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Pituitary Hormones. V. The Purification of Corticotropin-B by Ion-exchange Techniques

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Methods have been developed for the purification of concentrates of corticotropin-B by ion-exchange techniques. Corticotropin-B undergoes exchange on columns of the cation-exchange resin Amberlite IRC-50 buffered with sodium ions, while inert materials are eluted by washing with aqueous pyridine and aqueous acetic acid solutions. The active principle is then removed from the resin by dilute hydrochloric acid, and is recovered as a solid hydrochloride, which is free of inorganic salts, and which possesses an activity of 250 to 300 u./mg. Considerable purification is also obtained by fractionation on columns of oxycellulose. In these processes, the presence of a reducing agent such as sulfite or hydrogen sulfide has inhibited inactivation and has made possible better separations and more highly active products.

At the time that work on the ACTH problem was initiated in this Laboratory, it was considered that the active principle of the pepsin digests of the "ACTH protein"[1,2] might be a small peptide with a chain of six to eight amino acid units.[3] It seemed likely that such a molecule should be amenable to purification by ion-exchange techniques and, accordingly, studies to this end were begun. It now appears that corticotropin-B, a very potent active principle of pepsin-digested corticotropin, is of considerably higher molecular weight. White, Fierce and Lesh have recently suggested[4] that partially pepsin digested material behaved more like a protein than a small peptide during paper chromatography. A molecular weight in the range of 5,000 to 6,000 has been advanced on the basis of ultracentrifugal studies and the amino acid composition of highly purified corticotropin-B.[5] Despite this higher molecular weight, purification of corticotropin-B has been achieved by ion-exchange techniques by us and other workers. The excellent process for the purification of crude extracts of corticotropin to the 80-u./mg. level with oxycellulose probably operates by an ion-exchange mechanism.[6] Elsewhere, crude corticotropin and ACTH "protein hormone" were chromatographed on the cation-exchange resin Amberlite IRC-50, using a *p*H 6.8 phosphate buffer.[7] The adrenocorticotropic activity was separated in this way from the bulk of the proteinaceous materials, and was indicated by its behavior to be a basic substance. Although proteinaceous molecules are usually too large to undergo ion-exchange with ordinary resins, in isolated instances compounds of relatively high molecular weight have been successfully fractionated by chromatography on Amberlite IRC-50, as has been shown by the recently reported purifications of the basic proteins ribonuclease,[8] lysozyme[8] and cytochrome-c[9] by this method.

Early in our study of the use of ion-exchange resins, it was noted that the highly active principle of corticotropin-B concentrates underwent exchange almost quantitatively with the cation-exchanger Amberlite IRC-50 buffered with sodium ions; that it was tightly held on the resin, indicating a strongly basic character; and that it could be eluted with hydrochloric acid. It also soon became evident that troublesome inactivations, apparently oxidative in nature,[4,7] could be prevented or minimized by the presence of reducing agents. Sodium sulfite was used in neutral or weakly alkaline solutions, and hydrogen sulfide was added to acidic solutions. This precaution resulted in sharper separations during the ion-exchange fractionations, and gave products of higher activity than were obtained without the use of the reducing agents.

These observations led to procedures which gave some threefold enhancement of the activity of corticotropin-B concentrates beyond the 60- to 100-u./mg. level. The active material of such concentrates[10] was allowed to undergo exchange with a sodium-buffered column of Amberlite IRC-50. A substantial amount of inactive proteinaceous material was removed from the column by washing it with aqueous pyridine; and other proteins, pyridine and sodium ions were eluted with aqueous acetic acid. An active concentrate (*ca.* 150 u./mg.) was eluted by washing the column with 0.01 *N* hydrochloric acid solution (*p*H 2.0). Finally, the remainder of the corticotropin-B was removed from the column by elution with hydrochloric acid solution of *p*H 1.6 and was recovered as a white, amorphous solid with an activity of approximately 250 to 300 u./mg. When the process designated procedure A in Table I was used, hydrogen sulfide was present in all of the solutions employed during the resin purification. Procedure B differed only in that sodium sulfite was substituted for hydrogen sulfide in the steps prior to elution of the column with acid.

