ADRENALIN AND HYDROXYTYRAMINE IN THE PAROTID GLAND VENOM OF THE TOAD, BUFO MARINUS*

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Nearly all studies on the biosynthesis of adrenalin have concerned themselves with the ability of adrenal medullary tissue to convert supposed intermediates *in vitro* to adrenalin (*cf.* Blaschko, 1950). An exception is the work of Gurin and Delluva (1947) who demonstrated by a tracer technique the conversion of phenylalanine to adrenalin *in vivo*. The direct isolation of intermediates from adrenal tissue has not been reported. With the development, however, of methods of paper chromatography which can separate and identify adrenalin and related orthodiphenols (James, 1948) a more direct approach to the problem of adrenalin synthesis has been made available. The adrenals of man and several other animals have been investigated for the presence of possible adrenal precursors. In none of these cases, however, could any precursors be found except noradrenalin (Outschoorn, 1951; Goldenberg *et al.*, 1949; von Euler and Hamberg, 1949; Crawford, 1951). It appeared that orthodiphenols other than noradrenalin do not accumulate in quantities detectable by even this most sensitive of techniques.

It was with these facts in mind that this investigation was undertaken. The parotid gland venom of many toads has long been known to contain adrenalin (Abel and Macht, 1912; Chen and Chen, 1933). Since the amounts of adrenalin are truly enormous, it seemed possible that demonstrable quantities of precursor substances might be found in this material by chromatographic methods. Furthermore, Bacq and Lecompte (1947) using biological assay and colorimetric means, have shown that in *Bufo arenum* parotid venom there occur phenolic substances in addition to adrenalin. Their relationship to adrenalin was suggested by the fact that denervation of the parotid glands caused a decrease in the adrenalin content and a net increase in the content of phenolic substances. Bacq and Lecompte also noted that there did not seem to be present any sympathomimetic substances but adrenalin—*e.g.*, noradrenalin—though it is obvious that the biological assay for such substances in the presence of large amounts of adrenalin is difficult.

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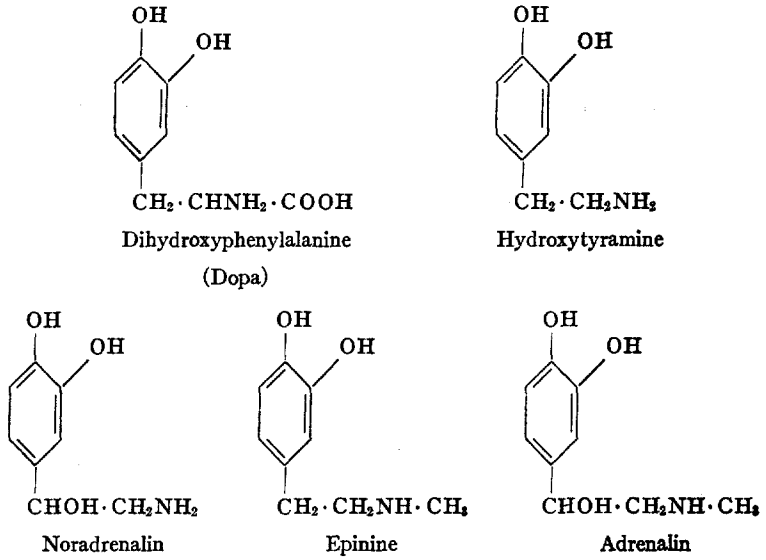

We obtained a number of specimens of the toad *Bufo marinus* from Puerto Rico in mid-March of this year.[1] The animals were kept in a cool aquarium containing moist sphagnum moss and a pan of water. They were force-fed with horsemeat about once a week and appeared to be in excellent condition. The parotid venom was collected by squeezing the gland while holding an inverted Petri dish over it at a distance of several inches. The venom is ejected from small openings on the surface of the gland and sticks to the dish as a white or yellow gum which quickly solidifies to a brittle mass. After drying the material in a desiccator for about a day, it is removed from the collecting dish and ground to a fine powder.

It is necessary to separate the phenolic substances of the venom from the steroids, indole derivatives, and proteins comprising the bulk of the material. Direct extraction with water leads to difficulties, and the following method of extraction was finally adopted.

One hundred mg. of the powdered secretion was extracted with 25 ml. of acidic ethanol (1 ml. concentrated hydrochloric acid per 100 ml. 95 per cent ethanol) by allowing the powder to stand in the solvent overnight at room temperature. At this point the ethanolic solution was usually yellow and the solid, originally yellow, was colorless. After filtering off and discarding the solid material, the ethanolic solution was evaporated to dryness under reduced pressure. Twenty-five ml. of water was added to the flask and the residue was shaken into suspension. After centrifuging the suspension and discarding the solid material, one obtains an aqueous extract from which the bulk of the proteins and steroids have been eliminated. There is still, however, enough extraneous material present to interfere with the proper develop-

[1] I am indebted to Mr. Juan A. Rivero-Quintero who made this investigation possible by securing these animals for me.

ment of a chromatogram. In order to free contained catechol derivatives further from interfering substances, the aqueous extract was subjected to the procedure of von Euler (1948) which relies upon the fact that certain catechol derivatives are adsorbed on aluminum hydroxide at pH 7. The details of the method are as follows:—

To the aqueous extract is added aluminum sulfate to make the final concentration 0.1 per cent. The pH is adjusted electrometrically to 7.5 by the dropwise addition of 2 N sodium hydroxide, and the precipitated aluminum hydroxide is centrifuged down. The supernatant solution is discarded and the precipitate containing the adsorbed catechol derivatives is dissolved in several milliliters of 6 N sulfuric acid. The acid solution is brought to pH 4.0 with 2 N sodium hydroxide, and four volumes of 95 per cent ethanol are added. The solution containing the precipitated salts is allowed to remain in the refrigerator overnight, and the salts are filtered off. The nearly colorless ethanolic solution is evaporated to dryness under reduced pressure. As much of the residue as will dissolve is taken up in 1 ml. of acidic ethanol. This is the final extract, from which aliquots were taken for chromatographic examination.

