PREVENTION BY HYDROCORTISONE OF CHANGES IN CONNECTIVE TISSUE INDUCED BY AN EXCESS OF VITAMIN A ACID IN AMPHIBIA

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Experiments described elsewhere have suggested that cortisone can protect the cartilage of rabbits from dissolution by an excess of vitamin A.[1] These findings are in keeping with the general hypothesis that vitamin A in excess releases protease(s) from lysosomes both *in vitro* and *in vivo*,[2-5] and that the action of lysosomal protease is responsible for the subsequent degradation of chondromucoprotein in cartilage matrix.[2,3] In contrast, cortisol and its analogues have been shown to retard the release of acid protease from lysosomes[6,7] and to protect cartilage matrix against an excess of vitamin A.[1,8] Thus it was unexpected, when hypervitaminosis A was induced in the larvae of *Xenopus laevis* by vitamin A alcohol,[9] to find that the simultaneous administration of hydrocortisone accelerated rather than retarded the damage caused by the vitamin.[10] One possible explanation for this phenomenon was that the steroid caused the release of vitamin A from liver stores into the circulation; such an action has been recently documented by McGillivray.[11] Were this effect sufficient to explain the acceleration by hydrocortisone of hypervitaminosis A in Xenopus, one would expect protection by the steroid against an excess of vitamin A acid. The acid form of the vitamin, in contrast to vitamin A alcohol which is stored as the ester, is not stored in liver to any degree.[12]

The experiments to be described below have shown that hydrocortisone did, in fact, protect connective tissue of *Xenopus laevis* against vitamin A acid in excess. The vitamin caused kyphoscoliosis, collapse of rostral tentacles, and frank hemorrhage in the tadpoles; all of these findings were significantly less frequent in larvae given hydrocortisone concurrently. To extend these observations, the effects of thyroxine and epsilon-amino caproic acid (EACA) on hypervitaminosis A in toad larvae were also studied. The former agent initiates metamorphosis, while the latter agent would inhibit the activators of plasmin which could be responsible for some of the lesions in hypervitaminotic larvae.

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Material and Methods

Adult toads (California Xenopus Exchange) were maintained in spring water and fed minced rabbit liver. Larvae were obtained by the breeding of adults in the laboratory, and reared as recommended by Nieuwkoop and Faber.[13] In groups of 12 or 24, they were kept in plastic tanks containing 2 liters of spring water and fed thrice weekly with an aqueous extract of nettle powder (*Herbae urticae*). At the beginning of each set of experiments, larvae were at stages 51 to 53 (late larval stage with early hind-limb protrusion).[13]

Vitamin A acid was obtained through the courtesy of the Hoffman-La Roche Company, Nutley, New Jersey. The yellow crystals were added directly to the feeding mixture in dosages detailed below.

Hydrocortisone sodium succinate (Solu-Cortef,® Upjohn) was dissolved in water immediately before feeding to a final concentration of 25 mg. per l. and given 3 times a week. Larvae were treated twice with the steroid before they were exposed to any other agent concurrently.

Thyroxine (U.S.P.), given twice weekly at 15 mg. per l., was ground by mortar and pestle and added directly to the tanks before feeding.

Epsilon-amino caproic acid (EACA, Lederle) was given daily in a concentration of 250 mg. per l. dissolved in tank water.

Experimental Design and Tabulation of Results. Animals were assigned to various treatment groups after they had been carefully matched for developmental stage. With each experimental group, an equal number of controls were studied; larvae given excess vitamin A acid alone were always included when the effects of several agents on hypervitaminosis A were to be studied. As a standard measure of the lethal effects of various agents alone and in combination, the day when mortality occurred in 50 per cent of the vitamin A-treated group was chosen as reference. On that day, skeletal lesions and hemorrhage in the several groups were compared. The day when 50 per cent of each group were dead was also determined. Experiments lasted a maximum of 30 days. At least 2 members of each group were fixed in buffered formalin; sections were stained with hematoxylin and eosin, and aqueous toluidine blue acidified 1:1,000 with glacial acetic acid.

Results

The Effects of Vitamin A Acid

Gross Features. When vitamin A acid was given in doses of 2.5 mg. per l. twice a week (Table I), changes became apparent on the fourth day of treatment. These were indistinguishable from those described after vitamin A alcohol[9,10] and consisted mainly of collapsed rostral tentacles and kyphoscoliosis (Figs. 1 and 2). No resorption of tail parenchyma appeared then, or at any time. By the sixth day, many of the treated group had sharp hemorrhagic areas in the skin of the head and at the tips of their extremities, which looked not unlike toes painted red with fingernail polish. By this time, a mucinous diarrhea like that previously described[9] was noted. Hyperpigmentation was pronounced, and prognathos appeared towards the end of the experimental period.[9,10] By the eleventh day all treated animals were dead.

