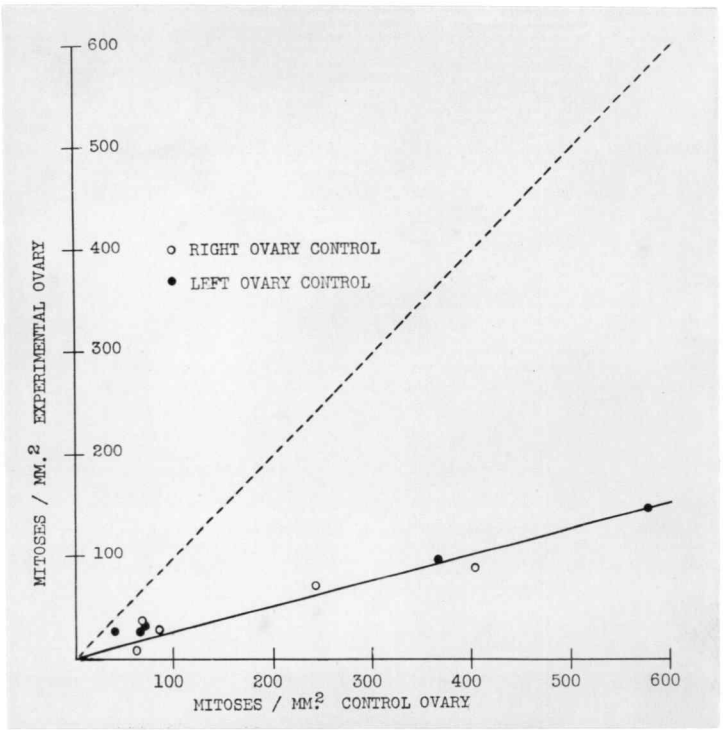
Treatment Remarks	Animal No.	Rate Right Ovary Mitoses/mm^2	Rate Left Ovary Mitoses/mm^2
Colchicine 8 hours	B1-A	5.33	7.04
	B1-B	74.0	78.0
	B1-C	31.6	42.0



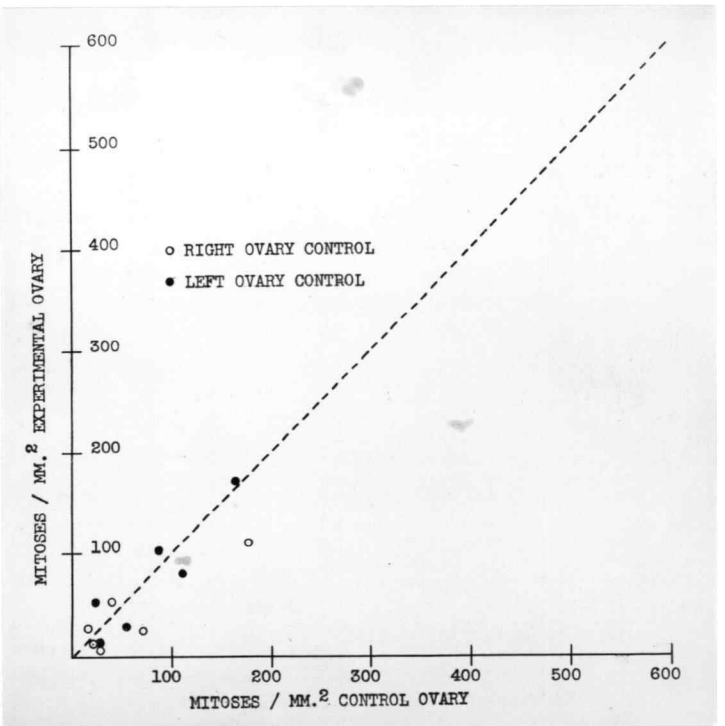
GRAPH 1. ADENYLIC ACID



GRAPH 2. GUANYLIC ACID



GRAPH 3. CYTIDYLIC ACID



GRAPH 4. URIDYLIC ACID

DISCUSSION

Both oöcytes and follicular cells arise from the germinal epithelium through differentiation of the indifferent cells. This depletion of cells from the germinal epithelium is taking place at a high rate in ovaries of the ages studied here (Slater and Dornfeld 1945). A simultaneous replacement of the germinal epithelium is proceeding by mitotic division of the remaining cells. It is this reproductive growth that is being studied in the present investigation. Certain physical and chemical factors affect the rate at which this proliferation occurs and these will be considered in the discussion that follows.

