total amount of N precipitated may greatly exceed that thrown down in the homologous reaction. Similar behavior has been noted by Kleczkovsky[22] in cross reactions of chemically altered horse serum globulin in antisera to unaltered globulin but not with Ea and its corresponding derivatives and their antisera, and was ascribed to a physical alteration in the antigen preventing the formation of soluble complexes with antibody. This cannot apply in the case of DnEa since excess of this antigen results in an inhibition zone with anti-DnEa. The failure of the DnEa-anti-PEa system to show inhibition with excess DnEa is more probably due to a relatively restricted range of combining proportions and a greater tendency of any soluble compound formed to dissociate.[23]

The specificity conferred upon Ea by the introduction of phosphoryl groups is not entirely a hapten specificity in the sense of Landsteiner[24] for anti-PEa sera are not precipitated by phosphates nor is the specific precipitation of PEa inhibited by as much as 0.15 M phosphate. On the other hand, one of three lots of phosphorylated Felton solution (water-precipitable globulin from anti-pneumococcus horse serum) and one lot of phos phorylated horse serum albumin[25] gave precipi tates with anti-PEa but casein (Merck) did not.

(22) A. Kleczkovsky, *Brit. J. Exptl. Path.*, **21**, 1, 98 (1940).

(23) *Cf.* M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **59**, 519 (1934).

Summary

The phosphorylation of crystalline egg albumin and its effect on the viscosity, titration curve, and other physical, chemical and immunological properties of the product are described and discussed. The lability of a portion of the phosphorus is noted and commented upon.

(24) K. Landsteiner, "The Specificity of Serological Reactions," C. C. Thomas, Springfield, Ill., 1936.

(25) Immunochemical studies on these products are now in progress.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE, AND THE GEORGE WASHINGTON UNIVERSITY SCHOOL OF MEDICINE]

Purification of the Pressor Principle of the Posterior Lobe of the Pituitary Gland by Electrophoresis

BY GEORGE W. IRVING, JR.,[1] HELEN M. DYER AND VINCENT DU VIGNEAUD

In a recent communication a simple method was presented by which fairly large amounts of a preparation containing 10 pressor and 10 oxytocic units per mg. could be readily obtained from the posterior lobe of the pituitary gland.[2] In a continuation of these preparative studies it was decided to explore the possibility of utilizing electrophoresis for purification of these principles. Our previous investigation of their electrophoretic behavior had demonstrated that under the conditions employed both migrated toward the cathode, the pressor principle traveling at a faster rate.[3,4] This work had given rise to the hope that by subjecting the material to electrophoresis, various impurities might be separated from the active material. The present communication deals with the development of such a preparative method for the pressor principle. A crude preparation, the "ether precipitate" previously described,[2] was partially purified by fractionations with various solvents and the resulting preparation was then subjected to electrophoresis in a newly designed apparatus. By this procedure 35 mg. of a preparation containing 200 pressor units per mg. was obtained per kilo of posterior lobes.

Since evidence has been presented which indicates that the pressor principle undoubtedly contains the disulfide linkage,[5,6,7] the cystine content of a series of fractions was determined to ascertain the effect of purification upon the content of this amino acid. Tyrosine analyses were carried out for the same reason since tyrosine also has been shown to be present in fairly high concentration in highly potent pressor preparations.[8,9]

Experimental

Prevention of Inactivation in the Cathode Cell.—Under certain conditions, inactivation of the posterior lobe prin-

(1) (a) Parke, Davis and Company Research Fellow. (b) Present address: The Rockefeller Institute for Medical Research, New York, N. Y.

(2) Irving and du Vigneaud, THIS JOURNAL, **62**, 2080 (1940).

(3) Du Vigneaud, Irving, Dyer and Sealock, *J. Biol. Chem.*, **123**, 45 (1938).

(4) Irving and du Vigneaud, *ibid.*, **123**, 485 (1938).

(5) Sealock and du Vigneaud, *J. Pharmacol.*, **54**, 433 (1935).

(6) Gulland and Randall, *Chemistry and Industry*, 422 (1936).

