barbital produced profound depression of the central nervous system at a dose of 100 mg./kg.

Acknowledgment.—The authors wish to thank Mr. Jerry L. Compton for synthesis of some of the intermediate compounds, and Messrs. Norbert E. Adler and John P. Braselton for the pharmacologic studies.

Large Scale Preparation and Purification of the Vasopressor Polypeptide, Substance A[1]

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Revised Manuscript Received June 2, 1961

A procedure for the large scale preparation of substance A is described. By means of precipitation techniques and chromatography on carboxymethylcellulose we have been able to purify substance A 70-fold. Using paper electrophoresis substance A has been shown to move as a discrete substance and by means of paper electrophoresis it has been shown to be an amphoteric substance with an isoelectric pH between 6–7.

We have reported previously that a pharmacologically active polypeptide provisionally designated as substance A can be produced by incubating an α-amylase preparation with fraction IV-4 of human plasma protein.[3,4] Chemical and pharmacological comparison with other known polypeptides led to the conclusion that substance A was very similar to angiotensin.[4] However, efforts to characterize adequately this material have been handicapped by inadequate amounts of substance A and by lack of material of sufficient purity

(1) Supported by a grant from the National Heart Institute, U. S. Public Health Service (Grant H-4873). A preliminary report of this work was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, University of Washington, Seattle, Aug. 22–25, 1960.

(2) This investigation was supported by a Senior Research Fellowship (SF-72) from the Public Health Service.

(3) C. G. Huggins and E. J. Walaszek, *Proc. Soc. Exp. Biol. N. Y.*, **100**, 100 (1959).

(4) E. J. Walaszek and C. G. Huggins, *J. Pharmacol.*, **126**, 258 (1959).

for identification purposes. The present work was directed toward the development of a method for the preparation of a relatively large amount of substance A.

Materials and Methods

Isolated Smooth Muscle.—Preparations of guinea pig ileum and rat uterus were used as previously described.[4] A 3-cm. section of rat duodenum, suspended in a 10-ml. bath containing deJalon's solution was used according to the method of Gaddum and Horton.[5] In some experiments atropine sulfate (10^{-6} g./ml.) and tripelennamine hydrochloride (10^{-7} g./ml.) were present in the Tyrode's solution and deJalon's solution. The contact time of the tissue with the polypeptide preparation was 60 seconds except in the duodenum assay where it was 30 seconds. The time interval between applications was 5 minutes.

Chemicals.—The α-amylase used for the large scale preparation of substance A was obtained from Nutritional Biochemical Corporation, NBC-7942. Fraction IV-4 of human plasma protein was obtained through the courtesy of the American Red Cross and E. R. Squibb and Sons. Animal charcoal was deactivated by the method of Peart,[6] before being used. Carboxymethylcellulose (0.88 meq./g.) was obtained from California Corporation for Biochemical Research. Bradykinin was prepared by the method of Prado *et al.*[7] All solvents and chemicals were reagent grade and were obtained from local chemical supply houses.

Units.—In this and subsequent papers we define unit as the activity contained in one mg. of dry *standard* substance A as assayed on the guinea pig ileum.

Experimental

Large Scale Preparation of Substance A.—In the formation of angiotensin Bean[8] has reported that "angiotensinase" activity is essentially abolished near 0° whereas renin remained active.[9] These observations have been confirmed by Clark *et al.*[10] Similar studies with respect to the formation and destruction of substance A were carried out by mixing equal volumes of 1% α-amylase and 1% fraction IV-4 both dissolved in Tyrode's solution and incubating at 0–4° for the prescribed time. In these experiments 5 ml. of incubation mixture was removed at various times and added to 2 ml. of 2*N* hydrochloric acid and then heated for 5 minutes in a boiling water bath. After cooling and centrifugation the supernatant fluid was neutralized and then analyzed for substance A on the guinea pig ileum. The data presented in Table I show that maximum activity was found after 60 min. of incubation. The active substance was partially destroyed in the incubation mixture after 4 hr., the inactivation being almost

