Studies on Pituitary Adrenocorticotropin. II. Paper Chromatography of Pepsin Treated Materials. (19159)

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Early work with paper chromatography of ACTH fractions in our laboratory(1) and similar work by Payne *et al.*(2) did not indicate any correlation between physiological activity and ninhydrin-reactive spots. More recently, however, we found the correlation to be complete and reproducible in fractions produced by peptic hydrolysis.

The reasons for our early difficulties probably include: (1) The heterogeneity of our early samples leading to poor resolution of ninhydrin-reactive spots; (2) Use of insufficient material to produce distinct coloration; (3) Operation under conditions which gave poor recovery of activity, and (4) Use of unsatisfactory technics for developing the ninhydrin color.

Methods. Whatman No. 1 and Whatman No. 4 paper have been used interchangeably with equal success. Most of the chromatograms have been made by the use of the ascending technic(3). All solvents were redistilled before use and those containing peroxides and/or aldehydes were first purified by the technic described by Newton and Abraham(4). Some technical grade solvents, such as sec-butanol, contain peroxides and completely destroy physiological activity on short contact. Purification, however, usually eliminates this difficulty. Because of this apparent susceptibility of ACTH to oxidative destruction, all chromatograms were run in an atmosphere of hydrogen. In order to eliminate the possible effect of heavy metals, all water used in making up chromatographic solutions and eluants was redistilled in pyrex glass and contained four milligrams of 8-hydroxyquinoline per liter. When assays were to be made on portions of the chromatogram, two identical runs were made on parallel 1½ inch sections of paper with a ½ inch section between. After the run was made and the solvent removed, the sections were cut apart and one was developed with ninhydrin(5). The second section was then cut into the desired segments. Each segment was further reduced by cutting into ⅛ inch strips for elution. Elution of activity was accomplished by immersing the paper in N/10 HCl for 15 minutes. Preliminary experiments on pepsin treated material of known potency showed that under these conditions all of the activity and ninhydrin-reactive material was removed from the paper. Since the amounts of material used on paper chromatograms are necessarily small and the eluates are dilute, such fractions were protected from oxidation prior to assay by the addition of a 0.05 M phosphate buffer at pH 7.0 containing the following additives per liter: 1.0 g glycine, 1.0 g cysteine hydrochloride and 4 mg 8-hydroxyquinoline. For application to the paper, all samples were made up at 10 mg/ml in redistilled water. In order to restrict the size of the original spot, only 0.01 ml was added at any one time. With most samples, 200 to 300 μg of material was required to give distinct ninhydrin spots. In such cases, the paper was thoroughly dried by an infra-red lamp between applications.

Starting materials. All samples used in this study were made from whole hog or sheep pituitary glands by the method outlined in a previous paper(1). The steps in this procedure include the initial extraction and fractionation to produce crude material at a potency of one to 3 times the International

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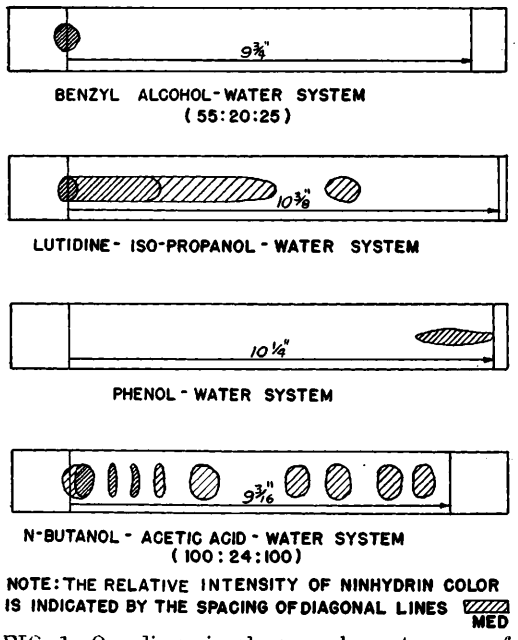

FIG. 1. One dimensional paper chromatograms of pepsin treated ACTH.