(7) Gulland, Partridge and Randall, *J. Chem. Soc.*, 419 (1940).

(8) Du Vigneaud, Sealock, Sifferd, Kamm and Grote, *Proc. Am. Soc. Biol. Chem.*, **27**, xciv (1933).

(9) Stehle and Fraser, *J. Pharmacol.*, **55**, 136 (1935).

ciples had been encountered in the cathode cell during electrophoresis.[3] Various experiments indicated that some change had taken place in the active material due to contact with the electrode. It may be recalled that Gulland and Randall[10] observed electrolytic inactivation of the oxytocic hormone. Experiments were accordingly designed to eliminate the possibility of a reaction at the cathode.

In the course of the work it was noted that when the cathode cell became sufficiently alkaline the active principles were unable to migrate into that chamber. It was decided, therefore, to take advantage of this behavior by placing the cathode in a chamber sufficiently alkaline at the start to prevent the active material from entering it, and thus contact between the active material and the electrode was circumvented. A so-called "alkaline barrier" was employed.[11] The one devised for this work is shown in Fig. 1. It consisted of a glass tube filled with 0.01 N sodium hydroxide. The negative electrode (platinum foil) was placed in one end of the tube while the other end was closed by means of a sintered glass disk and then was immersed in the cathode cell solution. Under these conditions the active principles were found to be unable to migrate into the chamber at this high pH.[12]

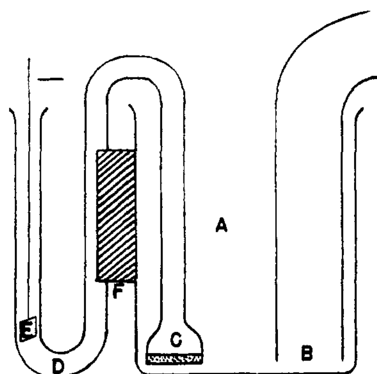

Fig. 1.—The outside electrode: A, 400-cc. beaker, Cell I; B, one arm of siphon connecting Cell I with Cell II; C, sintered glass filter, porosity Jena G3; D, 8-mm. Pyrex tubing; E, platinum electrode; F, grooved wooden block. The outside electrode is held against the beaker by means of a broad rubber band.

The efficacy of the "alkaline barrier" is indicated in the following typical experiment. Three hundred mg. of ether precipitate (3600 units of each activity) was dissolved in 325 cc. of water and the solution was placed in Cell IV of a 7-cell setup,[3] the cells being numbered consecutively, with the cathode cell as Cell I. The remaining cells were filled with water and the siphons were filled in the usual manner. The "alkaline barrier" was attached to Cell I as shown in Fig. 1. A carbon electrode was inserted in Cell VII to act as the anode and electrophoresis at 3000 to 6000 volts was carried out for seventy hours. The cell contents were then removed and assayed.[13] Ninety per cent. of the pressor activity was recovered. Cell I contained 70% of the starting activity while the remainder was distributed among Cells II, III, IV. The "alkaline barrier" solution contained only a trace of pressor activity. The cathode cell material obtained by this procedure proved to be relatively stable. The solution in Cell I was acidified with acetic acid (pH 3.0) and was distilled to dryness *in vacuo* at 35°. The residue was dissolved in glacial acetic acid and the active material was precipitated by the addition of peroxide-free anhydrous ether. Fifty mg. of a chalk-white powder was obtained which contained 50 pressor and 15 oxytocic units per mg. In parallel experiments without the "alkaline barrier" the corresponding product possessed less than half of this potency.

Attempts to further purify the 50 unit pressor material by re-electrophoresis and by chemical fractionation were ineffective. It was therefore decided to purify the ether precipitate chemically and then subject the partially purified material to electrophoresis. This resulted in the elaboration of the routine preparative procedure described below.

Chemical Fractionation Procedure.—A fractionation procedure based on that of Kamm, *et al.*, was used.[14,15] Approximately 50-g. lots of ether precipitate[2] were employed as the starting material and were carried through the following steps.

