THE EFFECT OF SODIUM MALONATE ON MITOTIC RATE IN THE GERMINAL EPITHELIUM OF RAT OVARIES GROWN IN VITRO

by

MORRIS EUGENE WEAVER

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF ARTS

June 1956

APPROVED:

[redacted]

Professor of Zoology
Chairman of Department of Zoology

In Charge of Major

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

Date thesis is presented May 2, 1956

Typed by Louise Ferguson

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. Ernst J. Dornfeld who as major professor and friend has been such a help with the necessary training, experimentation and completion of this work. Thanks should also be given Dr. Jerome Li for his suggestions concerning the statistical approach to this problem and the evaluation of the data.

TABLE OF CONTENTS

THE EFFECT OF SODIUM MALONATE ON MITOTIC RATE IN THE GERMINAL EPITHELIUM OF RAT OVARIES GROWN IN VITRO

INTRODUCTION

The chemical factors underlying the process of mitosis are only partially understood. This applies not only to the immediate mechanisms of nuclear and cell division, but also to the problem of mitotic stimulation or inhibition, i.e. the control of the mitotic rate. It is well known that specific tissues vary greatly with regard to rates of cell division, and the causes of such differences represent a fundamental problem of biology.

It appears obvious that the mitotic process requires energy which might be sought in carbohydrate metabolism. That such is indeed the case has already been established by a number of investigators studying a variety of cell-types. Considering the wide range of material investigated, it is not surprising that differences of detail have been found.

Eggs during cleavage have long been a favorite material for the study of cell division. As early as 1895, Loeb demonstrated a cessation of mitotic activity in the eggs of echinoderms under anaerobic conditions (13). Other studies showed that while some eggs require oxygen for cleavage, others have varying degrees of tolerance to anaerobic conditions (5).

The earliest attempt to measure respiration and so estimate the energy requirements of mitosis in eggs was that of Lyon in 1904 (14). Outstanding among later workers who measured the rate of respiration during egg cell division were Runnstrom who in 1933 studied sea urchin eggs (21), and Brachet who between 1932 and 1935 investigated frog eggs (2,3,4). Perhaps the most careful and extensive determinations have been made by Zeuthen in 1951, using a Cartesian diver respirometer (24). In general there has been agreement among workers that the respiration of eggs changes during cell division. However, because eggs are full of storage material and equipped for an existence largely independent of the environment, they are not very satisfactory for the experimental study of the metabolic requirements of mitosis. Mitosis is a chain of individual processes and metabolic studies will make sense only when related to the various components of cellular reproduction (23). Much of the older work on the metabolism of mitosis is rather meaningless because mitosis was treated as a unitary process and various criteria were used to measure growth and metabolism, i.e. increased area, oxygen consumption, carbon dioxide production, and actual mitotic activity.

In 1933 Laser found that chick fibroblasts would show some growth in area with exceedingly low oxygen tensions (12). Parker in 1936, noted differences in oxygen require-

ments of adult and embryonic tissues (19). Needham and Nowinsky had shown in 1937 that a chick embryo does not metabolize glycogen but relies instead on glucolysis for the necessary energy of mitotic activity (17). The term glucolysis is used in the writings of Needham and Nowinsky, Bullough, and Pomerat and Willmer to indicate the utilization of glucose and not glycogen in the Meyerhof scheme of anaerobic glycolysis (17,5,20).

The relation between carbohydrate metabolism and actual mitotic activity in chick fibroblasts in vitro was examined in detail by Pomerat and Willmer in 1939 (20). They found that factors which inhibit aerobic respiration have little or no immediate effect on mitosis. Evidently cell division in such cultures is not dependent on the tricarboxylic acid cycle. However, as soon as anaerobic glycolysis is inhibited by fluoride or iodo-acetate the mitotic activity is depressed. Thus it seems that in early growth the chick embryo is normally dependent on glucolysis only. There is a certain amount of evidence to indicate that glucolysis does indeed continue under aerobic conditions (18) and that some adult tissues normally possess the faculty to maintain mitotic activity in the absence of oxygen (10).

The first observations on mitotic activity in adult epidermis in vitro appear to be those of Medawar, who in 1947 and 1948 cultured rabbit epidermis in serum and

buffered saline (15,16). He found that the mitotic activity may vary directly with the oxygen tension over a fairly wide range. He also showed that when glycolysis is inhibited by concentrations of about 10^{-4} M. iodo-acetate the epidermal cells die.

