THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

EXPERIMENTAL IN VITRO STUDIES OF THE MAMMALIAN THYMUS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

1958

EXPERIMENTAL IN VITRO STUDIES OF THE MAMMALIAN THYMUS

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

I would like to express my thanks to Dr. Kenneth M. Richter of the Department of Anatomy for his guidance during the course of this study and for the use of the special research facilities of his laboratory. I would, also, like to thank Dr. James A. Hagans of the Department of Preventive Medicine and Public Health, Biostatistical Unit, for his assistance in the statistical analysis of the data obtained.

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EXPERIMENTAL IN VITRO STUDIES OF THE MAMMALIAN THYMUS[1]

CHAPTER I

INTRODUCTION

Although many workers have attempted to determine the functional significance of the thymus, there is still no general agreement concerning the precise role of this organ in the general physiology of the mammal (Keynes, '54, and Klein, '56). Considerable data have been obtained from in vivo studies indicating a functional relationship between the thymus and several of the endocrine glands, particularly the adrenal cortex and the thyroid (Dougherty, '52).

The present study was undertaken to obtain data on the individual effects of cortisone acetate and of DL thyroxine on the histology, cytology, and general growth characteristics of thymic tissue isolated in culture from other possible hormonal and general systemic factors.

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[1]Supported by Research Fellowships from The Graduate Medical Sciences Committee of The University of Oklahoma School of Medicine, and The National Cancer Institute of the Public Health Service.

CHAPTER II

MATERIALS AND METHODS

To obtain data on the individual effects of cortisone acetate (Cortone, Merck, Sharp & Dohme) and DL thyroxine (National Biochemicals Corporation) on the mouse[1] thymus in vitro, two entirely separate series of cultures were prepared and designated the Cortisone Acetate Series and the DL Thyroxine Series respectively. Each of the series was composed of several groups of cultures prepared from thymic fragments obtained from the thymus of a single animal. The groups were identified by the date at which the cultures were set up. Random selection was made of the cultures immediately after explanting the tissue fragments for control or experimental treatment. Parallel control and experimentally treated cultures were utilized for all technical procedures employed.

The thymic fragments were cultured by the split cover glass roller tube method using the conventional chicken plasma clot and fluid culture nutrient. The fluid culture nutrient was comprised basically of twenty-five percent chick embryo extract, twenty-five percent human ascitic fluid,[2] fifty percent Tyrode's saline.

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[1]Rockland all purpose female mice were used at twenty-five to forty-eight days of age. Rockland Farms, Rockland County, New York.

[2]No record of the pathology was available.

Cortisone Acetate Series

The control nutrient for the Cortisone Acetate Series was prepared as indicated above and varied for the experimentally treated cultures only by the addition of 100 micrograms of cortisone acetate per milliliter.[1] At this concentration, the cortisone acetate was in excess of solubility and imparted a distinct cloudiness to the culture nutrient as a consequence of the formation of micro-crystals of cortisone.

Representative fourteen day cultures were carefully washed in Tyrode's saline and placed in control fluid culture nutrient for microscopic examination by polarized light relative to the possible intracellular localization of cortisone acetate crystals. For the latter examinations, the cultures were maintained in a special sandwich type perfusion chamber (Richter and Woodward, '55).

The initial pH of the culture nutrients was adjusted to 7.5 to 7.6 with sterile 0.1 N HCl and checked with a Beckman glass electrode pH meter using a special plastic vessel for measurement of small samples (Richter, '56). The pH of the fluid culture nutrient was determined again after three and four days of cultivation. The differences between the pH of control and experimental fluid culture nutrients after cultivation were tested for statistical significance by the "t" test.

Comparative data on the histology, cytology, growth and degree of thinning of the main body of the original explant were obtained from

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[1] The use of 100 micrograms cortisone acetate per milliliter fluid culture nutrient was selected arbitrarily to insure a very high concentration of the hormone in keeping with reports concerning in vivo effects of hyperactivity of the adrenal cortex (Dougherty, '52).

cultures which had been fixed and stained in toto after four, five, seven, eight and fourteen days in vitro. Routinely the cultures were fixed in methyl alcohol and stained by a modified Giemsa staining procedure used in earlier studies on the rat thymus (Vernon, '55). Representative cultures were also fixed in 10 percent formalin and stained as follows:

Sudan IV (Mallory, '38) ———— 4 and 7 day cultures

Sudan Black B (Pearse, '54) ——— 7 and 9 day cultures

Orcein stain for elastic fibers (Lillie, '48) ———————— 7 day cultures

Verhoeff's elastic tissue stain (Mallory, '38) ———————— 8 day cultures

Periodic-acid-Foot stain for connective tissue (Lhotka and Myhre, '53) _____ 4,8, and 14 day cultures

In addition to the above procedures, one drop of a 1 percent saline solution of carmine red was added to the culture nutrient approximately eighteen hours prior to the termination of representative four and seven day cultures, to obtain data on the phagocytic capacity of the cells in the growth zones. The latter cultures were fixed in 10 percent formalin and stained with Ehrlich's hematoxylin and eosin.

Comparative quantitative data were obtained from the fixed and stained control and cortisone treated cultures relative to (1) the number of explants growing, (2) the number of growing explants with healthy thymocytes in the growth zones (frequency distribution of thymocytes), (3) the number of growing cultures with capillary-like structures in growth zones (frequency distribution of capillary-like structures),

and (4) the number of growing explants showing plasma clot liquefaction in the region of the growth zone (frequency of plasma clot liquefaction). The significance of these quantitative data was tested by the Chi-square method.

The effect of cortisone acetate on extent of growth and degree of thinning of the main body of the original explant was ascertained by a system of arbitrary units used to evaluate the individual cultures. Statistical treatment of these data was not possible.

DL Thyroxine Series

In the DL Thyroxine Series of cultures, a very slight alteration of the fluid culture nutrient was necessary for both the control and experimentally treated cultures due to the insolubility of DL thyroxine in water or Tyrode's saline. No alteration was made in the ratio of ingredients indicated above. The DL thyroxine was dissolved as completely as possible in 1 percent NaOH, as suggested by Vogelaar and Erlichman ('36), then diluted with triple distilled water so that a stock solution was obtained which had approximately 60 micrograms DL thyroxine per milliliter of 0.06 percent NaOH. Of the three separate lots of stock solution prepared, two were sterilized by autoclaving as suggested by Vogelaar and Erlichman ('36), and the other by filtration through a Seitz filter with a 0.1 micron pore asbestos filtering pad. The stock solution was diluted further, using sterile 0.06 percent NaOH as the diluent, so that working solutions were obtained containing 6, 0.6, and 0.06 micrograms DL thyroxine per milliliter of 0.06 percent NaOH respectively. A concentrated Tyrode's (Clark, '54), containing only two-thirds the required amount of

water, was then diluted to the concentration of normal Tyrode's by the addition of sterile 0.06 percent NaOH in the preparation of control culture nutrient, or sterile 0.06 percent NaOH with the desired concentration of DL thyroxine in the preparation of the experimental culture nutrients. Thus in the finally composed nutrient for the studies of DL thyroxine effects, the control and experimental culture nutrients each contained 0.01 percent NaOH. Using the three working solutions of DL thyroxine in 0.06 percent NaOH listed above, experimental culture nutrients were prepared which contained in their final form 1, 0.1, and 0.01 microgram DL thyroxine[1] per milliliter respectively.

The pH of the nutrients was adjusted with sterile 0.1 N HCl and checked with a Beckman glass electrode pH meter as indicated for the Cortisone Acetate Series. The initial pH of the fluid culture nutrient for the DL thyroxine series was varied at the following levels of DL thyroxine concentrations:[2]

1.0 microgram/ml. _____ pH 7.55 to 7.65; pH 7.9

0.1 microgram/ml. _____ pH 6.6 to 6.7; pH 7.6; pH 7.9

0.01 microgram/ml. _____ pH 7.6; pH 7.9

The pH of the culture nutrient was again determined, in representative groups of cultures, at termination of the cultivation period. The

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[1]The selection of 1, 0.1, and 0.01 microgram DL thyroxine per milliliter fluid culture nutrient was made on the basis of previously reported studies of thyroxine effects on other tissues in vitro (Vogelaar and Erlichman, '36, and Clark, '54).

[2]The variations in pH were selected in an effort to determine whether or not there was an optimal pH range for maximum activity of this hormone on thymic tissue in vitro as had been found by Zondek ('44) in his work on tadpoles and alluded to by Vogelaar and Erlichman ('36) in their work on human thyroid tissue in vitro.

differences between the pH of control and experimental culture nutrients after cultivation were tested for statistical significance by the "t" test.