1. "Ether precipitate" was dissolved in 98% acetic acid (50 cc. per g.) and the solution was filtered from a small, gummy residue. To the filtrate was added 2.5 volumes of peroxide-free anhydrous ether. The precipitate was filtered off, washed with ether and dried. This procedure was repeated a second time.

2. The dry precipitate from Step 1 was dissolved in 98% acetic acid (20 cc. per g.) and the solution was filtered from a very small insoluble residue. To the clear filtrate were added acetone and ether in the following amounts for each gram of solids present in the acetic acid solution: 8 cc. acetone, 8 cc. acetone, 5 cc. ether, 25 cc. ether, 100 cc. ether. After the addition of each portion of reagent, the precipitate which separated was filtered, washed with ether, and dried. The filtrate in each case (washes excluded) was then carried to the next step.

3. The fourth fraction obtained in Step 2 was further purified by a second fractionation. The material was dissolved in 98% acetic acid (33 cc. per g.) and several fractions were precipitated by the successive addition of the following reagents: 50 cc. acetone, 20 cc. acetone, 20 cc. ether, 200 cc. ether. The procedure used was analogous to that described in Step 2.

4. The third fraction obtained in Step 3 was dissolved in glacial acetic acid (140 cc. per g.) and the solution was filtered from a small insoluble residue. The clear filtrate was treated with 3 volumes of peroxide-free anhydrous

(10) Gulland and Randall, *Biochem. J.*, **29**, 378 (1935).

(11) Freeman, Gulland and Randall, *ibid.*, **29**, 2211 (1935).

(12) We have recently been able to show that both the pressor and oxytocic principles are amphoteric.

(13) Pressor assays were carried out upon dogs under chloretone anesthesia or upon cats under amytal. Assays for the oxytocic hormone were based on its depressor action upon the blood pressure of hens under urethan.[5]

(14) Kamm, Aldrich, Grote, Rowe, and Bugbee, THIS JOURNAL, **50**, 573 (1928).

(15) We wish to thank Dr. Oliver Kamm of Parke, Davis and Co., for placing at our disposal unpublished data concerning the fractionation of the posterior lobe hormones and for generous supplies of frozen posterior lobes.

ether and the precipitate was filtered off, washed with ether and dried. This procedure was repeated 3 times.

5. The final precipitate from Step 4 was dissolved in water (28 cc. per g.) and the aqueous solution was extracted 10 times with successive portions of 80% *n*-butanol (40 cc. per g.). The extractions were carried out by means of vigorous mechanical stirring. After each extraction the layers were separated by centrifugation and the butanol layer was pipetted off. The completely extracted aqueous solution contained the desired purified pressor material.

The distribution of activity in the various fractions of the first three steps in this chemical purification procedure is given in Tables I and II. The figures given represent averages taken from a large number of similar experiments. In each table the weight and activity of the starting material has been taken as 100% and the relative amounts of activity and solids found in the various fractions have been expressed as percentages of the total.

The partially separated pressor material obtained in Step 1 of the procedure (Pressor Fraction, Table I) contained approximately 72% of the initial pressor activity. It possessed the same pressor potency as the ether precipitate used as the starting material but the oxytocic potency was reduced to approximately half of its original value. The two acetic acid–ether filtrates contained the remaining oxytocic activity and a small amount of pressor activity.

TABLE I

PARTIAL SEPARATION OF THE PRESSOR AND OXYTOCIC PRINCIPLES (STEP 1)

Fraction	Activity, units/mg. Pressor	Activity, units/mg. Oxytocic	Weight of fraction	% of total activity in fraction Pressor	% of total activity in fraction Oxytocic
Starting material	10	10	100	100	100
Acetic acid insoluble			17	2.5	Trace
Pressor fraction	10	5	74	72	36
Oxytocic fraction	20	150	3	7	45
Recovery total			94	81.5	81

TABLE II

FRACTIONATION OF THE PRESSOR PRINCIPLE (STEP 2)