Condition of the Ovaries in Vitro.

In order to decide to what extent the results obtained *in vitro* are the responses of normal tissues, it is of importance to establish whether degenerative changes have occurred in the explants. The morphological condition of the tissues very accurately reflects any extensive changes in their functional capacities (Bloom 1937). Comparison of cultures with normal explants (in Figs. 3 to 8) shows no evidence of abnormality in the former. No significant modification of the ovaries occurred in the later B series which were cultured with added oxygen employing the roller tube method. This is probably due in some respect to the nature of the explant. The ovary as

excised represents a more or less complete functional unit. The ovarian capsule serves to protect the germinal epithelium from direct contact with the walls of the culture tube. The oviduct and its ostium retain their normal relations to the germinal epithelium. No extensive cut or damaged surfaces are present, as is the case where fragments of organs are cultured.

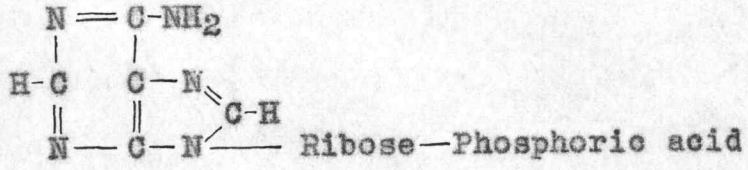
Variations in Mitotic Rate

Apart from the conclusions demonstrated by statistical analysis concerning the relative effects of the nucleotides on mitosis, certain other problems need comment. The sources of variation are of importance. Sufficient data are not yet available to show exactly the extent of the mitotic variation between right and left ovaries *in vitro* when cultured under the same conditions. It is apparent however that there is no consistent difference in favor of one side. Some data are presented showing the extent of these differences *in situ*. The largest absolute difference in these rates is 10.4 mitoses/mm^2. Stein (1947) has given total counts for mouse ovaries, over an 8 hour colchicine period, in which the greatest difference was 19%. Two ovaries of one pair were separately cultured under identical conditions in the present study and the absolute difference in mitoses was 3/mm^2, where the general mitotic rate was 50 mitoses/mm^2. This may be entirely

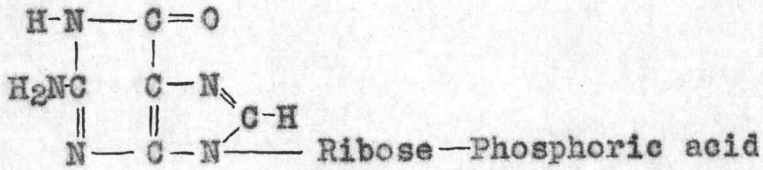
fortuitous. It is probable, however, that the variation between right and left ovaries *in vitro* is not greater than *in vivo*. If the variations between the control ovaries of different animals cultured under the same conditions are considered, it is seen that the greatest absolute difference is 380 mitoses/mm^2. This suggests that the differences between ovaries of different individuals are far greater than those of right and left ovaries of any single pair.

Action of Ribonucleotides on Cell Proliferation

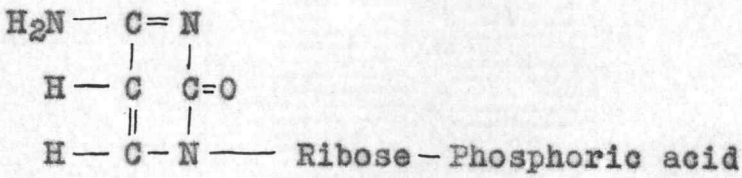
The results of these experiments have shown that the two purine ribonucleotides, adenylic acid and guanylic acid, and the pyrimidine ribonucleotide cytidylic acid inhibit mitoses in the germinal epithelium. The pyrimidine ribonucleotide uridylic acid showed no consistent inhibition or stimulation. The structural formulae of these compounds are shown below.



