Bull. Inst. Zool., Academia Sinica **1:** 63-72 (1962)

THE EFFECT OF CORTISONE ON FROG EMBRYOS[1]

CHIH-YÜN HSÜ[2] AND HSÜ-MU LIANG[3]

Received for publication November 23, 1962

The fact that cortisone inhibits animal development is not new (1-9). Most investigators, however, have concentrated their attention on birds and mammals. Comparatively few non-systematic observations have been made on amphibians.

Witschi and Chang in 1950 reported the induced transformation of ovaries into testis in larval frogs by cortisone (10). Wurmbach and Haardick in 1952 found that desoxycorticosterone acetate accelerated the increase in absolute wet weight and dry weight and in percentage of dry weight of tadpoles, and also noted a faster growth in length of the experimental tadpoles than of the controls (11). Kuusisto and Telkkar in 1953 demonstrated that cortisone exerted no influence on tadpole thyroid morphology and hence exhibited no effect on metamorphosis (12). Iwasawa in 1958 claimed that cortisone treatment hastened testicular but inhibited ovarian development (13). Dastoli and Tector in 1959 reinvestigated the problem of frog metamorphosis after cortisone treatment and came to the conclusion that this hormone, acting as a metamorphic inhibitory agent, exerted an antagonistic effect against thyroxin (14).

The present attempt was to study the reactions of the developing frog embryos to cortisone during their early stages with special emphasis on the phenomena of growth.

MATERIALS AND METHODS

The eggs of *Rana catesbeiana,* obtained in our laboratory by induced ovulation, were fertilized artificially.

Groups of 50 fertilized eggs were cultured in large Petri dishes, each containing 100 ml filtered pond water. Equal numbers of cultures were used for control and experimental purposes. In the experimental series, cortisone acetate (11-dehydro-17-hydroxycorticosterone-21-acetate, Upjohn) was added to the cultures when the eggs reached the blastula, gastrula, neurula and hatching stages. Cortisone was spread evenly by gentle shaking to give the eggs an equal chance of contact with the white particles. Corresponding cultures without cortisone were used as controls. The culture medium was renewed weekly and new cortisone was added each time.

During the first two weeks after fertilization the eggs and larvae were closely observed daily for developmental progress (Shumway stages, 15) and anomalies, if any.

Then the survived and control tadpoles were reared in glass aquaria with an allotment of pond water gradually increased up to 100 ml per tadpole. Both control and experimental tadpoles were fed boiled green vegetables. The culture medium was changed twice a week for 15 weeks but weekly cortisone treatment was continued for another three weeks only.

Total length (from mouth to tip of the tail), body length (from mouth to base of the tail) and body width were measured weekly for 14 weeks beginning at the second week after fertilization. In order to minimize unavoidable mortality due to manipulation, weekly measurement of body weight was not started until the eleventh week.

The control and the cortisone treated eggs, larvae or tadpoles of each experiment were kept under the same environmental conditions such as food, temperature, light, space, *etc.*

1. Supported in part by a grant from China Medical Board of New York, Inc.

2. Professor, Department of Biomorphics, National Defense Medical Center, Taipei, Taiwan, China.

3. Professor and head of the Department of Biomorphics, National Defense Medical Center, and Director of the Institute of Zoology, Academia Sinica, Taipei, Taiwan, China.

RESULTS

*Cortisone treatment at early blastula stage —Experiments FR1, FR2, FR3 and FR5**

A hundred fertilized eggs were used in each of the experiments FR1 and FR3, 50 as the control and the other 50 as the experimental eggs. In experiments FR2 and FR5 the number of eggs used was doubled. Early blastulae of 2, 4 or 8-cells (stage 3, 4 or 5) of the experimental series were treated with 12.5 mg of cortisone in 100 ml of culture medium. The control eggs of course received no cortisone.