Fraction	Reagent added, cc. per g.		Solids in pptd. fraction, % of total	Pressor content of pptd. fraction, % of total
First Fractionation[a]				
1	Acetone	8	46	20
2	Acetone	8	26	16
3	Ether	5	7	5
4[b]	Ether	25	9	49
5	Ether	100	5	7
Recovery total			93	97
Refractionation of Fraction 4 above (Step 3)[c]				
6	Acetone	50	35	23
7	Acetone	20	14	9
8[d]	Ether	20	29	45
9	Ether	200	18	15
Recovery total			96	92

[a] Values of fractions 1 through 5 are expressed as percentages of pressor fraction, Table I. [b] 55 pressor and 30 oxytocic units per mg. [c] Values of fractions 6 through 9 are expressed as percentages of fraction 4 as 100%. [d] 85 pressor and 30 oxytocic units per mg.

In the second step of the purification procedure 49% of the activity present in the partially separated pressor fraction was obtained as a fraction assaying 55 pressor units per mg. (Table II). A second fractionation of the 55 unit material (Step 3) yielded 45% of the pressor activity as a fraction assaying 85 pressor units per mg. (Table II).

Further purification of the 85 unit fraction (Step 4) yielded 90% of the pressor activity as a fraction assaying 110 pressor units per mg. Butanol extraction of an aqueous solution of the 110 unit fraction left 70% of the pressor activity in the aqueous layer which assayed 125 pressor and 20 oxytocic units per mg.

The 125 unit pressor fraction represents the highest potency which we have been able to attain by chemical purification. Approximately 120 mg. of this fraction can be obtained from 1 kg. of posterior lobes. A small additional amount of the potent pressor fraction can be obtained by reworking the combined low potency fractions. It should be noted that the potency given for this fraction and for the intermediate fractions represent averages taken from several similar experiments. In different preparations the potency of the final product was found to vary between 105 and 140 units per mg.

Electrophoresis of the Chemically Purified Pressor Fraction.—The aqueous solution from Step 5 above was subjected to electrophoresis from the center cell of the apparatus shown in Fig. 2. This apparatus consisted of a glass tube, closed at the ends and separated into 5 cells by means of sintered glass disks. The mercury wells, shown connected to the end cells in Fig. 2, were found necessary to counter-balance the electro-osmotic flow of water which was encountered. Cell I was filled with 0.01 *N* sodium hydroxide so that it could function as an "alkaline barrier." The remaining cells were filled with distilled water. After twenty-eight hours of electrophoresis at 600 to 3000 volts the cell contents were removed. The cell II solution was acidified with acetic acid (*p*H 4.5) and was distilled to dryness *in vacuo* at 40°. The residue was dissolved in approximately 2 cc. of 98% acetic acid and the active material was precipitated by the addition of 25 volumes of peroxide-free anhydrous ether. The precipitate, collected by centrifugation, was washed twice with ether, to yield a white, amorphous powder.

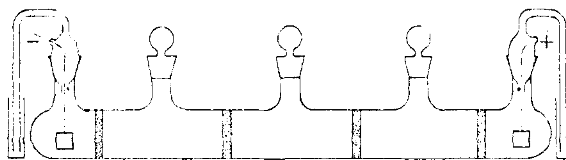

Fig. 2.—Sintered glass apparatus: the sintered glass disks are of Jena glass, porosity 3, diameter, 30 mm.; capacity of Cells I and V, 40 cc.; Cells II, III and IV, 80 cc.; platinum electrodes.

In a typical experiment an aqueous solution of 142 mg. (15,400 pressor units) of the chemically purified pressor fraction was subjected to electrophoresis in the sintered glass apparatus as described above. The results are given in Table III. Approximately 70% of the initial pressor activity was found in Cell II at the end of the run. The

solid fraction isolated from the Cell II solution weighed 40 mg. and assayed 198 pressor and 26 oxytocic units per mg. This compares favorably in potency with the purest preparations recorded by Kamm, *et al.*,[14] and by Stehle and Fraser.[9]

TABLE III

TYPICAL EXPERIMENT: ELECTROPHORESIS OF PURIFIED PRESSOR FRACTION

Fraction	Pressor activity, % of total
Cell IV at start of run[a]	100
Cell I (outside electrode)	4
Cell II[b]	67
Cell III	13
Cell IV	Trace
Cell V	0
Recovery total	84

[a] 108 pressor and 20 oxytocic units per mg. [b] Dry material isolated from Cell II represented 51% of the total activity and assayed 198 pressor and 26 oxytocic units per mg.