Control and thyroxine treated cultures were terminated by fixing and staining in toto to obtain comparative data on the histology, cytology and general growth of the cultures. The majority of the culture groups was terminated after four, eight, and fifteen days of cultivation. As in the Cortisone Acetate Series, the cultures were routinely fixed in methyl alcohol and stained by a modified Giemsa staining procedure. Representative cultures in the group set up 2-19-58, 1.0 /g./ml., pH 7.9 level, were fixed in 10 percent formalin and stained as follows:

Sudan Black B (Pearse, '54) ———— 4 day cultures

Periodic acid-Shiff reaction (Pearse, '54) ———— 15 day cultures

Orcein stain for elastic fibers (Lillie's '48) ———— 4 and 8 day cultures

Periodic-acid-Foot stain for connective tissue (Lhotka and Myhre, '53 ———— 4,8, and 15 day cultures

Other eight day cultures of this group, were fixed in 10 percent formalin-alcohol and stained by the Feulgen reaction (Pearse, '54). A few cultures from the group set up 1-8-58, 1.0 /g./ml., pH 7.6 level, were fixed in 10 percent formalin and stained by the Prussian blue reaction for iron (Cowdry, '48).

Comparative quantitative data were obtained on the following criteria from study of the fixed and stained cultures:

(1) The total number of control and thyroxine treated explants which were growing relative to the total number set up.

(2) The number of growing control and thyroxine treated cultures showing (a) healthy thymocytes in the growth zone (frequency distribution of thymocytes) (b) capillary-like structures in the growth zone (frequency distribution of capillary-like structures), and (c) liquefaction of the plasma clot in the region of the growth zone (frequency of plasma clot liquefaction). These data were checked for statistical significance by the Chi-square method.

(3) The relative growth increment of control and experimental cultures was determined by planimetric methods. Statistical evaluation was made by the "t" test.

(4) The mitotic frequency of thymocyte-like cells in the growth zones of control and thyroxine treated thymic explants. A population test was used to evaluate the data obtained.

(5) The number of growing control and thyroxine treated explants at the 0.01 μg./ml. level, showing, within their respective growth zones, (a) multinucleate giant cells and syncytial masses, and (b) cellular aggregates with a core of acidophilic crystalline appearing material. The data were treated statistically by the Chi-square method.

CHAPTER III

RESULTS

Cortisone Acetate Series

Quantitative and Objective Data

Fixed and stained control and experimental cortisone treated cultures showed a great deal of variation from one explant to another with regard to (a) the cell species present, (b) the histo- and cytologically differentiated components, (c) the frequency distribution of healthy thymocytes and capillary-like structures in the growth zones, (d) the frequency of plasma clot liquefaction, and (e) the degree and pattern of growth.

In general the cell species comprising the growth zones of both the control and cortisone treated cultures were qualitatively alike, in that all cell species characteristic of the control cultures were also characteristic of the cortisone treated cultures. The cell types present included (1) relatively large fibroblasts (Figures 6, 20), (2) typical fibroblasts (Figures 6, 19), (3) phagocytic macrophage-like cells (Figures 6, 19, 20), (4) endothelial cells (Figure 16), (5) mature and immature myelocytic cells (Figures 2, 3, 4), (6) mast cells (Figure 1), (7) large, medium and small thymocytes (Figure 6), (8) mature and immature fat cells, (9) multinucleate giant cells, (10) syncytial

masses (Figures 10, 12), and (11) large cells of unknown cytogenic relation having a strongly basophilic, vacuolated cytoplasm and acidophilic nucleus (Figure 5). In both control and experimental cortisone treated cultures, mitotic activity was shown by the fibroblast-like cells, typical fibroblasts, macrophage-like cells, thymocytes (Figure 7), and the myelocytic cell types (Figure 4).

Certain histologically differentiated components occurred in both control and cortisone treated cultures. The latter included (1) capillary-like structures (Figures 15, 16), (2) organized cellular aggregates, and (3) extracellular fibers.

Capillary-like components. The growth zones of cultures treated with cortisone acetate showed an insignificant decrease in the frequency distribution of capillary-like structures relative to control cultures after four and five days of cultivation (33 percent of 40 control explants; 25 percent of 38 experimental explants). A similar insignificant decreased frequency distribution of capillary-like structures was also found in seven and eight day cortisone treated cultures relative to the control cultures (36 percent of 70 control explants; 21 percent of 70 experimental explants). Combined consideration of all four to eight day control and cortisone treated cultures showed a significant decrease ($P < .05$) in the frequency distribution of capillary-like structures in the growth zones of cortisone treated cultures relative to the controls (38 percent of 110 control explants; 24 percent of 108 experimental explants). The development of the capillary-like structures in the growth zones of the cortisone treated cultures was less extensive than that of control cultures (Figures 17, 18). Actively mitosing

endothelial cells (Figure 16) were found only in the control cultures. Vascular structures within the main body of the original explant showed no visible alteration referable to cortisone acetate.

Cellular aggregates. Organized cellular aggregates occurred in the growth zones of both control and cortisone treated cultures. One type of aggregate, which seemed to be composed of the phagocytic macrophage-like cells, contained a core of acidophilic crystalline appearing material (Figures 13, 14). A second type similar to that shown in Figure 19 was occasionally found. This type of aggregate was comprised essentially of fibroblasts and macrophage-like cells which appeared to be walling off fragments of foreign material such as asbestos or cotton fibers. A third type occurred as relatively flat sheet-like aggregates of closely packed cells of different species (Figure 18). The latter type of cellular aggregate seemed to occur more frequently in seven to fourteen day cortisone treated cultures than in parallel control cultures.

Fibrous extracellular components. Control and cortisone treated cultures stained by the periodic-acid-Foot method for the demonstration of connective tissue fibers showed a progressive increase in the number and distribution of argyrophilic fibers in the growth zones during four, eight, and fourteen days of cultivation. There was no apparent qualitative or quantitative difference in the development of argyrophilic fibers in the cortisone treated cultures relative to the control cultures (Figures 22, 23).

Control and cortisone treated cultures stained with orcein after seven days of cultivation for the demonstration of elastic fibers (Figure 25), showed similar delicate corrugated networks of elastic fibers.

The physical structure of the elastic and argyrophilic fibers in the cortisone treated cultures was unaltered from that of their counterparts in control cultures.

Frequency distribution of healthy thymocytes. The frequency distribution of healthy, living thymocytes in the growth zones of cortisone treated cultures and control cultures was not significantly altered during four to five days of cultivation (75 percent of 40 control explants; 68 percent of 38 experimental explants). The number of explants containing healthy thymocytes in the growth zones of cortisone treated cultures was significantly decreased relative to the control series after seven to eight days in cultivation (90 percent of 70 control explants, 49 percent of 70 experimental explants; $P < .001$). This decreased frequency distribution was also accompanied by an apparent decrease in the abundance of healthy thymocytes.

General growth characteristics. The total number of cortisone treated explants growing relative to the total number of explants set up was not significantly different from that of the control cultures (100 percent of 110 control explants; 98 percent of 110 experimental cultures). The over-all extent of growth, as indicated by arbitrary unit evaluation of the growth zones, was relatively less in the cortisone treated cultures than in the controls (compare Figures 22 and 23) after seven to fourteen days of cultivation. At the same time, in general, the main body of the explant was thinner in cortisone treated than in control cultures as indicated by arbitrary unit evaluation.

Clot liquefaction. Liquefaction of the plasma clot in the region of the growth zone occurred with equal frequency in both

cortisone treated and control cultures. (11 percent of 110 control explants, 10 percent of 108 experimental explants).

Phagocytic activity. The macrophage-like cells in control and cortisone treated cultures showed similar levels of phagocytic activity after treatment with carmine red. Similar levels also were indicated in routinely fixed and Giemsa stained control and cortisone treated cultures relative to the phagocytosis of cellular debris.

Lipid content. The general lipid content as revealed by Sudan IV and Sudan Black B lipid stains varied from explant to explant within both the control and cortisone treated cultures. Variation was also noted in the lipid content within different cells of the same species in both the control and experimental cultures. The recognizable cell types within the growth zones of control and experimental cultures which were seen to contain lipid within their cytoplasm included (1) phagocytic macrophage-like cells (2) fat cells, (3) fibroblasts, (4) fibroblast-like cells and (5) myelocytic cells. Detailed studies with the Sudan IV stain revealed that in general there was a slight increase in the size of the individual lipid inclusions in the large macrophage-like cells in the cortisone treated cultures over those within the same cell species in the control cultures (Figures 20, 21). No other cell types showed changes in the lipid content referable to the influence of cortisone.

pH measurements. The pH of the fluid culture nutrient of both control and cortisone treated cultures showed an average increase from pH 7.6 to 7.8 after three and four days of cultivation.