Of particular interest have been the recent studies of Bullough beginning in 1951, who concentrated his attention on the epidermal mitoses in the ears of adult mice (5). A detailed examination was made of the relation between mitotic activity and respiration and it was found that the number of cells entering division at a given time was related to oxygen tension and glucose concentration (6,7,8). Lactate or pyruvate successfully replaced glucose in the culture medium suggesting that the Krebs cycle was providing energy for the mitotic activity. Moreover, intermediates in the citric acid cycle such as succinate, fumarate, and citrate were found to increase the rate of oxygen consumption and also the rate of cell division.

Since mitosis is physiologically dependent on maximum energy production, it was expected that any factor which interferes, even partially, with either glycolysis or the Krebs cycle will also interfere with mitosis. Bullough and Johnson obtained antimitotic effects with cyanide, azide, fluoride, iodo-acetate, malonate, and dinitrophenol --substances well known to interfere with the processes of

the Krebs cycle, the cytochrome system, and phosphorylation (8).

Though Bullough's work clearly establishes the dependence of mitosis on the Krebs cycle in adult epithelium, mitotic energy sources are still largely unexplored for other tissue types and may well be different. Embryonic fibroblasts, in fact, do not follow the adult epithelial pattern, as already pointed out.

The present study deals with the germinal epithelium of the ovaries of new-born rats. This tissue, which is neither of early embryonic nor adult type, therefore different from both chick fibroblasts and adult epithelium, represents a stage of rapid proliferative growth. It lends itself to successful in vitro culture, a condition favorable to experimental investigation (1). It was decided to study specifically the effect of malonate on the mitotic rate of this tissue, since such an approach would immediately provide information on the role of the tricarboxylic acid cycle in the mitotic activity. The results obtained would form the basis of further studies which would systematically explore the energy sources and requirements for mitosis in this and related tissues.

MATERIALS AND METHODS

Source and preparation of ovaries

Ovaries were obtained from female animals selected from litters of Holtzman strain albino rats. The litters ranged in age from a few hours to 24 hours *post partum*.

The animals were rendered unconscious by cervical dislocation and pinned out, ventral side up, on a large dissecting cork. A near-mid-line incision through the skin and abdominal musculature was made extending cranially to the sternum and caudally to the pubis. Lateral incisions were made above the level of the umbilicus extending well around the side below the angle of the ribs. The skin and musculature were pulled back and pinned, exposing the viscera. The oviduct was grasped with a fine forceps, the ovary was loosened from its body wall attachment by successive cuts with a scissors, completing the excision. The ovary and adnexa were transferred by means of a glass rod, to the culture tube. The opposite ovary was excised and treated in the same manner, care being exercised in handling the explants by their membrane attachments to avoid tissue injury.

The culture tubes used were 100x13 mm. rimless Pyrex tubes with a capacity of 8.5 ml. when closed by a rubber stopper. The empty tubes, chemically clean, were arranged

in pairs in a rack, fitted with rubber stoppers, and labeled according to the conditions of the experiment.

Culture medium and incubation

The culture medium used was a modified Krebs-Ringer solution developed by W. S. Bullough and associates (5). The solution was devised as follows:

0.90%	NaCl	100 ml.
1.15%	KCl	4 "
1.22%	CaCl	3 "
2.11%	KH_2PO_4	3 "
2.82%	$MgSO_4 \cdot 7H_2O$	1 "
1.30%	$NaHCO_3$	3 "

To 9 parts of this mixture was added 1 part of 0.1 M. phosphate buffer pH 7.4, and 0.3 M. glucose in the proportion of 8 ml. per 100 ml. of buffered saline. This mixture constituted the culture medium of the control group. The experimental media consisted of the further addition of sodium malonate, to a final concentration varying from 0.002 M. to 0.5 M. Both the control and experimental media were brought to pH 7.4 to 7.6 with 1N NaOH or HCl. One milliliter of culture medium was supplied to each tube.

Former studies have shown the necessity of utilizing an atmosphere of oxygen to maintain the ovary in good condition *in vitro* (5). The apparatus used in these experiments consisted of a commercial U.S.P. tank of oxygen connected by valve and pressure regulatory mechanism to a medium fine glass pipette. Addition of the oxygen atmosphere consisted of bubbling oxygen from the pipette through

the culture medium for 20 to 30 seconds and by slowly withdrawing the pipette expelling all air from the culture tube. The rubber stopper was then replaced.