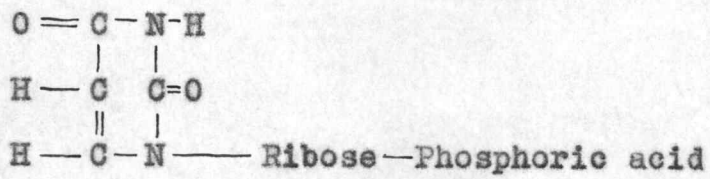
ADENYLIC ACID



GUANYLIC ACID



CYTIDYLIC ACID



URIDYLIC ACID

It will be noticed that the first three nucleotides mentioned above, which act as mitotic inhibitors, all have an amino group substituted on the purine or pyrimidine ring. Uridylic acid which has no substituted amino group, shows no inhibition. This suggests a correlation between structure and function in the biological activity of the compounds for the tissue studied here. No corroboration of these results is available in the literature.

Parsons, Gulland, and Barker (1947) injected mice with large doses of the nucleotides tested here. While no direct studies were made on cell proliferation, studies were made on the differential shifts of leucocytes in the circulating blood. This and other data on the resulting pathologies led these authors to conclude that the changes following treatment with the nucleotides very exactly paralleled the conditions resulting from X-radiation. Mitchell (1942) found that following both X- and gamma

radiation, cytoplasmic increases in ribonucleotide concentration occurred. According to Mitchell, the effect of radiation, which inhibits mitosis, does so by inhibiting the conversion of cytoplasmic ribonucleic acid into desoxyribosenucleic acid. Stowell (1945) found a slight decrease in desoxyribosenucleic acid in mammary carcinoma following X-radiation. Contrariwise, Brachet (1947) reviews evidence that in all of the many growing and dividing cells studied (Caspersson 1941, 1941a, 1941b; Brachet 1941), the concentration of cytoplasmic ribonucleic acid is high. During actual cell division this concentration drops, thus it was assumed that conversion to desoxyribosenucleic acid took place during cell division. Brachet strongly suggests that ribonucleotides in diffusing from one tissue to another might play a mitogenetic role by increasing the availability of this necessary cytoplasmic ribonucleic acid.

Thus two cases of cytoplasmic increase in ribonucleic acid components are seen, the one resulting from radiation, in which mitosis is inhibited, while the other occurs in rapidly proliferating tissue. The results of the present investigation seem to extend and clarify the data obtained from the radiation experiments.

It has been shown, at least under the conditions of this investigation, that ribonucleotides as such do not

in any way stimulate proliferation. This casts some doubt on their function as mitogenetic agents. It has not been shown in Brachet's case that the mononucleotides contribute to the ribonuclease removable basophilia on which he bases his hypothesis. Nucleic acid products other than these, possibly of higher molecular weight, can not yet be excluded from such activity. In the case of X- and gamma radiation effects Mitchell found some evidence that ribonucleotides were actually present. Jolles (1949) found that irradiation through a sieve gave that same effect as irradiation over the whole area, thus suggesting that the effect might be due to a readily diffusable substance. The lower molecular weight mononucleotides would exhibit such diffusion behavior. It seems very probable that the mitosis inhibiting effects of X-radiation are due to the action of the free monoribonucleotides.

SUMMARY

A technique has been described by means of which the immature rat ovary intact with its adnexa can be cultured *in vitro*. This technique maintained the ovaries of animals 6 hours to 2 days old in normal morphological condition and in an active state of growth over the 24-hour time period used in these experiments.

A method is described for the measurement of the rate of cell reproduction in the germinal epithelium. According to this method colchicine is allowed to act on the explant for a fixed period of time (8 hours), halting the mitoses in metaphase. The numbers of mitoses are counted in the serially sectioned material, and the area of the germinal epithelium is figured from projection tracings. The number of mitoses per unit area thus forms an accurate basis for comparison of cell proliferation in the germinal epithelium of different ovaries.

Using the methods summarized above, the effect of .0025 M concentrations of mononucleotides of yeast nucleic acid on proliferation of germinal epithelium was determined.

Under the conditions of these experiments it was found that the ribonucleotides adenylic acid, guanylic acid, and cytidylic acid inhibit mitoses, whereas uridylic acid has no significant effect in this respect.

A correlation between the structure and the action of the nucleotides is noted. The nucleotides adenylic acid, guanylic acid, and cytidylic acid have amino groups substituted on the purine or pyrimidine ring. These have an inhibitory action on mitoses. Uridylic acid which shows no such action has no amino group.

Brachet's suggestion that ribonucleotides may be effective in the stimulation of cell proliferation is not supported by the results of this study.