Clearing phenomenon and polarizing light studies. The

experimental nutrient which was initially cloudy due to the formation of micro-crystals of cortisone acetate, cleared up so completely after the first twenty-four hours of cultivation of the thymic tissue that it could no longer be visibly distinguished from control nutrient even on vigorously shaking the culture tubes. Some clearing occurred when the nutrient was replaced with fresh experimental nutrient, but not to the same extent as that of the first twenty-four hours of cultivation.

Polarized light studies of the native cortisone acetate crystals and the micro-crystals in the culture nutrient showed them to be birefringent. Polarized light studies of representative fourteen day cultures revealed the presence of nondescript intra- and extracellular birefringent material in the growth zones of both control and cortisone treated cultures. No evidence was obtained which indicated an intracellular localization or phagocytosis of micro-crystals of cortisone acetate.

DL Thyroxine Series

Fixed and stained control and experimental cultures of the DL thyroxine series revealed the same variations from explant to explant which were noted in the control and experimentally treated cultures of the Cortisone Acetate Series. In general, the cell species and histologically differentiated components characteristic of the growth zones in the Cortisone Acetate Series also were characteristic of the growth zones of the control and experimentally treated cultures in the DL Thyroxine Series. Mitotic activity was shown by the same species of cells in both the control and experimentally treated cultures of the DL Thyroxine Series as in the control cultures of the Cortisone Acetate

Series. (See pages 9 and 10).

Quantitative Results

Specific data bearing on (1) the growth response, (2) the frequency distribution of thymocytes in the growth zones, (3) the frequency distribution of capillary-like structures in the growth zones, (4) the frequency of plasma clot liquefaction and, (5) the mitotic frequency of thymocyte-like cells are presented in Tables 1 and 2, and in Chart 1. The data in Table 2 have been treated statistically by Chi-square analysis according to (1) the individual culture groups as indicated by the date set up, and (2) the combined culture groups at each hormone concentration. Actual values in percent were included for ease in viewing the data.

TABLE 1

DATA RELATIVE TO THE EFFECTS OF DL THYROXINE ON THE MITOTIC FREQUENCY OF THYMOCYTE-LIKE CELLS IN VITRO

Concentration of thyroxine	Days in vitro	Date set up	Treatment	Number cells counted	Number mitotic cells	Mitosing cells per 1000
0.01 γg/ml. pH 7.6	5	5-31-57	C	6000	24	4
			E	5309	23	4
0.1 γg/ml. pH 7.6	7	5-1-57	C	3886	7	2
			E	3748	2	1
1.0 γg/ml. pH 7.6	7	6-6-57	C	5116	6	1
			E	6329	7	1

Statistical treatment: P greater than .10

0.01 γg. DL thyroxine/ml. In general, the data showed no significant difference between the control cultures and the cultures treated

TABLE 2

DATA RELATIVE TO THE EFFECTS OF DL THYROXINE
ON THE GROWTH ZONES OF THYMIC EXPLANTS

Concentration and initial pH	Date set up	Age of animal (days)	Days in vitro	Number of explants		Number of explants growing		Actual value in %	
				Con.	Exp.	Con.	Exp.	Con.	Exp.
0.01 µg./ml.									
pH 7.6	5-31-57	31	5	100	100	95	100	95	100
Totals:				100	100	95	100	95	100
0.1 µg./ml.									
pH 7.6	5-1-57	43	7	50	50	47	45	94	90
	6-5-57	41	7	50	60	43	60	86	100
pH 7.9	4-16-57	47	7	20	20	17	19	85	95
Totals:				120	130	107	124	89	95
1.0 µg./ml.									
pH 7.6	6-6-57	42	7	40	50	40	48	100	96
	5-21-57	25	7	30	40	25	39	83	98
	1-8-58	34	7	70	100	66	98	94	98
	2-12-58	31	7	20	10	20	10	100	100
pH 7.9	2-19-58	38	4	20	30	20	29	100	97
	2-19-58	38	8	30	40	30	40	100	100
Totals:				210	270	201	264	96	98

Con., control explants; Exp., experimental explants

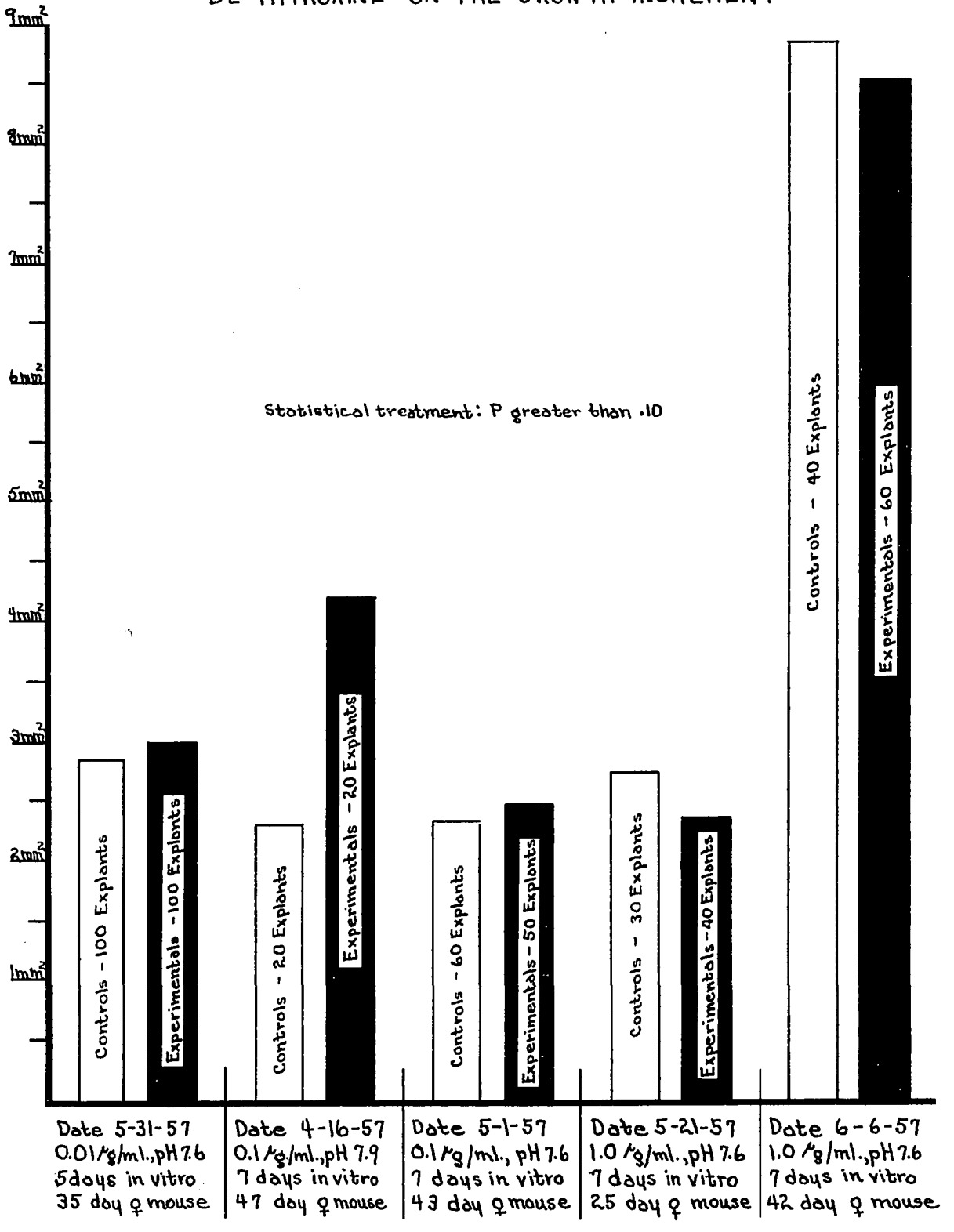
TABLE 2 - Continued

Of those explants growing, the number of growth zones showing:											
Healthy thymocytes		Actual value in %		Cap.-like structures		Actual value in %		Lique-faction		Actual value in %	
Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.
89	85[a]	94	85	33	45	35	45	7	6	7	6
89	85[a]	94	85	33	45	35	45	7	6	7	6
23	28	49	62	5	4	11	9	18	18	38	38
33	44	77	73	25	18[b]	58	30	16	40[b]	37	67
12	13	71	68	8	9	47	47	4	7	23	46
68	85	63	68	38	31	36	25	38	64[a]	36	52
37	40	92	83	23	27	57	56	4	21[c]	10	43
21	26	84	66	3	5	12	13	13	21	52	54
34	46	52	47	32	35	48	36	4	11	6	11
19	4[a]	95	40	12	5	60	50	3	1	15	10
5	7	25	24	1	4	5	14	1	4	5	14
16	20	53	50	10	10	33	25	7	16	23	40
132	143[b]	66	54	81	86	40	32	32	74[b]	16	28

Statistical treatment: a, $P < .02$; b, $P < .01$; c, $P < .001$

CHART 1

PLANIMETRIC DATA RELATIVE TO THE EFFECT OF DL THYROXINE ON THE GROWTH INCREMENT



with 0.01 /ᵘg. DL thyroxine/ml. nutrient relative to: (1) the general culture growth, (2) the frequency distribution of capillary-like structures in the growth zones, (3) the mitotic frequency of thymoctyes, and (4) the clot liquefying capacity of the explants. A significant decrease occurred in the number of growing thyroxine treated explants with healthy thymocytes in the growth zones relative to the control cultures (Table 2). Comparisons between control and experimental explants showing healthy thymocytes in the growth zones revealed no obvious differences in the total complement of thymocytes in control and thyroxine treated cultures.