(5) J. H. Gaddum and E. W. Horton, *Brit. J. Pharmacol.*, **14**, 117 (1959).
(6) W. S. Peart, *Biochem. J.*, **59**, 300 (1955).
(7) J. L. Prado, R. Monier, E. S. Prado, and C. Fromageot, *Biochim et Biophys. acta*, **22**, 89 (1956).
(8) C. Bean, *Am. J. Physiol.*, **136**, 731 (1942).
(9) L. A. Sapirstein, R. K. Reed, and F. D. Southard, *J. Lab. Clin. Med.*, **29**, 633 (1944).
(10) L. C. Clark, C. Winkler, F. Gollan, and P. R. Fox, *J. Biol. Chem.*, **206**, 717 (1954).

TABLE I

THE ENZYMATIC FORMATION AND DESTRUCTION OF SUBSTANCE A AT 4°

Sample	Incubation time in hours	Substance A activity[a]
1	0	0
2	1/3	140
3	1	350
4	4	185
5	7	75
6	24	75
7	48	50
8	72	50

[a] Expressed as μg. of standard substance A in 0.6 ml. of incubation mixture.

complete by 24 hr. of incubation. This may be contrasted with the complete inactivation obtained after approximately 2 hr. of incubation at 37°. In addition, it was found that relatively larger amounts of active material could be obtained from a mixture incubated at 0–4° than from those incubated at 37°. We have chosen 0–4° and 1.25 hr. as the temperature and time, respectively, for the incubation conditions in the large scale preparation of substance A.

The procedure for the large scale preparation of substance A was carried out as indicated by the flow sheet in Fig. 1. All manipulations were carried out at 0–4° in a cold room until the active material was extracted from the charcoal. Thereafter, the procedures were carried out at room temperature. Sixteen liters of a 1% solution of α-amylase and 16 liters of a 1% solution of fraction IV-4 both dissolved in Tyrode's solution were mixed and incubated with shaking for 1.25 hr. at 0–4°. At the end of this time, charcoal, 15 g./l., was added and the incubation mixture shaken for an additional 5 min. and then allowed to settle overnight. The suspended charcoal was removed by centrifugation in a Servall continuous-flow high-speed centrifuge and combined with that obtained by settling-out overnight. The combined charcoal sediments were washed in succession with 5 l. of 0.9% NaCl, distilled water and absolute ethyl alcohol. After each washing the charcoal that failed to settle was separated by centrifugation. The washed charcoal sediment was extracted three times with glacial acetic acid (4,4,3 liters) at room temperature. Each time the glacial acetic acid extract was clarified by filtering through a pad of Hyflo-Supercel. The glacial acetic extracts were combined and evaporated to a small volume (400 ml.) under reduced pressure and below 40°. The insoluble material was removed by high speed centrifugation and washed twice with 100 ml. of glacial acetic acid. The combined glacial acetic acid extracts (500 ml.) were mixed with 5400 ml. of anhydrous ethyl ether and after standing at 0–4° overnight the precipitate was separated by centrifugation. The precipitate was washed several times with anhydrous ethyl ether, the resulting dry tan powder was redissolved in glacial acetic acid and filtered through a bacteriological filter (Seitz filter) and the active material precipitated from the filtrate (400 ml.) by the addition of 4000 ml. of anhydrous ethyl ether. The precipitate after standing overnight at 0-4° was separated by centrifugation and