To prolong the experimental period, vitamin A was given in most experiments at a dosage of 1.25 mg. per l. twice a week (Mondays and

Thursdays). The results for the longest experiment are given in Text-figures 1 to 3, while the totals for all experiments are listed in Table I. In the former experiment 12 larvae were used; data for 10 are given (2 were fixed on the 20th day). Kyphoscoliosis, tentacle collapse, hemorrhage and death ensued, but later than with higher doses of vitamin A. No loose stools were seen, nor was tail resorption evident. Mean mortality (50 per cent) occurred on the twelfth day in 5 experiments involving 91 larvae.

Microscopic Features. These findings will not be described in detail; in most respects they resembled lesions produced by an excess of vitamin A alcohol.[9,10] The acid, at 2.5 mg. per l. produced not only cartilage changes (Fig. 4) consisting of loss of metachromasia from the matrix of chondrocranial cartilage, but also loss of metachromatic material in tail parenchyma (Fig. 6). In addition, mucous metaplasia of the gastrointestinal tract was noted (Fig. 8); this was similar to changes induced by vitamin A alcohol. Hemorrhagic lesions, in skin and extremities were numerous. It was not possible to assign a definite cause of death, on morphologic grounds, in these or other larvae.

Animals given the lower dose (1.25 mg. per l.) remained free of histologic lesions other than in cartilage matrix or of generalized hemorrhage. Loss of metachromasia in cartilage was evident, and the entire cartilage plate of the skull base was shrunken. Hemorrhages into the extremities were readily seen. No changes in the gastrointestinal tract could be consistently identified, nor was there any appreciable dissolution of metachromatic material in the tail.

The Effects of Hydrocortisone Administered Concurrently with Vitamin A Acid

Gross Features. In animals given the higher doses of vitamin A (2.5 mg. per l.) the concurrent administration of hydrocortisone effectively reduced the incidence of skeletal lesions by half, and substantially inhibited hemorrhage (Fig. 3). No mucinous diarrhea developed, and 50 per cent mortality was delayed to the tenth day.

To extend this sequence of events, vitamin A dosage was reduced, while hydrocortisone was given as before. Four such experiments were performed (Table I); the longest experiment is shown in Text-figures 1 to 3. Hydrocortisone protected all except 2 of the larvae from skeletal lesions, and prevented the appearance of hemorrhage. Protection against the lethal effects of the vitamin is seen in Text-figure 1. Hydrocortisone reduced the incidence of skeletal lesions in these larvae from 97 per cent to 13 per cent (Table I), and reduced the incidence of frank hemorrhage from 50 per cent to 6 per cent (Table I). The general develop-

