Chromatographic Studies With Pig Adrenocorticotrophin

By T. H. FARMER

Department of Experimental Biochemistry, The London Hospital Medical College, London, E. 1

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Oxycellulose concentrates of adrenocorticotrophin have been chromatographed on carboxylic-cation exchangers by a number of workers. Buffered Amberlite IRC-50 was used by White & Fierce (1953), Li, Geschwind, Dixon, Levy & Harris (1955), Dixon & Stack-Dunne (1955) and Dedman, Farmer & Morris (1957). Raben (1956) published a note on the use of carboxymethylcellulose and *m*-acetic acid in conjunction with a calcium chloride gradient. With concentrates obtained from pig pituitary glands, Dixon & Stack-Dunne (1955) obtained evidence for the presence of three active components which were designated corticotrophin A_1 , A_2 and A_3 , and further showed that alkali probably converted corticotrophin A_1 into A_2 by the hydrolysis of an amide group. Shepherd *et al.* (1956), from countercurrent-distribution studies, reported the presence of seven corticotrophins in oxycellulose concentrates. Partition chromatography utilizing an ammonium sulphate-zinc sulphate-2-ethoxy-ethanol-water system has given two highly active peaks (T. H. Farmer & C. J. O. R. Morris, unpublished work).

This paper reports the use of carboxymethylcellulose and diethylaminoethylcellulose in the chromatography of oxycellulose concentrates of pig adrenocorticotrophin.

EXPERIMENTAL

Preparation of crude corticotrophin. The oxycellulose (OC) concentrates were prepared, according to the method of Astwood, Raben, Payne & Grady (1951), from acetone-dried powder made from the anterior lobes of pig pituitary glands (Dedman *et al.* 1957).

Carboxymethyl- and diethylaminoethyl-cellulose. These ion-exchange reagents were prepared from 200–400 mesh, dry-sieved, Whatman ashless paper powder, standard grade, as described by Peterson & Sober (1956).

Buffer solutions. Sodium acetate, pH 5.9: sodium acetate ($\text{CH}_3\cdot\text{CO}_2\text{Na}\cdot 3\text{H}_2\text{O}$; 28.6 g.) with 5.45 ml. of 2*N*-acetic acid was made up to 2 l. with water. Sodium phosphate, pH 5.5: 950 ml. of 0.1 *N*- NaH_2PO_4 was mixed with 100 ml. of 0.05 *M*- Na_2HPO_4 . Sodium phosphate, pH 6.3: 80 ml. of 0.1 *M*- NaH_2PO_4 and 20 ml. of 0.1 *M*- Na_2HPO_4 were diluted to 1 l. with water. Sodium phosphate, pH 11.3: 50 ml. of 0.05 *M*- Na_2HPO_4 was added to 7.6 ml. of 0.1 *N*-NaOH. α -Picoline acetate, pH 5.7: 61 g. of α -picoline, distilled from BaO , together with 231 ml. of 2*N*-acetic acid was diluted to 2 l. with water.

Ion-exchange chromatography. Buffered carboxymethyl (CM)-cellulose was obtained by first converting the ion exchanger into its sodium salt by the addition of 7 ml. of 0.1 *N*-NaOH/g. of CM-cellulose, and then suspending this material several times, in portions of the relevant buffer solution. With the α -picoline acetate system the CM-cellulose was washed initially with aqueous 0.3% α -picoline before suspension in buffer solution. Diethylaminoethyl (DEAE)-cellulose was shaken with 0.1 *M*-phosphate buffer, pH 6.3, before being suspended in 0.01 *M*-phosphate, as equilibration with the dilute buffer solution took a considerable time. It was found necessary with both CM-cellulose and DEAE-cellulose to use a glass homogenizer to break up small aggregates of the exchanger. Columns were packed under a pressure of 15 cm. Hg, either in sections or as suggested by Porath (1956); there was no observed difference in the performance of columns packed by these two procedures. Before use columns were washed with buffer solution until the pH of the effluent was within 0.02 unit of pH of the developing solution. This usually took 24–72 hr., depending on the size of the column. To minimize the growth of micro-organisms in phosphate buffer solutions the solutions were saturated with toluene, and fractions were collected into tubes containing sufficient HCl to adjust to pH 3.0. The sample to be chromatographed was dissolved in the minimum volume of buffer solution by shaking for 20 min., any insoluble material being removed by centrifuging. Columns were developed under a constant head of buffer solution, flow rates of less than 25 ml./cm.²/hr. giving the best results. $E_{1\text{cm.}}$ at 275 $m\mu$ was used to estimate the amounts of various fractions eluted from columns with the sodium acetate and sodium phosphate buffer solutions. The α -picoline acetate buffer solution absorbed strongly at 275 $m\mu$ and $E_{1\text{cm.}}$ at 285 $m\mu$ had to be used. All extinctions were measured relative to the buffer solutions with a Unicam SP. 500 spectrophotometer.