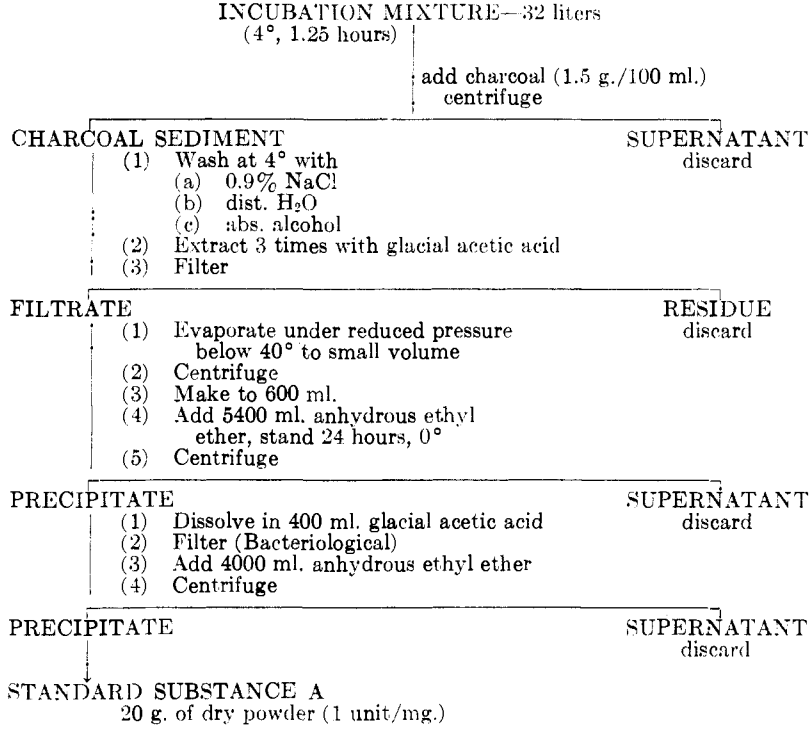

Fig. 1.—Flow sheet for the large scale preparation of substance A.

washed several times with anhydrous ethyl ether. A dry light tan powder was obtained, yield 20 g. (1 unit/mg.). Theoretical yield calculated from the biological assay of the incubation mixture was 25 g.

Column Chromatography.—Carboxymethylcellulose possessing a capacity of 0.88 meq./g. was used for the column chromatographic studies in a manner similar to that as described by Ward and Guillemin.[11] Chromatography was carried out in glass columns 22 × 375 mm. and packed to a height of 300 mm. with 20 g. of ion-exchanger previously equilibrated with ammonium acetate buffer, 0.02 *M* pH 6.0. After preparation of the column, 400 ml. of ammonium acetate buffer, 0.02 *M*, pH 6.0, was passed through the column and then 100 mg. of standard substance A, dissolved in 10 ml. of ammonium acetate buffer 0.02 *M* pH 6.0, was applied to the column. Development of the column was carried out using a gradient through a 500 ml. mixing chamber[12] to an ammonium acetate buffer, 0.2 *M*, pH 7.0. The flow rate was approximately 2.5 ml./min., and 10 ml. frac-

(11) D. N. Ward and R. Guillemin, *Proc. Soc. Exp. Biol. N. Y.*, **96**, 568 (1957).
(12) R. M. Bock and W. Ling, *Anal. Chem.*, **26**, 1543 (1954).

tions were collected by means of an automatic fraction collector which was connected to an ultraviolet absorption meter with a maximum at 280 mμ (Gilson Medical Electronics) for measuring the protein elution pattern. Biological activity was located by bioassay on the guinea pig ileum and the appropriate fractions were pooled and lyophilized. Figure 2 shows the elution pattern obtained