No difference in developmental rate between the control and experimental eggs was noticed earlier than stage 17, 18 or 19. Nor was any apparently abnormal egg found in the experimental groups. From then on the cortisone treated eggs showed developmental irregularities. Their development was at first retarded for a few stages when compared with that of the controls, and then hastened to catch up with that of the controls before stage 25.

During the retardation period the affected tadpoles began to develop grossly discernible edema which appeared at first under the heart and then spread gradually all over the body except the dorsum and the tail. When the development reached stage 25 the skin over the ventral and lateral parts of the body became so transparent that movements of heart, stomach and intestine could be readily made out. Their heads often curved upward with gills underdeveloped.

The edematous condition of the larvae varied in degree. The most seriously affected ones died quickly from the rapidly developed edema with their bellies burst and viscera exposed, while others suffered an insidious increase of edema and died eventually. Thus in the same experiment all grades of edematous larvae could be observed (Fig. 1). In rare occasions small blebs appeared either on the back or on the tail, in addition to the general edema.

The suffering larvae lay on the bottom of the dish on their sides, and the yolk was not much consumed. Before they began eating, the fecal material was very scarce. After feeding commenced, ingested cortisone was sometimes seen in the intestine readily and white fecal excreta was found on the bottom of the dish.

The control larvae of the same age, on the other hand, looked quite normal with no identifiable edema. At stage 25 they lay on the bottom of the dish on their bellies and had consumed most of their yolk. Brownish fecal material was found before they started eating. After feeding with green vegetables, the excreta was greenish in color and much more than those of the treated tadpoles.

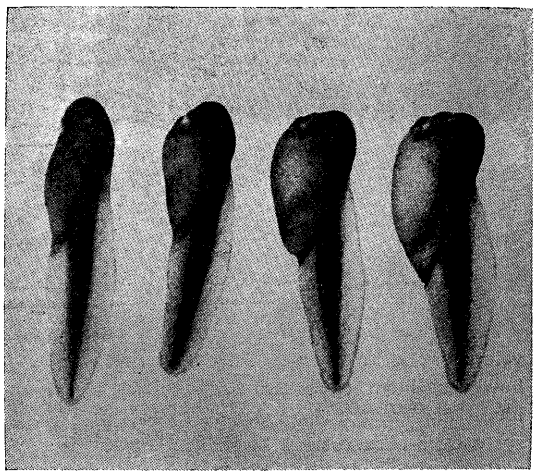

Fig. 1. Photograph of one normal tadpole (left) and of three edematous tadpoles (right) of increasing intensities from left to right. Enlargement 5×.

Table I summarizes the differences between control and cortisone treated eggs.

From Table I, it is clear that cortisone treatment caused a preliminary retardation of the development of the eggs with subsequent recovery, and the production of edema in the larvae. The retardation in development and the production of edema hence appeared to be somehow inter-related as if the former was followed by the latter. The percentage of edematous larvae and the condition of the edema varied in different experiments. Thus in experiments FR1 and FR2 the rate of edematous larvae was higher and the edematous condition more serious, whereas in experiments FR3 and FR5 the edema was less so and some of the abnormal larvae recovered.

Cortisone treatment at mid-blastula stage —Experiments FR4, FR6 and FR8

Four lots of fertilized eggs, 50 each, began to be treated with 50.000, 25.000, 12.500 and 3.125 mg of cortisone in 100 ml of pond water respectively, at mid-blastula stage (stage 8). The control eggs, also 50, were reared in plain pond water. The results are shown in Tables II and III.

* Denotes code number of female frogs from which fertilized eggs were obtained.