The over-all yield for the complete purification process may be summarized as follows. From 1 kg. of posterior lobes, chemical fractionation yields 120 mg. of a product possessing a potency of 125 pressor units per mg. Electrophoresis of this weight of material yields 35 mg. of the 200 unit pressor material. The procedure outlined has been used routinely in our laboratory for the preparation of this high potency material with consistent and easily duplicated results.

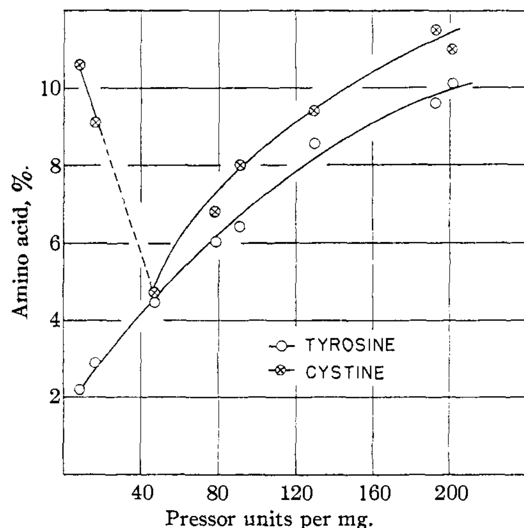

Fig. 3.—Analysis of pressor fractions.

Analysis of Pressor Fractions for Cystine and Tyrosine.—Analytical results upon a series of fractions ranging in potency from 8 to 200 pressor units per mg. are given in Fig. 3. Tyrosine was determined by the micro method of Bernhart.[16] Cystine was determined upon 1.5 to 2.0 mg. samples by the Sullivan method. The hydrochloric-formic acid hydrolytic procedure described by Miller and du Vigneaud[17] was employed. Inspection of Fig. 3 reveals that the tyrosine content increased steadily as the potency of the fraction increased, the most potent fraction containing approximately 10% tyrosine. This value corresponds closely to those found by du Vigneaud, *et al.*,[8] and Stehle and Fraser[9] in preparations of equivalent potency. In the case of cystine the initial steps in the purification resulted in a removal of impurities which had a high cystine content, while in the subsequent purification steps, the data indicate that the cystine content increased with increasing potency. The most potent pressor fraction contained approximately 11% cystine. In the analytical work on pressor fractions reported by du Vigneaud and co-workers[8] an accurate cystine value by the Sullivan or Folin–Marenzi method was not obtained due to the presence of a chromogenic substance in the hydrochloric acid hydrolyzate which gave a yellow color when the solution was made alkaline. The latter occurred during the sodium cyanide and sodium sulfate treatments in the Sullivan and in the Folin–Marenzi methods, respectively. In the present work it was found that hydrolysis with hydrochloric acid–formic acid prevented the formation of the chromogenic substance and allowed the cystine content to be more accurately determined.

The authors wish to thank Mr. Theodore W. Loring for his invaluable assistance.

Summary

A procedure for obtaining a highly potent preparation of the pressor principle of the posterior lobe of the pituitary gland (200 units per mg.) by the electrophoresis of a chemically purified pressor fraction (125 units per mg.), has been developed.

A modified electrophoresis apparatus, which makes use of sintered glass filter disks, has been devised for this work and certain precautions which must be observed to prevent inactivation of the pressor principle during electrophoresis have been presented.

Data have been presented to indicate that tyrosine and cystine are present in the pressor principle and that these two amino acids account for approximately 20% of the best pressor fraction.

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(16) Bernhart, *Proc. Am. Soc. Biol. Chem.*, **32**, x (1938).

(17) Miller and du Vigneaud, *J. Biol. Chem.*, **118**, 101 (1937).