Oxidation of corticotrophin A_1 . Corticotrophin A_1 was oxidized by aqueous H_2O_2 as described by Dedman *et al.* (1957).

Reduction of oxidized corticotrophin A_1 . Oxidized corticotrophin A_1 was reduced with cysteine as described by Dedman *et al.* (1957).

Action of sodium phosphate solution, pH 11.3, on fraction 4 and fraction 5 (A_1) from column 4. Solutions of 2.7 mg. of fraction 4 and 3.6 mg. of fraction 5 in 0.8 ml. of sodium phosphate solution, pH 11.3, were allowed to stand under N_2 at 4° for 15 hr. and were then adjusted to pH 5.5 by the addition of *m*- H_3PO_4 .

Bioassay. The adrenocorticotrophic potency was measured by the adrenal ascorbic acid-depletion method of Sayers, Sayers & Woodbury (1948), as modified by Morris (1951). The results, summarized in Table 1 show the mean depletions of four rats.

Desalting. Salts were removed from solutions by the use of Dowex-50($\times 16$) and Dowex-2($\times 8$) as described by Dedman *et al.* (1957).

RESULTS

Fig. 1 (column 1) illustrates the resolution obtained by chromatographing an OC concentrate on a preparative column in sodium acetate buffer, pH 5.9. The faster-running components were better separated than on Amberlite IRC-50, whereas the two major peaks, fractions 4 and 5, showed a resemblance to those obtained with Amberlite IRC-50 chromatography. The material corresponding to these peaks was highly potent in the adrenal ascorbic acid-depletion assay (Table 1). Fractions 2 and 3 were desalted, freeze-dried, and rechromatographed on CM-cellulose in sodium phosphate buffer, pH 5.5; the results are shown in Fig. 1 (columns 2 and 3, respectively). Fractions 1-4 from column 2 were weakly active biologically, fractions 5 and 6 being moderately active. Fractions 1, 2 and 3 from column 3 were highly potent (Table 1).

To eliminate possible hydrolytic effects of unbuffered Dowex-50($\times 16$) in the desalting procedure [Amberlite IR 120 having been reported to hydrolyse

insulin (Davies & Harris, 1958)], it was thought desirable to use a volatile buffer solution. Dixon (1956) had successfully used pyridine acetate in conjunction with Amberlite IRC-50, and α -picoline acetate, pH 5.7, was found suitable for use with CM-cellulose. Fig. 2 shows the results obtained with a preparative column. The picture was similar to that obtained with column 1 although the resolution appeared to be of a lower order. The effluent was bulked as indicated, freeze-dried and the fractions were assayed for biological activity. Fractions 4 and 5 were highly active; fraction 3 was moderately active, whereas fractions 1 and 2 had negligible activity (Table 1). Samples of these fractions were then rechromatographed on CM-cellulose in the sodium phosphate buffer, pH 5.5. The results are summarized in Fig. 3. The first two peaks in