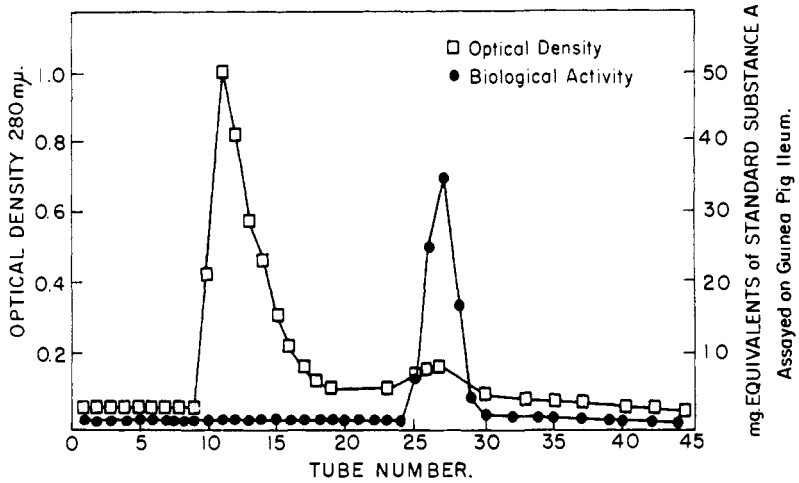

Fig. 2.—Chromatography of substance A on carboxymethyl cellulose, 0.88 meq./g.: 100 mg. with potency of 1 unit/mg. was applied to the column (22 × 375 mm.) in 0.02 *M* ammonium acetate buffer, pH 6, gradient to 0.2 *M* ammonium acetate buffer pH 7, through 500 ml. mixing chamber, 10 ml. fractions were collected: □, optical density at 280 mμ; ●, contractile activity on isolated guinea pig ileum.

under these conditions. It can be seen that the major ultraviolet absorption peak does not correspond to the peak possessing the greatest degree of biological activity. The activity in this latter peak represents approximately 85 mg. of standard substance A. It is of interest that almost 60% of the total activity was found in tubes 26 and 27. The eluates contained in tubes 25–30 were pooled and lyophilized. The dry residue was then taken up in a small amount of distilled water, centrifuged, and filtered and again lyophilized. A white powder weighing 15 mg. with an activity of 5.3 units/mg. was obtained. Varying degrees of purification have been obtained using one passage through a carboxymethylcellulose column. We have obtained preparations containing 4–16 units/mg. of material (see Table II). In an effort to purify further the column-purified substance A we have used the 1-butanol extraction procedure of Elliott and Peart.[13] The column-purified substance A (5.3 units/mg.) was dissolved in 0.5% trichloroacetic

(13) D. F. Elliott and W. S. Peart, *Biochem. J.*, **65**, 246 (1957).

TABLE II

PURIFICATION OF SUBSTANCE A

Treatment	Units/mg.
Standard substance A	1
Column chromatography on carboxymethylcellulose	4–16
Column chromatography on carboxymethylcellulose plus 1-butanol extraction	20
Column chromatography on carboxymethylcellulose. Two successive passages	62

acid and extracted into 1-butanol which was diluted with ethyl ether and the active material extracted from the organic solution with distilled water. Lyophilization of this extract resulted in a product possessing 20 units/mg. Recently, Elliott, Lewis and Horton[14] have reported that passage of impure bradykinin through two successive carboxymethylcellulose columns results in a relatively pure preparation. We have found also that two successive passages of substance A through this type of column results in a product that has a relatively high degree of activity, almost 70 units/mg. of material.

In our earlier studies on the pharmacological activity of standard substance A it was found that a biphasic response was obtained on the rat duodenum, that is, relaxation of the rat duodenum was followed by a contraction. Since Gaddum and Horton[6] have reported that bradykinin produces a relaxation of the rat duodenum, it was believed that substance A was contaminated with material possessing bradykinin activity. This was shown to be the case, since, when standard substance A was purified by chromatography on carboxymethylcellulose the purified material produced only a contractile response on the rat duodenum. If the column chromatography was extended past tube 45 (see Fig. 2), a biologically active fraction was obtained which produced a relaxation of the rat duodenum. The bradykinin-like activity in this fraction represented approximately 10% of the total biological activity as assayed on the guinea pig ileum.

Paper Electrophoresis.—This was carried out on strips of Whatman #3 paper 18 × 57 cm. in an apparatus similar to that described by Markham and Smith[15] and Davidson and Smellie[16] as modified by Hokin and Hokin.[17] The paper was irrigated for 48 hours with 4 *N* acetic acid before use. Chlorobenzene was used as the coolant in which the paper was immersed during the high voltage electrophoresis. The potential drop along the paper was approximately 45 volts/cm. and the buffer solutions employed were: pH 2.0, 5% (v./v.) formic acid; pH 2.5, 30% (v./v.) acetic acid; pH 4.0 and 6.0, acetic acid—sodium acetate (0.01 *M*); pH 8.0, HCl/sodium barbital (0.04 *M*); pH 9.5, glycine/NaOH (0.1 *M*). There was no rigid control of ionic strength. Substances were spotted in the center of the dry paper with the amount applied varying according to the quantity, concentration and activity of each sample. At the end of the run