The results from a number of such purifications are summarized in Table I. The results indicated above and those described in detail in the Experimental section represent typical *good* experiments. At times, the results were less satisfactory, with lower yields and products of lower activity. One factor which has on occasion affected the success of the process has been contamination of the starting materials with heavy metals. Such samples showed

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dark precipitates in the presence of hydrogen sulfide, and the products from these preparations tended to be low in activity. Another cause of occasional difficulty may have been the presence in samples of starting material of inactivation products so closely resembling corticotropin-B in properties as to make separation difficult. It must be strongly emphasized that due to variations and uncertainties in the bioassay, the values for activities and yields are to be regarded as approximate, even though replicate assays were obtained on some samples. Despite these difficulties, this type of purification procedure has been easy to carry out and adaptable to the handling of large quantities of material. The highly active products were readily recovered in stable form as the dry, solid hydrochloride, free of inorganic contaminants.

TABLE I

PURIFICATION OF CORTICOTROPIN-B CONCENTRATES ON AMBERLITE IRC-50

Starting material G.	u./mg.	Procedure	Product Mg.	u./mg.	Recovery of activity, %
280 mg.	80	A[a]	45	300[d]	60
300 mg.	80	A[a]	60	300(?)	75(?)
			40	300[d]	50
1.6	90	A[b]	198	200	27
			306	350[d]	75
1.0	80	B[b]	100	150	20
			40	375[d]	19
			50	275	17
2.0	60	A[b]	190	240	38
			140	300	35
1.3	76	A[b]	225	250	58
1.0	50	A[b]	210	150	63
300 mg.[e]	80	A[b]	Low activity		(?)
2.6[e]	75	A[c]	450	150	35
1.2	75	A[b]	Low activity		<20
1.0	60	B[b]	130	300	65
1.9	60	B[b]	235	260	60
1.7	75	B[b]	375	275	80
2.0	40	B[b]	310	140	54
1.5	40	B[b]	210	220	77
			200	130	40
1.45	55	B[b]	80	200	20
			500	130	82

[a] A 50-ml. buret was used for the column. [b] The column size was 80 × 2 cm. [c] The column size was 120 × 2 cm. [d] These samples were assayed three or four times, and an average value reported. [e] These samples appeared to be contaminated by heavy metals.

By the use of similar ion-exchange methods, quite crude corticotropin-B concentrates of activity 5 u./mg. or less were converted to preparations approximating 100 u./mg. in over-all yields which averaged about 35%.

In addition to the column techniques which have been described, a simple batchwise procedure for the purification of corticotropin-B concentrates with Amberlite IRC-50 was developed. The activities of preparations of potency about 80 u./mg. were doubled in approximately 80% yields by this method.

The methods involving ion-exchange resins for the preparation of concentrates of corticotropin-B in the 60 to 100-u./mg. activity range in our over-all isolation procedure were later largely replaced by an oxycellulose purification step. Astwood, Raben, Payne and Grady[6] had obtained corticotropin concentrates (not pepsin-digested) in greater yields and with less effort. Pepsin digestion of the oxycellulose product gave concentrates of corticotropin-B of activity 60 to 100 u./mg. which served as starting materials for further purification.

Fractionation of 100-u./mg. samples of corticotropin-B on oxycellulose (10.4% carboxyl)[11] columns resulted in doubling the activity. In this process, the material was put on the oxycellulose column in 0.1 *N* acetic acid solution and the column was washed with the same solvent and with dilute hydrochloric acid at *p*H 1.58, thereby removing impurities of low adrenocorticotropic activity. Elution of the column with hydrochloric acid at *p*H 1.40 removed the corticotropin-B, which was recovered as the solid hydrochloride at potency levels of about 200 u./mg. All solutions contained hydrogen sulfide.