TABLE I

THE EFFECTS OF VARIOUS AGENTS ON *Xenopus laevis* LARVAE

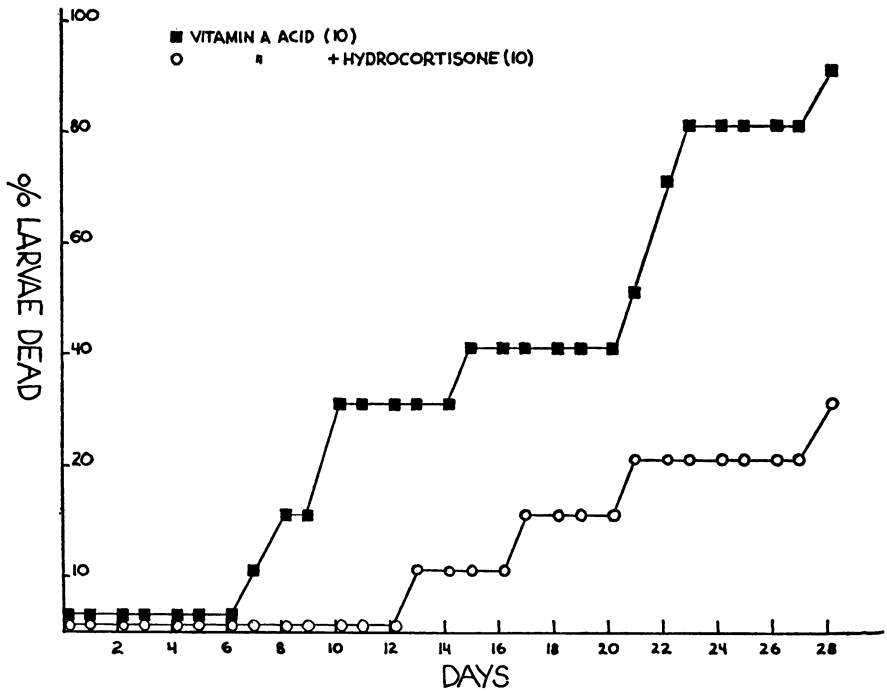
Treatment	No. of individual experiments	Total no. of larvae	Mean day of 50% mortality	Larvae with skeletal lesions		Larvae with hemorrhage		Other observations
				No.	Per cent	No.	Per cent	
Vitamin A acid (2.5 mg./l. 2 ×/wk.)	2	24	7	24	100	18	72	Mucinous diarrhea
Vitamin A acid (2.5 mg./l. 2 ×/wk.) *plus* Hydrocortisone (25 mg./l. 3 ×/wk.)	1	12	10	6	50	0	0	No diarrhea
Vitamin A acid (1.25 mg./l. 2 ×/wk.)	5	91	12	88	97	45	50	No diarrhea; prognathos
Vitamin A acid (1.25 mg./l. 2 ×/wk.) *plus* Hydrocortisone (25 mg./l. 3 ×/wk.)	4	67	17	9	13	4	6	No prognathos; retarded development
Hydrocortisone (25 mg./l. 3 ×/wk.)	2	36		0	0	0	0	Retarded development
Vitamin A acid (1.25 mg./l. 2 ×/wk.) *plus* Thyroxine (15 mg./l. 2 ×/wk.)	2	36	7	25	70	19	53	Accelerated development
Thyroxine (15 mg./l. 2 ×/wk.)	2	36	13	0*	0	2	6	Accelerated development
Vitamin A acid (1.25 mg./l. 2 ×/wk.) *plus* EACA † (250 mg./l. daily)	1	24	10	24	100	0	0	
EACA (250 mg./l. daily)	1	24		0	0	0	0	
None	6	217		0	0	0	0	

* Resorption of tentacles in those larvae undergoing full metamorphosis.

† EACA = epsilon-amino caproic acid.

ment of such larvae was somewhat slower than controls, they were shorter, and their forelimb development much delayed.

Microscopic Features. Animals given higher doses of vitamin A acid (2.5 mg. per l.) and hydrocortisone failed to develop mucous metaplasia of the intestine (Fig. 9). In 5 of 6 animals examined, protection against

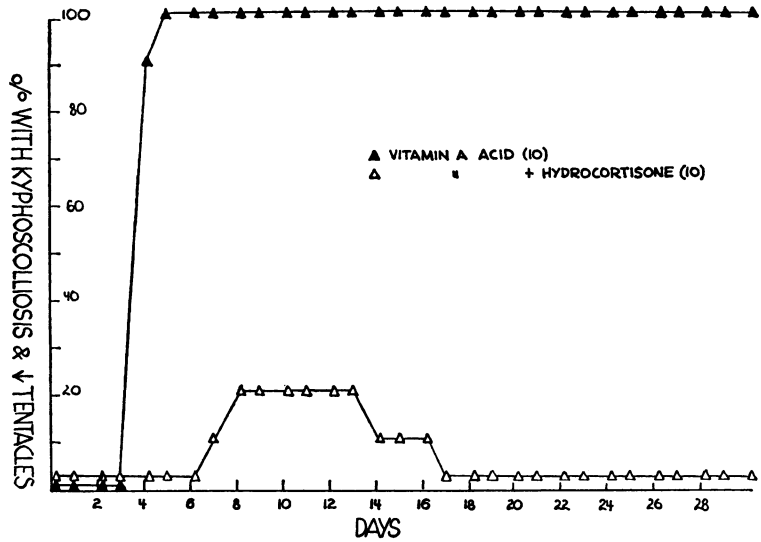


TEXT-FIGURE 1. Deaths of *Xenopus laevis* larvae. Vitamin A acid (1.25 mg. per l.) was given twice weekly beginning on day 1. Hydrocortisone (25 mg. per l.) was given for 2 feedings before day 1, thrice weekly thereafter.