Untabulated data relative to the frequency distribution of multinucleate giant cells and syncytial masses, considered together, showed no significant differences between control cultures and experimental cultures treated with 0.01 /ᵘg. DL thyroxine /ml. (35 percent of 95 control explants; 42 percent of 100 experimental explants). Also there were no significant differences found in the distribution of cellular aggregates containing a core of acidophilic crystalline appearing material (32 percent of 95 control explants; 43 percent of 100 experimental explants).

The average pH of the control nutrient increased from the initial pH of 7.6 to 7.8, and that of the experimental nutrient from 7.6 to 8.0 after five days of cultivation. The difference between the terminal pH of the control and experimental culture nutrient was not significant.

<u>0.1 /ᵘg. DL thyroxine/ml</u>. The data relative to thymic fragments cultured in the presence of 0.1 /ᵘg. DL thyroxine/mL nutrient at an initial pH of 7.6 and 7.9, as compared with parallel control cultures,

showed no significant differences in (1) the general culture growth, (2) the frequency distribution of thymocytes, (3) the frequency distribution of capillary-like structures, or (4) the mitotic frequency of thymocyte-like cells. There was a significant increase in the frequency of plasma clot liquefaction in the region of the growth zones of thyroxine treated cultures relative to control cultures (Table 2). None of the above quantitated features in the control and/or experimentally treated cultures could be related to a variation of the initial pH from 7.6 to 7.9 (Table 2, Chart 1).

Using nutrient with an average initial pH of 7.6, the average terminal pH of control nutrient was 7.8 and that of the parallel experimental cultures was 7.6. The difference between the terminal pH of control and experimentally treated cultures was not significant. The nutrient of both control and experimentally treated explants cultured at an initial pH of 7.9 had an average terminal pH of 7.8.

Explants treated with 0.1 μg. DL thyroxine/ml. at an average initial pH of 6.7 (total of 170 experimental explants) failed to grow for the entire seven day cultivation period, whereas 87 percent of the parallel control cultures did grow (total of 120 control explants). The average terminal pH of the nutrient of both control and experimental cultures increased from the average initial pH of 6.7 to 7.4.

1.0 μg. DL thyroxine/ml. Quantitative data relative to (1) the general cultural growth, (2) the frequency of capillary-like structures in the growth zones, and (3) the mitotic frequency of thymocyte-like cells showed no significant differences between control cultures and parallel cultures treated with 1.0 μg. DL thyroxine/ml. nutrient. There

was a significant decrease in the number of growing thyroxine treated explants showing healthy thymocytes in the growth zones relative to control cultures (Table 2). There were no obvious differences in the relative abundance of healthy thymocytes in the growth zones of the experimental and control cultures which did show the presence of healthy thymocytes in their growth zones.

No quantitative differences were found in control and/or thyroxine treated cultures directly referable to a difference in the initial pH of the culture nutrient from 7.6 to 7.9 (Table 2). Both control and experimentally treated culture nutrients, adjusted to an average initial pH of 7.6, had an average terminal pH of 7.7. The terminal pH was not determined on the fragments cultured at an initial pH of 7.9 at the 1.0 /μg. DL thyroxine/ml. level.

Objective Data

Vacuolation. Multinucleate giant cells and syncytial masses were more highly vacuolated in the thyroxine treated cultures at all three thyroxine concentrations relative to their counterparts in parallel control cultures (Figures 10, 11, 12). The large phagocytic macrophage-like cells showed a greater tendency toward vacuolation in cultures treated with 1.0 /μg. DL thyroxine/ml. nutrient than cells of the same species in control cultures (Figures 6, 9).

Cell size. There was an apparent over-all diminution in the size of cells of all species present in the growth zones when cultured under the influence of 1 /μg. DL thyroxine/ml. relative to comparable cells in the growth zones of control cultures (Figures 6, 9).

Phagocytic activity. The level of phagocytosis on the part of macrophage-like cells in the growth zones of thyroxine treated cultures appeared to be entirely comparable to that of the same species of cell in control cultures, as indicated by phagocytosed cellular debris in routinely fixed and Giemsa stained cultures. Histochemical demonstration of iron within the cytoplasm of macrophage-like cells, as well as the histochemical demonstration of phagocytosed Feulgen positive material within the cytoplasm of this same species of cell, also, indicated similar levels of phagocytic activity in control and thyroxine treated cultures. The extent of migration of the macrophage-like cells did not seem to be affected by thyroxine relative to control cultures.

Fibrillogenesis. Both control cultures and cultures treated with 1 /ug. DL thyroxine/ml. nutrient, which were stained by the periodic-acid-Foot stain for connective tissue after four, eight, and fifteen days of cultivation, showed a progressive increase in the abundance and distribution of argyrophilic fibers with no apparent qualitative or quantitative differences between the control and thyroxine treated cultures.

Control and experimental cultures treated with 1 /ug. DL thyroxine/ml., which were stained with orcein after four and eight days of cultivation for the demonstration of elastic fibers, showed entirely comparable corrugated networks of fibers in control and experimentally treated cultures. There was an increased abundance and distribution of these fibers in the growth zones of both control and thyroxine treated cultures after eight days of cultivation as compared with the four day cultures.

The physical structure of argyrophilic and elastic fibers did not appear to be affected by treatment with DL thyroxine when compared

CHAPTER IV

DISCUSSION

Studies of the mammalian thymus by means of tissue culture methods have been reported by a number of workers (Popoff, '27; Bellone, '43; Mendelsohn, '54; Törő, '52, Vadász, '54, Vernon, '56; and others). It is not evident from the literature, however, that other workers have investigated the effects of cortisone acetate or DL thyroxine on thymic fragments cultured by the more conventional tissue culture methods used in the present study.

Effects of Cortisone Acetate

The clearing phenomenon occasioned in the experimental culture nutrient during the first twenty-four hours of cultivation, was a constant finding which could not be related to specific factors such as a simple settling out of the excess cortisone acetate crystals. Polarized light studies provided no information on the possible role of phagocytosis of the cortisone crystals in this clearing phenomenon. It seems significant that clearing of the fluid culture nutrient of cortisone acetate crystals does not occur in cultures of embryonic chick heart containing 100 μg. cortisone acetate/ml. nutrient (Richter and Ritcheson, '58), for it suggests that thymic tissue has a special physiologic

affinity for this substance.

The present studies indicate that cortisone acetate does not demonstrably alter any of the basic cytophysiologic processes involved in bringing about changes in the pH of the nutrient media or in the clot liquefying capacity of the explants.

Not all workers have indicated clearly which form of cortisone was used in their in vitro studies, but there seems to be uniform agreement that a variety of tissues grow in culture in the presence of cortisone (Ruskin et al., '51; Paff and Stewart, '53; Holden et al., '51; Geiger et al., '56; and others). There has, however, been considerable disagreement as to whether cortisone has an inhibitory (Ruskin et al., '51) or no effect (Sacerdote de Lustig and Mancini, '51) on the growth and migration of cells. Evidence has been presented relative to the culture conditions under which cortisone may or may not be inhibitory (Geiger et al., '56). Undoubtedly the controversy has been enhanced by differences in technical methods and source of the tissues cultured. In the present studies, the findings of (1) a decreased extent of growth, (2) an increased degree of thinning of the main body of the original explant, and (3) a decrease in the degree of development of capillary-like structures in the cortisone treated cultures after seven to fourteen days of cultivation as compared with the control cultures, would seem to reflect a delaying action on the growth and migration of the cells in general, much like that observed in wound healing under the influence of cortisone (See Lukens, '54; Shwartzman, '53) rather than a specific inhibition of fibroblasts (Heilman, '45a, Holden et al., '51) and/or

macrophages (Paff and Stewart, '53).