The further fractionation of a corticotropin-B preparation which had been purified by ion-exchange to an activity of approximately 300 u./mg. was attempted using chromatography on oxycellulose by the method described above. About 60% by weight of the material was eluted in the manner of a single substance, as represented in Fig. 1. It may be noted that fractions represented by points on both branches of the curve had the same biological activity, within the limitations of the assay method. A peak fraction was examined for homogeneity by a twenty-transfer countercurrent distribution in the system *s*-butyl alcohol/0.5% aqueous trichloroacetic acid.[12] Comparison of the observed and calculated distribution curves[13] revealed the presence of only about 5% of substances detectable as impurities by this method.

Other fractions of corticotropin-B purified by ion-exchange and having activities of about 200 to 300 u./mg. were examined in more extensive countercurrent distributions of 200 and 450 transfers. The results of these investigations, which revealed the presence of other components in addition to corticotropin-B, are described in the accompanying article.[12] An electrophoretic examination[14] of a highly active fraction from the oxycellulose fractionation of 300-u./mg. material was carried out at *p*H 4.6 in acetate buffer. Two components having mobilities 2.8×10^{-5} and 3.4×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} were detected. This finding also suggests that the column-purified sample of corticotropin-B was not entirely homogeneous, although other interpretations involving for example, dissociation or partial interaction with the buffer are not impossible.

The differences in behavior of corticotropin-B (pepsin-digested) and corticotropin (not pepsin-digested) have been mentioned.[15] The non-di-

(11) We wish to thank Dr. R. W. Miller of Tennessee Eastman Co., who kindly supplied us with this material.

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(14) The authors are indebted to Dr. John B. Conn of this Laboratory for the determination.

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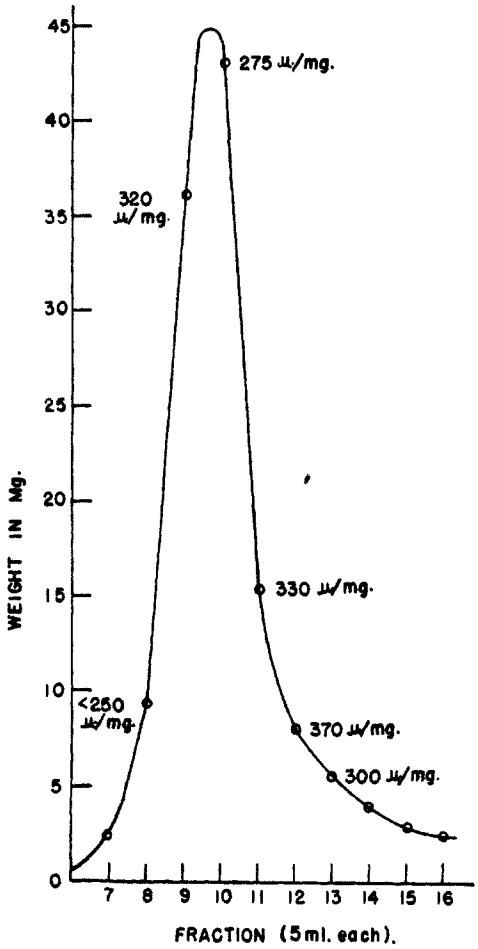

Fig. 1.—The fractionation of a corticotropin-B concentrate (300 u./mg. activity) on the oxycellulose column.

gested material did not undergo exchange efficiently with the resin under the conditions employed for the pepsin-digested corticotropin-B fractions. The oxycellulose column purification technique which gave corticotropin-B at potencies of 200 to 300 u./mg. yielded material of activity only 125 u./mg. when corticotropin was used even in the presence of reducing agents. The possibly larger molecular size of corticotropin may account for these differences. It is of interest in this connection that corticotropin-B is quite soluble in 5% aqueous trichloroacetic acid, while corticotropin concentrates are not.