The relation of these findings to the effects of X- and gamma radiation on tissues has been discussed. It is suggested that the free ribonucleotides released by the action of radiation are the active agents in the observed blocking of mitoses.

EXPLANATION OF FIGURES

Abbreviations used on the figures are as follows:

cap.	capsule
g.e.	germinal epithelium
mit.	mitotic figure(s)
nec.	necrosis
ost.	ostium
od.	oviduct
s.l.	suspensory ligament

Figure 1. Ovary A1CY, age 1 day post partum. Cultured 24 hours with oxygen at atmospheric concentration. Most of the ovary is necrotic, although many mitoses are noted in the oviduct. X 150.

Figure 2. Ovary A3AR, age 5 days post partum. Cultured 24 hours with 98% oxygen. Culture not rotated. Note necrotic region where ovary rested against tube wall. X 140.

Figure 3. Ovary B4-7, age 1 day post partum. Cultured 24 hours with 98% oxygen. Culture rotated. Note normal appearance of ovary. Compare with Fig. 4. X 150.

Figure 4. Ovary A1AX, age 1 day post partum. Normal uncultured ovary. X 150.

Figure 5. Ovary A1AX, age 1 day post partum. Normal uncultured ovary. X 2000.

Figure 6. Ovary B3-7, age 1 day post partum. Cultured 24 hours with 98% oxygen. Culture rotated. Compare appearance of cells with Fig. 5. X 2000.

Figure 7. Ovary A5AR, age 1 day post partum. Cultured 24 hours with 98% oxygen. Culture not rotated. Compare appearance of cells with Fig. 8. epithelium. X 850.

Figure 8. Ovary B1AR, age 1 day post partum. Uncultured ovary taken from animal injected with colchicine for 8 hours. X 850.

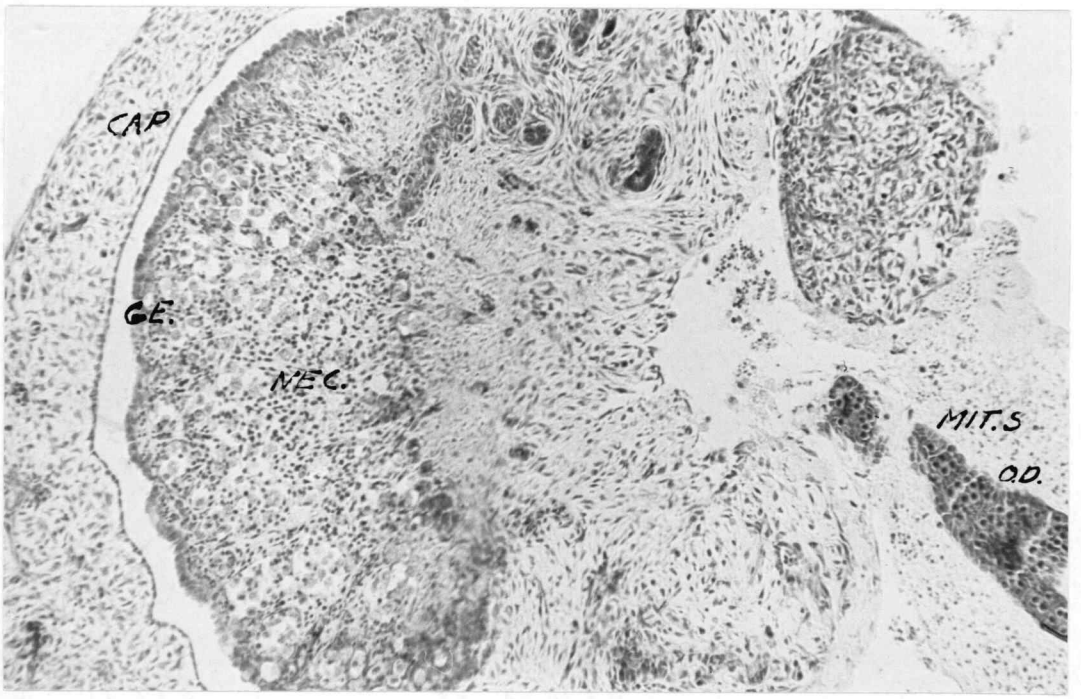


FIGURE 1.

