A STUDY OF THE EFFECTS OF HISTAMINE, FOLIC ACID, ACRIFLAVINE AND INDOLEACETIC ACID ON THE MITOTIC ACTIVITY OF EMBRYONIC CHICK HEART FIBROBLASTS GROWN IN VITRO

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In order to study the effects of chemicals on mitosis, four chemicals, acriflavine, folic acid, histamine and indoleacetic acid were selected. The effects of these chemicals on mitotic activity were determined by observation, by counting the phases and using the formula devised by Wilson and Leduc.

$$\text{Mitotic activity} = \frac{\text{number of mitotically active cells}}{\text{area of section} \times \text{nuclear density of section}}$$

The results indicated that the highest mitotic activity was in the explants which contained 50μ g/ml of indoleacetic acid. All concentrations of acriflavine were strong mitotic inhibitors. Folic acid and histamine were mitotic stimulants at 50 μg/ml concentration. But 10μ g/ml and 100μ g/ml concentration had no effect at all.

The use of indoleacetic acid, a plant growth hormone, augmented the growth of chick heart fibroblasts. The similarity of effects of indoleacetic acid on plant and animal cells, may be due to the similar mechanism of nuclear division.

Introduction

Harrison[5] first observed the development of nerve fibers that were grown in hanging drop preparations. Since that time considerable progress has been made in the development of this technique and various others have been developed.

Harrison's tissue culture technique has been used extensively in the study of the effects of various chemicals on mitosis, mitotic activity and physiology of the cells.

Blumental[1] reported on the influences of cyanides on developing *Arbacia punculate* embryos. He placed the eggs in the cyanide solutions of various concentrations at various stages of development and found that the eggs continue mitotic activity at all concentrations.

Creech[2] studied the action of methylcholanthrenecholeic acid, acenaphthenecholeic acid, dibenzanthracenecholeic acid, and phenanthrenecholeic acid on the rate of mitosis of connective tissue fibroblasts, which were taken from embryonic mice. He added the chemicals to the tissue cultures in various concentrations ranging from 0.1 mg/ml to 0.001 mg/ml.

As a result of these experiments, Creech reported an increase of 2% in the mitotic count from the cultures which contained the lowest amount of acenapthenecholeic acid and methylcholanthrenecholeic acid. He further reported that dibenzanthrenecholeic acid caused an increase in the mitotic count by 7.9%, while phenanthrenecholeic acid reduced the count by 34.9% at 0.1 mg/ml concentration. Ten-fold increase in concentration caused a retardation of mitosis.

Woodard and Estes studied the effects of 1.0 mg/ml colchicine solution on mitosis in the neural tube of chick embryos.

The results reported by the investigators, showed that the percentage of all phases except metaphase, eventually declined (prophase from 2.8% to 1.1%, anaphase from 0.2% to 0.0% and telophase from 4.6% to 0.0%). In metaphase it increased 2.4% to 20.5%. This rise in the number of metaphase stages was accounted for by the accumulation of nuclei in this phase. In their conclusion they indicated that colchicine at this concentration i.e.1 mg/ml was not mitotic stimulant but produced a metaphase block.

In the study of the action of drugs on mesenchymal tissue of chick embryos, Lettre,[7] added ammonium carbonate, adenosine, adenosine triphosphate, pyocyanine, acriflavine, colchicine, and adernaline solutions to the culture media in various concentrations.

The study showed that ammonium carbonate, adenosine, and adenosine triphosphate have produced vacuolization of cytoplasm, and pyocynine increased uptake of oxygen by the cells and inhibited cell division. While the acriflavine produced well-known phenomenon of sticking and clumping of chromosomes during mitosis.

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The addition of colchicine solution of 1.0 μg/ml arrested 30% of dividing cells in metaphase. A threshold dose i.e. 90 μg/ml of adernaline brought about binucleated division. Thus he concluded that acriflavine arrested the nuclear division by interfering with the biochemical mechanism of nuclear division, here as in the case of adernaline, cytokinesis was inhibited.

O'Conner[8] studied the effect of acridine derivatives on cells of chick midbrain grown *in vitro*. Four acridine compounds, 5-amino-2-trifluoromethylacidinium hydrochloride, 5-amino-10-methylacidinium bromide, 5-amino-1-phenyl-10 methylacidinium bromide, and 5-amino-acridine hydrochloride were selected and used in this investigation.