A few experiments with other resins have been made. Such sulfonic acid resins as Amberlite IR-100 and IR-120, of small pore size, were unsatisfactory for the purification of corticotropin-B concentrates because of failure of the material to undergo exchange. Duolite C-10 and a Dowex-50 sample with 2% cross-linkages[16] held the active material so firmly that no satisfactory method for elution was found. Pepsin digests were not absorbed on the strong anion-exchange resins such as Amberlite IRA-400 and XE-75 when these were on the acetate cycle. When on the hydroxide cycle, the resins absorbed the activity, but satisfactory purification was not achieved.

(16) We wish to thank Dr. W. C. Bauman of Dow Chemical Company for this sample of Dowex-50.

Experimental[17]

Purification of Corticotropin-B Concentrates of Low Activity on Amberlite IRC-50.—Two columns (3.6 × 120 cm.) were employed. The first column contained 350 g. of Amberlite IRC-50 which had previously been treated with 7 g. (2%) of sodium hydroxide. The second column contained 350 g. of resin (20 to 60 mesh) in the hydrogen form. The exit tube at the bottom of the first column was connected to the top of the second column by tygon tubing. The solutions were first run into the column buffered with sodium ions and from it into the second column. A solution of a pepsin digest (10 g., 5 u./mg.) in 1 l. of water was passed through the columns at the rate of 3 ml./min. The columns were washed with 1 l. of water at the rate of 6 ml./min. and eluted with 3 l. of 10% aqueous acetic acid at the rate of 30 ml./min. Two liters of hydrochloric acid (*p*H 1.90) was run through the columns at 20 ml./min. All of these fractions were found to contain little activity and were discarded. The column was then eluted with hydrochloric acid (*p*H 1.60) at a rate of 6 ml./min. The most active material broke through the column just as the *p*H of the eluate dropped below 1.9. One liter of hydrochloric acid was required before this *p*H change occurred, and the fraction gave 210 mg. of product with an activity of 15 u./mg. With the second liter of hydrochloric acid (*p*H 1.50), 1.9 g. of material active at 30 u./mg., and representing a recovery of 58%, was collected. Finally, 2.5 l. of acid was passed through the columns to elute 1.6 g. of a concentrate of potency 15 u./mg.

The material active at about 30 u./mg. was further purified to a level of about 100 u./mg. by application of procedure A described below.

Purification on Amberlite IRC-50: Procedure A.—For the preparation of the resin buffered with sodium ions, 100 g. of Amberlite IRC-50 (30 to 60 mesh) on the hydrogen cycle was rapidly stirred in 1 l. of water while a solution of 2 g. of sodium hydroxide in 100 ml. of water was slowly added. After the addition of sodium hydroxide was completed, the resin was stirred for one-half hour. The resin, as a thick slurry in water, was poured into the top of a column 80 cm. long and 2 cm. in diameter filled with water. After the resin had settled by gravity, most of the supernatant water was removed and more resin was added. The column had a volume of 240 ml. and contained about 100 g. of resin. Before use, the column was washed with water containing hydrogen sulfide. The starting material was a corticotropin-B concentrate of activity 90 u./mg. derived from hog pituitary glands. A solution of this concentrate (1.6 g.) in 25 ml. of water saturated with hydrogen sulfide was passed through the column at the rate of 10 ml./hour. The column was washed over a period of two hours with 150 ml. of water containing hydrogen sulfide. It was rapidly eluted (800 ml./hour) with 1 l. of 10% aqueous pyridine, followed by 2.5 l. of 10% aqueous acetic acid. These eluants were prepared by diluting one volume of either pyridine or acetic acid to ten volumes with water, and then passing a slow stream of hydrogen sulfide into the solutions for five minutes. All of the solutions collected to this point were found to contain little adrenocorticotropic activity, and were discarded.

