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Chromatographic Studies on Corticotropin

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Adrenocorticotrophic hormone has been assayed and defined in several ways, but of late the term has been restricted to substances active in the ascorbic acid depletion test of Sayers, Sayers & Woodbury (1948). It is with factors active in this test by intravenous injection into hypophysectomized rats that this paper is concerned. Corticotropin A has been chemically characterized (White, 1953) after isolation from pig pituitaries and shown to possess activity. The designation A is to distinguish it from corticotropin B, isolated after treatment of concentrates with pepsin by Brink *et al.* (1952). As we, in agreement with Bell (1954), are reporting the presence of more than one active substance in such concentrates before hydrolysis, it is at present convenient to give these designations A₁, A₂ etc. The corticotropin B group may require similar subdivision.

Preliminary results (Dixon, Moore, Stack-Dunne & Young, 1951) suggested the use of ion-exchange chromatography with a carboxylic resin, because of the success of this method with the basic proteins ribonuclease and lysozyme (Hirs, Moore & Stein, 1953; Tallan & Stein, 1953). Some of our earlier results have been published, in particular the

separation of the active material from a preparation of the protein hormone which had been thought to be the active substance. Extension of the method to concentrates gave better results than with crude materials (Dixon, Stack-Dunne & Young, 1953). Many other workers have since used ion-exchange chromatography with ACTH, and the chief successes of the method have been the isolation of corticotropin A (White & Fierce, 1953) and α -corticotropin (Li *et al.* 1954).

METHODS

Materials

(1) *Pituitary powder.* Whole pig pituitaries were collected in acetone and dried with acetone after mincing. The pituitaries were removed within 1 hr. of death, and the final drying was within 10 days. Potency, 0.1–0.2 i.u./mg.

(2) *Crude corticotropin and oxycellulose-purified corticotropin (OPC preparation).* These were prepared from whole-pituitary powder by the methods of Payne, Raben & Astwood (1950) and Astwood, Raben, Payne & Grady (1951).

(3) *Fraction L (crude prolactin).* This was prepared by a modification of the method of Lyons (1937). The advantage of raising the acetone concentration during the extraction was retained by adding further acetone, before separating the solids, after extraction at a low acetone concentration. The preparation was carried out at 0–3°.

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Pituitary powder (250 g.) was stirred with 1.5 l. of acetone, 0.94 l. of water and 70 ml. of 11.6*N*-HCl (a final concentration of 60%, v/v, acetone) overnight. Acetone (2.5 l.) was added over 1 hr. with stirring (acetone concentration 80%, v/v), the mixture stirred for 15 min. and the supernatant separated by centrifuging. The solids were re-extracted with 1.25 l. of 80% (v/v) acetone for 0.5–1 hr. and separated. The combined extracts (about 4.8 l.) were mixed with 9.3 l. of acetone and allowed to stand overnight. The precipitated solids were dried with acetone. Weight yield 7–10%. ACTH potency 1–2 i.u./mg.

(4) *Fraction OL*. This preparation was made at room temperature. Fraction L (20 g.) was dissolved in at least 250 ml. of 0.1*N* acetic acid and centrifuged if necessary. Sufficient Amberlite IR-4B acetate was stirred with the solution to remove the chloride originally present in L, and the solution filtered. The solution was treated with 2.0 g. of oxycellulose in a total volume of 1 l. of 0.1*N* acetic acid, and the preparation continued as described by Astwood *et al.* (1951). Weight yield, 2.0–2.5%. Potency, 15–40 i.u./mg.

Chromatography

The carboxylic resin Amberlite IRC-50 in the ground form XE-64 was used after the cleaning and preparation described by Hirs *et al.* (1953). By alternate stirring and settling two fractions were selected. The one used for most of the work was of such particle size as to give a flow rate of about 5 ml./cm.²/hr. under gravity alone when packed in a column of 30–40 cm. height. The finer fraction required a pressure of 15–25 cm. Hg to maintain half this rate of flow. The buffer used for most of the work was 0.2*M* in phosphate and 0.3*N* in Na (pH 6.7). The sodium salt of the resin was equilibrated with the buffer, when it was in suspension in 0.2*M* phosphate, by the addition of 0.2*M*-Na₂HPO₄ or 0.2*M*-NaH₂PO₄ until the pH of a sample of filtrate from the suspension was within 0.01 unit of that of the buffer. Care is needed (cf. Hirs *et al.* 1953) to ensure that equilibrium has been attained. Phenol (0.2%, v/v) was added to the buffer to act as a preservative.

No antioxidant has been found necessary for the chromatography of concentrates at the relatively high loads described. Thiodiglycol was previously used (Dixon *et al.* 1951) in the chromatography of crude extracts of ACTH, but one sample of thiodiglycol was found to destroy the biological activity.

Columns were prepared and operated according to the methods of Hirs *et al.* (1953). A flow rate of 2–4 ml./cm.²/hr. was used, and a load of 5–20 mg./cm.² of oxycellulose-purified concentrates, or about a third of this for the re-chromatography of single components. The sample was applied for chromatography in a volume of buffer corresponding to 1.0–1.5 ml./cm.² of the column. Fractions, each of about 1.5 ml./cm.², were collected.

Expression of chromatographic results. The concentration of solute in the eluate was determined by the photoelectric ninhydrin methods of Moore & Stein (1948, 1954). This was plotted against the volume of eluate, *V*, from the application of the sample, corrected to what it would have been for a 100 ml. column resin bed. $V_{100} = 100V_{\text{obs}}/v$, where *v* = resin bed volume. The fraction size was checked by weighing the tubes and the resin bed volume accurately determined.

