Brit. J. Pharmacol. (1953), **8**, 435.

THE PURIFICATION, POTENCY, AND AMINO ACID CONTENT OF MELANOPHORE-EXPANDING, PRESSOR, AND OXYTOCIC PREPARATIONS FROM BEEF PITUITARY GLAND

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(RECEIVED JUNE 12, 1953)

Of the amino acid composition of the hormones of the posterior lobe of the pituitary gland least is known of that of the melanophore-expanding hormone. The need for more knowledge concerning this hormone has increased recently because of publications whose authors think it is related to ACTH. In this paper the amino acid composition of a melanophore preparation is presented, and some observations are made upon its relation to ACTH.

In recent years Du Vigneaud and his collaborators have made notable progress in isolating the pressor and oxytocic hormones of the pituitary gland and in determining the constituent amino acids of the products (Livermore and Du Vigneaud, 1949; Turner, Pierce, and Du Vigneaud, 1951; Popenoe, Pierce, Du Vigneaud, and Van Dyke, 1952; Pierce and Du Vigneaud, 1950a, b). Their method of isolation, countercurrent distribution, has yielded only small quantities of the hormones. For the analyses they used starch column chromatography.

In this paper the results of Du Vigneaud *et al.* are confirmed by entirely different methods. In addition, it is shown that these hormones may be obtained in larger amounts by a simpler method than countercurrent distribution.

MATERIALS AND METHODS

Melanophore-expanding Hormone Preparation.—The preparation used was 150 times as potent as standard powder. A note by R. L. Stehle concerning its preparation will be found at the end of this paper.

The preparation was hydrolysed and the hydrolysate analysed by paper chromatography. From the intensities of the spots produced by the reaction with ninhydrin (observed by the eye directly) an attempt was made to get semi-quantitative information about the quantities of each acid. An effort to get more exact quantitative results by the use of a densitometer, as described by Block (1950), was abandoned after a test of its applicability using mixtures of pure amino acids.

A picrate like that from which this preparation was derived was also analysed. This picrate consisted of fine irregular crystals which decomposed at about 150–160°.

Pressor and Oxytocic Hormone Preparations.—The work was done partly on preparations put at the disposal of the writer by Dr. Stehle; these had been the subject of several communications by him and his collaborators. In addition, the author has isolated similar products from raw material following the technique of Stehle and Fraser (1935).

Paper Chromatography of the Unhydrolysed Preparations.—Pilot experiments were first carried out with 1-mg. amounts on Whatman No. 1 filter paper, and with 3-mg. amounts on Whatman No. 3 filter paper. One dimensional chromatography was by the ascending technique using a mixture of N-butanol glacial acetic acid, and water in the proportions 4:1:5. On developing the strip with ninhydrin various zones appeared, and the one containing the hormone was located by bioassay (Fig. 1 *a*). Two dimensional chromatography was carried out first in water-saturated phenol, and then in the mixture of N-butanol and acetic acid.

To obtain large quantities of the hormones paper chromatography on a larger scale was then applied, using 40 mg. of material on Whatman No. 3 filter paper, run in the mixture of N-butanol and acetic acid by the ascending technique. The material was dissolved in 0.1 ml. of 0.25% acetic acid and was placed on a line 20 cm. long in separate spots with a micropipette. It was run for 24 hours, after which the sheet was dried in air and cut in horizontal strips, using as a guide small vertical strips from both ends, which were developed with ninhydrin. The horizontal strips were then eluted with 0.25% acetic acid by running 10 ml. from a microburette through each strip during a period of not less than one hour. The solution was evaporated in a desiccator over calcium chloride and the residue dissolved in 0.5 ml. of water. After adding 5 ml. of methanol the material was precipitated with 45 ml. of ethylacetate.

Hydrolysis.—3–5 mg. was dissolved in 0.3–0.5 ml. 6 N-hydrochloric acid and heated in a sealed ampoule at 110–112° for 20 hours. The

content of the ampoule was then evaporated to dryness in a vacuum desiccator over calcium chloride and potassium hydroxide. The residue was dissolved in enough water to make a solution which contained about 0.15 mg. in 0.02 ml., this being the amount of solution placed upon the paper.

The picrate was treated in the same fashion ; the picric acid moved faster than the amino acids in all of the solvents, and did not affect their distribution.

Paper Chromatography of the Hydrolysed Preparations.—Chromatograms were made as follows :

1. Two dimensional, ascending, the first run with secondary butanol, 150 parts ; 3% ammonia, 60 parts. This was repeated. The 90° run was with secondary butanol, 150 parts ; 88% formic acid, 30 parts ; water, 20 parts (Hausmann, 1952). This procedure is suitable for alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, oxyproline, phenylalanine, proline, threonine, tyrosine, and valine.