Table 1. Adrenal ascorbic acid depletions produced by chromatographic fractions

Column no.	Fraction	Dose (μ mg./100 g.)	Mean ascorbic acid depletion (μ g./100 mg. of adrenal tissue) ($\pm$ s.e.)
1	4	10	36 (± 3)
	5	10	61 (± 10)
2	1	25	Inactive
	2	25	17 (± 4)
	3	25	19 (± 7)
	4	25	40 (± 8)
	5	25	50 (± 6)
3	6	25	94 (± 10)
	1	25	125 (± 5)
	2	25	100 (± 12)
4	3	25	87 (± 9)
	1	25	Inactive
	2	25	Inactive
	3	10	25 (± 6)
	4	10	93 (± 10)
10	5	10	106 (± 13)
	A ₁	10	48 (± 13)
12	A ₂	10	49 (± 6)
	A _{1a}	10	74 (± 8)
13	A ₂	20	87 (± 9)
	A _{1a}	20	95 (± 12)
14	A ₃	18	80 (± 17)
	A ₂	24	125 (± 14)
21	1	20	Inactive
	1	10	85 (± 11)
	(reduced)		
	2	20	120 (± 5)
	2	10	92 (± 12)
	(reduced)

Fig. 1. Column 1: 5 g. of CM-cellulose, 42.5 cm. $\times$ 1.2 cm., sodium acetate, pH 5.9; 100 mg. of OC concentrate in 10 ml. of buffer solution was used and 3.6 ml. fractions were collected. Column 2: 1.5 g. of CM-cellulose, 90.6 cm. $\times$ 0.35 cm., sodium phosphate, pH 5.5; 5.9 mg. of fraction 2 from column 1 in 1 ml. of buffer solution was used and 1.55 ml. fractions were collected. Column 3: 1.5 g. of CM-cellulose, 90.6 cm. $\times$ 0.35 cm., sodium phosphate, pH 5.5; 4.8 mg. of fraction 3 from column 1 in 1 ml. of buffer solution was used and 1.55 ml. fractions were collected.

columns 6-9 were due to a non-retarded substance, possibly an α -picoline oxidation product, and α -picoline, respectively. The surprising heterogeneity of fraction 4 from column 4 is shown in column 8.

In an attempt to clarify the relationships between fractions 5, 4 and 3 from column 4 and corticotrophins A_1 , A_2 and A_3 , fractions 5 and 4 from column 4 were treated with sodium phosphate buffer (Dixon & Stack-Dunne, 1955), pH 11.3, and rechromatographed on CM-cellulose in sodium phosphate buffer, pH 5.5. Figs. 4 and 5 summarize the results. The alkaline buffer solution appears to have transformed approximately 50% of fraction 5 into a component with a retention volume equal to that of the faster-running component of fraction 4. Fig. 5 illustrates the effect of the pH 11.3 buffer solution on fraction 4 from column 4; fraction 3 from column 4 was chromatographed for comparison. Here it was seen that the more retarded component of fraction 4 was converted into material which was chromatographically similar to the less-retarded component, together with another faster-running substance. Both products had high biological activity (Table 1). Column 15 shows the results obtained on chromatographing a sample of the moderately active fraction 3 from column 4.

For comparison, fractions designated A_1 , A_2 and A_4 , which had been obtained by chromatographing OC concentrates on Amberlite IRC-50 at pH 8.3 (Dedman *et al.* 1957), were analysed on CM-cellulose columns with sodium phosphate buffer, pH 5.5. Columns 16 and 17 revealed the heterogeneous nature of the A_4 and A_2 fractions, respectively, whereas column 18 showed that the A_1 fraction contained only one major component (Fig. 6). These results are summarized in Table 2.

The possibility of using DEAE-cellulose as an alternative method for fractionating corticotrophin

concentrates was investigated, 0.01M-sodium phosphate, pH 6.3, being used. Fig. 7 (column 19) shows some typical results. Biological activity was distributed throughout the chromatogram, the last two peaks exhibiting moderate activity. However, large losses in biological activity were sustained and this method was discarded for preparative purposes. A sample of fraction 5 from column 1 was analysed in this system and gave the symmetrical peak shown in Fig. 8. The resolving power of the DEAE-cellulose

Fig. 2. Column 4: 8 g. of CM-cellulose, 62.2 cm. $\times$ 1.2 cm., α -picoline acetate, pH 5.7; 100 mg. of OC concentrate in 12 ml. of buffer solution was used and 3.6 ml. fractions were collected.