In elution chromatography it can be shown that $E - F = bK$, where *E* is the retention volume (the volume of

effluent from the application of the sample to the emergence of the solute in maximum concentration), *F* is the volume of liquid in the bed, *b* is the volume of stationary phase, and *K* is the partition coefficient of the solute between the stationary phase and the liquid, in favour of the stationary phase. Also $N = E(E - F)/S^2$, where *N* is the number of theoretical plates in the column, and *S* is the standard deviation of the distribution curve representing the concentration of solute in the effluent as measured in terms of effluent volume. These expressions may be derived as follows (Stack-Dunne, 1954):

If the column is considered as a series of 'plates' where the concentrations of solute in the mobile and static phases are brought into equilibrium with each other in each plate separately, and the mobile phase in each plate is then transferred quantitatively to the next one along, the operation is analogous to that of a countercurrent machine operated according to the single withdrawal scheme of Craig (1950), and the same mathematical treatment can be applied.

The symbols already defined are used, and also the following: *T* = the number of transfers up to the point of emergence of the solute in maximum concentration, and *p* = the fraction of the solute in the mobile phase of any plate at equilibrium. Then

$$(1 - p)/p = Kb/F \quad (1)$$

since the factor *b/F* corrects the partition coefficient for the different volumes of the phases.

After *t* transfers the distribution of solute in the plates is given by the terms of the binomial expansion of

$$(p + [1 - p])^t,$$

and of these the (*pt* + 1)th term is the largest. The solute therefore travels at *p* times the rate of the solvent.

Thus after *T* transfers the solute emerges from the column at maximum concentration (at effluent volume *E*), where $pT = N$, and

$$p = F/E = N/T. \quad (2)$$

By combining eqns. 1 and 2 we obtain $E - F = bK$. This relationship has also been derived by Duin (1952).

The standard deviation of the distribution in the tubes, i.e. $(p + [1 - p])^t$, in terms of number of tubes, is given by

$$S_T = \sqrt{[tp(1 - p)]}. \quad (3)$$

The distribution of the solute in the effluent is a reflexion of the distribution on the column. When the peak concentration emerges, $t = T$, and it can be assumed that because *N* is large, the width of the distribution relative to *N* will be small, and that all the emergent material has been subjected to *T* transfers. In fact, the leading portions will have had less than, and the trailing portions more than, *T* transfers; these effects tend to cancel out.

If the peak of the distribution is moved the distance of one plate on the column, an effluent volume of *E/N* must emerge, and the contents of one plate will be in this volume. Hence

$$S_T = SN/E \quad \text{or} \quad S = ES_T/N \quad (4)$$

This equation may be rewritten, by using eqn. 3 and putting $t = T$, as

$$S = \frac{E}{N} \sqrt{[Tp(1 - p)]},$$

from eqn. 2 and eliminating *T* and *p* this becomes:

$$N = \frac{E(E - F)}{S^2}.$$

This equation is somewhat similar to one derived by Mayer & Tompkins (1947). It can be applied to determine the resolving power from the plot of any elution chromatogram, with or without knowledge of the units in which the effluent is measured. The relations between the terms here used and the conventional R values are:

$$R = v/E \quad \text{and} \quad R_F = F/E.$$

Isolation of products after chromatography (desalting).

(1) By ion exchange. The solution was adjusted to pH 3.0 and allowed to run through a 5 cm.-high column of the same fraction of Amberlite IRC-50 (XE-64) that was used in chromatography. The resin was in its acid form. The load of solution was 10 ml./cm.² and the flow rate 10 ml./cm.²/hr. The column was washed with 1 ml./cm.² of 0.1N acetic acid and the hormone displaced with 1M-NH₃ solution. The fractions close to the break-through of alkali were combined and dried while frozen. The whole process was carried out at 0–5° and the columns were cooled with jackets of ice and water. This method has been found to cause modification of the A₁ component and its use has therefore been abandoned.

Modifications of this method may be used for purifying crude material. Extracts of pituitaries in 0.2N-HCl may be adjusted with sodium acetate to pH 3 and run through columns of Amberlite IRC-50 (beads or ground form). The active material is retained and inactive matter may be washed out with 0.1N acetic acid. Elution with M ammonium acetate solution of pH 9.5 displaces further inactive material first, and the active material shortly before the alkaline break-through. This method has not been developed further. The active material was not adsorbed by oxycellulose from the medium of ammonium acetate after acidification with HCl (at pH 3.0 or 4.5).

Aqueous pyridine (25%, v/v) did not displace the active material from a desalting column, in agreement with the findings of Richter, Ayer, Bazemore, Brink & Folkers (1953). A solution of pyridine (30%, v/v) and acetic acid (4%, v/v) rapidly displaced active material without modification after the resin had been washed with pyridine. Inorganic material, which had evidently been adsorbed together with the hormone, was, however, simultaneously displaced.

(2) By phenol extraction. The principle of White, Fierce & Lesh (1951) has been used as follows:

Reagents: Phenol, redistilled under reduced pressure, equilibrated with 0.01N-HCl; 0.01N-HCl saturated with phenol; 0.01N-HCl; peroxide-free wet ether. The fraction was adjusted to pH 3.5 with conc. HCl. This acidified solution (100 ml.) was extracted with 30 ml. of phenol and twice more with 20 ml. of phenol. Each phenol extract was washed six times with 10 ml. of 0.01N-HCl (saturated with phenol) by passing it in turn through six separating funnels. The phenol extracts were combined (60 ml.) and to them were added 60 ml. of 0.01N-HCl and 320 ml. of ether. After equilibration, the aqueous phase was passed through two washes, each of 60 ml. of ether, and the three ether phases extracted twice in turn with 60 ml. of 0.01N-HCl. The combined aqueous phases were treated with Amberlite IR-4B acetate or Amberlite IRA-400 bicarbonate to remove the HCl and were re-extracted with 60 ml. of ether. The clean resin had been washed shortly before use with water, ethanol, ether, ethanol, water and, for the acetate, dilute (0.1%) acetic acid. Part of the dissolved ether was

removed by boiling the solution under reduced pressure before freezing and drying while frozen. Thiodiglycol is not removed in this process.