A 400-ml. volume of 0.01 *N* hydrochloric acid containing hydrogen sulfide was put through the column at a rate of 200 ml./hour (fraction 1), and was followed by 400 ml. (fraction 2) and then 600 ml. (fraction 3) of hydrochloric acid at *p*H 1.55–1.60 (*ca.* 0.028 *N*), all containing hydrogen sulfide. Sufficient Amberlite IRA-400 on the carbonate cycle was added to each solution to adjust the *p*H to 2.5 to 3.0. After removal of the resin by filtration, the eluates were frozen and lyophilized. All operations including lyophilization was conducted in a hood. The products recovered in the three fractions were: (1) 198 mg., 200 u./mg.; (2) 134 mg., 350 u./mg.; and (3) 172 mg., 370 u./mg.

(17) Samples were assayed in hypophysectomized male rats by a modification of the method of M. A. Sayers, G. Sayers and L. A. Woodbury, *Endocrinology*, **42**, 379 (1948). For a more detailed description of the assay as used in this Laboratory, the reader is referred to part III of this series, THIS JOURNAL, **74**, 480 (1952). Results are reported in terms of U. S. P. units of corticotropin, although a secondary house standard was actually used during most of the work. Because of the variations in the assay, all values should be regarded as approximate.

Purification on Amberlite IRC-50: Procedure B.—A column 66 cm. high and 2 cm. in diameter was filled with buffered Amberlite IRC-50 as described under procedure A. One gram of a corticotropin-B concentrate of potency 80 u./mg. and which had been prepared from commercial acid–acetone powder was dissolved in 20 ml. of aqueous sodium sulfite solution (200 mg. of sodium sulfite/l.) and passed into the column at a rate of 7 ml./hour. The column was washed with 200 ml. of the sodium sulfite solution. One liter of 10% aqueous pyridine containing 500 mg. of sodium sulfite was passed through the column in 1.5 hours, followed by 2.5 l. of 10% aqueous acetic acid at the same rate. The acetic acid eluate and the following acidic eluants contained hydrogen sulfide. The column was placed in a cold-room (0°) overnight. It was then eluted with 450 ml. of hydrochloric acid (*p*H 2.0, 0.01 *N*), which removed 60 mg. of material of potency about 150 u./mg. The following fractions were collected with hydrochloric acid of *p*H 1.58 and isolated as described in procedure A: fraction 1 (150 ml.), 41 mg., 170 u./mg.; fraction 2 (150 ml.), 40 mg., 375 u./mg.; fraction 3 (675 ml.), 50 mg., 275 u./mg.

Batchwise Purification on Amberlite IRC-50.—A mixture of 5 g. of Amberlite IRC-50 which had been buffered by 2% of sodium hydroxide, 500 mg. of a 75-u./mg. corticotropin-B concentrate and 50 ml. of a 0.2 *M* sodium sulfite solution was stirred for four hours. The resin was collected on a filter and washed with three 40-ml. portions of 10% aqueous acetic acid. The resin was slurried three times into 40-ml. portions of 0.1 *N* hydrochloric acid with occasional stirring on the filter and then sucked dry. The hydrochloric acid filtrates were adjusted to *p*H 2.5–3.0 with IRA-400 on the carbonate cycle and lyophilized. The colorless solid product weighed 210 mg. The recovery of activity at a level of 150 u./mg. was 84%. When a pepsin digest (80 u./mg.) was treated in the same manner except that 10% aqueous pyridine containing hydrogen sulfide was used in place of a sodium sulfite solution, a product of 180 u./mg. activity was recovered in an 80% yield.