Fig. 3. CM-cellulose (1.35 g.) column, 79.2 cm. $\times$ 0.35 cm., sodium phosphate, pH 5.5; 1.55 ml. fractions were collected. Column 5: 1.3 mg. of fraction 1 from column 4 in 0.5 ml. of buffer solution. Column 6: 1.5 mg. of fraction 2 from column 4 in 0.8 ml. of buffer solution. Column 7: 3.0 mg. of fraction 3 from column 4 in 1.0 ml. of buffer solution. Column 8: 4.1 mg. of fraction 4 from column 4 in 2.0 ml. of buffer solution. Column 9: 2.2 mg. of fraction 5 from column 4 in 1.0 ml. of buffer solution.

columns, as calculated by the method of Simpson & Wheaton (1954), was approximately 4 plates/cm. compared with 20 plates/cm. obtained with the CM-cellulose columns. It was, however, decided that the behaviour of a mixture of oxidized and non-oxidized corticotrophin A_1 on a DEAE-cellulose column might prove instructive. These substances had been previously separated on Amberlite IRC-50 (Dixon & Stack-Dunne, 1955) and CM-cellulose (Farmer & Morris, 1956). Fig. 9 illustrates the asymmetrical peak obtained. The effluent was bulked as indicated and assayed before and after reduction with cysteine. Fraction 1 was inactive before, but was highly active after cysteine treatment, whereas fraction 2 was highly active before reduction (Table 1).

DISCUSSION

In agreement with the results of previous investigators (White & Fierce, 1953; Li *et al.* 1955, Dixon & Stack-Dunne, 1955; Dedman *et al.* 1957) chromatography of OC concentrates of pig adrenocortico-

trophin on CM-cellulose has shown that the major active component accounted for approximately 30% of the total material. In addition, evidence has been obtained for the presence of three other highly active components (Fig. 1, columns 1 and 3). The major component corresponded to corticotrophin A_1 , as was shown by column 18. Fraction 4 from column 4 was found to be complex, three peaks other than one due to A_1 were obtained on chromatography in the sodium phosphate buffer, pH 5.5 (column 8). Columns 12 and 14 indicated that the fastest-running peak corresponded to A_2 , and the other two were therefore named A_{1a} and A_{1b} , respectively. As it has been suggested by Dixon & Stack-Dunne (1955) that the conversion of A_1 into A_2 was accompanied by the hydrolysis of an amide group, and since A_{1a} and A_{1b} were also converted into less-retarded substances by the action of alkali (columns 12 and 14), it seemed probable that A_{1a} and A_{1b} were also amides. Bell, Howard, Shepherd, Finn & Meisenhelder (1956) have shown that biologically active molecules containing 28, 30 and 31 amino acid residues may be prepared from the 39 amino acid residue β -corticotrophin by pepsin digestion, the C-terminal sequence being attacked under these conditions. The amino acid composition of corticotrophin A_1 , obtained from Amberlite IRC-50

Fig. 4. CM-cellulose (0.9 g.) column, 40.0 cm. $\times$ 0.5 cm., sodium phosphate, pH 5.5; 1.55 ml. fractions were collected. Column 10: 3.0 mg. of fraction 5 from column 4 in 0.6 ml. of buffer solution. Column 11: 3.0 mg. of fraction 4 from column 4 in 0.6 ml. of buffer solution. Column 12: 3.6 mg. of fraction 5 from column 4, pH 11.3, for 15 hr.

Fig. 5. CM-cellulose (0.9 g.) column, 40.0 cm. $\times$ 0.5 cm., sodium phosphate, pH 5.5; 1.55 ml. fractions were collected. Column 13: 2.7 mg. of fraction 4 from column 4 in 0.6 ml. of buffer solution. Column 14: 2.7 mg. of fraction 4 from column 4, pH 11.3, for 15 hr. Column 15: 2.8 mg. of fraction 3 from column 4 in 0.6 ml. of buffer solution.