In the preparation of this media, O'Conner used various concentrations of the above-mentioned compounds, which ranged from 0.7 mg/100 ml to 4.0 mg/100 ml in a saline solution.

As a result of this experiment, the investigator observed that all acridine derivatives inhibited the mitosis at the concentrations ranging from 0.7 mg/100 ml to 1.8 mg/100 ml.

Jacobson[6] performed experiments on the effects of folic acid antagonists, aminopterin and A-methopterin on cells. He used various tissues from 12-day old embryos grown *in vitro*, and incubated the cultures for 24–48 hr. The explanted tissues were taken from the heart, frontal bone, skin and spinal cord.

Jacobson reported two types of effects, short-time exposure effects and long-time exposure effects. During the short-time exposure, a solution of 1: 2000 concentration of animopterin was allowed to act on cells for 15 min, which increased the metaphase stage from 39% to 70% in experimental cultures. The total percentage of mitosis was decreased. The dilution of the solution to 1:5000 and 1:10,000 caused a decrease in the total number of mitosis similar to that described above. The total mitotic count in four control cultures was 1205 and in the experimental cultures in 1:5000 and 1:10,000 was 976 and 544 respectively. The metaphase count of the experimental explants rose from 38% of the control to 70% and 64% respectively.

A prolong exposure of the experimental cultures to the reagents for 24 hr in various concentrations produced transitory effects on them, therefore there was normal distribution of mitotic phase and their total number. Similar observation was made with the folic acid antagonist, A-methopterin.

Richter[9] studied the effects of histamine dihydrochloride and chlortrimenton maleate (antihistamine) on the growth of the chick heart fibroblast. Solutions of 10 μg/ml concentration were added to the culture media.

In these experiments, Richter observed that the growth was dense and extensive in the control and experimental groups, when the amount of histamine and both histamine and antihistamine was 10 μg/ml. The growth of the tissue at 100 μg/ml level of histamine, antihistamine, both histamine and antihistamine and at 10 μg/ml concentration of histamine was uniformly thin. This indicated that the rate of growth was low in 100 μg/ml concentrations and high in 10 μg/ml.

In the conclusion, the investigator expressed his opinion that antihistamine at 10 μg/ml and 100 μg/ml inhibited growth, while histamine at the same concentration had no effect but strongly inhibited growth at 100 μg/ml. Their joint administration at 10 μg/ml stimulated fibroblast growth, while at 100 μg/ml the more profound effect of histamine was partially supressed.

Elliot[4] tested the effects of three plant hormones, 3-indoleacetic acid, β-3-indolepropionic acid and γ-indolebutyric acid on three species of protozoa in pure cultures. Protozoa used in this investigation were *Euglena gracilis*, a chlorophyll-bearing small flagellates, *Khawkinea halli*, a colorless-euglenoid flagellate and *Colpidium striatum*, a holotrichous ciliate.

In order to study the effects of hormones on the rate of multiplication, different concentrations were prepared. They were 1/10,000, 1/100,000, 1/1,000,000 and 1/10,000,000 parts per ml. The solutions of these concentrations were added to the media.

The result showed a reduction in the final count of *Colpidium striatum* and *Khawkinea halli* in all the solutions of all the three phytohormones. In the case of *Euglena gracilis* the count was more than in control except at 1/10,000 concentration where the count was less than the control.

The purpose of this investigation was to determine the influence of four chemicals, acriflavine, folic acid, histamine and indoleacetic acid on the mitotic activity of the chick-heart fibroblast grown *in vitro*. Special reference is made to the use of the indoleacetic acid, a plant hormone, on chick fibroblasts.

Materials and Methods

In this investigation, the hanging drop method of tissue culture as developed by Harrison[5] was used and explants of heart tissue from 6-day-old chick embryos were prepared in each series of experiments. The medium for the control cultures consisted of chicken plasma, an equal amount of embryonic extract with distilled water and 2% of 95% alcohol. The medium for experimental purposes contained four chemicals, namely, acriflavine, folic acid, histamine and indoleacetic acid in addition to the reagents used in the control cultures. Ten milligrams of each chemical was dissolved in 0.5 ml of 95% ethyl alcohol and was diluted to 25 ml with distilled water, thus the concentration of each solution was 400 μg/ml. The chemicals were supplied by Matheson Coleman and Bell, East Rutherford, New Jersey.