TABLE I

Developmental stage and edema incidence rate of control larvae and those treated with cortisone at blastula stage

Expt.	Cortisone dosage mg/100 ml	Developmental stage and percentage of edematous larvae after cortisone treatment					
		2nd day	3rd day	4th day	6th day	8th day	10th day
FR1	12.5	S 16 E 0	18 0	19 100.0	19 100.0	25 100.0	25 100.0
	0	S 16 E 0	19 0	20 0	22 0	25 0	25 0
FR2	12.5	S 14 E 0	17 0	18 100.0	20 100.0	22 100.0	25 100.0
	0	S 14 E 0	18 0	19 0	21 0	23 0	25 0
FR3	12.5	S 14 E 0	16 0	17 0	21 91.9	25 78.3	25 78.3
	0	S 14 E 0	16 0	18 0	22 0	25 0	25 0
FR5	12.5	S 14 E 0	18 0	21 100.0	25 55.5	25 10.5	25 10.5
	0	S 14 E 0	19 0	22 0	25 0	25 0	25 0
Overall average final % of edema	12.5						72.2
	0						0

S: Shumway stage of development.
E: % of edematous larvae.

TABLE II

Developmental stage of control larvae and those treated with cortisone at mid-blastula stage

Expt.	Cortisone dosage mg/100 ml	Developmental stage after cortisone treatment				
		2nd day	3rd day	4th day	6th day	8th day
FR4	50.000	14	17	19	20	25
	25.000	14	17	19	20	25
	12.500	14	18	20	22	25
	3.125	14	18	20	22	25
	0	14	18	20	22	25
FR6	50.000	18	19	20	25	25
	25.000	18	19	20	25	25
	12.500	18	20	23	25	25
	3.125	19	22	23	25	25
	0	19	22	23	25	25
FR8	50.000	17	20	23	24	25
	25.000	17	20	23	24	25
	12.500	17	20	23	24	25
	3.125	17	20	23	24	25
	0	17	20	23	24	25

TABLE III

Edema incidence rate of control larvae and those treated with cortisone at mid-blastula stage

Expt.	Cortisone dosage mg/100 ml	Percentage of edematous larvae after cortisone treatment					Average final % of edema of all treated eggs
		2nd day	3rd day	4th day	6th day	8th day	
FR4	50.000	0	0	97.6	100.0	Dead	51.5
	25.000	0	0	95.1	95.1	95.1	
	12.500	0	0	52.2	34.8	10.7	
	3.125	0	0	6.4	4.3	0	
	0	0	0	0	2.0	2.0	
FR6	50.000	0	67.4	100.0	Dead	—	58.8
	25.000	0	97.9	97.9	89.2	83.0	
	12.500	0	50.0	55.1	45.5	45.5	
	3.125	0	0	0	6.7	6.7	
	0	0	0	0	0	2.3	
FR8	50.000	0	0	63.8	100.0	100.0	42.0
	25.000	0	0	58.3	68.1	68.1	
	12.500	0	0	4.1	4.0	0	
	3.125	0	0	0	0	0	
	0	0	0	0	0	0	
Overall average final % of edema	50.000					100.0	
	25.000					82.1	
	12.500					18.7	
	3.125					2.2	
	0					1.4	

Data in Table II indicate clearly that the stage of development was much affected by the concentration of cortisone in the culture medium. The higher doses of cortisone delayed the development much more than lower ones. It also shows that the developmental stage of the three experiments differed from each other at the same age. It remains to be seen however, whether such variations were the result of differences in parentage of the eggs used, in season of the experimentation, and/or in temperature of the environment.

Cortisone dosage also exerted identical effects on the rate of appearance of edematous larvae (Table III). In the case of the highest dose, *i.e.* 50 mg of cortisone in 100 ml of culture medium, all of the treated eggs developed edema and all of the edematous larvae died within a short period. When the dosage was decreased the edema incidence rate dropped accordingly.

With higher doses of cortisone, like 50 and 25 mg per culture, the edema incidence rate showed a strong tendency to increase against time; while with smaller doses of 12.5 and 3.125 mg the frequency of edema decreased. Apparently it was also because of different parentage that experiment FR6 appeared outstanding when compared with the other two.

The above findings confirmed the effect of cortisone on eggs treated at the early blastula stage as shown in Table I, giving additional support to the role of development in edema production.

It is interesting to note that edema developed also in the controls of FR4 and FR6, but at later stages and with much smaller frequency than in the treated ones.