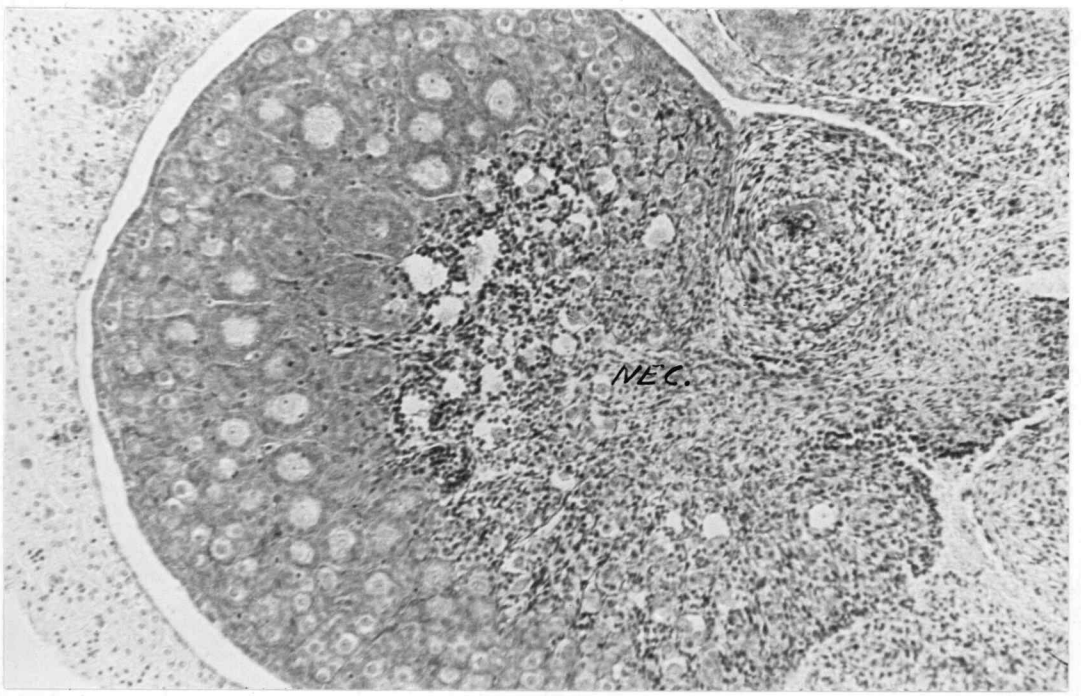
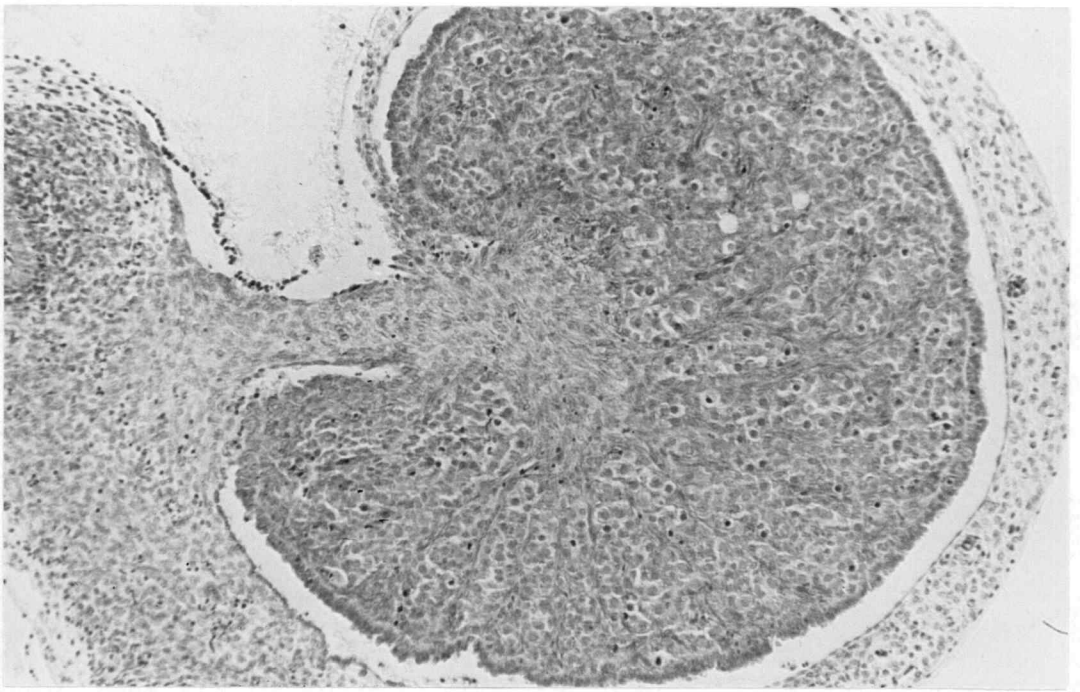


FIGURE 2.