After addition of the oxygen atmosphere, the culture tubes were placed in an incubator maintained at 38 degrees C., which was equipped with a rotating mechanism. The tubes were held in a slanted position making an angle of about 10 degrees with horizontal, and were rotated at 20 to 25 r.p.m. This served to keep the explant from adhering to the wall of the tube and facilitated gaseous exchange with the oxygen atmosphere.

Addition of colchicine

After one hour of incubation the culture tubes were opened and 0.01 ml. of stock colchicine solution was added by micro-pipette so that the resulting concentration was 0.004 mg. colchicine per ml. culture medium. The cultures were then reoxygenated as previously described and returned to the incubator. Four hours after the addition of the colchicine the ovaries were removed from the culture tubes and placed in fixative.

Processing the cultured ovary

The ovaries were placed in Zenker-formol solution, slightly warmed to increase penetration, and left for a period of 8 hours or overnight. Washing was accomplished by five 15-minute changes of water in the same vial used for fixation, and was followed by five changes of dioxan

for dehydration. Iodine was added to the first dioxan change to remove any mercuric crystals due to Zenker fixation. After rinsing for 2 minutes in xylol, the ovaries were infiltrated by 4 changes of paraffin and embedded. Sectioning was done at 10 micra and the ovaries were mounted serially. The sections were mordanted for 5 minutes in 2.5% ferric ammonium sulfate and stained for 10 minutes in Heidenhain's hematoxylin. A saturated aqueous solution of picric acid was used for destaining.

Methods of measurement

The mitoses occurring in the last four hours of incubation were stopped in metaphase by the action of the colchicine. Since the ovary was mounted serially, tabulation of the total number of mitotic figures present in the entire ovary was possible by microscopic examination. A projection tracing was made of the germinal epithelium of every fifth section and the total length in centimeters determined by the use of a map measurer. This length, when corrected for projection magnification and section thickness, represented one fifth of the area of the germinal epithelium. The total number of tabulated mitoses, divided by 5, gave the number within the above area, allowing simple calculation of the average number of mitoses per square millimeter. All mitotic data were recorded in these terms.

DATA

In these experiments the effects of varying concentrations of sodium malonate (sodium malonate · H_2O, Matheson Co.) were tested. The concentrations used included 0.002 M., 0.01 M., 0.02 M., 0.1 M., 0.15 M., 0.2 M., and 0.5 M. A total of 93 pairs of ovaries were tested using 4 to 12 pairs in each concentration group. When examined cytologically, the tissues treated with malonate up to and including 0.1 M. appeared to be healthy, cellular detail not differing noticeably from that of the controls (plates I and II). Concentrations higher than this proved lethal, the cells of the germinal epithelium appearing ragged and not sharply defined (plate III).

The mitotic effects obtained, summarized in Table I, have been expressed in two types of graphs for each non-lethal concentration group. Graphs in text figure 1 treat each pair of ovaries by showing the actual spread due to the difference of mitotic counts of the control and experimental specimens. The average (arithmetic mean) of the control and experimental values is represented by a broken and solid line, respectively, for each concentration group. Graphs in text figure 2 treat the differences of the mitotic counts percentage-wise. All of the control groups are listed as 100% and the experimental values are in terms of reduced percentages. From this the percent of inhibi-

tion is seen as the extent of reduction from the control value. For each concentration group, the average inhibition is represented by a broken line.

TABLE I

INHIBITION OF MITOSIS BY MALONATE

Litter	Animal	Conc. of Malonate	Mitoses/mm^2 Untreated	Mitoses/mm^2 Malonate	% of Control
288	a		99	84	85
"	b		84	80	95
"	c		73	57	78
"	d		111	55	50
299	a		87	82	94
"	b	0.002 M	98	48	49
"	c		113	68	60
"	d		87	70	80
272	a		124	140	113
"	b		138	123	89
"	c		96	100	104
"	d		153	84	55
26	a		310	79	25
"	b		272	56	21
"	c		215	92	43
"	d	0.01 M	266	85	32
"	e		217	147	68
"	f		299	167	56
10	a		232	90	39
"	b		176	98	56
281	a		94	54	57
"	b		80	32	40
"	c		83	19	23
"	d	0.02 M	107	47	44
282	c		64	26	41
"	d		73	32	44
"	e		69	18	26
292	a		196	92	47
"	b		240	114	48
"	c		142	77	50
"	d		234	111	48
"	e	0.1 M	196	59	30
293	a		104	89	86
"	b		231	94	41
"	c		130	95	73
"	e		141	77	55

Figure 1

Mitotic inhibition by sodium malonate as indicated by the spread between the mitotic counts of the control and experimentally treated ovaries.