Ionophoresis on paper

The method of Gordon, Gross, O'Connor & Pitt-Rivers (1952) has been used. Following these authors, veronal buffer (0.05M) of pH 8.8 was used and Whatman no. 3 filter paper. The paper was dipped in the buffer and blotted. Samples of 50–400 µg. (usually 200) were applied in 10–20 µl. of buffer or water. A potential of about 2.5 v/cm. and a time of 16 hr. were found to give optimum separation. Staining with bromophenol blue (Durrum, 1950) and spraying with 0.2% ninhydrin in *n*-butanol were used for the detection of spots. Samples were applied close to the equilibrium positions of their components (where migration was balanced by flow, which was due to capillary replacement of evaporation in addition to electro-osmosis) to attain the sharpest spots (cf. Durrum, 1950).

Pepsin hydrolysis

Samples were incubated at a concentration of 1 mg./ml. in 0.01N-HCl with 2% of their weight of crystalline pepsin (Armour), at 37°. Incubation times of 1.5, 3 and 24 hr. were studied. There was little inactivation under these conditions in 3 hr. The hydrolysate was heated in 2–3 min. to 80°, maintained at this temperature for 5 min. and cooled in 2–3 min. Excess of Amberlite IR-4B acetate was added to remove chloride and the solution filtered and dried while frozen. Weight yield, about 90%.

Bio-assay

The ascorbic acid depletion method of Sayers *et al.* (1948) was used with minor modifications (Stack-Dunne, 1953). Wistar albino rats of 200–350 g. weight were used. The ascorbic acid was determined by electrometric titration.

Amino acid analysis

A sample of 5 mg. was boiled under reflux for 24 hr. in a mixture of 5 ml. 10N-HCl and 5 ml. of 90% (v/v) formic acid. The hydrolysate was analysed by chromatography on a column of the sulphonic resin Dowex-50, with N-, 2.5N- and 4N-HCl as eluents (Stein & Moore, 1949). The fractions of eluate were evaporated to dryness and allowed to react with ninhydrin under the conditions specified by Troll & Cannan (1953).

RESULTS

Chromatographic patterns. Chromatography of OPC preparations (oxycellulose concentrates of acetic acid extracts of pituitaries) showed resolution of both activity and ninhydrin-reacting material into two main peaks (Fig. 1) and a minor peak. There was, in addition, an unretarded peak of ninhydrin-reacting material which had small but definite activity. A similar pattern was found with preparation OL (an oxycellulose concentrate of an acid-acetone extract of pituitaries) (Fig. 2).

The positions of the peaks agreed well between chromatograms when columns of 0.8–1.0 cm.

Fig. 1. Chromatography of oxycellulose concentrates of glacialacetic acid extracts of pituitaries. Continuous line: ninhydrin colour. Blocks: biological activity. Column C66 was 3.51 cm.² × 39.5 cm. and the load was 188 mg. of an OPC preparation made by using the usual nitrogen tetroxide oxycellulose with 11% of CO₂H. Column C62 was 0.68 cm.² × 40.0 cm. and the load was 10 mg. of an OPC preparation made by using periodate chlorite oxycellulose with 8% of CO₂H.

Fig. 2. Chromatography of an oxycellulose concentrate of corticotropin (preparation OL). Analytical column C113 was 0.6 cm.² × 34.2 cm. and the load was 15 mg. Preparative column C187 was 13.4 cm.² × 29.2 cm. and the load was 205 mg.

Table 1. *Effects of changes in the chromatographic system on the retention volumes of corticotropin peaks*

Columns of 0.8–1.0 cm. diameter and 20–40 cm. height (except last)						
Sodium phosphate buffer		<i>E</i> values (ml./100 ml. resin bed) for			Notes	Column no.
Normality of Na†	pH	A ₁	A ₂	A ₃		
0.3	6.70	190	129	98	OL sample	C113
		192	136	103	A ₁ sample modified by ion-exchange desalting	C119
		188	133	103	Same sample after heating in glacial acetic acid	C120
		182	128	—	OL sample; 1% (v/v) benzyl alcohol added to buffer	C122
		188	131	104	OL sample; 0.5% BRIJ 35† added to buffer	C123
	Means	186	132	99	A ₁ sample modified by ion-exchange desalting	C125
		183	130	—	OL sample; zinc acetate added to sample	C173
		192	135	102	OPC sample	C174
		188	132	102		
		226	156	120	OL sample; phosphate 0.207M	C148
0.3	6.79	174	125	87	OL sample; phosphate 0.194M	C147
0.35	6.70	130	104	—	OL sample; phosphate 0.232M	C142
0.25	6.70	362	216	140	OL sample; phosphate 0.168M	C145
0.366	7.42	77	Not resolved		OL sample; phosphate 0.2M; system suitable for some corticotropin B components; Na ₂ HPO ₄ / NaH ₂ PO ₄ =5 (molar ratio)	C193
0.3	6.70	283	187	—	A ₁ sample modified by ion-exchange desalting	C177*
		283	190	139	OL sample	C186*
0.1	9.0	600	—	—	OL sample; system of White & Fierce (1953) (bicarbonate)	C201
0.103	8.5	239	143	—	{ OPC sample; column in collaboration with E. B. Astwood, M. S. Raben & I. Rosenberg for study of intermedin and adipokinin; column 1.5 cm. diam. × 70 cm.	D3‡

* Finer resin sample used.
† A polyoxyethylene lauryl alcohol (Atlas Powder Co., Wilmington, Delaware).
‡ Using Amberlite IRC-50 (XE-97).

diameter were used (Table 1, top section), but preparative columns of 2 or 4 cm. in diameter showed more variable and larger retention volumes, and this is illustrated in Table 2 (cf. Figs. 1 and 2). The peaks are named A_1 , A_2 and A_3 as shown in Fig. 2. After peak A_1 further active material could be displaced from the resin with N ammonia solution. This material is analogous to the types II and III ACTH of White & Fierce (1953).