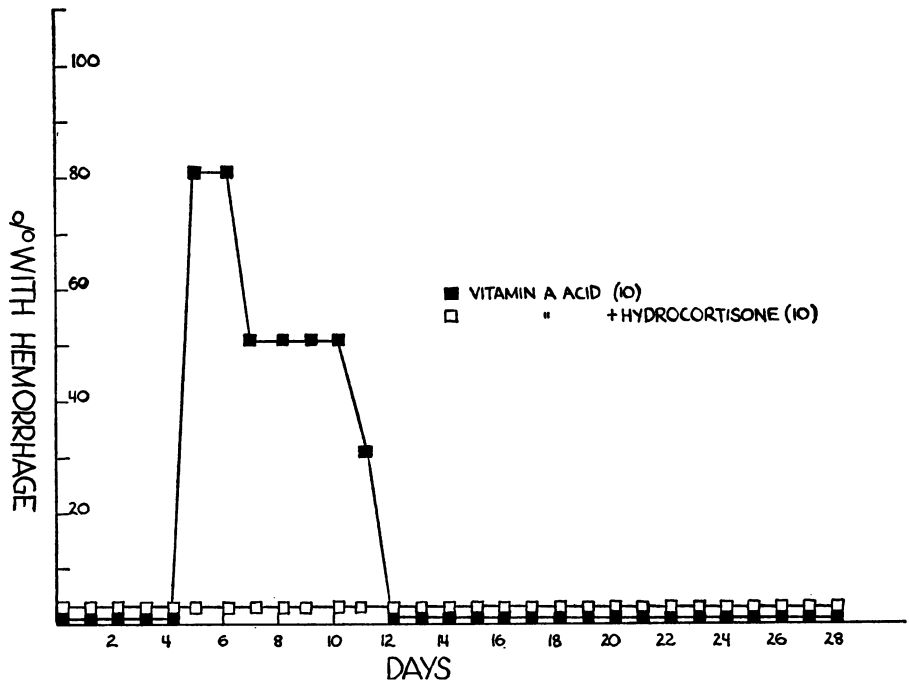
the loss of metachromatic material in tail and cartilage was observed (Figs. 5 and 7). In all essential respects, these animals did not differ histologically from control animals. One of the 6 animals showed changes indistinguishable from larvae not treated with hydrocortisone.

The Effects of Hydrocortisone Alone on Xenopus Larvae

Gross and microscopic characteristics of this treatment have been described before.[10] However, some new observations on the action of hydrocortisone during metamorphosis of the larvae have been made. Six control larvae and 6 given hydrocortisone throughout the period of tail resorption were examined. Treatment was begun when forelimb rudiments were just evident. The final development of these structures was delayed by as much as 2 weeks over controls, which took 6 to 7 days for



TEXT-FIGURE 2. Incidence of skeletal lesions in *Xenopus laevis* larvae. Dosage as in Text-figure 1. Figures 2 and 3 demonstrate the gross appearance of hypervitaminotic larvae and their hydrocortisone-treated mates.



TEXT-FIGURE 3. Incidence of hemorrhage in *Xenopus laevis* larvae. Dosage as in Text-figure 1.

development. Whereas each of the control larvae took only 3 to 4 days to resorb its tail (measured from early tip friability) the hydrocortisone treated-larvae did not resorb their tails until the 14th to 18th days. Histologic examination of the thyroid of the hydrocortisone-treated animals revealed less colloid, and a more cuboidal appearance of the thyroid cells, than in control animals.

The Effects of Thyroxine and Vitamin A Acid

If hydrocortisone slowed metamorphosis, and inhibited hypervitaminosis A, it might be expected that thyroxine, which has a well-known metamorphotic action, would accelerate hypervitaminosis A. Animals given thyroxine together with vitamin A died much earlier than those given either vitamin A or thyroxine alone (Table I). Grossly, only those lesions already described for hypervitaminosis A alone were noted and these occurred earlier. However, the blood vessels, which were easily visible through the transparent skin, were markedly dilated, and hemorrhages which extended to the pericardial sac were more pronounced than in animals given the vitamin alone. Development was accelerated. Microscopically there were hemorrhages into the capsule of the liver and into the pericardium; there was also loss of metachromasia in cartilage. Animals given this dose of thyroxine alone had the expected acceleration of development towards full metamorphosis. These showed resorption of rostral tentacles, as in the later stages of normal metamorphosis.

The Effects of EACA and Vitamin A Acid

No gross or microscopic lesions were seen in larvae given EACA alone. When EACA was given with excess vitamin A, all of the skeletal lesions regularly brought about by vitamin A in excess were seen, but no hemorrhages were noted (Table I). This was confirmed histologically; disappearance of cartilage metachromasia was as readily apparent in these larvae as in animals given vitamin A alone; no microscopic hemorrhages were seen.