Cortisone treatment at dorsal lip stage —Experiment FR7

A hundred fertilized eggs, divided in two dishes, were treated with cortisone (12.5 mg per culture) beginning at the dorsal lip stage (stage

TABLE IV

Developmental stage and edema incidence rate of control larvae and those treated with cortisone at dorsal lip stage

Cortisone dosage mg/100 ml	Developmental stage and % of edematous larvae after cortisone treatment				
	2nd day	3rd day	4th day	6th day	8th day
12.5	S 19	20	23	25	25
	E 0	7.2	39.2	40.2	40.2
0	S 20	22	23	25	25
	E 0	0	0	0	0

10). Another 100 eggs, also divided into lots of 50, served as duplicate controls. The result is shown in Table IV.

The data in Table IV reproduced the results shown in Tables I, II and III, in that in cortisone treated eggs retardation of development preceded the appearance of edema, and the retarded larvae caught up in development with the controls before reaching stage 25.

Cortisone treatment at the stages of early blastula, dorsal lip, neurula and hatching on eggs of the same parentage —Experiment FR9

Since the appearance of edema and the rate of development possibly varied with different parentage, it was deemed very necessary to solve this question of genetic differences due to individual frogs by carrying out the following experiments on eggs obtained from the same source.

Two sets of five groups of 100 fertilized eggs, each group divided in two dishes, at early blastula and dorsal lip stages were treated with 50.000, 25.000, 12.500, 6.250 and 3.125 mg of cortisone per dish respectively. The 6th group of 100 eggs was also reared in two dishes, but with pond water only, as the controls.

Two sets of 100 larvae, equally divided in two dishes, at neurula (stage 15) and hatching (stage 20) stages were treated with a single costisone concentration of 12.500 mg per dish. Another two sets of 100 larvae, divided equally and receiving no cortisone, served as the controls.

Developmental stage and incidence of edematous larvae. The results are shown in tables V and VI.

As this experiment (FR9) was performed with eggs collected from a single female at one time and under practically identical environmental conditions, valid comparison of results obtained from cortisone treatment at different developmental stages with different doses became possible. One could readily observe that at the stages of early blastula and dorsal lip the developmental rate of the treated eggs was related directly to the dosage of cortisone (Table V). This dosage-dependent phenomenon holds true, and is even more evident, in the incidence rate of edematous larvae (Table VI). However, cortisone treatment at neurula stage caused only a very low incidence of edema, while treatment at hatching stage produced neither change of developmental rate nor appearence of edema. Therefore it is obvious that there was a difference in sensitivity of the eggs at different stages toward cortisone treatment; a small dose of 12.5 mg of cortisone per culture, which sufficed to exert harmful effect in younger larvae, was comparatively inactive for older ones (Fig. 2).

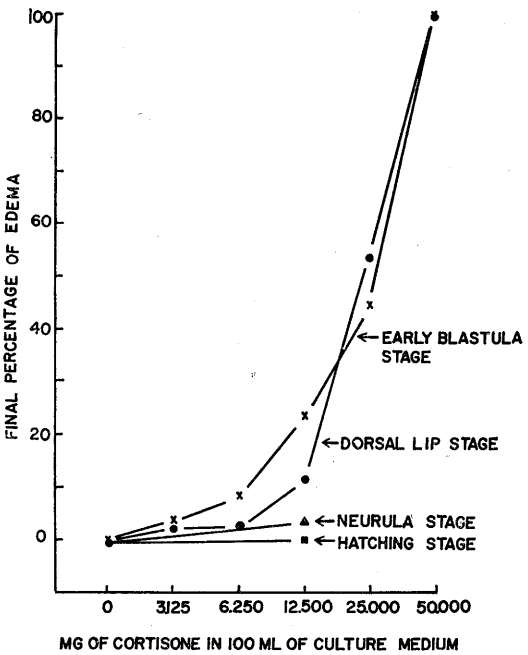

Fig. 2. Edema incidence rate in frog larvae after various doses of cortisone treatment at different developmental stages.