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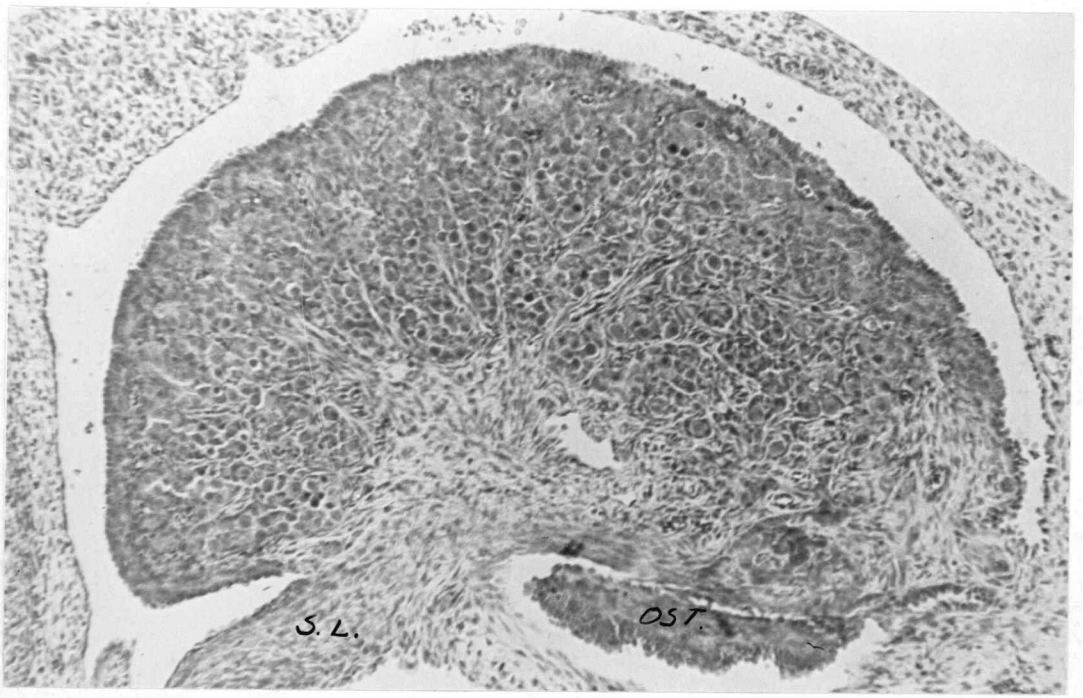

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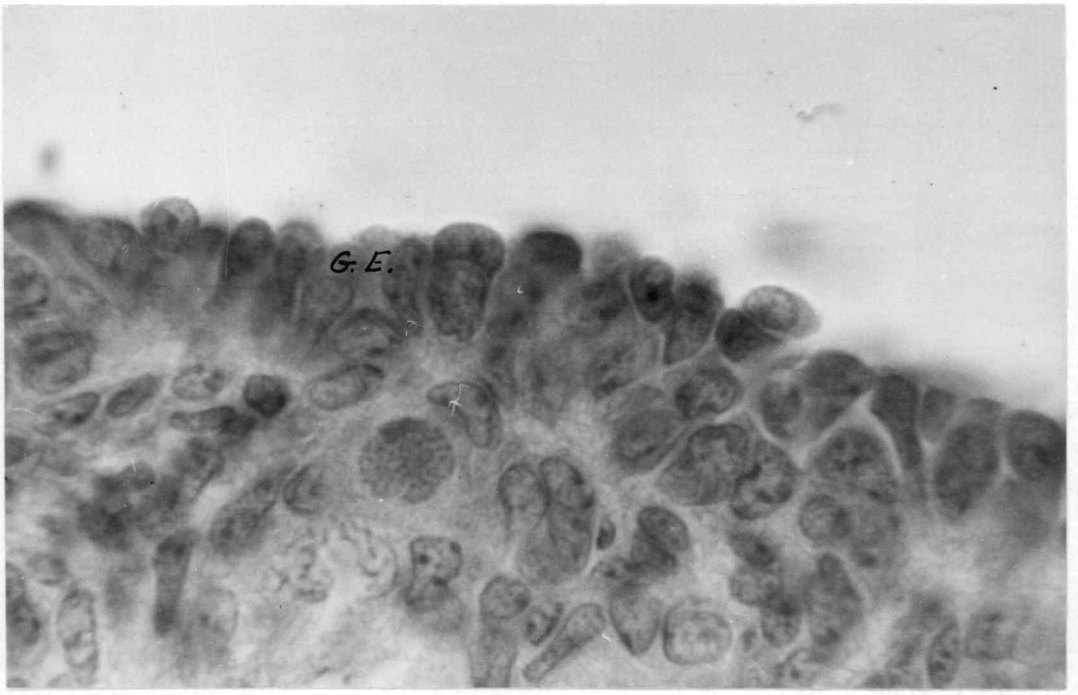