2. One dimensional, ascending, with 90% formic acid, 15 parts ; water, 15 parts ; tertiary butanol, 70 parts. This is the technique of Block (1950) for cystine. The colour is developed with isatin (Acher, Fromegeot, and Jutisz, 1950) for the detection of oxyproline and proline.

3. One dimensional, descending, for five days, with water-saturated tertiary amyl alcohol, the water layer containing 1% diethylamine (Work, 1949). This is suitable for isoleucine, leucine, methionine, phenylalanine, and valine.

4. One dimensional, descending, for four days, with organic phase obtained by mixing normal butanol, acetic acid, and water in the ratio 4 : 1 : 5 (Partridge, 1948). For arginine, cystine, histidine, and lysine.

5. One dimensional, descending, for two days, with water-saturated phenol, the water layer containing 1% ammonia. Before starting, the cystine is oxidized to cysteic acid (Dent, 1947). For aspartic acid, cystine, glutamic acid, glycine, serine, and threonine.

The sheets were dried in air, sprayed with 0.1% ninhydrin in water-saturated normal butanol, and placed in the dark for 24 hours. To get an approximation to the amount of each amino acid from the colour intensities, the chromatograms were compared with those obtained with a 0.07 μM solution of amino acids in 0.6 N-hydrochloric acid.

Tryptophane was determined by the method of May and Rose (1922), as modified by Stehle (1945) to permit the use of small quantities of material.

Assays of Potency.—The pressor hormone was tested by measuring the blood pressure effect in dogs anaesthetized with chlorbutol.

Oxytocin was tested on the isolated uterus of the virgin guinea-pig suspended in Locke solution.

Results

Melanophore-expanding Hormone

The constituent amino acids of the melanophore hormone preparation are listed in Table I.

TABLE I

CONSTITUENT AMINO ACIDS OF THE MELANOPHORE-EXPANDING HORMONE

Present in largest amounts:	
Arginine	Proline
Glutamic acid	Serine
Glycine	Tryptophane
Histidine	Tyrosine
Lysine	Valine
Phenylalanine	
Present in smaller amounts:	
Alanine	Leucine
Aspartic acid	
Present in traces:	
Cystine	Methionine
Isoleucine	Threonine

Pressor and Oxytocic Hormones

The two dimensional chromatography of the crude preparations (Stehle and Fraser, 1935) gave the following picture (Fig. 1 *b*, *c*): R_f vasopressin in phenol, 0.85–0.90 ; in butanol-acetic acid, 0.15–0.18. R_f oxytocin in phenol, 0.85–0.90 ; in butanol-acetic acid, 0.35–0.40. Impurities—very probably lysine, arginine, and lower peptides—had an R_f value of 0.15–0.40 in phenol and 0.05–0.14 in butanol-acetic acid. If the ninhydrin-sprayed sheet was placed in an oven at 100° for a few minutes, the hormone spots gave a yellow colour that turned purple on prolonged heating. They gave a purple colour with ninhydrin on standing in the dark for 24 hours.

To remove the impurities, a one dimensional chromatography with 40 mg. of each of the impure preparations was carried out in butanol-acetic acid. The constituent amino acids of these preparations are listed in Tables II and III. The main constituents are identical with those reported by Du Vigneaud and his collaborators (Turner *et al.*, 1951 ; Popenoe *et al.*, 1952 ; Pierce *et al.*, 1950a, b). The spots of the amino acids present in traces were extremely faint in comparison with the others.

A picrate which consisted of fine irregular crystals was obtained from the pressor hormone.