Discussion

The experiments described above show that manifestations of an excess of vitamin A acid in larvae of *Xenopus laevis* can be largely prevented by the simultaneous administration of hydrocortisone. This observation is in contrast to the acceleration by hydrocortisone of hypervitaminosis A induced by vitamin A alcohol in toad larvae.[10] In other species, cortisone has been found to release vitamin A from liver stores into the circulation.[11,14] The results are therefore compatible with the

hypothesis that acceleration of vitamin A alcohol toxicity by hydrocortisone was caused by the exposure of larval tissues to a continuous circulation of higher doses of the vitamin than could be antagonized by the steroid.[10] An alternate explanation may be equally possible. In rabbits, the re-synthesis of cartilage matrix after dissolution by papain is effectively inhibited by cortisone.[15] Thus, in the long-term experiments with vitamin A alcohol, breakdown of connective tissue caused by excess vitamin A could be augmented by a failure of the cortisone-treated larva to re-synthesize its own chondromucoprotein-like materials. The net result would be an apparent acceleration of hypervitaminosis A by cortisol. In the present, shorter experiments with vitamin A acid, this action of hydrocortisone may not have been a factor.

These experiments also support the findings of Thomas, McCluskey, Li and Weissmann,[1] who showed protection by cortisol and its analogue of rabbit cartilage *in vivo* from the toxic effects of vitamin A palmitate and acid. The experiments also support the findings of Fell and Thomas[8] who described protection by hydrocortisone of chick cartilage *in vitro* against excess vitamin A. Disruption of lysosomes by vitamin A, with release of hydrolytic enzymes, has been held responsible for the dissolution of matrix by excess vitamin.[3-5] This has been considered to result from the direct action of vitamin A on lysosomes. However, excess vitamin A may also act upon the surfaces of erythrocytes[16] and on other subcellular particles;[17] an effect on lysosomes may be a single manifestation of a general biologic action of the vitamin on membranes.

Pre-metamorphotic larvae such as those of Xenopus provide an excellent *in vivo* system in which to examine the effects of various agents on susceptible lysosomes. These organelles appear to be associated with the autodigestive and resorptive processes of metamorphosis.[18,19] Thus, tentacles, tails and chondrocranium, which undergo resorption and remodeling in metamorphosis are the special targets for the action of vitamin A in excess. Protection of these structures by hydrocortisone would lend support to the hypothesis that cortisol may act, at least in part, by the stabilization of lysosomes against injury by several agents.[5-7]

Our experience with vitamin A acid in excess parallels the preliminary findings of Thompson and Pitt.[20] These workers reported that vitamin A acid caused bone resorption and hemorrhages in rats more rapidly and at lower doses than did other forms of the vitamin. We have also found the acid to be more toxic at lower doses and to produce lesions earlier than the alcohol. These observations support the speculation[17] that vitamin A acid is more directly active on tissues (other than eye or gonads) than are alcohol, aldehyde or esterified forms of the vitamin.

At lower doses of vitamin A acid (1.25 mg. per l.), little damage to

connective tissue of larval tails could be identified histologically, nor was there evidence of mucous cell metaplasia in the intestine. Both of these changes have been described as due to an excess of vitamin A alcohol,[10] and were readily elicited by higher doses of vitamin A acid (2.5 mg. per l.). Resorption of tail substance was observed only after 3 weeks in vitamin A alcohol-treated larvae; by this time most A-acid-treated larvae were already dead.

Acceleration by thyroxine of vitamin A acid-induced damage to connective tissue might be interpreted as indicating an additive effect of thyroid hormone and the vitamin on lysosomes. It may also reflect a generally increased metabolic activity of the affected larvae, or simply be the result of an additive toxicity of hormone and vitamin upon membrane systems in general. The deleterious effects of thyroxine on mitochondria have been extensively studied; no data are available suggesting an effect upon lysosomes as well. No evidence for such an action has been obtained from experiments with lysosome-rich fractions of rabbit liver.[21] Acid hydrolases were not more readily released from a large granule fraction of rabbit liver if this had been prepared from animals pretreated with thyroxine. The morphologic findings described above, therefore, cannot be taken as evidence of direct lysosomal effects of thyroxine, since accelerated metabolic activity caused by the hormone may be reflected by a number of secondary phenomena.

Inhibition by EACA of hemorrhages induced by hypervitaminosis A would suggest that these are caused, at least in part, by an effect upon the activators of plasmin.[22] Since other evidences of vitamin A-induced changes in cartilage or connective tissue were not diminished by EACA, alterations in the activators of plasmin may not play a role in the production of these lesions.