The eggs for the experiments were secured from Georgia State Hatchery, Atlanta, Georgia and allowed to incubate at 39°C for 6 days. When the eggs were placed in the incubator, each egg was marked with the date.

As a measure of precaution against bacterial and fungal infection, all equipment used for culturing was cleaned and sterilized. Before each experiment was started, all glassware such as micropipettes, Petri dishes, slides, coverslips, finger bowls and beakers, as well as, the scissors, scalpels, forceps, and arrow-head needle were washed in Alconox. After washing, they were rinsed thoroughly in distilled water and allowed to dry in the air. The glasswares and instruments were placed in cannisters and sterilized at 150°C for 90 min.

Chicken plasma (No 457709) was obtained from Difco Laboratories, Detroit, Michigan. It was reconstituted to its original volume by adding, under aseptic conditions, 4.5 ml of reconstituting fluid (No. B 352). Chick embryonic extract was prepared from 6 day-old embryos. The embryos were removed from the shells and rinsed in Earle's salt solution (Nos. 4577866 and 4577867). They were transferred into a sterilized syringe and crushed with pressure. An equal amount of Tyrode solution was added to the macerated embryos. After waiting for 30 min the mixture was transferred to centrifuge tubes, and centrifuged for about 30 min, at a speed of 2000 rev/min. The supernatant fluid was withdrawn as the chick extract.

In the preparation of the control and experimental media, various combinations of chemicals, embryonic extract and solvents were used.

The number of mitotically active cells in a given area was counted under a 95× objective with an ocular micrometer. The nuclear density was determined by counting the total number of nuclei in that area. The mitotic activity was

TABLE 1.—THE VARIOUS COMBINATIONS OF CHEMICAL SOLUTIONS AND EMBRYONIC EXTRACT USED AS THE CONTROL* AND EXPERIMENTAL MEDIA.

Series	Name of chemical substance	Amount of chemical solution (ml)	Amount of Extract (ml)	Amount of mixture (ml)	Concentration of chemical in medium (μg/ml)
I	Acriflavine	0.025	0.975	1	10
	Folic acid	0.025	0.975	1	10
	Histamine	0.025	0.975	1	10
	Indoleacetic acid	0.025	0.975	1	10
II	Acriflavine	0.125	0.875	1	50
	Folic acid	0.125	0.875	1	50
	Histamine	0.125	0.875	1	50
	Indoleacetic acid	0.125	0.875	1	50
III	Acriflavine	0.25	0.75	1	100
	Folic acid	0.25	0.75	1	100
	Histamine	0.25	0.75	1	100
	Indoleacetic acid	0.25	0.75	1	100

* Control experiments were performed with a mixture of distilled water and alcohol.

then computed according to the formula used by Wilson and Leduc.[10]

$$\text{Mitotic Activity} = \frac{\text{number of the mitotically active cells}}{\text{area of the section} \times \text{nuclear density of the section}}$$

Results

In this investigation a series of experiments were conducted. There were three series in the control, as well as in the experimental cultures. Each control and experimental series consisted of 10 and 40 explants respectively. In each experimental series, solutions of four different chemicals, acriflavine, folic acid, histamine and indoleacetic acid were added to the media. The addition of the alcohol and distilled water to the control media provided a uniform condition for the growth of all the cultures, except the presence of chemicals in the experimental cultures. The control and experimental series were divided on the same basis i.e. according to the concentration of the chemicals.

TABLE 2.—THE AVERAGE COUNT OF THE MITOTICALLY ACTIVE CELLS AND THE DIFFERENT PHASES IN THE CONTROL MEDIA, INCUBATED FOR 36 HR.