A modification of the OL preparation was made, designed to shorten or make milder every step used after the acetone drying of the glands. Half the quantity of hydrochloric acid was used in the extraction and the final pH was 2.1, in place of the usual value of 1.5–1.7. The product was isolated and dried on the same day instead of being set aside overnight. In the treatment with oxycellulose the whole process was performed at 0–3° and the elution with hydrochloric acid was shortened to a total of 2 hr. Chromatography showed the typical

pattern for OL and typical weight yields of the peaks were obtained (C209 in Table 3). It is therefore unlikely that any interconversion of peaks occurred during the preparation of OL from acetone-dried glands.

Table 2. *Comparison of retention volumes of corticotropin peaks on wide and narrow columns*

Sodium phosphate buffer, 0.3N-Na, pH 6.70 (0.2M phosphate). Retentions in ml./100 ml. resin bed. $\pm$ S.D. between columns. Analytical columns 0.8–1.0 cm. in diameter, 20–40 cm. in height; 8 columns; various samples (Table 1). Preparative columns 3.9–4.2 cm. in diameter, 29–34 cm. in height; 5 columns; OL samples.

Peak	Retention volumes	
	Analytical	Preparative
A_1	188 $\pm$ 4	212 $\pm$ 12
A_2	132 $\pm$ 3	141 $\pm$ 5
A_3	102 $\pm$ 3	105 $\pm$ 5

Table 3. *Potencies of fractions isolated from OL by chromatography*

Fractions A_1 and A_2 consist of the resolved peaks A_1 and A_2 . Fraction A_3 contains the imperfectly resolved peak A_3 and fraction A_4 signifies the unretarded material. Potencies are expressed in i.u./mg. followed in brackets by the fiducial limits for $P=0.95$.

Potency of sample	Fraction	Weight yield (%)	Potency	Activity yield (%)	Remarks
18 (11–30)	A_4	5.9	1.0 (0.3–3)	0.35	C115. Desalted by ion exchange
	A_3	4.7	—	—	
	A_2	10.9	17 (12–23)	10.6	
	A_1	14.8	30 (22–40)	26	
		36			
18 (11–30)	A_4	6.8	—	—	C127. Desalted by ion exchange. (Before desalting A_1 had potency of about 36)
	A_3	5.6	—	—	
	A_2	11.9	—	—	
	A_1	21.4	20 (8–53)	26	
		46			
17 (9–30)	A_4	6.5	—	—	C161. Desalted by ion exchange
	A_3	2.4	17 (8–36)	3	
	A_2	10.0	24 (11–49)	14	
	A_1	18.5	39 (23–67)	44	
		38		61	
17 (9–30)	A_4	2.2	—	—	C164. Desalted by ion exchange. Column run at 0°
	A_3	2.0	—	—	
	A_2	7.9	—	—	
	A_1	13	29 (11–73)	22	
		25			
33 (19–56)	A_4	Negligible	—	—	C182. Phenol desalted
	A_3	7	—	—	
	A_2	13	23 (9–60)	9	
	A_1	26	42 (21–86)	33	
		46		42	
33 (19–56)	A_3	5	—	—	C187. Phenol desalted
	A_2	26	—	—	
	A_1	25	35 (24–50)	—	
		56			
	A_3	3	—	—	
	A_2	13	—	—	C209. Phenol desalted. OL rapidly prepared
	A_1	21	32 (15–68)	—	

When the OL preparation, instead of being made from acetone-dried pituitaries, was made from frozen pituitaries, or pituitaries which had been allowed to autolyse for one day, a different chromatographic pattern was obtained. The A_2 peak was smaller, only 10% of the A_1 peak in ninhydrin reaction, in place of the usual 30–50%. A similar result was obtained when acid-acetone was used for drying the glands. Fig. 3 shows the

Fig. 3. Comparison of oxycellulose concentrates from glands dried with acetone and with acid-acetone. Column C236 was $0.6 \text{ cm.}^2 \times 39.8 \text{ cm.}$ and the load was 6.2 mg. of OL from pituitaries dried with acetone alone. Column C235 was $0.6 \text{ cm.}^2 \times 39.8 \text{ cm.}$ and the load was 5.3 mg. of OL from pituitaries dried with acid-acetone.

comparison of OL preparations made from one batch of frozen glands, 25 g. of which (about 100 glands) were dried for 6 days in 250 ml. of acetone, and another 25 g. of which were dried in 250 ml. of acetone containing 1.5 ml. of 10N hydrochloric acid. Half the usual quantity of hydrochloric acid was added in the subsequent extraction with 60% acetone of the batch previously treated with acid. These results suggest that the hormone is more predominantly in one form in frozen glands, glands dried with acid acetone and glands allowed limited autolysis than it is in the acetone-dried glands used for the greater part of this work. The small A_2 peak of the acid-acetone-dried preparation possessed biological potency comparable with A_1 with respect to ninhydrin colour.

On rechromatography the isolated components ran true with respect to retention volume (Fig. 4). The A_1 peak shown (C184) had been desalted by extraction into phenol, since ion-exchange desalting modified this material. The A_2 sample (C224) was derived from a preparative chromatogram of OL and the tails of its peak probably represent heterogeneity; an A_2 peak without such

tails is shown in Fig. 6. The A_3 peak (C144 of Fig. 4) was derived from a chromatogram of the A_2 and A_3 peaks of several columns.