Summary

Hypervitaminosis A was induced orally by means of vitamin A acid in larvae of *Xenopus laevis*. Grossly, this treatment resulted in kyphoscoliosis, resorption of rostral tentacles, and hemorrhages into the skin and extremities. Microscopically, loss of metachromasia in cartilage and connective tissue was found. These changes were largely prevented by the simultaneous administration of hydrocortisone. Differences between the effects of hydrocortisone upon the lesions induced by vitamin A alcohol and those induced by vitamin A acid were related to the liberation of excess vitamin A from liver stores by the former. Thyroxine accelerated the vitamin A-induced changes in connective tissue; epsilon-aminocaproic acid prevented hemorrhage. The actions of hydrocortisone were compatible with the hypothesis that this agent stabilizes lysosomes against damage by an excess of vitamin A.

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[*Illustrations follow*]

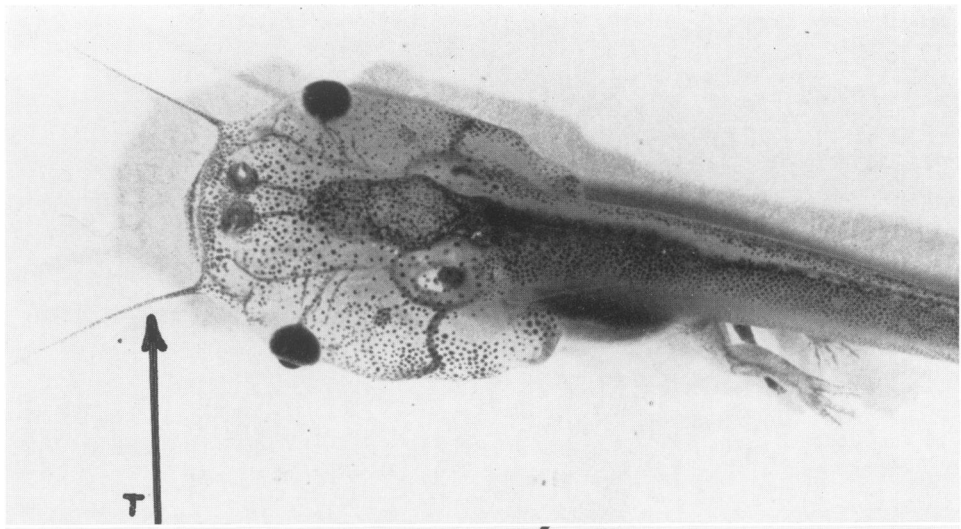
Legends for Figures

Photomicrographs were prepared from sections stained with toluidine blue.

Fig. 1. Normal larva of *Xenopus laevis*, stage 56. The back is straight and rostral tentacles (T) are erect. × 4.

Fig. 2. Larva of *Xenopus laevis* exposed to vitamin A acid (2.5 mg. per l.) for 7 days. There are collapse and resorption of tentacles (T), hemorrhage in the head (H), and marked kyphoscoliosis (K). Hyperpigmentation is also manifest. Stage 56. × 4.

Fig. 3. Larva of *Xenopus laevis* exposed to vitamin A acid (2.5 mg. per l.) and hydrocortisone (25 mg. per l.) for 7 days. Tentacles are erect (T) and the back relatively straight. No hemorrhages are seen. Stage 56. × 4.



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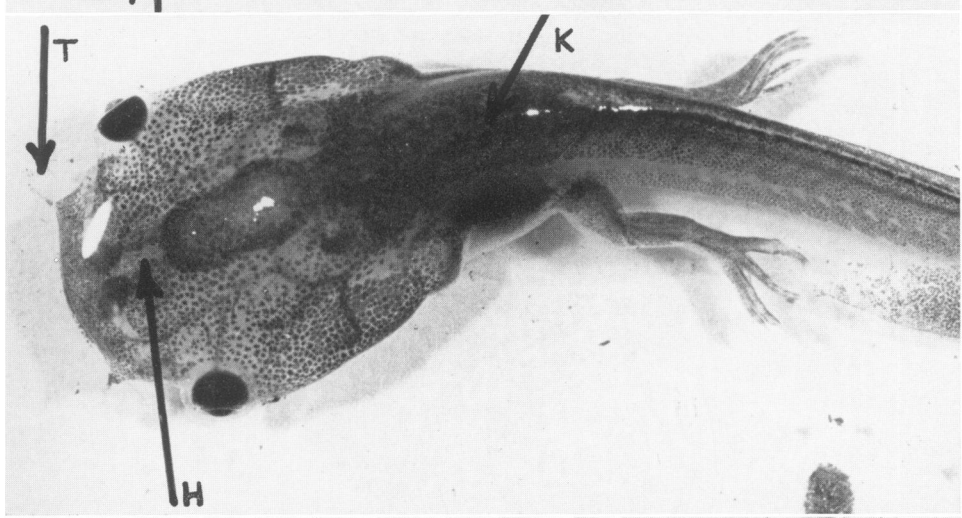