Culture S. *No		Pro-phase	Meta-phase	Ana-phase	Telo-phase	%	Total nuclei	M.A.†	Total% of M.A.C.‡
Control series I	1	2	0	0	0	2.6	76		
	2	0	3	0	0	4.7	63		
	3	3	0	0	0	5.5	58		
	4	1	1	0	0	1.5	128		
	5	2	0	0	0	1.5	131		
	6	1	1	0	0	2.3	85	0.02	2.5%
	7	1	0	1	0	1.8	107		
	8	1	0	1	0	2.2	90		
	9	2	0	0	0	4.7	42		
	10	0	1	0	1	2.0	100		
	Total	13	6	2	1=22		880		
II	1	1	0	1	1	7.0	38		
	2	0	1	2	0	2.9	109		
	3	2	0	0	0	2.1	89		
	4	1	0	0	0	2.0	52		
	5	1	0	1	0	2.1	83		
	6	3	2	0	1	8.1	74	0.03	3.3%
	7	1	0	0	0	1.2	83		
	8	2	0	0	0	3.5	57		
	9	2	0	0	0	1.8	104		
	10	1	2	0	0	5.7	52		
	Total	14	5	4	2	= 25	741		
III	1	3	1	0	0	2.6	153		
	2	1	1	0	1	6.6	45		
	3	2	0	0	1	3.5	56		
	4	2	0	0	0	3.5	58		
	5	3	0	0	0	3.0	97	0.03	3.3%
	6	1	0	0	0	1.2	80		
	7	1	0	3	0	6.7	59		
	8	1	2	0	0	4.4	68		
	9	1	2	0	0	2.8	105		
	10	2	0	0	0	1.4	135		
	Total	17	6	3	2	= 28	856		

* Serial number. † Mitotic activity. ‡ Total percentage of mitotically active cells.

Following are the results obtained during the study of the control and experimental explants.

Control Series

Series I.—The medium of the first control series contained 0.025 ml of the solvents and 0.975 ml of embryonic extract per ml. The average mitotic activity of the series was 0.02 (Table 2).

Series II.—In the medium for the cultures of this series the amount of the solvents and embryonic extract was 0.125 ml and 0.875 ml per ml respectively. The average mitotic activity was 0.03 (Table 2).

Series III.—In this series 0.25 ml of the solvents and 0.75 ml of the embryonic extract was present in 1 ml of the medium. The mitotic activity was recorded as 0.03 (Table 2).

Experimental Series

Series I.—In this series of experimental explants, solutions of 10μg of each chemical, acriflavine,

TABLE 3.—THE AVERAGE COUNT OF THE MITOTICALLY ACTIVE CELLS AND THE DIFFERENT PHASES IN THE EXPERIMENTAL MEDIA, WHICH CONTAINED FOLIC ACID IN 10 μg/ml, 50 μg/ml AND 100 μg/ml CONCENTRATIONS AND INCUBATED FOR 36 HR.

Culture S. No.*		Pro-phase	Meta-phase	Ana-phase	Telo-phase	%	Total nuclei	M.A.†	Total % of M.A.C.‡
Expt'l Series§	1	0	0	1	0	2.0	50		
	2	1	0	0	1	2.6	75		
I	3	3	0	1	0	4.7	85		
	4	1	1	0	1	3.3	90		
	5	1	0	0	0	1.0	95	0.02	2.5%
	6	1	0	0	1	1.8	105		
	7	0	2	0	0	2.0	97		
	8	1	0	0	0	2.8	35		
	9	3	0	0	0	3.7	105		
	10	0	0	1	1	2.0	51		
Total		11	3	3	3 = 20		788		
II	1	1	2	0	1	2.8	145		
	2	1	2	1	0	3.9	102		
	3	2	1	0	1	5.3	75		
	4	1	1	1	0	3.0	99		
	5	1	0	0	0	4.4	45	0.04	4.4%
	6	2	2	0	1	4.2	119		
	7	2	1	0	0	5.3	56		
	8	3	0	0	1	5.1	78		
	9	4	0	0	1	10.0	46		
	10	2	0	1	0	6.9	43		
Total		19	9	3	5 = 36		808		
III	1	2	0	0	0	3.2	62		
	2	1	3	0	0	8.0	50		
	3	0	2	0	0	2.1	95		
	4	2	1	0	0	4.0	75		
	5	0	2	0	0	5.8	35	0.04	4.0%
	6	1	1	0	0	4.0	50		
	7	1	0	0	0	1.7	57		
	8	1	2	1	0	4.0	99		
	9	0	2	0	0	4.0	27		
Total		8	13	1	0 = 22		550		

* Serial number. † Mitotic activity. ‡ Total percentage of mitotically active cells. § Experimental series.

folic acid, histamine and indoleacetic acid, were added to the media. The average mitotic activities were 0.00, 0.02, 0.02 and 0.04 respectively (Tables 3–5).

