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Testosterone Propionate Minimum for Induction of Male Development in Anurans; Comparative Data from Other Vertebrates.*

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After the discovery[1,2] that administration of male sex hormone to anuran tadpoles can induce testicular development in genetic females, it became of some interest to study the sensitivity of the system in terms of the smallest quantity of androgen which could effect such a change. In previous investigations in which tadpoles were raised from the time of hatching to metamorphosis in water containing graded concentrations of testosterone propionate, male differentiation irrespective of genetic constitution was observed in *Rana sphenocephala, Rana pipiens*, and *Pseudacris triseriata* at 0.01 mg (10 μg) of hormone per liter of aquarium water, a concentration of 1:100,000,000.[3,4] From all indications, however, it appeared that the minimal effective dose might well be considerably below any so far employed. Such has since been found to be the case (preliminary report by Mintz and Witschi[5]).

Materials and Methods. *Rana sylvatica* eggs were collected near New Haven, Conn. in March and April and treatments were initiated 6-8 days after hatching. Controls preserved at this time were entirely undifferentiated as to sex. Groups of 50-100 larvae, including untreated controls, were each kept in 10 liters of aerated water. Solutions of crystalline testosterone propionate† in 95% ethyl alcohol were added daily from calibrated syringes to the following concentrations in different groups: 1,000, 200, 40, 10, 2, 1, 0.1, and 0.01 μg/liter. The alcohol volume measured 0.15 ml/liter of water. These animals were preserved in Bouin's fixative approximately 30 days later during metamorphosis when one foreleg perforated the opercular covering. Individuals that died were preserved only if they appeared to be still in good condition. The temperature of the amphibian room averaged 20°C but occasionally dropped down to 16°C.

Results. As seen in Table I, tadpoles exposed to 2 μg or more of hormone/liter emerge exclusively as phenotypic males indistinguishable from control males, with the exception of 2 male-like intergrades and one female at 10 μg/liter. It is possible that the latter non-conformist was accidentally transferred from one of the lower dosage jars. The amount of 2 μg/liter, still inducing complete masculinization of all genetic females, corresponds to a concentration of 1:500,000,000. One half of this quantity, 1 μg/liter or 1:1,000,000,000, represents the threshold value for masculinization inasmuch as it produces a preponderance of males and masculine intersexes.

Among the controls, we observe a moderate excess of females. In a population of this size, the probability of obtaining a deviation from the expected 1:1 ratio of males : females (actually 58 males : 58 females) due to the error inherent in random sampling would bring the acceptable range to the extremes of

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† The author wishes to thank the Ciba Pharmaceutical Products, Inc., for supplying testosterone propionate (Perandren).

1 Gallien, L., *C. R. Acad. Sci.*, 1937, **205**, 375.

2 Witschi, E., and Crown, E. N., *Anat. Rec. Suppl.*, 1937, **70**, 121.

3 Witschi, E., *Cold Spring Harb. Sympos. on Quant. Biol.*, 1942, **10**, 145.

4 Witschi, E., Foote, C., and Mintz, B., *Anat. Rec. Suppl.*, 1942, **84**, 455.

5 Mintz, B., and Witschi, E., *Anat. Rec. Suppl.*, 1946, **96**, 30.

TABLE I.
Sex distribution in *Rana sylvatica*.

Treatment	♀	♀	♂ +	♂ •	♂
Testosterone propionate					
1000 μg/l	—	—	—	—	30
200 "	—	—	—	—	38
40 "	—	—	—	—	46
10 "	1	—	—	2	131
2 "	—	—	—	—	77
1 "	13	2	3	7	66
0.1 "	45	2	4	6	40
0.01 "	47	—	2	2	37
Controls	68	—	4	—	44

47 : 69 in favor of either sex, as calculated by 3 P.E. or 2 S.E. The observed distribution of 48 males and intersexes : 68 females falls within these limits but is close to the extremes and, together with the occurrence of 4 hermaphrodites, may therefore indicate a slight deviation toward the semi-differentiated racial type.

Discussion. It is logical to assume that the tadpole does not utilize all of the hormone available in its ambient environment. This would certainly appear to be indicated judging from Foote's[6] measurements of the slow rate of disappearance of estrone from the aquarium water in which *Rana pipiens* tadpoles were kept. Although it may well be that the animals are able to concentrate the absorbed hormone to critical levels, we observe at any rate that they exhibit a remarkable ability to "detect" exceedingly minute amounts of androgen to which they respond with a marked alteration in morphogenesis. This testifies to the extreme lability of the embryonic sex inductor system in anurans.

The method outlined might serve as an assay procedure for ascertaining the presence of very small quantities of testosterone not otherwise determinable. A series of experimental groups treated with successively diluted aliquots of the preparation in question would indicate that proportion of the total which contained the minimal effective dose for male differentiation.

Table II summarizes data on sensitivity of some of the more widely studied vertebrates to testosterone propionate. Because of the different modes of hormone administration, the differing extents of changes produced as well as total weight of the test animal, and, presumably, differences in economy in utilizing the hormone, we must of course regard such comparisons with some degree of reservation. The tests cited indicate more recently reported lowest levels at which an effect can still be obtained; credit is not here necessarily given to the originator of the test. Literature on quantitative bioassays has by now become impressive in volume, and includes some disagreement on threshold dosages. For purposes of comparison, the threshold dose for masculinization of *Rana sylvatica* is expressed as μg/ml since this amount of solvent is considerably closer to the volume administered to other test animals than is the "per liter" designation.