Fig. 4. Rechromatography of A_1 , A_2 and A_3 materials. Column C184 was $0.68 \text{ cm.}^2 \times 29.2 \text{ cm.}$ and the load was 3.3 mg. of A_1 material phenol-desalted from a preparative chromatogram. Column C224 was $0.6 \text{ cm.}^2 \times 40.6 \text{ cm.}$ and the load was 1.8 mg. of A_2 material phenol-desalted from a preparative chromatogram. Column C144 was $0.68 \text{ cm.}^2 \times 25.6 \text{ cm.}$ and the load was 3.2 mg. of A_3 material from a second chromatogram of several A_2 and A_3 fractions (desalted by ion exchange).

Modification occurred when A_1 material was desalted by ion exchange. Chromatography after such desalting showed the appearance of A_2 and A_3 peaks (the latter in only small amounts). Different amounts appeared in the different samples desalted. A sample of A_1 from a preparative chromatogram of an OL sample was divided and parts were desalted by the two different methods. Rechromatography (Fig. 5) showed production of A_2 in the sample desalted by ion exchange, but not in that extracted into phenol. A second ion-exchange

desalting of the A_1 that had survived the first produced further conversion into A_2 , as shown in C135 of Fig. 5, showing that there was no part of the A_1 which resisted the change. The assays shown in Fig. 5 suggested that no change of biological activity accompanied the modification.

After standing overnight at pH 11.3 in buffer (Na_2CO_3 , 0.28M; Na_3PO_4 , 0.043M; Na_2HPO_4 ,

0.007M), A_1 material was completely converted into A_2 as C225 (Fig. 6) shows. A sample of 5.2 mg. of A_1 was used, and in the first 5 hr. $1\text{--}1.5\ \mu\text{M}$ of volatile base was given off and the evolution was nearly complete. This result was consistent with the loss of one group of amide ammonia from a substance of molecular weight 4000–5000.

Fig. 5. Modification of A_1 peak on desalting by ion exchange. Comparison of columns C129 and C130 shows the modification. C135 shows that the residual A_1 of C130 can also undergo the change. C129 was $0.68\text{ cm.}^2 \times 30.1\text{ cm.}$ and the load was 10 mg. of A_1 peak from a preparative column desalted by the phenol method. C130 was $3.5\text{ cm.}^2 \times 29.7\text{ cm.}$ and the load 56 mg. of A_1 peak from a preparative column desalted by ion exchange. Assays (Sayers *et al.* 1948) on marked tubes are shown below. The fractions were tested at a dose equal with respect to their ninhydrin colour.

Tube no.	Ascorbic acid change (mg./100 g.)	S.E.M.	No. of rats
42	-97	15	4
45	-88	11	5
48	-94	16	4
51	-77	9	4
60	-102	12	4
66	-90	13	5
69	-67	7	4

Conclusion: no significant change in biological activity occurred on conversion of A_1 peak into A_2 . C135 was $0.68\text{ cm.}^2 \times 41.0\text{ cm.}$ and the load 16 mg. of the A_1 peak from C130 desalted by ion exchange.

Fig. 6. Chromatography of corticotropin A_2 produced by alkaline treatment of A_1 material. Column C225 was $0.6\text{ cm.}^2 \times 40.6\text{ cm.}$ and the load about 3 mg. of phenol-desalted product after corticotropin A_1 had stood for 15 hr. in solution in buffer of pH 11.3.

The asymmetry of the A_1 peak (e.g. Fig. 2, C187) suggested that two partially resolved components might be present. Accordingly, the forward and backward parts of this peak from a preparative chromatogram were separately desalted and rechromatographed. Fig. 7 shows the results with the preparative column and the analytical columns. As usual the retention volumes were larger with the wider column, but agreement of position of the peak is shown between the two analytical columns, despite the failure of the fraction collector to give regular fractions in C191. This result suggests that the asymmetry of the peak was not due to inhomogeneity of A_1 .

Characterization of peak materials. Table 3 gives the potencies of the peak materials isolated from chromatograms. Potencies of 30–40 i.u./mg. have been achieved for corticotropin A_1 .

There was no evidence of heterogeneity of A_1 material on electrophoresis at pH 8.2 in glycine-NaCl buffer (mobility $-1.3 \times 10^{-5}\text{ cm.}^2\text{v}^{-1}\text{ sec.}^{-1}$ at $18\text{--}19^\circ$; $I=0.1$; glycine 0.0086M; NaCl 0.0986M), or at pH 5.1 in acetate-NaCl buffer (mobility $+0.4 \times 10^{-5}\text{ cm.}^2\text{v}^{-1}\text{ sec.}^{-1}$ at 0° ; $I=0.2$; acetate 0.0265M; NaCl 0.09M). This is consistent with an isoelectric point between these pH values, as Li *et al.* (1954) have reported for sheep α -corticotropin.

Ultracentrifuging at pH values of 7.6 and 2.7 revealed only one component, with sedimentation values of 0.85 and 0.72 Svedberg unit respectively (corrected to water at 20°).

Fig. 7. Chromatographic comparison of leading and trailing edges of A_1 peak. The positions of the A_1 peaks in columns C190 and C191 agree. As usual, the peaks are less retarded than in the wider column C187. Preparative column C187 was 13.4 cm.² × 29.2 cm. and the load 205 mg. of OL fraction. Column C190 was 0.6 cm.² × 26.2 cm. and the load 1.48 mg. of phenol-desalted fraction 1 from C187 (above). Recovery of ninhydrin colour, 81%. Column C191 was 0.6 cm.² × 29.8 cm. and the load 1.36 mg. of phenol-desalted fraction 3 from C187 (above). Recovery of ninhydrin colour, 78%.