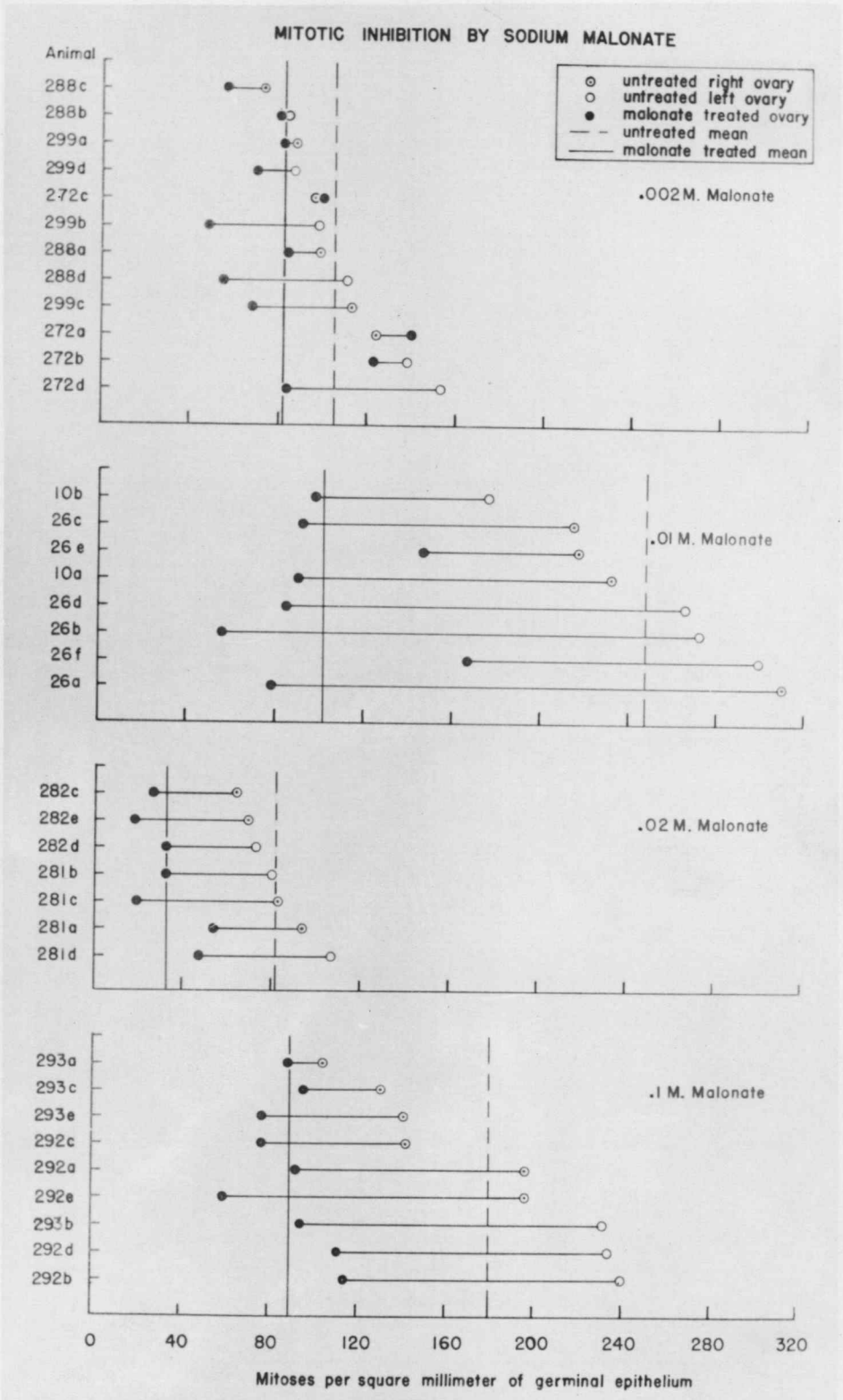