The finding reported here indicates that cortisone acetate has no effect on the phagocytic capacity of macrophage-like cells, and is in agreement with that of Heilman ('45a) on cultures of the rabbit spleen. Her observation that cortisone stimulates the migration of macrophages is not in agreement with the present studies.

Although many workers have not mentioned specific histo- or cytomorphic effects, Trowell ('53) has pointed out the absence of any effect on lymphocytes. Similarly, Barski and de Brion ('52) found cortisone to have no effect on a variety of tissues in culture. The finding recorded here that mast cells did not seem to be affected by cortisone acetate is in rather sharp contrast to the findings of Asboe-Hansen ('52) in his in vivo studies of the mast cells in the skin of several mammalian species. He found that cortisone acetate injections into the intact animal caused a decrease in the number of mast cells, degranulation, and vacuolation. There may be some general systemic factors involved in these differences.

Fibrillogenesis has been reported to be inhibited by cortisone but not by cortisone acetate by Sacerdote de Lustig and Mancini ('51) although Barski and de Brion ('52) reported that cortisone did not visibly influence the formation of collagen in vitro. Castor and Baker ('50), in studies of the effects of prolonged application of cortisone to the skin of rats, noted that while collagenous fibers became more homogeneous and compact, the elastic fibers remained unaffected. In the present studies, no effect was noted on the fibrillogenesis or

physical appearance of the argyrophilic or elastic fibers.

The often reported atrophy of lymphoid tissue in general, and the thymus in particular, after injection of adrenocorticotropin, extracts of the adrenal cortex, or certain specific hormones such as cortisone (Baker et al., '51; Simpson et al., '43; Wells and Kendall, '40; Ringertz et al., '52; Winter et al., '50) has lead a number of workers to investigate the possibility of a direct effect upon lymphoid cells by in vitro methods. Robertson ('48) reported that the adrenal cortical extracts he used had no effect on cell suspensions from the inguinal lymph nodes of rats after eight to twelve hours incubation except in the presence of heterologus serum. Feldman ('50) found that while the adrenal cortical extract he used had a rapid and complete cytotoxic activity, compound E, among others, was without effect on cell suspensions from the thymus, spleen and lymph node of rabbits, rats, guinea pigs, and humans up to twenty-four hours in vitro. Schrek ('49, '51a and '51b) found a decreased cell survival rate in suspensions of cells from the thymus of rabbits incubated for somewhat longer intervals than those of Feldman ('50) and Robertson ('48) in the presence of adrenal cortical extract as well as a number of purified compounds including cortisone. Hechter and Johnson ('49) reported that lymphocytes obtained from pooled fragments of spleen, thymus, and cervical lymph nodes were broken down more quickly by some of the many agents tested, but that compound E was without effect on the rate of lymphocytolysis. The great differences between the technical procedures used by these several workers in the preparation and maintenance of their cell suspensions, and those used in the present study, do not permit a just comparison of findings. The findings reported here relative to a

decreased frequency distribution and an apparent numerical decrease of thymocytes in the growth zones of thymic cultures after seven days of cultivation are, however, in general agreement with those findings of Trowell ('53) and Heilman ('45b) who used more conventional methods of tissue culture. Trowell ('53) found that ten micrograms cortisone per milliliter had killed at least 45 per cent of the lymphocytes in cultures of lumbar and sacral lymph nodes of rats within two days of cultivation as determined by a method of pycnotic counts. Heilman ('45b) found decreased migration and increased degeneration of the lymphocytes in cultures of rabbit mesenteric lymph nodes in the presence of cortisone for twelve to eighteen hours. Since the majority of the free cells which flow out of thymic fragments upon explanting, die very quickly leaving a background of pycnotic and degenerating nuclei (Vernon, '55; Bellone, '43), it could not be determined from the present studies whether the decrease in frequency distribution and abundance of thymocytes noted here, was due to a cytolytic effect as suggested by Trowell ('53) and Schrek ('49, '51a and '51b), to an increased rate of lymphocytolysis as suggested by Hechter and Johnson ('49), or to a controlled maturation and mitotic rate as suggested by Robertson ('48). The failure to note any effects on the four and five day cultures in the present studies as compared with the findings of Trowell ('53) and Heilman ('45b) of effects within the first two days, may have been due to differences in the form of cortisone used and/or to differences between thymic tissues and lymph nodal tissues.

Effects of DL Thyroxine

Clark ('54) and Semura ('31) have reported stimulation of fibroblast growth at concentrations comparable to the lowest used in these studies and inhibition at concentrations comparable to the highest used in the present studies. The data recorded here on the thymus, however, indicate that thyroxine at the various concentrations used (0.01, 0.1, and 1.0 μg/ml.) had no significant effect on the over-all growth in these cultures or specifically on the growth of fibroblasts or fibroblast-like cells therein under normal levels of pH (7.6 and 7.9). However, at pH levels of 6.6 to 6.7, and at a concentration of 0.1 μg/ml., growth is completely inhibited and this is in keeping with the findings of Clark ('54) of a decreased survival rate in cultures of embryonic chick heart as the pH of the culture medium decreased at 0.1 and 0.5 microgram/ml. levels. This finding is in rather sharp contrast, however, to those of Zondek ('44) in studies of tadpole metamorphosis, who suggested that the thyroid hormone might have a greater physiologic activity at a more acid pH than at the pH of blood. Although Vogelaar and Erlichman ('36) alluded to this point of Zondek's as a possible explanation for the differences between their findings and those of Semura ('31), specific data on pH were not included in their report, nor did they state whether they had made cultural pH determinations. The thyroxine concentrations used in this study were not found to effect significant changes in the pH of the fluid culture medium or to effect growth, as was found by Clark ('54) in cultures of embryonic chick heart. This lack of agreement may be a reflection both of fundamental differences

between embryonic and adult tissue metabolism and/or of specific metabolic differences between heart and thymic tissues per se.

Increased vacuolation of syncytial masses and of giant multinucleate cells at all concentrations of DL thyroxine at normal pH levels indicate some stimulation by thyroxine of this cellular capacity. A similar stimulative effect was noted in the phagocytic macrophage-like cells at the one microgram/ml. level.

The increased frequency of plasma clot liquefaction at the 1.0 and 0.1 μg/ml. levels, while not always noticeable within individual culture groups, was statistically significant at both thyroxine concentrations upon collective consideration of all the culture groups at these thyroxine concentrations (Table 2). These findings suggest that thyroxine stimulates the responsible cellular activity at a concentration of 1.0 and 0.1 μg/ml.

It has been reported that thyroid hormone may cause an increased rate of metamorphosis in the tadpole with an over-all diminution in size (Zondek, '44). It also has been reported in connection with cellular differentiation, that thyroxine may effect an over-all diminution in cell size (Clark, '54; Fell and Mellanby, '55; and Richter, '44). A similar diminution in size of thymocytes and the several other species of cells in thymic cultures, was noted also in the present studies at a DL thyroxine concentration of 1 μg/ml. It is not definitely known whether changes of the type reported here relative to diminution in cell size are referable to a specific developmental function on the part of thyroxine, or to altered energy metabolism or both (Barker, '51).

Little, if any, work has appeared relative to the influence of DL thyroxine or other thyroid substances on the growth of capillary structures in culture. The present data considered collectively indicate that thyroxine at the higher concentrations of 0.1 and 1.0 γg/ml. has a tendency toward inhibition of capillary growth. Although the collective differences noted (Table 2) are not statistically sound, the experimental group set up 6-5-57 showed a statistically significant inhibition of capillary-like growth. In contrast, the data on the lowest concentration used (0.01 γg/ml.) indicate a tendency toward stimulation of capillary-like growth, although the differences evident are not statistically significant.

The present study has demonstrated that thymocytes have the capacity to reproduce mitotically at rather constant rates in vitro under the conditions used in these studies. Although a few other workers have reported mitotic activity on the part of thymocytes in vitro (Bellone, '43; Papenheimer, '12- '13; Tschassownikow, '27), it is not known if other workers have attempted to determine the mitotic frequency of thymocytes in vitro. The present study shows further, that the mitotic frequency of thymocytes in vitro was neither enhanced nor inhibited by DL thyroxine. This fact, however, cannot be taken to mean that thyroxine had no effect on thymocytes. At the 1 γg/ml. level of concentration, thymocytes as well as the several other species of cells present in the growth zones of thymic cultures showed a slight decrease in size. It has also been demonstrated that at the 0.01 and 1.0 γg/ml. levels of DL thyroxine concentration (Table 2), there was a significant

decrease in the number of growing thyroxine treated explants containing healthy thymocytes in the growth zones. The fact that thymocytic frequency distribution may be decreased under the influence of thyroxine without a coincident reduction in the thymocytic mitotic index, suggests that thyroxine may have specific effects on the heteroplastic transformation of thymocytes and/or their progenitor stem cells, the thymic reticulum cells --- an effect which is consistent with findings of others on the role of thyroxine in cellular differentiation (Richter, '44; Schneider, '39a, and '39b; Fell and Mellanby, '55).