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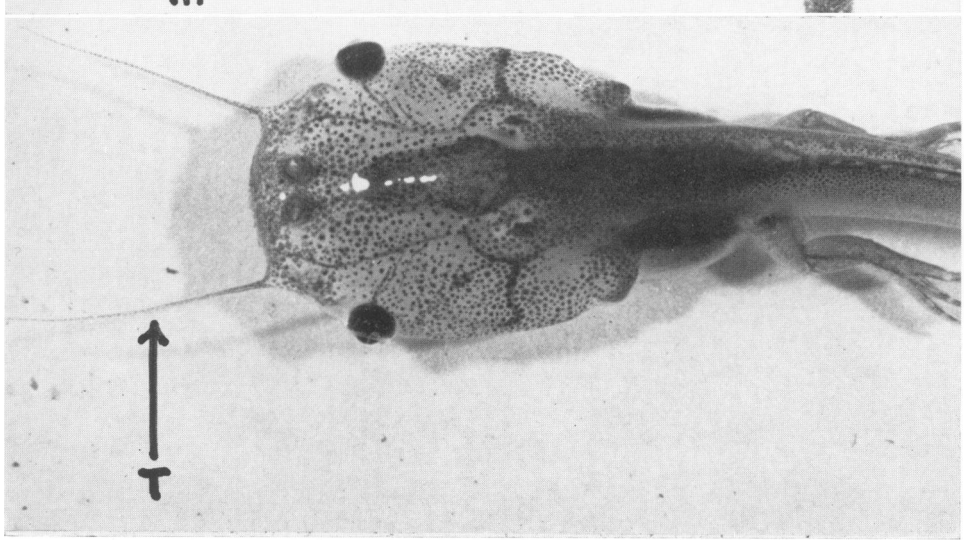

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FIG. 4. Chondrocranial cartilage from larva of *Xenopus laevis* exposed to vitamin A acid (2.5 mg. per l.) for 7 days. Progressive loss of metachromasia and crowding of cells are apparent. × 80.

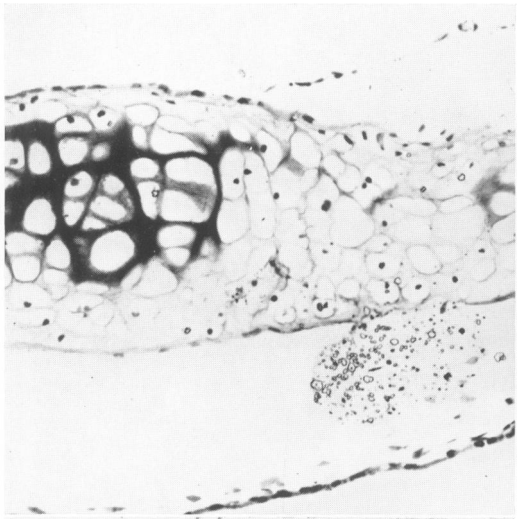
FIG. 5. Chondrocranial cartilage from larva of *Xenopus laevis* exposed to vitamin A acid (2.5 mg. per l.) and hydrocortisone (25 mg. per l.) for 7 days. Abundant metachromatic matrix appears between chondrocytes. × 96.

FIG. 6. Tail of *Xenopus laevis* larva exposed to vitamin A acid (2.5 mg. per l.) for 7 days. No metachromatic material is seen. × 125.

FIG. 7. Tail of *Xenopus laevis* larva exposed to vitamin A acid (2.5 mg. per l.) and hydrocortisone (25 mg. per l.) for 7 days. Abundant metachromatic material (M) is present; this section is indistinguishable from controls. × 96.

FIG. 8. Gastrointestinal tract of *Xenopus laevis* larva exposed to vitamin A acid (2.5 mg. per l.) for 10 days. There is overgrowth of metachromatic goblet cells in a segment of proximal intestine. × 960.

FIG. 9. Gastrointestinal tract of *Xenopus laevis* larva exposed to vitamin A acid (2.5 mg. per l.) and hydrocortisone (25 mg. per l.) for 10 days. Cells are entirely normal in appearance. × 960.