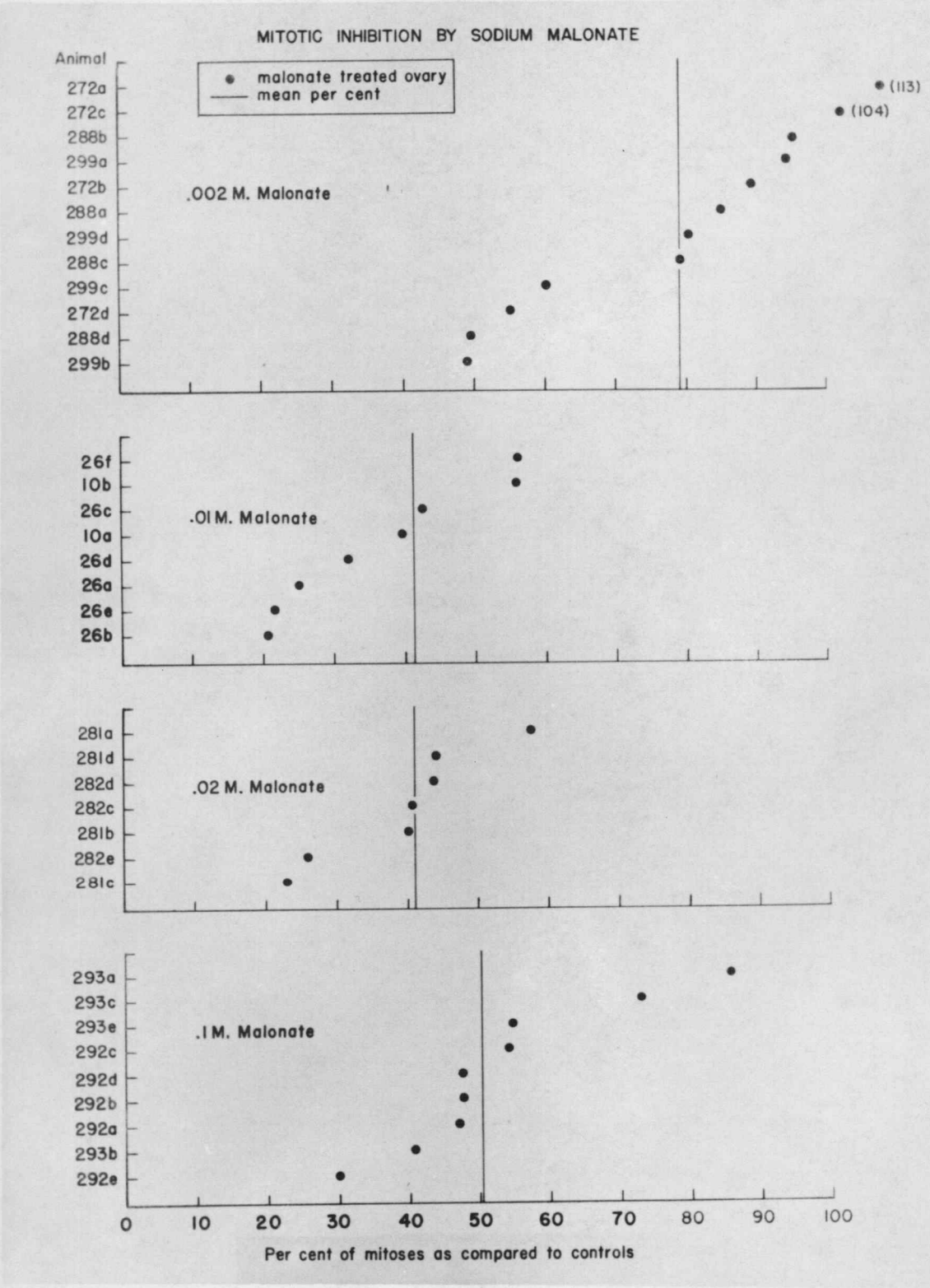