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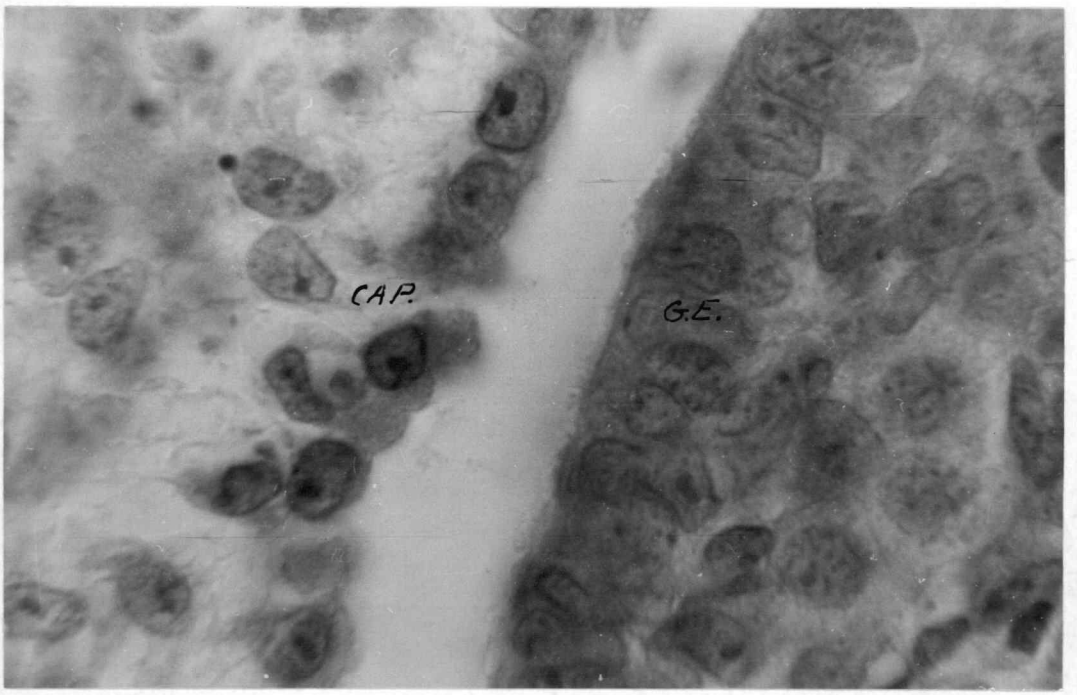