CHAPTER V

GENERAL SUMMARY

Comparative studies were made relative to the continuous individual effects of cortisone acetate and of DL thyroxine on thymic tissue of the mouse isolated in culture from other hormonal and systemic factors. Conventional tissue culture methods were used. The cultured fragments were fixed and stained by routine and special methods after varying periods of cultivation ranging from four to fifteen days.

Cortisone acetate was found to have no histo- or cytomorphologic effects on thymic cultures even after fourteen days of cultivation. Cortisone effected no changes in the pH of the culture medium after three and four days of cultivation. A slight inhibitory or delaying effect on the over-all growth of the cultures was occasioned after seven to fourteen days in vitro. The latter effect was accompanied by an increased thinning of the main body of the original explant. Cortisone treated cultures showed less extensive and less abundant growths of capillary-like structures. Thymocytes were significantly reduced in abundance and in frequency distribution in cortisone treated cultures after seven to fourteen days of cultivation. Cortisone had no effect on the plasma clot liquefying capacity of thymic explants.

DL thyroxine completely inhibited growth at a concentration of

0.1 microgram/ml. when cultured at an initial pH of 6.6 to 6.7. At concentrations of 1.0, 0.1, and 0.01 microgram/ml., DL thyroxine had no effect on the growth of thymus when cultured at an initial pH of 7.6 and 7.9. DL thyroxine had no effect on the pH of the fluid culture nutrient as compared with that of control cultures. There was apparently an increased cytophysiologic activity in thyroxine treated cultures as evidenced by (1) an increased degree of vacuolation of multinucleate giant cells and syncytial masses at all concentrations of thyroxine used, (2) an increased tendency toward vacuolation of phagocytic macrophage-like cells at the one microgram/ml. level, and (3) an increased frequency of plasma clot liquefaction with increasing concentration of the hormone. Thyroxine was found also to effect an over-all diminution in cell size at the one microgram/ml. level. Quantitative studies revealed a significantly decreased frequency distribution of thymocytes in the growth zones of cultures treated with 0.01 and 1.0 microgram thyroxine/ml. nutrient as compared with control cultures. Thyroxine treated cultures which did contain healthy thymocytes in their growth zones, showed the normal complement relative to control cultures containing healthy thymocytes in their growth zones.

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APPENDIX A

All figures are unretouched photomicrographs of thymic cultures which were fixed and stained "in toto." A 35 mm. Leica camera equipped with a Micro-Ibso attachment and a 1/3X conical extension tube was used to take the photomicrographs.

Abbreviations

M, macrophage-like cells
F, fibroblasts
FL, fibroblast-like cells
E, endothelial cells
CA, cellular aggregate
AB, asbestos fiber

PLATE I

Explanation of Figures

1. Mast cells in growth zone of a 7 day control culture of the thyroxine series, pH 7.6. Giemsa stain. 500X

2. Numerous myelocytic cells in growth zone of 4 day control culture of the cortisone acetate series. Carmine red, Ehrlich's hematoxylin and eosin stain. 500X

3. Myelocytic cells and thymocytes in growth zone after 7 days cultivation under the influence of 1 μg. thyroxine/ml., pH 7.6. Giemsa stain. 1000X

4. Myelocytic cells deep in area of original explant after 7 days cultivation under the influence of 1 μg. thyroxine/ml., pH 7.6. Note the two mitotic figures at the arrows. Giemsa stain. 1000X

5. Relatively large cells of unknown cytogenic relation characterized by a vacuolated basophilic cytoplasm and acidophilic nucleus with a distinct nuclear membrane. Control culture of the thyroxine series, pH 7.6 after 7 days cultivation. Giemsa stain. 1000X

PLATE I

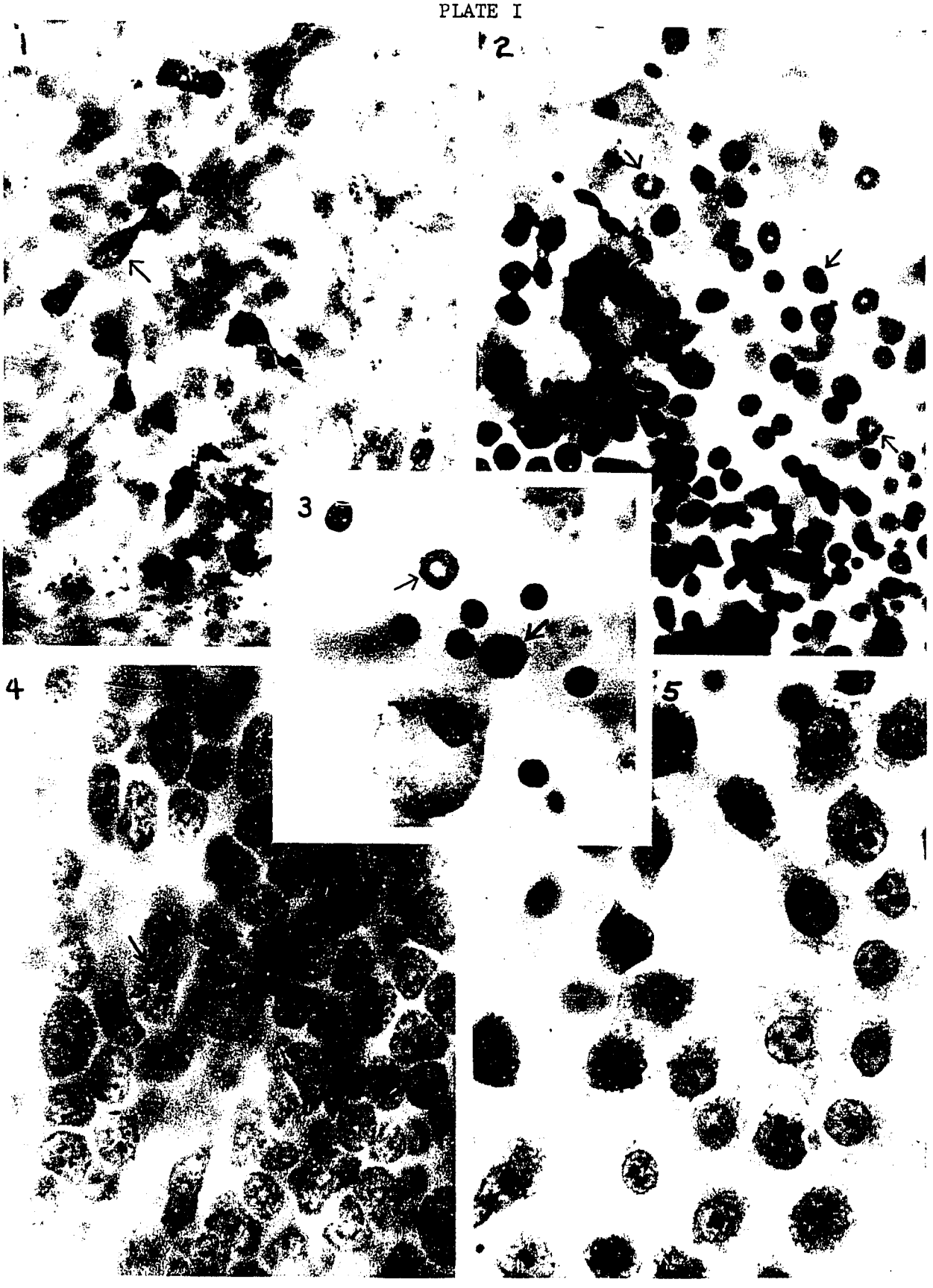


PLATE II

Explanation of Figures

6. Seven day control culture of the thyroxine series, pH 7.6 showing numerous healthy thymocytes in clusters in the growth zone along with phagocytic macrophage-like cells, fibroblast-like cells and more typical fibroblasts. Giemsa stain. 500X

7. Thymocyte in mitosis in growth zone of a 5 day control culture of the thyroxine series, pH 7.6. Giemsa stain. 1000X

8. Phagocytic macrophage-like cells two of which had particles of iron within the cytoplasm. Seven day control culture of the thyroxine series, pH 7.6. Prussian blue reaction and Meyer's paracarmine. 1000X