Table 2. Retention of corticotrophin peaks (columns 10–15, 17, 18)

The effluent volumes refer to a CM-cellulose column, 40.0 cm. × 0.5 cm., with sodium phosphate, pH 5.5, as eluent.

Material chromatographed	Effluent vol. to peak maxima (ml.)				
Fraction 5 from column 4	—	—	—	—	149
Fraction 5 from column 4, pH 11.3	—	—	59	—	143
Fraction 4 from column 4	—	—	65	104	—
Fraction 4 from column 4, pH 11.3	—	45	64	—	—
Fraction 3 from column 4	39	—	—	—	—
Corticotrophin A ₃ *	37	—	67	101	—
Corticotrophin A ₁ *	—	—	—	—	138

* Obtained by chromatography of OC concentrates on Amberlite IRC-50 (Dedman, Farmer & Morris, 1957).

Fig. 6. Column 16: 4.5 g. of CM-cellulose, 86.5 cm. × 0.7 cm., sodium phosphate, pH 5.5; 28.5 mg. of A₄ (from a column of Amberlite IRC-50) was chromatographed in 3 ml. of buffer solution and 3.6 ml. fractions were collected. Columns 17 and 18: 0.9 g. of CM-cellulose, 40.0 cm. × 0.5 cm., sodium phosphate, pH 5.5; 1.55 ml. fractions were collected. Column 17: 6.2 mg. of A₃ (from a column of Amberlite IRC-50) in 1.3 ml. of buffer solution. Column 18: 1.7 mg. of A₂ (from a column of Amberlite IRC-50) in 0.5 ml. of buffer solution.

Fig. 7. Column 19: 7.5 g. of DEAE-cellulose, 61.2 cm. × 0.9 cm., 0.01M-sodium phosphate, pH 6.3; 10 mg. of OC concentrate was chromatographed in 1 ml. of buffer solution and 1.55 ml. fractions were collected.

Fig. 8. Column 20: 2 g. of DEAE-cellulose, 62.8 cm. × 0.5 cm., 0.01M-sodium phosphate, pH 6.3; 1.9 mg. of A₁ was chromatographed in 0.5 ml. of buffer solution and 1.55 ml. fractions were collected.

chromatography differed only slightly from that of β -corticotrophin (Dedman *et al.* 1957), and it seemed likely that corticotrophins A_{1a} and A_{1b} differed from A₁ in the C-terminal sequences. Further studies are needed to elucidate this point.

The action of alkali on fraction 4 from column 4 produced another highly active corticotrophin which was probably identical with corticotrophin A₃. This material had a retention volume similar to that of fraction 3 from column 4 (Fig. 5), and it appeared probable that the activity of fraction 3 was due to the presence of A₃, since A₂ had been detected on OC concentrates by Dixon & Stack-Dunne (1955). The action of alkali on A₁ did not produce material similar to A₃ (column 12), and this result suggested that A₃ was a product of A_{1a} or A_{1b}. However, no evidence has been obtained to support this suggestion.

Chromatography of fractions designated corticotrophins A₁ and A₂, and fraction A₄, obtained from columns of Amberlite IRC-50 (Dedman *et al.* 1957), gave the results illustrated in Fig. 6. A₁ was found to contain one major component chromatographically identical with the A₁ obtained from CM-cellulose chromatography. However, the A₂ fraction obtained from Amberlite IRC-50 columns gave a picture similar to that obtained with fraction 4

Fig. 9. Column 21: 6 g. of DEAE-cellulose, 85.6 cm. $\times$ 0.7 cm., in 0.01 M-sodium phosphate, pH 6.3; the mixture of 4 mg. of oxidized A_1 and 3.1 mg. of A_1 was chromatographed in 0.7 ml. of buffer solution and 1.55 ml. fractions were collected.

from column 4, indicating that corticotrophin A_{1a} and A_2 were present. The term corticotrophin A_2 should therefore be used for material which has the same retention volume, when chromatographed on CM-cellulose in the sodium phosphate buffer, pH 5.5, as the product formed on the treatment of corticotrophin A_1 with alkali. As was expected, fraction A_4 , the least-retarded material on Amberlite IRC-50 at pH 8.3, proved to be composed of many components.