In examining these data, it is apparent that the anuran larva displays a much greater reactivity to testosterone propionate as compared with other vertebrates studied, and that the nature of the response, involving the primary sex characters, is a relatively more drastic one.

There are indications of the existence of species differences in response among anurans. Treatment of a small number of Hyla arenicolor larvae with the 2 μg/liter dose resulted in approximately equal numbers of males and females, plus a few hermaphrodites.

Summary. An amount of testosterone propionate as small as 1 μg/liter of aquarium water, a concentration of 1 : 1,000,000,000, is sufficient to induce male development in a

[6] Foote, C. L., *J. Exp. Zool.*, 1941, **86**, 291.

TABLE II.
Testosterone Propionate, Minimal* Effective Dosages.

Test animal	Diagnostic effect	Method of administration	No. of days	Dosage	Reference
Brown Leghorn capon	Comb growth	Inj. (intramus.) daily	4-5	5-25 μg/d	Koch[7]
Brown Leghorn capon	,, ,,	Local application, daily	10	.01-.1 μg/d	,,
English sparrow capon	Bill blackening	Inj. (intramus.) daily	6	.2 μg/d	Witschi[8]
English sparrow capon	,, ,,	Local application, daily	4-16	.06 μg/d†	Pfeiffer, Hooker, and Kirschbaum[9]
Rat ♀§ (8 days old)	Prostate	Inj. daily	6	1 μg/d	Price[10]
Rat ♂§ (8 days old)	Prostate, seminal vesicles	,, ,,	6	1 μg/d	,,
Rat ♀, pregnant	Masculinization of secondary sex char's. of ♀ embryos	Inj. once	—	2500 μg	Greene and Ivy[11]
Hamster ♀, pregnant	Masculinization of secondary sex char's. of ♀ embryos	,, ,,	—	1000 μg	Bruner and Witschi[12]
Mouse ♀, pregnant	Masculinization of secondary sex char's. of ♀ embryos	,, ,,	—	1000 μg	Turner[13]
Rana sylvatica	Male development	Immersed	30‡	0.001 μg/ml	

* Not all workers on mammals listed have tried doses below those given, so that the minima in some cases may be lower.
† Minimum for testosterone rather than testosterone propionate. Positive results with 2 μg/d of testosterone propionate for 4-5 days but minimum not determined.
‡ The critical time has not been determined. Possibly the results could be read sooner.
§ Hermaphrodite.
7 Koch, F. C., 1946, personal communication.
8 Witschi, E., *Anat. Rec. Suppl.*, 1943, **87**, 478.
9 Pfeiffer, C. A., Hooker, C. W., and Kirschbaum, A., *Endocr.*, 1944, **34**, 389.
10 Price, D., *Physiol. Zool.*, 1944, **17**, 377.
11 Greene, R. R., and Ivy, A. C., *Science*, 1937, **86**, 200.
12 Bruner, J. A., and Witschi, E., *Am. J. Anat.*, 1946, **79**, 293.
13 Turner, C. D., *J. Morph.*, 1939, **65**, 353.

large majority of *Rana sylvatica* larvae raised in such a solution from hatching to metamorphosis. The relationship of these results to similar work on other vertebrates is discussed.

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Concerning the Relation Between Pituitary Adrenocorticotrophin and the Circulating Blood Platelets.

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Although it has been known for some time that infection, trauma, hemorrhage, asphyxia, etc. result in rather marked alterations in the number of circulating blood platelets, the exact mechanism controlling the number of platelets is as yet unknown. With the discovery by Dougherty and White[1] that pituitary adrenocorticotrophin caused a dissolution of lymphoid tissue and a peripheral lymphopenia a new line of investigation was opened up. Since the conditions under which changes in the platelets have been reported are those which would stimulate a release of pituitary ACTH, and since this hormone has been shown to affect other blood elements through the adrenal, it did not seem too unreasonable to believe that the platelets might also be at least to some extent under the control of corticotrophin.

Consequently experiments were undertaken in both rats and humans to determine whether any effect of ACTH upon the blood platelets could be demonstrated. As far as could be determined by these experiments, there is no effect upon the platelets of either species by this hormone.

Experimental. Sixteen 100-150 g, hooded, male rats were injected subcutaneously with 80 mg of a suspension of acetone-dried hog pituitary after two control counts had been made, and determinations of the platelets* and RBC were made from the capillary tail blood every hour for 8 hours. No significant change was seen to occur in either blood element.

Six rats were injected in a similar manner with 40 mg of hog pituitary powder every morning for 3 days. Again there was no significant change in the number of platelets.

Four rats were injected with 10 mg of a purified pituitary corticotrophin preparation and counts made every hour for 8 hours with similarly negative results.

Following these experiments a preparation of pituitary ACTH† which was quite pure was made available to us, and it was decided

[1] Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

* Three methods were used to count the platelets: (1) that of Rees and Ecker[2]; (2) a direct chamber count using 3.8% sodium citrate as the diluting fluid; (3) an indirect count of a wet preparation containing platelets stained with brilliant cresyl blue. The direct counts were used chiefly on the rats and the indirect on the human subjects.

[2] Rees, H. M., and Ecker, E. E., *J.A.M.A.*, 1923, **80**, 621.

† Kindly supplied by Dr. John R. Mote of the Armour Laboratories, Chicago, Ill. It was believed that each vial in the lot supplied (Lot G-59703-H) contained the equivalent of 12 mg of the Armour ACTH standard. After the completion of these experiments Dr. Mote informed us that on re-assay each vial contained only the equivalent of 5.2 mg of their standard. The doses given in this paper conform to the final assay.