9. Healthy thymocytes growing in clusters in 7 day culture under the influence of 1 μg. thyroxine/ml., pH 7.6. Note the increased vacuolation of the macrophage-like cells and the apparent over-all diminution of cell sizes as compared with those in Figure 6. Giemsa stain. 500X

PLATE II

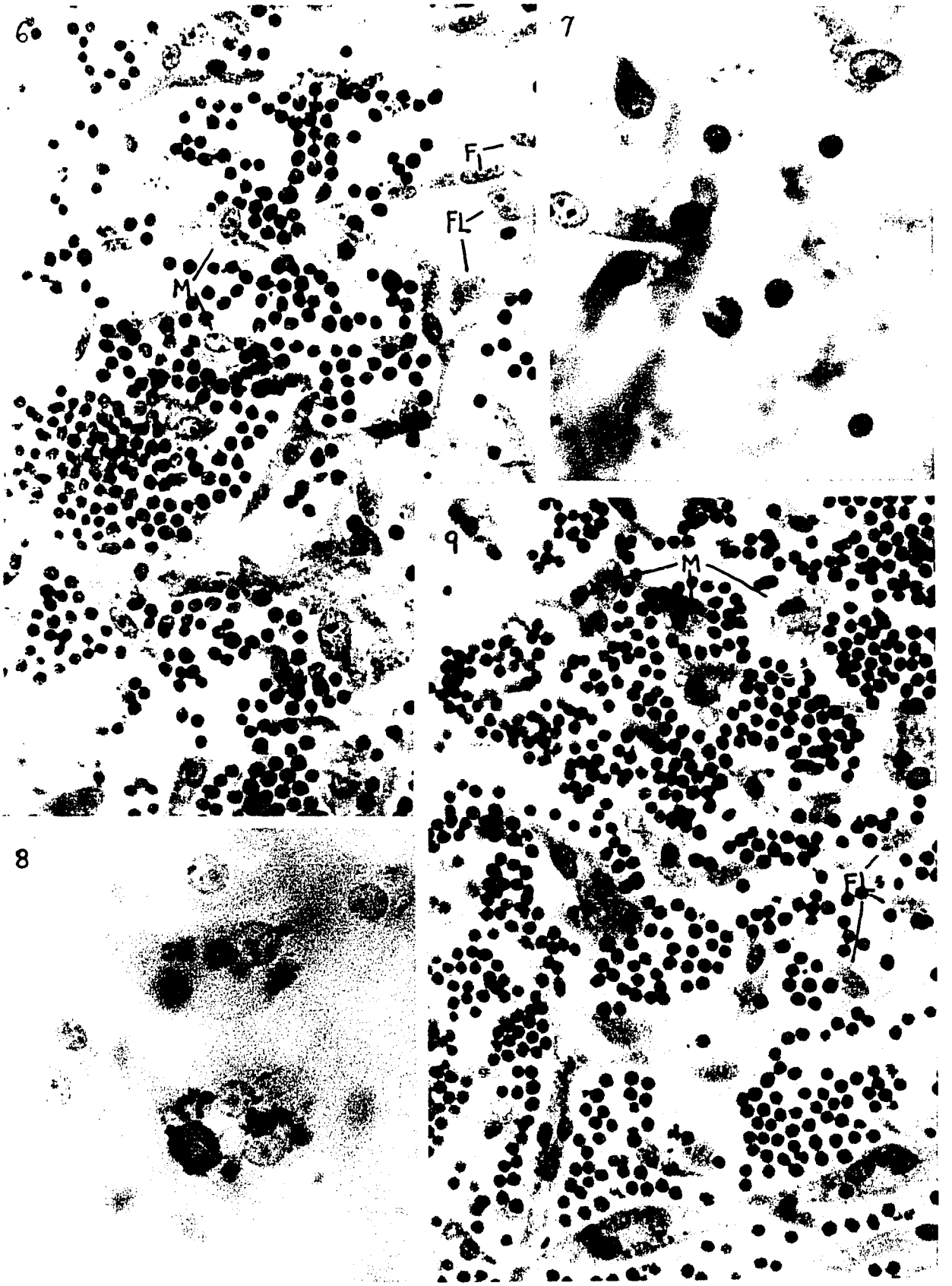


PLATE III

Explanation of Figures

10. Vacuolated syncytial mass in a 5 day control culture of the thyroxine series, pH 7.6. Giemsa stain. 500X

11. Vacuolated syncytial mass in a 5 day culture under the influence of 0.01 µg. thyroxine/ml. at pH 7.6. Giemsa stain. 500X

12. Syncytial mass of 5 day control culture of the thyroxine series, pH 7.6. Giemsa stain. 500X

13. Cellular aggregate with a core of acidophilic crystalline material in a 5 day control culture of the thyroxine series, pH 7.6. Giemsa stain. 1000X

14. Same structure as in Figure 13 at a different level of focus.

PLATE III

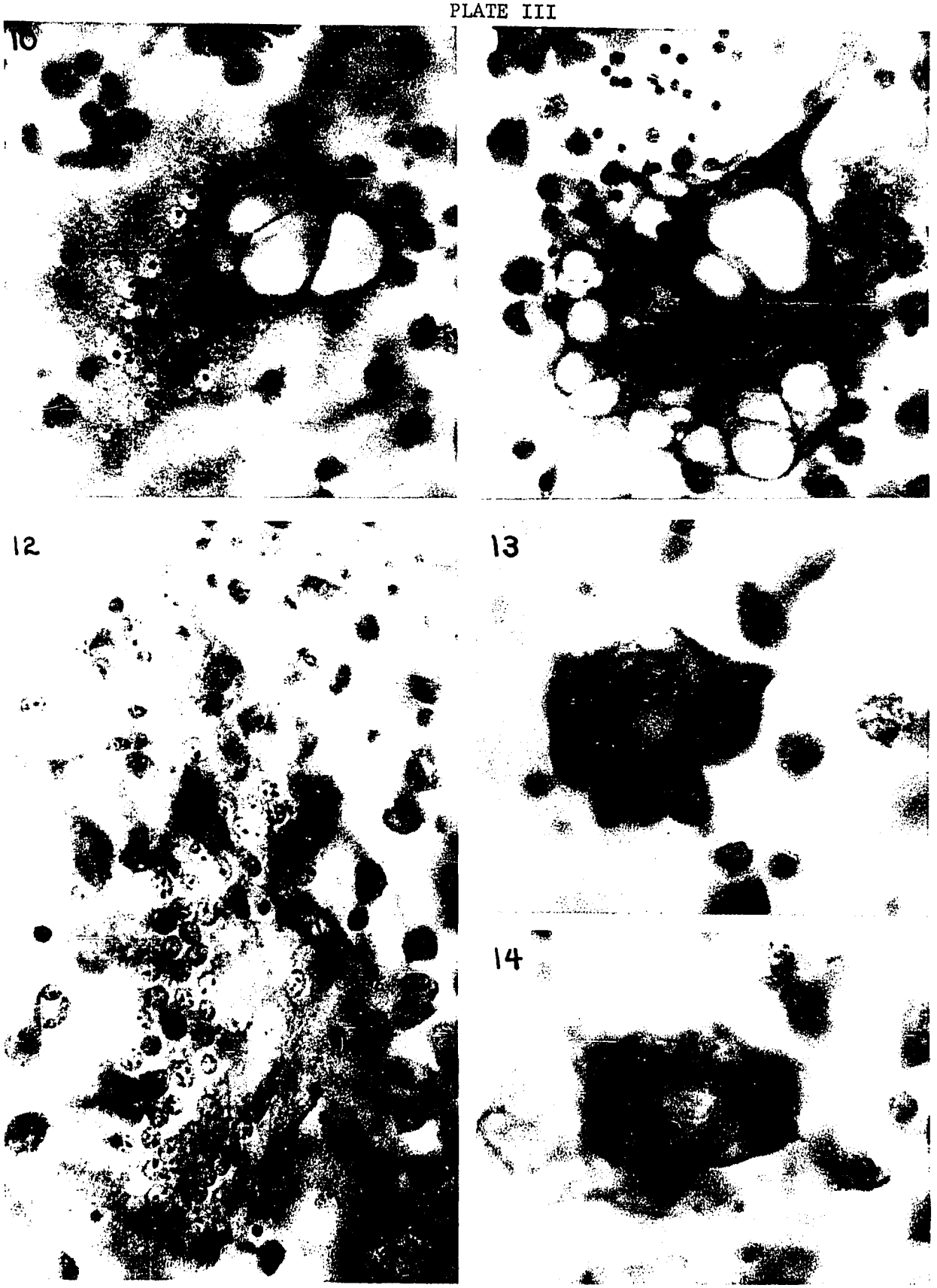


PLATE IV

Explanation of Figures

15. Area of a 7 day control culture of the thyroxine series. pH 7.6 showing growth of capillary-like structures. Giemsa stain. 100X