TABLE V

Developmental stage of control larvae and those treated with cortisone at different stages

Stage when treatment began	Cortisone dosage mg/100 ml	Developmental stage after cortisone treatment					
		2nd day	3rd day	4th day	6th day	8th day	10th day
Early blastula	50.000	S12	S16	S18	S19	S23	S25
	25.000	S12	S17	S19	S20	S23	S25
	12.500	S12	S17	S19	S20	S23	S25
	6.250	S12	S17	S19	S20	S23	S25
	3.125	S12	S17	S19	S20	S23	S25
	0	S12	S17	S19	S20	S23	S25
Dorsal lip	50.000	S17	S18	S19	S21	S23	S25
	25.000	S17	S18	S20	S22	S24	S25
	12.500	S17	S18	S20	S22	S24	S25
	6.250	S17	S18	S20	S22	S24	S25
	3.125	S17	S18	S20	S22	S24	S25
	0	S17	S18	S20	S22	S24	S25
Neurula	12.500	S17	S18	S20	S22	S24	S25
	0	S17	S18	S20	S23	S25	S25
Hatching	12.500	S22	S23	S23	S25	S25	S25
	0	S22	S23	S23	S25	S25	S25

TABLE VI

Edema incidence rate of control larvae and those treated with cortisone at different stages

Stage when treatment began	Cortisone dosage mg/100 ml	% of edematous larvae after cortisone treatment				Average final % of edema of all treated eggs
		4th day	6th day	8th day	10th day	
Early blastula	50.000	44.0	98.7	100.0	100.0	
	25.000	31.1	37.8	44.6	44.6	
	12.500	21.1	23.7	23.7	23.7	
	6.250	7.8	7.8	7.8	7.8	
	3.125	0	3.5	3.5	3.5	35.9
	0	0	0	0	0	
Dorsal lip	50.000	100.0	100.0	100.0	100.0	
	25.000	42.0	50.0	53.5	53.5	
	12.500	19.8	16.5	11.8	11.8	
	6.250	4.7	2.4	2.4	2.4	
	3.125	0	0	2.2	2.2	33.9
	0	0	0	0	1.0	
Neurula	12.500	0	0	3.1	3.1	3.1
	0	0	0	0	0	
Hatching	12.500	0	0	0	0	0
	0	0	0	0	0	

In Table VI, it is clear that edema did develop in the control larvaes but it did not appear earlier than stage 25 when the mesonephroi had already started their development (18).

Fig. 2 is a graphic representation of the data in Table VI, showing clearly the susceptibility of the larvae at different stages to cortisone treatment.

Accelerated growth rate of the survived tadpoles after cortisone treatment. All the eggs treated with 50 mg of cortisone developed edema and died within two weeks after fertilization. With 25 mg of cortisone the incidence of edema was lower; but the edematous larvae died first nevertheless and the remaining apparently normal ones also died within three weeks. Still fewer eggs treated with 12.5 mg or less of cortisone developed edema, and some died subsequently, but the normal ones and those which recovered from edema survived. These survivals could withstand a further cortisone treatment for another three weeks and even outgrew the controls. This could be seen from the data on weekly measurement of body length, body width and body weight as shown in Fig. 3 and Fig. 4. Under the influence of cortisone, the increase in body width was more significant than the increase in body length as shown by Table VII.