Ionophoresis on paper by the method of Gordon *et al.* (1952) was suitable for characterizing certain of the concentrates. Fig. 8 shows the patterns given by the different desalted fractions from a chromatogram. These fractions were desalted by ion exchange, and consequently the A_1 peak contained A_2 material. Other samples have shown only the spot which moves least from the origin. The chromatographically unretarded fraction is shown to be heterogeneous, and the A_2 and A_3 fractions are not distinguished. The spot corresponding with that from A_1 which is present in the A_2 fraction has been repeatedly found in A_2

samples from preparative chromatograms of OL that by chromatographic criteria are free from A_1 , and it is thought to represent not merely contamination with A_1 material.

Samples of A_1 material were exposed to the different conditions of ion-exchange desalting and analysed by paper electrophoresis in order to discover which of the conditions involved had caused the modification. Contact with N ammonia, with or without the presence of Amberlite IRC-50, was found to effect the change into A_2 material.

Fig. 8. Ionophoresis on paper of fractions (desalted by ion exchange) from a preparative chromatogram of OL. Samples (200 μ g.) applied in 10 μ l. Ionophoresis in 0.05M veronal buffer, pH 8.8. O, OL sample; U, unretarded fraction; A_1 , A_2 and A_3 , peak fractions.

The material was isolated by drying while frozen after most of the ammonia had been evaporated off. Only the faster-moving spot was present on electrophoresis. Other alkaline conditions were found to cause the change, such as standing in solution adjusted to pH 12 with sodium hydroxide. The reagent was removed with Amberlite IR-120 before freeze-drying. Ethylamine (25%) produced the change, but not 50% pyridine (after being set aside overnight at room temperature). Only partial change occurred in 2 hr. at 0° in N ammonia. After alkali had thus been found to effect the change the alteration was confirmed chromatographically as described above (Fig. 6). In this case too only the faster-moving spot was visible after analysis of the sample by paper electrophoresis.

A preliminary study of the products of peptic hydrolysis of corticotropins A_1 and A_2 has been

made, and the results are illustrated in Fig. 9. Families of more basic materials have been produced from both, and comparison (EC 58) shows that the products from A_1 are more basic (moved further towards the cathode) than those from the less basic A_2 . The former are termed members of the corticotropin B_1 family and the latter members of the B_2 family. At least two of the products from A_1 were converted by standing in

Modifications of the chromatographic system. Increase and decrease of the pH of chromatography, with a constant sodium-ion concentration in the buffer, respectively decreased and increased the retention volumes. The results of this and of other changes in the system are presented in Table 1. This change was expected, since increase of pH should neutralize the positive charges on the solute molecules. Another result was that increase of sodium-ion concentration decreased the elution volumes, and decrease of concentration similarly raised the volumes. This is explained by competition between solute and sodium ions for the adsorbent. The change in $(E-F)$ and hence of the partition coefficient was greater than inversely proportional to the cation concentration, as was also found for cytochrome *c* by Boardman & Partridge (1953).

The use of a finer sample of the resin greatly increased the elution volumes (Table 1). This effect could be explained if only a layer on the exterior of the particles, or fissures close to the outside, were available for the retention of substances of relatively high molecular weight. In this case '*b*' would be less than $(v-F)$, and decrease of particle size would, through the increase of surface, increase the value of '*b*', the effective volume of adsorbent. This explanation is consistent with the observation that the factor by which $(E-F)$ is increased is the same for peaks A_1 , A_2 and A_3 . The unretarded peak emerged at $V_{100}=50$, so that the value of 50 for F was unaltered. This excluded the possibility that more resin had been packed into the same bed volume.

The effects of increasing the pH and decreasing the sodium-ion concentration will tend to counteract each other in passing to the bicarbonate system of White & Fierce (1953) (Table 1). The difference with the sample of Amberlite IRC-50 (XE-97) in the bicarbonate system is probably largely due to a difference in particle size.

The use of a veronal buffer of pH 8.0 was partially successful, but some modification of the A_1 peak occurred during chromatography.

Resolving power of chromatograms. Application of the formula given for N shows that the preparative columns of 2-4 cm. diameter gave a resolution of about 5 plates/cm. [HETP (height of equivalent theoretical plate) = 2 mm.] for components A_1 and A_2 . The analytical columns of 0.8-1.0 cm. diameter showed a resolution of about 10 plates/cm. (HETP = 1 mm.). The finer batch of resin showed a significantly greater resolution of about 40-100 plates/cm. (HETP = 0.1-0.25 mm.) (Figs. 10, 12). There was no evidence of resolution of the A_1 peak into further components in such columns of 1000-4000 plates. The mean particle diameter was 30-40 μ . in the coarser fraction of resin and 10-20 μ . in the finer.

Fig. 9. Ionophoresis on paper of pepsin-treated materials (see Methods), in 0.05M veronal buffer, pH 8.8. Samples of 200 μ g. Sample 7 shows the change of B_1 components into B_2 components, analogous to the change of A_1 into A_2 .

ammonia solution into products with the same mobility as those from A_2 (Fig. 9, EC 60). This suggests that the property of being altered by alkali to a less basic substance survived the peptic hydrolysis. If amide loss is the change concerned these results may be explained by the structure put forward by Bell (1954). These conditions of peptic hydrolysis (see Methods) caused little or no inactivation.

Load on chromatographic columns. When the load on chromatograms was below 5 mg./cm.² of OL, unsatisfactory patterns were obtained. Fig. 10 shows two such examples. The resolution appears decreased and the peaks spread backwards. It is possible that the lower limit of satisfactory load is reached earlier in cases such as these, where the peaks are more retarded (in one case owing to finer resin and in the other owing to decreased cation concentration).