Purification with Oxycellulose.—A column 0.9 × 90 cm. was packed with fresh, washed oxycellulose (10.4% carboxyl) suspended in 0.1 *N* acetic acid. The sample of corticotropin-B to be purified weighed 600 mg. and had an activity of 100 u./mg. All of the solutions used in this procedure contained hydrogen sulfide, and all were passed through the column at a rate of 6 ml./hour. The sample was applied to the column in solution in 10 ml. of 0.1 *N* acetic acid. The column was washed with an additional 50 ml. of the same solvent, and then with 250 ml. of hydrochloric acid at *p*H 1.58. The material eluted to this point, about 350 mg., showed assay values of less than 20 u./mg. The most active corticotropin-B was then eluted by hydrochloric acid at *p*H 1.40, and collected in 5.6-ml. fractions with the aid of an automatic fraction collector. The tenth and eleventh 5.6-ml. fractions yielded 48 mg. of product active at 330 u./mg.; fractions 12 and 13 gave 21 mg. of activity 320 u./mg.; and fractions 13 and 14 gave 7.5 mg. with an assay value of 210 u./mg.

Although activities of 300 u./mg. or more were obtained in this experiment, the procedure more frequently gave products of not more than 200 u./mg.

Repeated Oxycellulose Treatment of 300 u./mg. Corticotropin-B.—An 80 × 0.9-cm. column was filled with oxycellulose (10.4% carboxyl) which had been suspended in 0.1 *N* acetic acid containing hydrogen sulfide. A solution of 194 mg. of a corticotropin-B fraction of activity 300 u./mg. was introduced into the top of the column. All of the eluting solvents contained hydrogen sulfide. All of the fractions were collected at the rate of 6 ml./hour, and with the aid of a drop-counting automatic fraction collector adjusted to take 5.0-ml. fractions. The column was washed with 30 ml. of 0.1 *N* acetic acid, yielding 6 mg. of material of low activity. Elution with 125 ml. of hydrochloric acid at *p*H 1.58 gave 9 mg. of essentially inactive product. Finally, the active corticotropin-B was removed with hydrochloric acid at *p*H 1.40. As may be seen from Fig. 1, some 60% of the weight of the starting material was recovered in fractions 9 through 13 at activities of the order of 300 u./mg.

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Pituitary Hormones. VI. The Purification of Corticotropin-B by Countercurrent Distribution

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The isolation of corticotropin-B from its concentrates has been accomplished by the countercurrent distribution technique. In the solvent system employed, *s*-butyl alcohol and 0.5% aqueous trichloroacetic acid solution, the trichloroacetate salt of corticotropin-B was characterized by a distribution coefficient (C_{org}/C_{aq}) of 0.5 to 0.6. This component of high adrenocorticotropic activity behaved as a pure substance in countercurrent distributions of 200 to 450 transfers. Despite this, some loss of biological activity in the isolation of the most potent material has been indicated. The presence of two other components in all corticotropin-B concentrates subjected to countercurrent distribution has been observed. One of these appeared to have a low order of adrenocorticotropic activity.

Several groups of investigators have employed the countercurrent distribution technique for the fractionation of corticotropin and corticotropin-B concentrates. A phenol–ether/water system when applied to a pepsin digest of corticotropin with activity 18 u./mg. gave a fraction of potency 120 u./mg. based upon total nitrogen.[1] Other workers subjected 20 u./mg. of corticotropin concentrates which had initially been purified by cellulose chromatography to countercurrent distributions with butanol or *s*-butyl alcohol/aqueous organic acid systems and in some cases secured products of assay values about 100 u./mg. The best purifications were achieved with picric, sulfosalicylic and benzenesulfonic acids.[2] A recent report[3] has described the use of a countercurrent distribution system prepared with 2,4,6-collidine and water which separated pepsin-treated material from either sheep or porcine sources into at least two components. In neither case was the increase in activity as great as that expected from the separation of solids.

The solvent systems mentioned and a number of others which have been tried with apparently less success had offered one or more serious disadvantages: emulsification, extensive destruction of adrenocorticotropic activity, unfavorable distribution coefficient, difficulty of locating the adreno-

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