MITOTIC INHIBITION BY SODIUM MALONATE
Animal
288c
288b
299a
299d
272c
299b
288a
288d
299c
272a
272b
272d
untreated right ovary
untreated left ovary
malonate treated ovary
untreated mean
malonate treated mean
.002 M. Malonate
10b
26c
26e
10a
26d
26b
26f
26a
.01 M. Malonate
282c
282e
282d
281b
281c
281a
281d
.02 M. Malonate
293a
293c
293e
292c
292a
292e
293b
292d
292b
.1 M. Malonate
0
40
80
120
160
200
240
280
320
Mitoses per square millimeter of germinal epithelium

Figure 2

Mitotic inhibition by sodium malonate as indicated by presenting the mitotic count of the experimentally treated ovary as a percent of the mitotic count of the control ovary of the pair.


MITOTIC INHIBITION BY SODIUM MALONATE
Animal
malonate treated ovary
mean per cent
(113)
(104)
272a
272c
288b
299a
272b
288a
299d
288c
299c
272d
288d
299b
.002 M. Malonate
26f
10b
26c
10a
26d
26a
26e
26b
.01 M. Malonate
281a
281d
282d
282c
281b
282e
281c
.02 M. Malonate
293a
293c
293e
292c
292d
292b
292a
293b
292e
.1 M. Malonate
0
10
20
30
40
50
60
70
80
90
100
Per cent of mitoses as compared to controls

DISCUSSION

In these experiments it has been shown that the mitotic rate of ovarian germinal epithelium is inhibited by the presence of sodium malonate. The measurement of mitotic rate was based on the action of colchicine, a plant alkaloid which blocks mitosis in the metaphase stage. Colchicine itself does not prevent cells from entering mitosis (9). Bullough and Johnson have shown that mitoses already underway are completed normally after malonate and other respiratory inhibitors are added (7). They conclude that the inhibition occurs at some point prior to the visible prophase, which they have designated as "antephase" (5).

Addition of colchicine to the medium was at the end of one hour of incubation when it was judged that all mitoses begun before exposure to malonate must have passed the metaphase stage of development. This allowed blocking of only those mitoses which had begun while under the action of the substance being tested.

The chemical action of malonate is well known. It was first investigated by Szent Gyorgyi (22) who demonstrated that succinate accumulates in respiring muscle poisoned by malonate. The action of malonate was later shown to be one of competitive inhibition. That the inhibition of succinic dehydrogenase by malonate is specific,

competitive, and dependent upon the ratio of succinate to malonate has been demonstrated by noting the extent to which pyruvate and intermediate compounds of the Krebs cycle are utilized (11). Figures 1 and 2 show a definite decrease in the number of mitoses in the malonate-treated tissues. This mitotic inhibition is in accordance with the similar results of other workers on rat ear epithelium (5), chick fibroblasts (20), and chick embryo blood and brain tissue (18). It gives further support to the finding that part or all of the energy necessary for mitosis comes from carbohydrate metabolism via the Krebs cycle.

Comparison of the graphs (figures 1 and 2) further shows a variation of mitotic rate seemingly due to the concentrations of malonate. Such a conclusion would be in line with other work (8,11) and would support the hypothesis that the degree of inhibition may increase with concentration of malonate to a maximum and then decline, due to toxicity and cell injury. This maximum inhibition would represent the percent of energy necessary for mitosis that is contributed by the Krebs cycle in this type of material.

Statistical analysis of the data, more specifically the analysis of variance, showed a significant decrease in the number of mitoses of the experimental tissue due to the action of malonate. That the decrease in mitotic rate varied with concentration of malonate appeared likely but the evidence was inconclusive.

Further work along this line is planned with a definite randomization of individuals to determine the quantitative effect of the concentration of malonate on the mitotic rate as a basis for study of other energy sources in the ovarian tissue of newborn rats.

SUMMARY AND CONCLUSIONS

1. In testing the effect of sodium malonate on the mitotic rate of ovarian germinal epithelium, ninety-three pairs of ovaries were used in seven different concentrations of sodium malonate.

2. Of each ovary pair one ovary was used as control while the other was experimentally treated. The culture period was five hours in an artificial medium of balanced salt solution, phosphate buffer, and glucose under an atmosphere of oxygen. The mitoses were halted in metaphase by colchicine which allowed accurate tabulations of mitoses after histological fixation, serial sectioning, and staining. Numbers of mitoses were calculated per unit area of germinal epithelium.

3. The malonate treated tissues showed a marked decrease in the mitotic rate, indicating the activity of the Krebs cycle as an energy source for the initiation of cell division.

BIBLIOGRAPHY

1. Berrian, James H. and Ernst J. Dornfeld. Cellular proliferation in the germinal epithelium of immature rat ovaries. An in vitro method for the study of mitotic rate. Journal of experimental zoology 115:493-512. 1950.