The chromatograms were run according to the method of James (1948). 0.02 to 0.03 ml. of the final extract was applied to the filter paper as a spot several millimeters in diameter. Control solutions of adrenalin, hydroxytyramine, etc. were prepared in acidic ethanol and applied as spots containing approximately 10 μg. of the free base each. The developer used was a mixture of 88 per cent phenol and 12 per cent water in which the chromatograms were run for 18 to 30 hours. In every case the chromatogram was freed from the developing solvent by heating at 90–100°C. for 1 hour before spraying with an indicator.

Three indicators were used to demonstrate the presence of orthodiphenols. One was the potassium ferricyanide reagent of James (1948) which oxidizes the phenols to colored pigments and easily demonstrates 5 μg. The second was a 1 per cent solution of ferric chloride in 95 per cent ethanol, capable of detecting about 10 μg. The reagent gives a green color with many phenols. The third reagent was a mixture of equal volumes of 0.1 N silver nitrate and 5 N ammonia such as has been used to detect reducing sugars by Partridge and Westall (1948). It has not been used previously in chromatography for indicating orthodiphenols. The sensitivity is approximately that of the ferric chloride reagent.

Appearance of the colored spots was instantaneous with all the reagents. In the case of the silver nitrate indicator it was necessary to wash the chromatogram with distilled water and then with running tap water as soon as the spot became visible if a permanent record was desired. With the ferricyanide reagent the chromatogams showed five spots. The pertinent information involving each indicator is shown in Table I. The spots are numbered from top to bottom as they appeared on an ascending chromatogram.

Of the five spots Nos. 2 and 3 were identified by their congruity with the positions of control applications of adrenalin and hydroxytyramine. Spot 1 showed an R_f which compares well with that of lactyl-adrenalin. The formation of this compound is discussed in detail by Crawford (1951). Following the

procedure of this investigator we hydrolyzed an aliquot of the final venom extract and found that this spot did, in fact, disappear. It may be an artefact of our extraction procedure, but in that case its formation was a fortuitous advantage, since it served to enhance the separation of the adrenalin and hydroxytyramine spots by moving most of the adrenalin in its combined form high onto the chromatogram.

We can offer no suggestions as to the identity of spots 4 and 5 for they do not correspond in position with any compound with whose chromatographic behavior we are familiar. Their relationship to adrenalin or hydroxytryamine is problematical. They appear to be orthodiphenols, but the color reactions alone cannot make even this certain.

TABLE I

Chromatograms of Orthodiphenols from Toad Venom

Spot No.	R_f range	Color with ferricyanide	Color with FeCl	Color with $AgNO_3$
1	0.79–0.86	Red → lavender	Light green	Black
2	0.55–0.64	Red → yellow	Light green	Black
3	0.49–0.59	Lavender	Dark green	Black
4	0.44–0.55	Reddish brown	Light green	Dark brown
5	0.40–0.48	Lavender	Light green	Black

We thought that some difference in the chromatographic pattern might be seen in venom taken from actively regenerating glands. To test this hypothesis the venom from eight animals was collected, and 9 days later collected again. The total weight of the dried venom at the first collection was 1.7 gm. and at the second 0.75 gm., indicating approximately 50 per cent regeneration. There was no obvious difference on the chromatogram between extracts separately prepared from the two batches of venom.

We have shown therefore that both hydroxytyramine and adrenalin are present in the normal parotid venom of *B. marinus*. There is no evidence for the presence of noradrenalin, which is surprising in view of the fact that noradrenalin has been found associated with adrenalin in every vertebrate adrenal so far examined. This may indicate either an unusually efficient conversion of hydroxytyramine to adrenalin or a route of synthesis in *B. marinus* different from that in mammals. Holtz and Kroneberg (1949) propose that in mammals the synthesis of adrenalin proceeds *via* the pathway hydroxytyramine → noradrenalin → adrenalin. An alternative pathway in *B. marinus* might be hydroxytyramine → epinine → adrenalin; yet there was no indication of epinine in our venom extracts.

While this work was in progress we learned of the investigation of Goodall (1950) who has very recently shown by chromatographic means the presence

of hydroxytyramine in addition to noradrenalin and adrenalin in adrenals from normal sheep, and also of dopa in those from thyroidectomized sheep. This is therefore a second instance of the occurrence of hydroxytyramine in association with adrenalin in an animal tissue.[2]

SUMMARY

Preparations from the parotid gland venom of the toad *Bufo marinus* have been analyzed by paper chromatography for the presence of adrenalin precursors. In addition to adrenalin itself, hydroxytyramine was identified and a second compound which appeared to be an adrenalin ester—perhaps lactyladrenalin. Two other compounds which appeared on the chromatogram could not be identified. Neither noradrenalin nor another possible precursor, epinine, appeared to be present.

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[2] *Note Added in Proof.*—H. M. Lee and K. K. Chen (*J. Pharmacol. and Exp. Therap.*, 1951, **102,** 286) report the occurrence of noradrenalin in the venom of the Chinese toad (Chan Su). About 1 per cent as much noradrenalin was found as adrenalin. If noradrenalin were present in *Bufo marinus* in comparably small amounts, it might readily have been missed in the present experiments.