ACTH Standard, pH fractionation giving potencies of 5 to 10 times, followed by peptic digestion and trichloroacetic acid extraction giving potencies of 10 to 20 times standard. *Assay.* Physiological activity was determined by the Munson modification of the adrenal ascorbic acid depletion method of Sayers, Sayers, and Woodbury(6). The precision of this assay technic is such that a single determination, using 9 animals at one level compared to the same number of animals on each of 2 levels of standard, is subject to a standard error of 30%. In most of the work reported here, use of duplicates and repetition of experiments has considerably reduced this error. The reference standard used was the International ACTH Standard.*

Results. Preliminary runs (Fig. 1) using both sheep and hog preparations were made with the solvent systems commonly used for amino acids and peptides with the following results: (1) Benzyl alcohol-water. The ninhydrin-reactive material did not move from the point of application. (2) Lutidine-isopropanol-water (55:20:25)(7). Most of the ninhydrin-reactive material remained at the point of application but a somewhat better resolution was obtained than with the benzyl alcohol-water system. (3) Phenol-water. The ninhydrin-reactive material did not form bands but concentrated in a single streamer reaching to the solvent front. Further work with this system has shown that all fractions made from peptic digest material, whether active or inactive biologically give the same pattern. (4) N-butanol-acetic acid-water (100:24:100). Excellent resolution is obtained with this system. Pepsin treated ACTH from hog and sheep pituitary glands give similar patterns. Activity has been recovered only from the area corresponding to the slowest moving ninhydrin-reactive spot. Within the limits of error of the assay, all of the activity placed on the strip was recovered from this area, and no activity was recovered from any other portion. In these experiments the sensitivity of the animal assay was such that 1% of the total activity originally put on the strip was detectable.

In view of the excellent resolution obtained with the butanol-acetic acid-water system, a chromatopile(8) was run using 11 cm Whatman No. 1 paper discs. Three hundred mg of a peptic digest preparation from hog pituitary glands was taken up in water and diluted to 10 ml. Five-tenths ml of this solution was applied to each of 20 discs of the filter paper. The chromatopile was assembled with 40 blank discs at the top, then the 20 containing the sample, and finally 600 more blank discs. Starting at the top, a tagged disc was inserted after each group of 40. Hence, the sample was included in the second group of 40 discs.

The solvent was allowed to flow for 20 hours at room temperature in a confined space and during that time the solvent progressed through about 500 discs. The pile was disassembled, sectioned as tagged, and the discs were air dried. Each section was eluted by

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* Formerly designated as ARMOUR LA-I-A. These assays were carried out in the Biological Control Section with assistance from Mr. J. D. Fisher.

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TABLE I. Biological Activity of Fractions from Chromatopile Treatment of Pepsin Treated ACTH.

Fraction	Wt lyophilized solids (mg)	Original solids, %	Potency (× standard)	Total activity, int. units*	% activity of starting material
S M	300	100	15	4500	100
1	<1	<1	<.5	<.5	<.01
2	24	8	26	624	14
3	25	8.3	56	1400	31
4	20	6.7	34	680	15
5	9	3	<1	<9	<2
Total	78	26		2704	60

* One international unit is the activity of one mg of Int. Standard.

immersing the pack of 40 discs in 100 ml of N/10 HCl and agitating for 15 minutes. The discs were then transferred to a Buchner funnel and with the application of a vacuum were washed, first with the excess eluting solution and then with 100 ml of water. The eluates were adjusted to pH 3.5 with NaOH and concentrated *in vacuo* in a stream of hydrogen to about 25 ml. The concentrates were freed of salt by extracting biologically active material with liquid phenol and then reconstituting it in a fresh aqueous phase by the addition of ether. The resulting aqueous solutions were lyophilized and assayed.

In Table I is shown a summary of the fractions obtained from one such chromatopile in which 300 mg of a peptic digest material with a potency of 15 times the International Standard was used. Concentration of activity in fractions 2, 3, and 4 occurred with 30% of the total activity appearing in fraction 3 at a four-fold increase in potency. Other experiments have given similar results with recoveries of activity up to 100%.