2. Brachet, J. Sur la consummation d'oxygene pendant la mitose chez l'oeuf de Grenouille. Comptes rendus de seances de la Societe de Biologie et de ses Filiales, Paris 110:562-564. 1932.

3. Brachet, J. Le metabolisme de l'oeuf de Grenouille pendant la mitose. Archiv fur experimentelle Zellforschung besonders Gewebezüchtung 15:46-53. 1934.

4. Brachet, J. Etude du metabolisme de l'oeuf de Grenouille (Rana fusca) au cours du developpment. II. La respiration de l'oeuf pendant la fecundation et la mitose. Archives de biologie, Paris 46:2-45. 1935.

5. Bullough, W. S. The energy relations of mitotic activity. Biological reviews 27:133-168. 1952.

6. Bullough, W. S. and Monica Johnson. Epidermal mitotic activity and oxygen tension. Nature 167: 488. 1951.

7. Bullough, W. S. and Monica Johnson. A simple technique for maintaining mammalian epidermal mitosis in vitro. Experimental cell research 2:445-453. 1951.

8. Bullough, W. S. and Monica Johnson. The energy relations of mitotic activity in adult mouse epidermis. Proceedings of the Royal Society, B 138: 562-575. 1951.

9. Buschke, W., J. S. Friedenwald, and W. Fleischmann. Studies on the mitotic activity of the corneal epithelium. Methods, the effect of colchicine, ether, cocaine, and ephedrin. Bulletin of Johns Hopkins Hospital 73:143-167. 1943.

10. Eisa, E. A. The formation of lactic acid in the vagina of the adult rat. Acta endocrinologica 4:285-290. 1950.

11. Krebs, H. A. and L. V. Eggleston. The oxidation of pyruvate in pigeon breast muscle. Biochemical journal 34:442-459. 1940.

12. Laser, H. Der Stoffwechsel von Gewebekulturen und ihr Verhalten in der Anaerobiose. Biochemische Zeitschrift 264:72-86. 1933.

13. Loeb, J. Untersuchungen über die physiologischen Wirkungen des Sauerstoffmangels. Pflügers Archiv fur die gesamte Physiologie des Menschen und der Tiere 62:249-294. 1896.

14. Lyon, E. P. Rhythmns of CO_2 production during cleavage. Science 19:350-353. 1904.

15. Medawar, P. B. The behavior of mammalian skin epithelium under strictly anaerobic conditions. Quarterly journal of microscopic science 88: 27-37. 1947.

16. Medawar, P. B. The cultivation of adult mammalian skin epithelium in vitro. Quarterly journal of microscopic science 89:187-196. 1948.

17. Needham, J. and W. W. Nowinsky. Intermediary carbohydrate metabolism in embryonic life. Biochemical journal 31:1165-1184. 1937.

18. O'Connor, R. J. The effect on cell division of inhibiting aerobic glycolysis. British journal of experimental pathology 31:449-453. 1950.

19. Parker, R. C. Cultivation of large quantities of adult tissue in fluid media. Science 83:579-581. 1936.

20. Pomerat, C. M., and E. N. Willmer. Studies on the growth of tissues in vitro. VII. Carbohydrate metabolism and mitosis. Journal of experimental biology 16:232-249. 1939.

21. Runnstrom, J. Stoffwechselvorgänge während der ersten Mitose des Seeigeleies. Protoplasma 20: 1-10. 1933.

22. Szent Gyorgyi, Albert. Biological oxidation and vitamins. Harvey lectures 34:265-279. 1939.

23. Willier B. H., P. A. Weiss, and V. Hamburger. Analysis of development. Philadelphia, Saunders, 1955. 735 p.

24. Zeuthen, E. Segmentation, nuclear growth, and cytoplasmic storage in eggs of echinoderms and amphibia. Pubblicazioni Stazioni Zoologica di Napoli, Milan, supplement 23:47-69. 1951.

APPENDIX

EXPLANATION OF PLATES

Plate I

One-day rat ovary, cultured 5 hours *in vitro* without malonate. Note the mitotic figures arrested by colchicine. 92X, 352X.

Plate II

One-day rat ovary, cultured 5 hours *in vitro* with 0.002 M. malonate. Note reduction in mitotic figures arrested by colchicine. 92X, 352X.

Plate III

One-day rat ovary, cultured 5 hours *in vitro* with 0.5 M. malonate. Note lethal effect of high malonate concentration as shown by ragged cell boundaries and lack of cellular detail. 92X, 352X.