16. Same area as indicated by the rectangle in Figure 15 with an endothelial cell in mitosis at the arrow. 500X

17. Seven day control culture of the cortisone acetate series showing growth of capillary-like structures. Carmine red, Weigert's acid iron hematoxylin and eosin. 100X

18. Seven day experimental culture of the cortisone acetate series showing decreased numbers and extent of growth of capillary-like structures as compared with Figure 17. Also compare increased thinning of the area of the original explant. Giemsa stain. 100X

PLATE IV

PLATE V

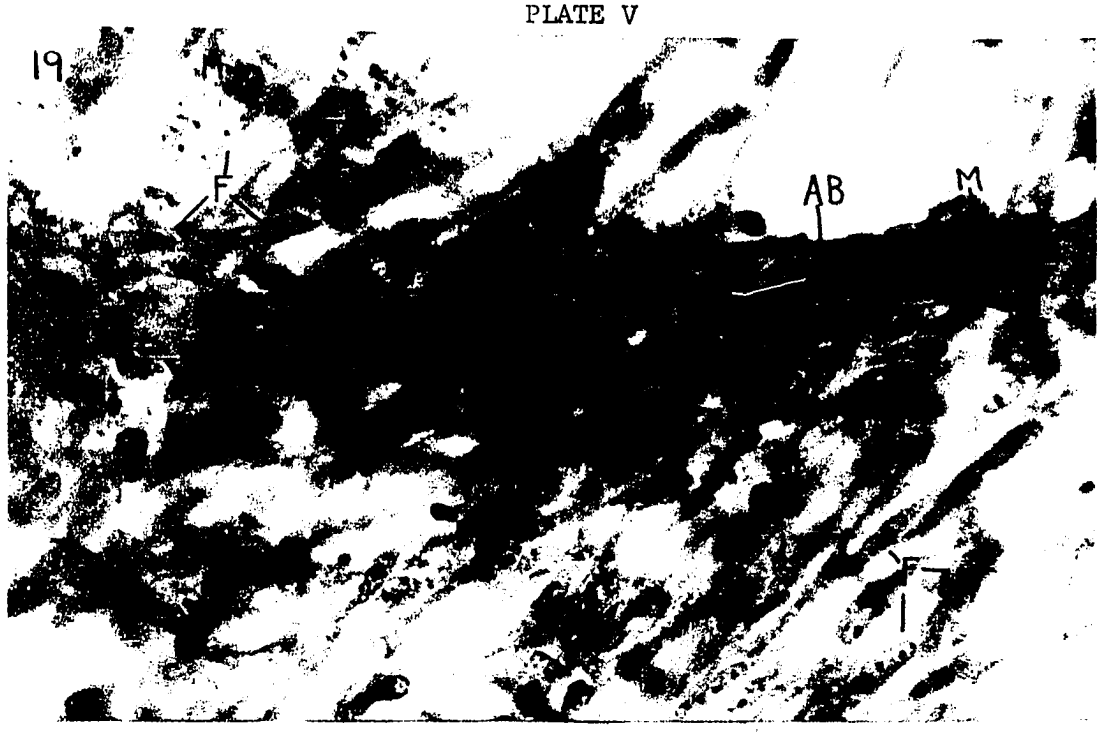
Explanation of Figures

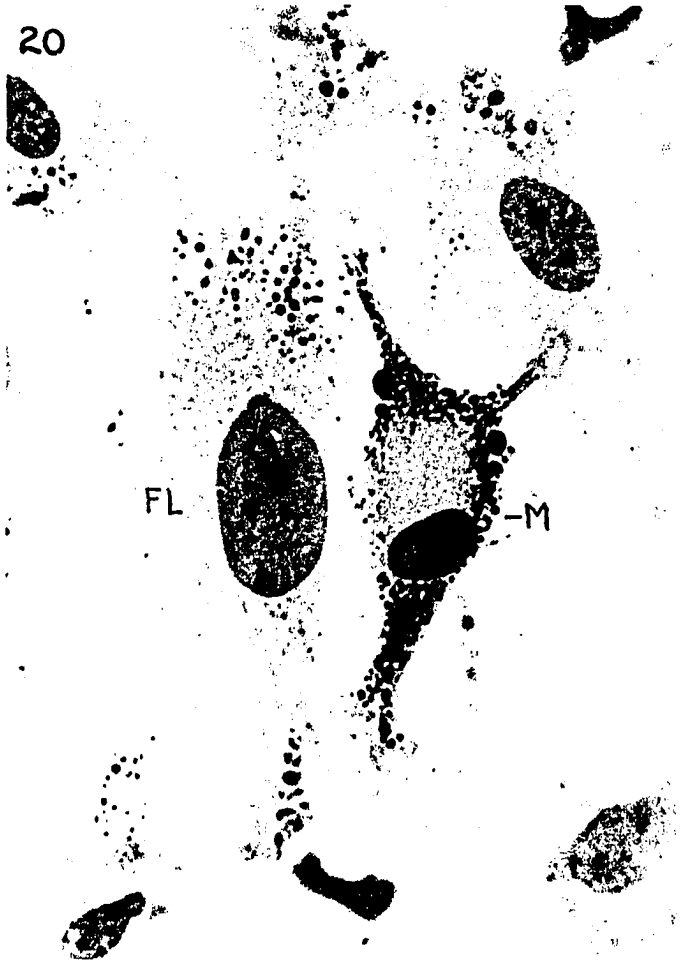
19. Five day control culture of the thyroxine series, pH 7.6, showing fibroblasts (F) and macrophage-like cells (M) walling off an asbestos fiber (AB) which was trapped in the plasma clot. Giemsa stain. 500X

20. Seven day control culture of the cortisone acetate series showing lipid droplets in large fibroblast-like cells (FL) and phagocytic macrophage-like cells (M). Sudan IV and Ehrlich's hematoxylin. 1000X

21. Seven day experimentally treated culture of the cortisone acetate series showing the slightly increased size of lipid droplets in the macrophage-like cells as compared with those of Figure 20. Sudan IV and Ehrlich's hematoxylin. 1000X

PLATE V





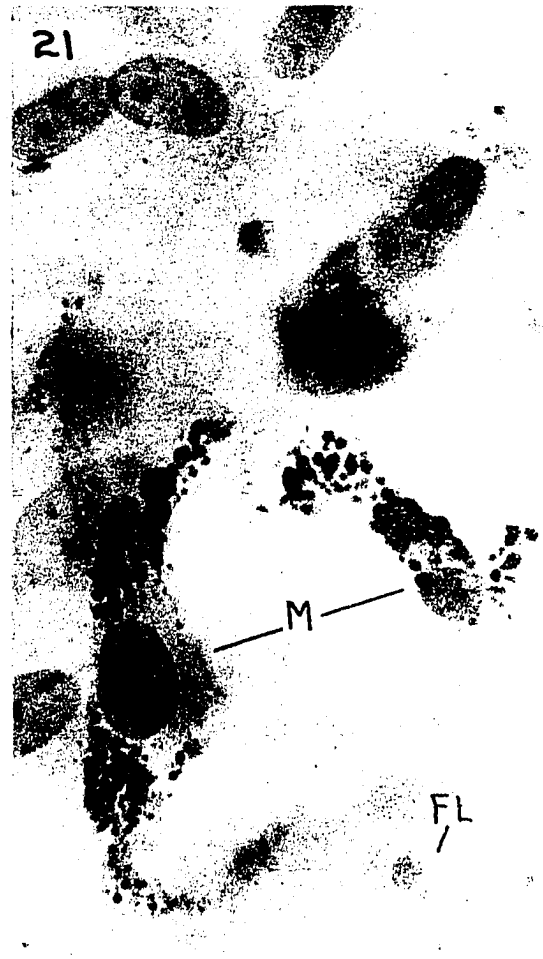


PLATE VI

Explanation of Figures

22. Eight day control culture of the cortisone acetate series showing the growth and extension of argyrophilic fibers into the growth zone. Periodic-acid-Foot stain. 100X

23. Eight day experimentally treated culture of the cortisone acetate series showing the growth and extension of argyrophilic fibers into the growth zone. Compare over-all extent of growth with that of Figure 22. Periodic-acid-Foot stain. 100X

24. Eight day control culture of the cortisone acetate series showing the very delicate as well as more coarse argyrophilic fibers. Periodic-acid-Foot stain. 500X

25. Elastic fibers in a 14 day control culture of the thyroxine series, pH 7.6. Orcein stain. 500X

PLATE VI