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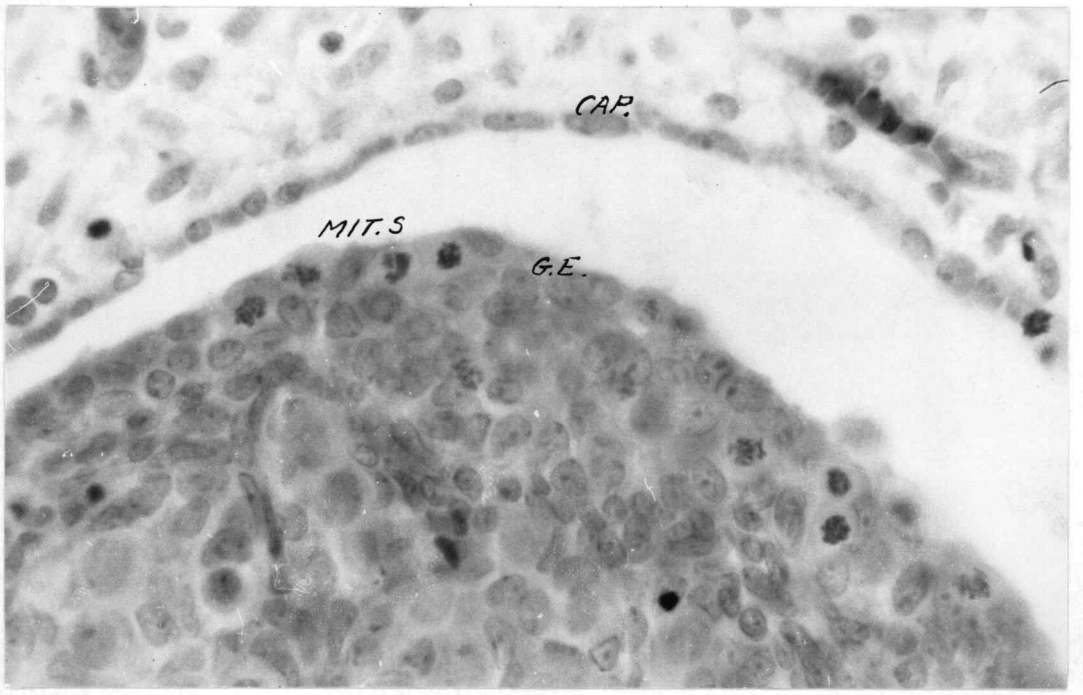

FIGURE 7.

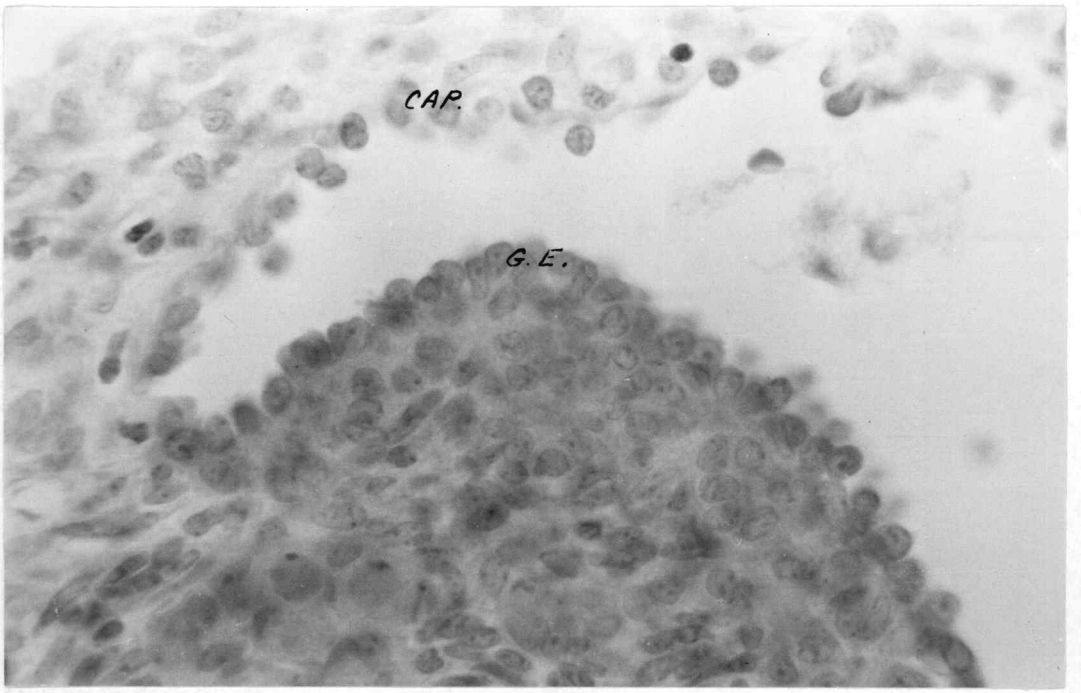

FIGURE 8.

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