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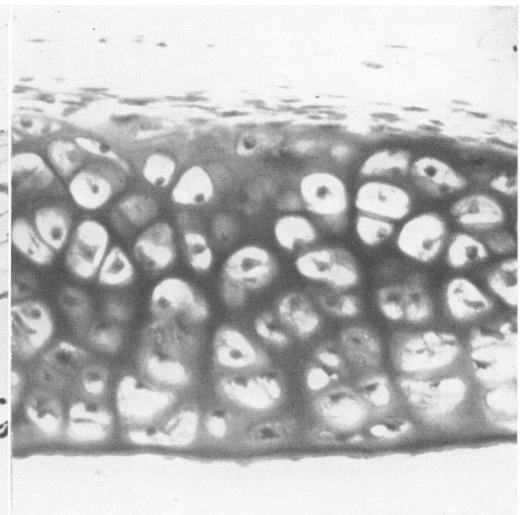

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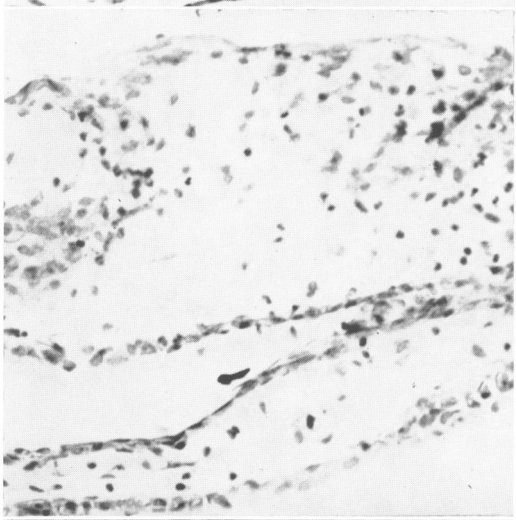

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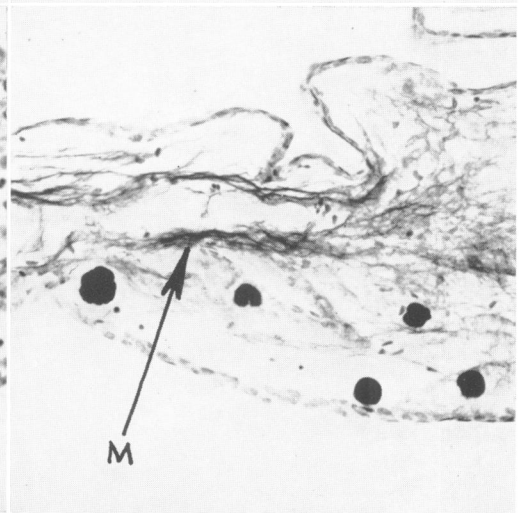

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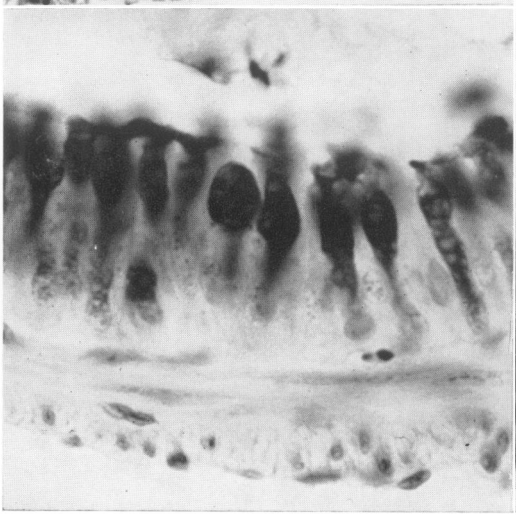

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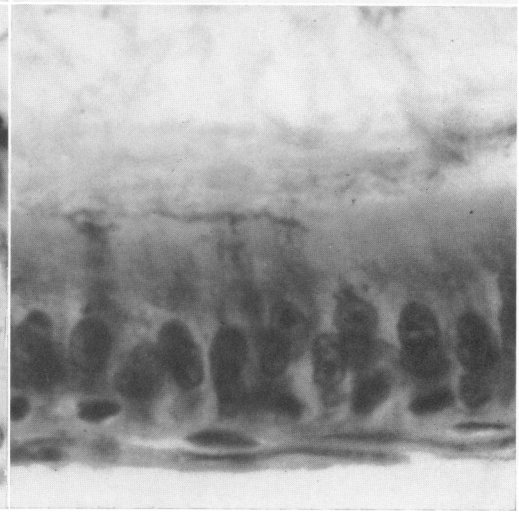

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