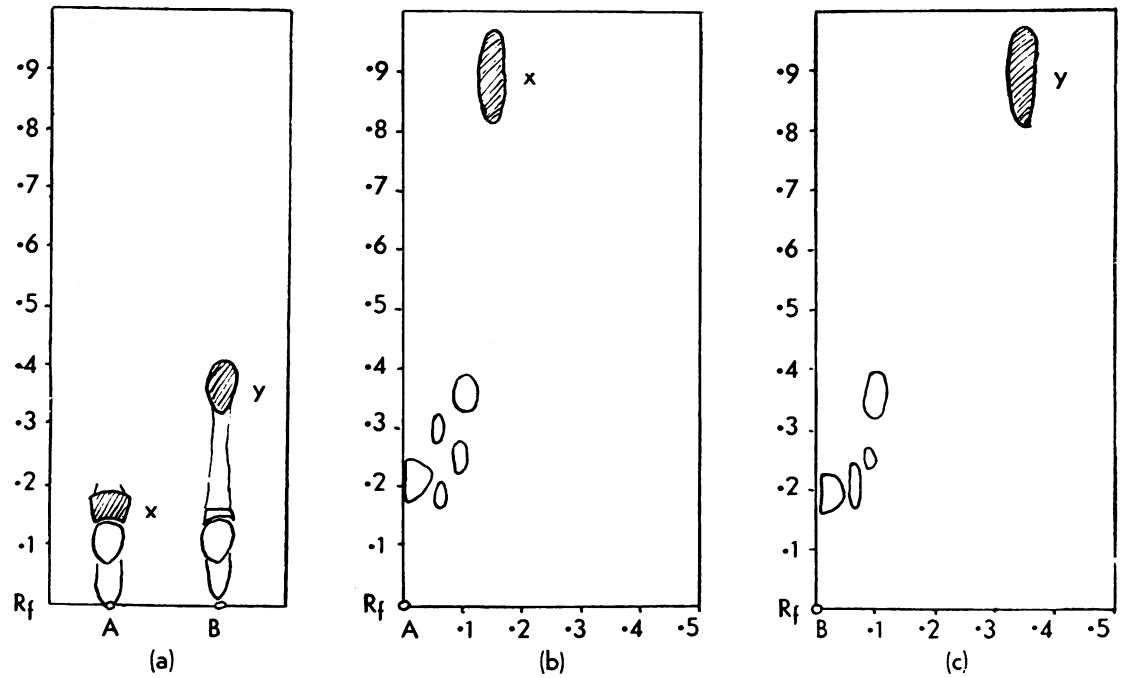

FIG. 1.—(*a*) One dimensional paper chromatography of the pressor and the oxytocic hormone (Stehle and Fraser, 1935) in N butanol-acetic acid-water, 4: 1: 5. A, point of application; x, pressor hormone after development. B, point of application; y, oxytocic hormone after development. (*b*) Two dimensional paper chromatography of the pressor hormone (Stehle and Fraser, 1935) in water-saturated phenol (vertical) and N butanol-acetic acid-water, 4: 1: 5 (horizontal). A, point of application; x, hormone. This material when purified by chromatography showed only the hormone spot. (*c*) Two dimensional paper chromatography of the oxytocic hormone (Stehle and Fraser, 1935) in water-saturated phenol (vertical) and N butanol-acetic acid-water, 4: 1: 5 (horizontal). B, point of application; y, hormone. This material when purified by chromatography showed only the hormone spot.

TABLE II

CONSTITUENT AMINO ACIDS OF THE PRESSOR HORMONE

Present in large amounts:

Arginine	Glycine
Aspartic acid	Phenylalanine
Cystine	Proline
Glutamic acid	Tyrosine

Present in traces:

Alanine	Leucine
Valine	

TABLE III

CONSTITUENT AMINO ACIDS OF THE OXYTOCIC HORMONE

Present in large amounts:

Aspartic acid	Isoleucine
Cystine	Leucine
Glutamic acid	Proline
Glycine	Tyrosine

Present in traces:

Alanine	Valine

It was prepared by adding a saturated aqueous solution of picric acid to a 1% solution of the pressor hormone; it precipitated instantly. The precipitate was separated by centrifugation and freed of excess picric acid by repeated washings with ethylacetate. It decomposed at 175–190°, and, when hydrolysed and chromatographed, gave the same chromatogram as the starting material.

The potency of the vasopressin was 200 units per mg. before chromatography and 330 units per mg. afterwards. This preparation had an oxytocic activity of about 15 units per mg. The potency of oxytocin was 250 units per mg. before chromatography, and remained unchanged by chromatography within the limits of error of the assay. 50 units had no pressor activity.

DISCUSSION

Melanophore-expanding Hormone

The conclusion of Sulman (1952) and of Johnsson and Hoegberg (1952), based upon the melanophore-expanding action of ACTH preparations, that the melanophore hormone is identical with ACTH has been denied by Geschwind, Reinhardt, and Li (1952); and by Reinhardt, Geschwind, Porath, and Li (1952). Recently, however, Brink, Boxer, Jelinek, Kuehl, Richter, and Folkers (1953), and Winter, Brink, and Folkers (1953), have stated that their corticotropin-B has a melanophore-expanding action; and since they claim that their material is pure the question appears to be still open.