Series.II—The concentration of the solutions used in this series was 50 μg/ml. The mitotic activities recorded were 0.00, 0.04, 0.05 and 0.075 respectively (Table 3–5).

Series III.—The concentration of the chemicals was increased to 100 μg/ml for this series. The average mitotic activities were 0.00,0.04,0.038 and 0.036 respectively (Tables 3–5).

Discussion

The results of this investigation indicate that the effects of the chemicals on mitotic activity

TABLE 4.—THE AVERAGE COUNT OF THE MITOTICALLY ACTIVE CELLS AND THE DIFFERENT PHASES IN THE EXPERIMENTAL MEDIA, WHICH CONTAINED HISTAMINE IN 10 μg/ml, 50 μg/ml AND 100 μg/ml CONCENTRATIONS AND INCUBATED FOR 36 HR.

Culture S. No.*		Pro-phase	Meta-phase	Ana-phase	Telo-phase	%	Total Nuclei	M.A. †	Total % of M.A.C. ‡
Expt'1 Series§	1	0	0	0	0	1.4	67		
	2	2	0	0	0	5.6	35		
I	3	1	0	0	0	1.7	57		
	4	1	1	1	1	5.1	78		
	5	1	0	1	0	4.0	49	0.02	2.7%
	6	0	1	0	0	1.3	77		
	7	1	0	0	0	1.1	85		
	8	1	2	0	0	3.3	89		
	9	0	0	0	2	4.0	50		
	10	1	0	0	0	3.9	28		
	Total	9	4	2	3 =	18	615		
II	1	5	0	1	0	12.0	50		
	2	3	1	0	0	4.1	96		
	3	1	2	0	1	3.5	120		
	4	1	0	0	1	4.1	48		
	5	2	2	1	1	4.0	147	0.05	5.1%
	6	2	2	0	0	2.9	135		
	7	4	1	0	1	12.5	48		
	8	3	1	1	0	10.5	47		
	9	1	1	0	0	3.0	67		
	10	0	1	0	0	2.8	36		
	Total	22	11	3	4=40		794		
III	1	2	0	1	0	2.9	102		
	2	2	1	1	0	3.6	55		
	3	0	1	1	0	2.1	92		
	4	3	1	0	0	4.2	95		
	5	2	2	1	2	6.1	130	0.038	3.8%
	6	0	1	0	0	1.8	55		
	7	1	0	0	0	2.3	43		
	8	1	0	1	1	6.0	49		
	9	1	1	0	0	3.0	67		
	10	1	1	0	0	3.0	67		
	Total	12	9	5	3=29		770		

* Serial number. † Mitotic activity. ‡ Total percentage of mitotically active cells. § Experimental series.

of the cells grown *in vitro*, varied with the nature of the chemical and its concentration in the media.

The addition of acriflavine solution to the medium did not show any cell division in all concentrations, 10 μg/ml, 50 μg/ml and 100 μg/ml, thus indicating an inhibiting effect on mitosis in embryonic chick fibroblasts. These results are in agreement with the investigation of Lettre,[7] in which he found that acriflavine arrested nuclear division.

Jacobson[6] reported that folic acid antagonists, aminopterin and A-methopterin have short and long time exposure effects. During the short exposure for 15 min of the experimental cultures

TABLE 5.—THE AVERAGE COUNT OF THE MITOTICALLY ACTIVE CELLS AND THE DIFFERENT PHASES IN THE EXPERIMENTAL MEDIA, WHICH CONTAINED INDOLEACETIC ACID IN 10 μg/ml. 50 μg/ml AND 100 μg/ml CONCENTRATIONS AND INCUBATED FOR 36 HR.