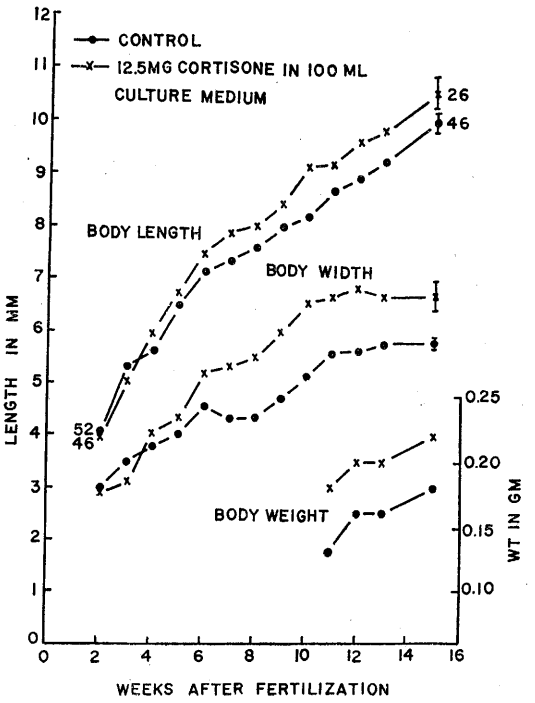

Fig. 3. Growth curves of control and of cortisone treated tadpoles at early blastula stage. The numbers at left and right sides of the top curves denote initial and final number of tadpoles of the experiment respectively. Vertical bars indicate standard errors.

Effect of cortisone on tadpole pigmentation

Besides the effects of cortisone on the developing eggs and larvae stated above, a change on pigmentation of the treated tadpoles was also observed.

Larvae and tadpoles of *R. catesbeiana* before stage 25 were dark, almost black in color. However the cortisone treated tadpoles were paler than the controls during the period of immersion. This lightening effect was more readily demonstrated by injecting cortisone into the peritoneal cavity of older tadpoles. No matter how it was produced, the lightening of pigmentation would disappear and the tadpoles would take up their original shade after cortisone treatment was stopped.

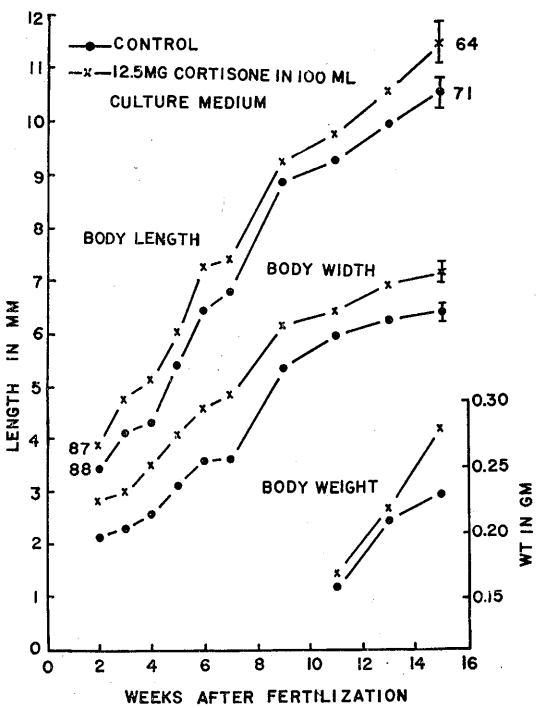

Fig. 4. Growth curves of control and of cortisone treated tadpoles at neurula stage. The rest is the same as that of Fig. 3.

TABLE VII

Differences of body width and body length between control and cortisone treated tadpoles

	Cortisone-treatment stage	Exptl	Control	Difference
Av. final body width in mm±S. E.	Early blastula	6.69±0.27	5.78±0.13	0.91, $P<0.01$
	Neurula	7.16±0.23	6.40±0.18	0.76, $P<0.01$
Av. final body length in mm±S. E.	Early blastula	10.56±0.33	10.07±0.22	0.49, $P=0.22$
	Neurula	11.55±0.38	10.60±0.28	0.95, $P=0.04$

DISCUSSION

The present experiments indicate obviously that the frog embryos reacted to cortisone by four manifestations, *viz.*, retardation of development at early stages, production of edema often leading to death, accelerated growth of the survivals and the lightening effect on pigmentation. While the last manifestation may be independent, the first three appear to be inter-related in some way.