Fig. 10. Poor resolution exhibited by chromatograms at low loads of active material (OL samples). The columns had the following dimensions and loads: C186, 0.68 cm.² × 38.3 cm. (load, 8.1 mg.); C185, 0.68 cm.² × 39.4 cm. (load, 3.08 mg.); C145, 0.86 cm.² × 17.0 cm. (load, 9.2 mg.); C143, 0.86 cm.² × 17.2 cm. (load, 2.3 mg.). Columns C186 and C185 were of the finest resin sample (Na⁺, 0.3N; pH, 6.70); columns C145 and C143 were of normal resin (Na⁺, 0.25N; pH, 6.70).

A greater decrease of load prevents analysis by the ninhydrin reaction, but the sensitive bio-assay may be used to examine the effluent. When 250 μg. of OL were put on columns of 0.95 cm.², a greater decrease of resolution was observed, with increased retardation of the activity as well as spreading. This is shown in Fig. 11. This might have been due to the existence in the resin of some easily saturated sites which differed from the rest of the resin in affinity for the active substances. To test this possibility a sample of resin was treated with salmine sulphate (0.5 mg./ml. of resin bed, added in solution to a stirred suspension of the resin in the buffer) and a chromatograph run at low load

with this resin. The ambiguous result is shown in C180 in Fig. 11.

This failure of the system at low loads contrasts with the behaviour of ribonuclease (Hirs *et al.* 1953). In that study, however, the load was somewhat higher in the successful chromatography of the enzyme from acid extracts of pancreas. The present failure explains the poor resolution in the chromatograms of crude extracts which has been previously reported (Dixon *et al.* 1951, 1953).

Fig. 11. Poor resolution exhibited by chromatograms at very low loads of active material (OL samples). Arrows $\leftrightarrow$ mark $\pm$ s.e.m. $\rightarrow$ shows normal position of A₁. Data from columns C176 and C178 are pooled. C176 was 0.95 cm.² × 22.5 cm. and C178 was 0.95 cm.² × 24.3 cm. The recovery of activity was about 40%. C180 was 0.6 cm.² × 27.4 cm. and the resin had been treated with salmine (see text). The recovery of activity was about 70%. The load on each column was 0.25 mg.

Phenol desalting. The method is satisfactory for the peak material as discussed above. An OL sample was submitted to the procedure, and some of it was lost in the aqueous phase (not extracted by phenol) and some in the ether phase (extracted together with phenol by ether), but appreciable amounts of peak material were not lost. After dissolving and desalting, the OL material recovered was chromatographed. The ninhydrin recovery of the chromatogram was 70% in place of the usual 40–50%, and this is probably due to the loss of components in the desalting which are retained by the resin on chromatography. The unretarded peak was also decreased. When the method was used with unretarded material from OL chromatograms little was recovered, and its initial presence stabilized phenol–water emulsions.

Chromatography of materials inactivated by treatment with hydrogen peroxide. A solution of OL in phosphate buffer was inactivated by adding 8% (v/v) of '100 volume' hydrogen peroxide and allowing 30 min. for reaction. The hydrogen

peroxide was decomposed by adding a solution of catalase, and a sample of the material was chromatographed. Over 99% of the activity was destroyed by this treatment. In two cases the A_1 peak showed retention volumes of 181 and 182 (C214, 215) in contrast with volumes of 195 and 197 (C217, 222) obtained with the same batch of resin with the original sample of OL. In order to show separation of the active and inactivated substances, a solution of corticotropin A_1 was treated with hydrogen peroxide, and further corticotropin A_1 dissolved

(1945) confirmed these figures (except for histidine, which reacted with the FDNB). Difficulties were met with other amino acids, but the following values were suggested by the results: serine 1-3, glutamic acid 4-5, valine 2-3, tyrosine 1-2, lysine 3-4, arginine 2-3, phenylalanine 2-4 and methionine present. The ultraviolet absorption spectrum in alkali (Beaven & Holiday, 1952) suggested: tyrosine 2, tryptophan 1.

Amide groups were determined by hydrolysis at 100° in 2N-HCl for 3 hr. and estimation of the

Fig. 12. Separation of active A_1 from A_1 inactivated by treatment with H_2O_2 . Column C220 (very fine resin sample) was 0.68 cm.² × 44.1 cm. 6.8 mg. of A_1 was dissolved in 1.25 ml. of buffer; 0.1 ml. of 100-vol. H_2O_2 was added; after 30 min. 0.02 ml. of catalase solution (0.006M in catalase haemin) was added and 6.5 mg. of A_1 ; 1.00 ml. on column.

after the peroxide had been destroyed with catalase. With the normal resin batch a clear-cut separation was not obtained, although the shape of the peak suggested two components, but the separation was satisfactory when the finer sample of resin was used as shown in Fig. 12. Assay showed that the faster peak was inactive and the slower active.

Reactivation of material inactivated by peroxide was not achieved at room temperature with H_2S or 4% aqueous potassium borohydride (which did not inactivate active material). In confirmation of the results of Dedman, Farmer & Morris (1955), inactivated corticotropin A_1 (25 μ g./ml.) was found to be reactivated by heating with cysteine (0.06M) at pH values of 3.4 and 1.9 at 74° for 18 hr.

Amino acid analysis. Only one sample of corticotropin A_1 was analysed and one check of recoveries made, so that reliable analytical figures were not found for all the amino acids. The following numbers of amino acids gave the best fit to the results, assuming a molecular weight of 4600. Glycine 3 (3.04), alanine 3 (2.96), aspartic acid 2 (1.76), leucine 2 (2.08) and histidine 1 (1.01). No cystine or isoleucine was present. Analysis of another sample after treatment with 1-fluoro-2,4-dinitrobenzene (FDNB) by the method of Sanger

Fig. 13. Titration curve of corticotropin A_1 in 0.7M-NaCl. Corticotropin A_1 (5.30 mg.) in 1.00 ml. of water, 0.10 ml. of 0.1N-NaOH and 1.10 ml. of 1.4M-NaCl titrated with 0.05N-HCl in 0.65M-NaCl. A blank titration was used to give corrections. 0.7M-NaCl was used to minimize the discrepancy between the measured pH and $-\log_{10} [H^+]$ (Danckwerts, 1952).

ammonia colorimetrically after microdiffusion in Conway units. The amide nitrogen was 1.9% of the total nitrogen, suggesting that one amide group was present.