Culture S. No.*		Pro-phase	Meta-phase	Ana-phase	Telo-phase	%	Total nuclei	M.A.†	Total % of M.A.C. ‡
Expt'l Series§	1	0	0	1	0	1.0	97		
I	2	1	2	0	0	2.8	101		
	3	5	0	0	0	6.0	83		
	4	3	0	1	0	6.1	65		
	5	2	0	0	1	2.7	101	0.04	4.2 %
	6	1	1	2	0	5·3	75		
	7	0	2	0	0	3.0	67		
	8	1	0	2	1	8.3	48		
	9	5	0	0	0	4·5	107		
	10	2	1	1	0	4.7	85		
Total		21	6	7	2=36		829		
II	1	2	0	0	0	5.6	35		
	2	2	1	0	0	13.0	23		
	3	5	0	0	1	13.0	44		
	4	5	1	0	0	10.0	59		
	5	1	1	2	0	7.6	52	0.075	7·5%
	6	3	0	1	0	3·7	107		
	7	2	1	0	0	8.5	35		
	8	4	0	0	0	8.0	50		
	9	1	1	0	2	5·3	75		
Total		25	5	3	3=36		480		
III	1	0	0	1	0	1.6	60		
	2	1	1	0	0	2.5	80		
	3	1	1	1	0	6.4	47		
	4	0	2	1	0	2.4	125		
	5	2	0	0	1	5.6	53	0.036	3.6%
	6	2	0	1	0	4.6	65		
	7	1	0	1	0	3·3	60		
	8	2	2	0	0	5.0	79		
	9	0	0	1	0	2.0	50		
	10	1	2	0	0	4.0	75		
Total		10	8	6	1=25		694		

* Serial number. † Mitotic activity. ‡ Total percentage of mitotically acitve cells. § Experimental series.

to the reagents, a marked accumulation of metaphase stages took place and the total percentage of mitosis was decreased as compared with those in control cultures. A prolonged exposure for 24 hr, in various concentrations of folic acid antagonist solutions, produced transitory effects.

The effects of folic acid were different from those of acriflavine and folic acid antagonists. The mitotic activity of the cells grown in the concentrations, 50 μg/ml and 100 μg/ml, was higher than that of the control and 10 μg/ml level. Such results reveal that folic acid is a mitotic stimulant at higher concentrations.

According to Richter,[9] the use of histamine in 10 μg/ml concentration did not effect the rate of growth of tissue, but when the concentration was increased to 100 μg/ml, it inhibited the growth. In this investigation it was found that a concentration of 50 μg/ml stimulated the mitotic activity. The other two concentrations, 10 μg/ml and 100 μg/ml, had no effect at all.

Elliot[4] found that among the three species of protozoa, *Euglena gracilis*, *Kawkinea halli* and *Colpidium striatum*; *Euglena gracilis*, a chlorophyll-bearing flagellate, responded to the action of the three phytohormones, β-3-indolepropionic acid, γ-indolebutyric acid and 3-indoleacetic acid. The rate of multiplication of *Euglena gracilis* cultures was more than that of the control and other experimental cultures.

The use of the indoleacetic acid gave some interesting results. The rate of mitotic activity was higher than those of the control and other experimental explants. The highest mitotic activity was observed at 50 μg/ml concentration. A high concentration, 100 μg/ml, inhibited mitosis. Appropriate concentrations of the indoleacetic acid may act as mitotic stimulants and are as effective on animal cells.

Summary and Conclusions

In order to study the effects of four chemicals, acriflavine, folic acid, histamine and indoleacetic acid, three series of control and three series of experimental cultures were prepared. Cultures in each series were allowed to grow for 36 hr.

The conditions for growth of the embryonic chick fibroblasts in all of the series were uniform except in the experimental cultures to which three different concentrations, 10 μg/ml, 50 μg/ml and 100 μg/ml, of the chemicals were added.

Acriflavine completely inhibited mitosis. Folic acid, histamine and indoleacetic acid stimulated mitosis in the 50 μg/ml concentration. They were not effective at the 10 μg/ml level. Histamine and indoleacetic acid inhibited mitosis at a concentration of 100 μg/ml.

The highest mitotic activity was recorded in the explants in a medium to which 50 μg/ml of indoleacetic acid were added.

The use of indoleacetic acid on animal tissue was successful. This effect may indicate a similar basic biochemical mechanism of nuclear division in plant and animal cells.

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