The retardation effect obtained in the present study is not an uncommon result after cortisone treatment because other organisms such as sea urchin eggs (4), chick embryos (1, 2, 3, 6 and 9) and rat embryos (7 and 8) also showed the same effect, the mechanism of which was claimed by Cavellero (16) to be the interference of protein synthesis and by Ross (17) the inavailability of energy utilization due to accumulation of excessive glycogen. The observation in the present experiments that the experimental larvae consumed less yolk and excreted less fecal material favors their view.

As edema in the treated larvae always developed during the period of retardation, it is apt to presume that occurrence of edema and developmental retardation were closely related.

Since edema mostly appeared arround the region of heart and abdomen and seldom on the back or tail, it seems that the excessive fluid gradually accumulated in the coelom. The origin of the fluid and its mode of exudation were unknown. Nevertheless it was a result of an unbalanced fluid exchange mechanism where water intake exceeded output. This might be attributed to the possibility that either intake mechanism was excited or output device suppressed.

The most probable organ or organs involved in water output mechanism may be either the nephric system or the posterior pituitary or both. With the eggs treated at earlier stages edema always appeared at stage 17-20 when pronephroi were just beginning to form (18). Furthermore, since retardation of development coincided with edema, the development of this nephric system was likely inhibited so that water output would be suppressed, leading to the production of edema which caused the death in cortisone treated tedpoles.

On the other hand, when larvae were treated at neurula or hatching stage while pronephroi had been well on their way of development, they became less sensitive to the detrimental effect of cortisone, and therefore the incidence of edematous larvae was very low or non-existent. However the true picture remains to be determined by microscopic study.

Another possible cause of edema is that cortisone might have disturbed water metabolism so that sodium and water were retained in the tissues and fluid finally accumulated in places of least resistance.

It is interesting to note that occasional edema were also observed in the normal controls at advanced developmental stages (at stage 23 for Experiment 4 and after stage 25 for Experiments 6 and 9). The edema in these cases had apparently a different cause.

According to White, the hypophyseal-adrenal cortical mechanism played an important role both in affecting the mobilization of protein reserves in tissue and in systhesizing new protein; he therefore considered that cortisone caused either an anabolic or a katabolic change in protein metabolism depending on its dosage (19). Ingle observed that whereas large doses of steroid hormone might cause a great loss of body weight, cortisone in small doses promoted growth in adrenalectomized rats (20).

In the present experiments, a large dose like

50 mg per culture definitely retarded growth, but with a small dose such as 12.5 mg per culture the retardation effect was less significant or even not observable (Experiments 4, 8 and 9). It was this lower dosage that accelerated growth. This fact corroborates White's and Ingle's findings.

The fact that cortisone caused a more significant increase of body width than of body length points to the possible differential growth effect of this hormone. Wurmbach and Haardick also observed that desoxycorticosterone had a strong effect on growth in breadth, so that spherical bodies resulted (11). Moscona and Karnofsky noticed that the susceptibility of various organs in the chick embryo to cortisone was specific (9). Cortisone might favor certain tissue or organ and at the same time depress others, accounting for more breadthwise increase than lengthwise increase in tadpoles.

Tadpole pigmentation is generally regulated by expansion and contraction of melanocytes and guonocytes. The former are controlled by MSH and melatonin (21). Lerner observed that ACTH could darken frog skin whereas hydrocortisone inhibited the release of MSH leading to a lightening effect (21). These observations lend themselves to explain the lightening effect of cortisone on tadpole pigmentation.

SUMMARY

Fertilized eggs of *R. catesbeiana* were treated with cortisone acetate beginning at early blastula, dorsal lip, neurula and hatching stages, for a period of two weeks with higher doses and five weeks with lower doses respectively. The treated tadpoles showed retarded development, production of edema, accelerated growth of the survivals and lightening effect on pigmentation. The degree of retardation and the percentage of edematous larvae depended on the dosage of cortisone and on the developmental stage when treatment began. The effects of retardation, edema production and accelerated growth appear to be definitely inter-related while the change of pigmentation is interpreted as an independent phenomenon.

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