It would seem that one way to try to settle the matter would be to test the corticotrophic action of the melanophore-expanding hormone, but this has not been done. The preparations which have had an action upon melanophores could have been contaminated preparations. Folkers *et al.* state that 0.1 μg. of their preparation expanded the melanophores of pieces of frog skin bathed in Ringer solution. Jores (1933), however, has found that 3.3 μg. of standard pituitary powder, which is equivalent to 0.022 μg. of the preparation studied in this work, is effective in December when the sensitivity is greatest. It is not stated when Folkers *et al.* made their tests. 0.01 μg. of the preparation studied in this work produced a strong effect, and 0.005 μg. produced an easily detectable effect when intact frogs, stored during the winter months, were injected in May. It would obviously not take much contamination of ACTH with such material to confer a melanophore-expanding action upon it.

Apart from testing the melanophore hormone for corticotrophic action, the solution of the problem might be advanced by giving attention to the tryptophane content of ACTH preparations. This amino acid seems to be a constituent of the melanophore hormone ; in preparations of different potencies the potencies run parallel to the tryptophane content (Stehle, 1945).

The amino acid constituents of ACTH as published (Geschwind, Porath, and Li, 1952 ; Li, 1952 ; Brink, Boxer, Jelinek, Kuehl, Richter, and Folkers, 1953), and of the melanophore hormone as given here, agree to a considerable extent except for tryptophane.

Pressor and Oxytocic Hormones

Biological assays of pituitary hormones have not a high degree of accuracy, and this may lead to claims of potency that are too high.

In their most recent publication, Pierce, Gordon, and Du Vigneaud (1952) conclude that, for their oxytocic hormone, 450–500 units per mg. is probably more nearly correct than the 800–865 units per mg. reported earlier (Livermore and Du Vigneaud, 1949 ; Pierce and Du Vigneaud, 1950b). On the other hand, they now state that their pressor preparation has 600–650 units per mg. (Popenoe, Pierce, Du Vigneaud, and Van Dyke, 1952) rather than the 400–450 units per mg. reported earlier (Turner, Pierce, and Du Vigneaud, 1951).

The potencies of the preparations described by Stehle and Fraser (1935) were given as 200 units per mg. for the pressor and 250 units for the oxytocic hormone. Dr. Stehle has informed the author that these potencies were given because his object was to assign values below which the potencies probably did not fall. Many individual assays gave higher values.

The pressor preparation mentioned above, which was obtained by chromatography from one of Stehle's preparations, and of which the chromatogram of the hydrolysate agreed with the composition given by Du Vigneaud, had only 330 units per mg. The corresponding oxytocic preparation had only 250 units per mg. If an underestimate of the potencies given for these preparations is as great as the overestimate of the oxytocic preparation first reported by Du Vigneaud, then his preparations and ours may be about equal in potency, and therefore in purity.

Summary

1. The component amino acids of a preparation of the melanophore-expanding hormone of the pituitary gland were determined. It appears to be distinguishable from ACTH by its content in tryptophane.

2. There is as yet no reason for believing that the melanophore hormone is identical with ACTH. Simple contamination of the latter with the former is an adequate explanation of the experimental results at present available.

3. A simple method is described for obtaining relatively large quantities of the pressor and oxytocic hormones of the pituitary gland in a highly purified state.

4. The amino acid composition of the products agrees with that given by Du Vigneaud *et al.*

5. The view is expressed that the potencies of these hormones may not be as high as is sometimes stated.

6. The pressor hormone may be precipitated as a crystalline picrate.

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ADDENDUM

by

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This preparation was obtained in May, 1952, from a picrate similar to those described before (Stehle, 1944). After the removal of the picric acid 638 mg. of the material was dissolved in 25 ml. of water and 150 ml. absolute alcohol was added. Turbidity but no flocculation occurred. 50 ml. of ethylacetate was then added. The turbidity increased and the solution was put aside for the night. By morning a precipitate had formed. This was removed by centrifugation and dried *in vacuo* (184 mg.). Ethylacetate to make 500 ml. was added to the solution. A precipitate formed slowly and after several hours was removed by centrifuging and dried (313 mg.). Ethylacetate to make 900 ml. was then added to the solution. The next day the precipitate which had formed was centrifuged and dried (62 mg.). This last precipitate had about twice the tryptophane content and was about twice as potent upon frogs as was the 1944 preparation.

Lack of material prevented a repetition of the work, but from trials with such residues as were available it seemed clear that the outcome depends upon removing the second precipitate at the right time. If this is delayed too long the hormone comes down with it and one obtains no precipitate on final addition of ethylacetate. It was a surprise, and more or less a disappointment, to find that the potency of the 1944 preparation could be increased.