A recent publication by Farrell *et al.* (1958) has reported that Bell's δ_1 -corticotrophin (Shepherd *et al.* 1956) was more active than β -corticotrophin in mobilizing aldosterone in the decerebrate dog. In the light of these results, it would be interesting to know which fraction obtained from the CM-cellulose chromatography of OC concentrates corresponded to δ_1 -corticotrophin.

DEAE-cellulose columns, run in 0.01 M-sodium phosphate buffer, pH 6.3, resolved OC concentrates into a number of peaks. However, although activity was distributed throughout the chromatogram, a large loss of biological activity made this system unsuitable for preparative purposes. The most active peak had the greatest retention volume. Chromatography of a sample of fraction 5 from column 1 (A_1) in this system gave a single peak, as illustrated in Fig. 8. An attempt to separate oxidized and non-oxidized A_1 was partially successful, although two peaks were not obtained. In this experiment the oxidized A_1 ran faster than the non-oxidized A_1 , as on CM-cellulose, indicating that mechanisms other than ion exchange were contributing to the process of chromatography.

SUMMARY

1. Carboxymethylcellulose has been used to prepare corticotrophin A_1 and show that at least

three other chromatographically distinct corticotrophins are present in oxycellulose concentrates. One of these has been identified with corticotrophin A_2 and the other two have been named corticotrophins A_{1a} and A_{1b} .

2. In agreement with previous workers alkali has been shown to convert corticotrophin A_1 into A_2 , and corticotrophins A_{1a} and A_{1b} into less-retarded substances on carboxymethylcellulose columns. One of these products has been tentatively identified with corticotrophin A_3 .

3. Diethylaminoethylcellulose has been used to fractionate oxycellulose concentrates, although large losses in biological activity were observed. Corticotrophin A_1 behaved as a homogeneous substance on diethylaminoethylcellulose, and oxidized A_1 and A_1 were partially separated.

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REFERENCES

- Astwood, E. B., Raben, M. S., Payne, R. W. & Grady, A. B. (1951). *J. Amer. chem. Soc.* **73**, 2969.
- Bell, P. H., Howard, K. S., Shepherd, R. G., Finn, B. M. & Meisenhelder, J. H. (1956). *J. Amer. chem. Soc.* **78**, 5059.
- Davies, J. W. & Harris, G. (1958). *Arch. Biochem. Biophys.* **74**, 229.
- Dedman, M. L., Farmer, T. H. & Morris, C. J. O. R. (1957). *Biochem. J.* **66**, 166.
- Dixon, H. B. F. (1956). *Biochem. J.* **64**, 47r.
- Dixon, H. B. F. & Stack-Dunne, M. P. (1955). *Biochem. J.* **61**, 483.
- Farmer, T. H. & Morris, C. J. O. R. (1956). *Nature, Lond.*, **178**, 1465.
- Farrell, G. L., Fleming, R. B., Rauschkolb, E. W., Yatsu, F. M., McCally, M. & Anderson, C. H. (1958). *Endocrinology*, **62**, 506.
- Li, C. H., Geschwind, I. I., Dixon, J. S., Levy, A. L. & Harris, J. I. (1955). *J. biol. Chem.* **213**, 171.
- Morris, C. J. O. R. (1951). *Analyst*, **76**, 470.
- Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
- Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.
- Raben, M. S. (1956). *Proc. Soc. exp. Biol., N.Y.*, **93**, 338.
- Sayers, M. A., Sayers, G. & Woodbury, L. A. (1948). *Endocrinology*, **42**, 379.
- Shepherd, R. G., Howard, K. S., Bell, P. H., Cacciola, A. R., Child, R. G., Davies, M. C., English, J. P., Finn, B. M., Meisenhelder, J. H., Moyer, A. W. & van der Scheer, J. (1956). *J. Amer. chem. Soc.* **78**, 5051.
- Simpson, D. W. & Wheaton, R. M. (1954). *Chem. Engng Progr.* **50**, 45.
- White, W. F. & Fierce, W. L. (1953). *J. Amer. chem. Soc.* **75**, 245.