(14) D. F. Elliott, G. P. Lewis, and E. W. Horton, *Biochem. J.*, **78**, 60 (1961).
(15) R. Markham and J. D. Smith, *ibid.*, **52**, 552 (1952).
(16) J. N. Davidson and R. M. S. Smellie, *ibid.*, **52**, 599 (1952).
(17) L. E. Hokin and M. R. Hokin, *Biochim. et Biophys. acta*, **13**, 401 (1954).

(usually 60 min.) the paper was dried in a current of warm air. Duplicate spots of each sample were run in order that one strip might be stained by either the ninhydrin method or the Pauly reagent of Ames and Mitchell.[18] Electroendosmosis was followed by using glucose as the neutral marker, the glucose being stained with aniline phthalate.[19] At the end of a run, successive one-half inch sections of the paper were eluted with Tyrode's solution and tested on the isolated guinea pig ileum to discover the site of biological activity. Using this technique it was possible to obtain reproducible results at pH values from 2–10 with the biological activity present in a confined area and readily eluted from the paper. In Fig. 3, the movement of substance A relative to glucose was plotted at various pH values, showing that substance A is amphoteric, with an isoelectric range of pH 6–7. It is of interest in this connection that substance A is eluted from the carboxymethylcellulose column between pH 6–7.

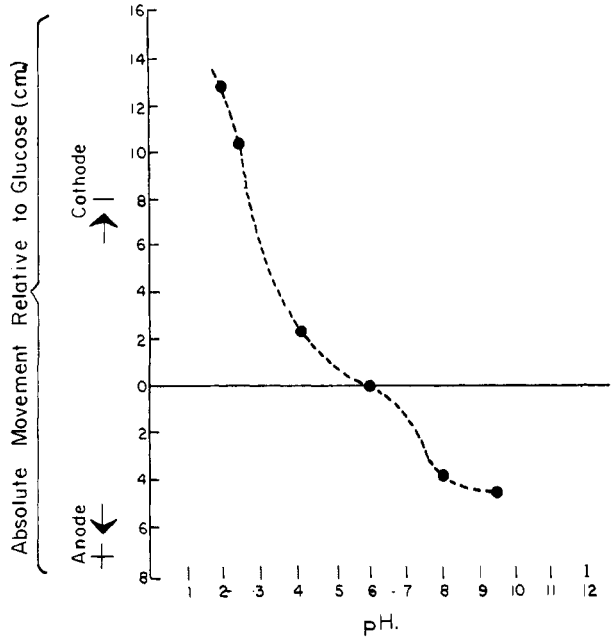

Fig. 3.—Paper electrophoresis of substance A, showing the movement of substance A relative to glucose at various pH's, run for 60 min. at 45 volts/cm.; 300 μg. of column purified substance A was applied and the activity was located by biological assay on eluates from successive 1.3 cm. strips of the paper.

(18) B. N. Ames and H. K. Mitchell, *J. Am. Chem. Soc.*, **74**, 252 (1952).
(19) S. M. Partridge, *Nature*, **164**, 443 (1949).

Discussion

Certain difficulties present themselves in releasing substance A from its substrate by the action of α-amylase. Consideration of the various factors to determine the most satisfactory method of obtaining relatively large amounts of crude substance A have indicated that the incubation of fraction IV-4 of human plasma protein with α-amylase at 0–4° could be utilized to provide the optimal conditions. The data presented indicate that the enzyme responsible for the formation of substance A is similar to renin in that the latter enzyme has been reported[8,9,10] to retain its activity near 0°. In addition, it would appear that the enzyme of destruction is similar to "hypertensinase" in that the latter is also inhibited by incubation at low temperature.

In the method presented, the crude substance A is obtained as a fraction contaminated by bradykinin and inactive polypeptides. Chromatography of this crude fraction on carboxymethylcellulose results in ready separation of substance A from the bradykinin-like polypeptide.