The titration curve (Fig. 13) shows the binding of eight μ equiv. of acid by 5.3 mg. corticotropin A_1 between pH values of 2.5 and 6. This is consistent with the presence of about seven free carboxyl groups in corticotropin A_1 , assuming that its molecular weight is 4600 as suggested by the formula of Bell (1954) as well as by the analysis reported here. From its method of preparation the sample was unlikely to contain acetate.

DISCUSSION

Gradient elution or a discontinuous change in eluent are useful for the chromatographic separation of substances of widely differing affinities for the absorbent. Higher resolution is to be expected in elution chromatography under conditions where

the eluent is in equilibrium with the absorbent, since the substance chromatographed will move at constant speed down the column. Under these conditions there is no fear that a single sharp peak of several substances of greatly differing affinity for the resin will be displaced by a sudden change of eluent, such as may occur when gradient elution is attempted with resins of high buffering power. Amberlite IRC-50 buffers less at higher pH values, such as those used by White & Fierce (1953) or by Li *et al.* (1954), but modification of the A_1 component is a possible danger under these conditions. The satisfactory resolution achieved in the present experiments may be attributed to the equilibrium conditions and low pH.

The chromatographic results, especially those with columns of very fine resin and high resolving power, suggest that corticotropin A_1 is an essentially homogeneous material. This is supported by the claim of White (1953) that a similar chromatographic product contained only 15% of impurity, for the published plot of his chromatogram shows lower resolution than that of the preparative columns described here.

The main observation against this claim of homogeneity is the low potency of the products (Table 3). The values previously reported include 100–150 i.u./mg. (White, 1953) and 80–100 i.u./mg. (Bell, 1954) in contrast with the present values of up to 50 i.u./mg. The standardizations originally made against Armour preparation 84–85 H have been checked against U.S.P. corticotropin and Armour preparation 60–61. Apart from a possible systematic error in the assay of potent materials, these low potencies might be explained by the presence of considerable amounts of inactive material. In view of the rigour of the methods used for separation, such material would most probably be inactivated ACTH rather than chance contaminant. It would have to differ from the active substance less than the material produced by treatment with hydrogen peroxide, since no peak corresponding with this material was observed on chromatography of normal concentrates (Fig. 10). A decrease of elution volume on Amberlite IRC-50 on treatment with peroxide has also been reported for ribonuclease (Ledoux, 1954), but in that case is referable to the oxidation of thiol groups.

The consistently lower potency of isolated corticotropin A_2 in comparison with A_1 (Table 3) may not be significant. The A_2 peak from chromatograms of OL is probably heterogeneous (electrophoretically, Fig. 8, and chromatographically, Fig. 10, C186) and some contaminant may be inactive. The equal potency of the A_2 produced from A_1 (Fig. 5) is not contradictory to this. No evidence has been obtained about the relative potency of purified A_3 , since the conditions of the

preparative columns are not suitable for obtaining this component in a pure state. Its peak clearly overlaps a considerable ninhydrin-reacting background.

The preliminary amino acid analysis for corticotropin A_1 , with its points of agreement with that of White (1953), in particular the absence of cystine and isoleucine [cf. also Bell (1954) and Li *et al.* (1954) for sheep material, and Brink *et al.* (1953) for corticotropin B] bears out the suggestion from the chromatographic data that we are concerned with the same material, corticotropin A. It is probable from its predominance in the extracts that corticotropin A_1 is also the β -corticotropin of Bell (1954).

The nature of the change of corticotropin A_1 to other products is not explained. Loss of amide ammonia is almost certainly a factor. But the production of A_3 and the fact that A_2 prepared chromatographically from OL shows two spots on paper ionophoresis (in contrast with the single spot shown by A_2 prepared by alkaline treatment of A_1) suggest that other changes may be involved. Since only one amide group is present, other forms of unmasking of acid groups or masking of basic groups (e.g. by acyl migration) would be needed to explain the change unless amino acid residues were lost by the molecule.

Acetone-drying causes partial modification of A_1 material, but, even with the conditions of collection which at present seem best, some active A_2 material exists in the glands. Increasing the time of autolysis did not greatly increase the amount of the A_2 material, so the hour which elapses between the death of the pig and removal of the gland is probably not responsible for its presence.

SUMMARY

1. Corticotropin concentrates have been chromatographed on a carboxylic resin in sodium phosphate buffer. Active components have been resolved. The elution volumes are reproducible on chromatography of the isolated components.

2. Methods have been used for isolating the active components from salt solutions, such as the buffers used in chromatography, which depend on the adsorption of the material on a carboxylic resin at pH 3 or on extraction of the material into phenol.

3. The main active component has been designated corticotropin A_1 . Other components (corticotropins A_2 and A_3) are present which are less retarded on the resin columns and their amount may be increased under certain conditions. Alkali converts corticotropin A_1 into A_2 .

4. Corticotropins A_1 and A_2 can be distinguished by ionophoresis on paper.

5. When corticotropin A_1 is treated with pepsin, inactivation does not occur, but a group of new substances, termed collectively corticotropin B_1 , is produced. The B_1 components are separable by ionophoresis on paper. A different family (B_2) is produced from corticotropin A_2 .

6. Alkali converts the corticotropin B_1 family into the B_2 family, a change analogous to that of A_1 to A_2 .

7. The effects of the cation concentration and pH of the buffer and of the particle size of the resin on the chromatographic elution volumes are described (Table 1).

8. From the method of isolation it seems that corticotropin A_1 is identical with the corticotropin A previously described by other workers.

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