The fractions from the chromatopile were tested by subjecting them to one dimensional paper chomatography. In Fig. 2 is shown the performance of Fractions 2 to 5 in the system n-butanol-acetic acid-water. Fractions 2 and 3 appear free from any ninhydrin-reactive material except that contained in the spot where the activity is concentrated. In view of the higher potency of Fraction 3, most of the subsequent work was done with this fraction. In the systems benzyl alcohol-water, collidine-water, lutidine-isopropanol-water, and methyl cellosolve-water (9:1)(9) Fraction 3 gave only one spot and that at the point of application. Phenol-water again gave a single ninhydrin-reactive area in the form of a streamer reaching to the solvent front.

Discussion. An ACTH fraction has been obtained which is not resolved in the solvent systems commonly used in the separation of amino acids and peptides. The R_f value of this material, however, is very low in all systems except phenol-water. The formation of a long streamer in the phenol-water system is not typical of the low molecular weight peptides reported in the literature(10,11). In fact, the appearance more closely resembles

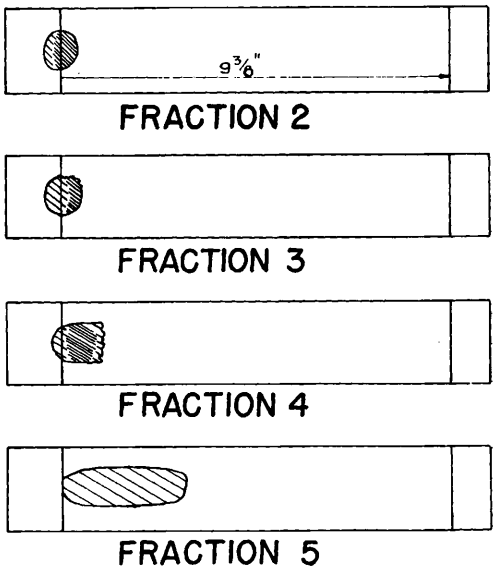

FIG. 2. One dimensional paper chromatograms of fractions from the chromatopile.

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the patterns obtained with proteins(12). This suggests the possibility that the product of the chromatopile has a higher molecular weight than the peptides so far studied in the solvent systems reported in this paper. This possibility is strengthened by the observation that the ninhydrin color produced by Fraction 3 is about one-tenth that obtained from an equal weight of an amino acid. A quantitative determination of ninhydrin coloration by the method of Moore and Stein(13) showed that 1 mg of Fraction 3 gives a color equal to that produced by about 0.06 mg alanine. If a mole-for-mole equivalence of color can be assumed, this would indicate a molecular weight of about 1500 for Fraction 3. Similar determinations on the starting materials for the chromatopile give a value of about 1200. Attempts are being made to devise new systems capable of resolving Fraction 3. Meanwhile, such samples provide excellent starting materials for studying the effects of enzymatic and acid hydrolysis.

Summary. Pepsin-treated ACTH materials at approximately 10 times standard were fractionated in a chromatopile using the system butanol-acetic acid-water. The activity was found to be associated with a slow-moving ninhydrin-positive area from which fractions having potencies of about 50 times standard were recovered. No further chromatographic resolution of these fractions was obtained in the solvent systems ordinarily used for amino acids and peptides. In all cases, the activity was associated with ninhydrin-positive areas of the chromatograms.

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Relationship of Ascorbic Acid to Secretion of Adrenocortical Hormones in Guinea Pigs.* (19160)

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Ascorbic acid was first isolated from animal tissues by Szent-Gyorgi who found the vitamin in significant quantities in the adrenal gland (1). Subsequently it was shown that ascorbic acid is present in greater concentration in the adrenal cortex than in any other tissue(2). It had been observed also that the adrenals hypertrophy in scurvy. This change has come to be recognized as one of the pathologic criteria of vit. C deficiency(3-5). These facts have stimulated interest in the relationship of ascorbic acid to adrenocortical function with the result that numerous investigations have dealt with the problem(6-13).

This paper reports the results of a study in which the secretion of adrenocortical hormones was determined in guinea pigs on

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