Summary. A method is described for the preparation in high yield, from whole sheep pituitary glands, of purified ACTH, substantially free from growth, gonadotropic, thyrotropic, antidiuretic and pressor activities. Clinical and laboratory evidence indicates that ACTH prepared by this method is therapeutically and metabolically effective in patients with rheumatoid arthritis.

1. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1943, v149, 413.

2. Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, 1943, v149, 425.

3. Li, C. H., Simpson, M. S., and Evans, H. M., *J. Biol. Chem.*, 1942, v146, 627.

4. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1948, v42, 379.

5. Reinhardt, W. O., and Li, C. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 836.

6. Thorn, G. W., Forsham, P. H., Frawley, T. F., Hill, S. R., Jr., Roche, M., Staehelin, D., and Wilson, D. L., *New England J. Medicine*, 1950, v242, 783.

Received October 31, 1951. P.S.E.B.M., 1951, v78.

Chromatographic Behavior of Adrenocorticotropic Peptides in Diatomaceous Earth and Starch Column.* (19176)

R. MENDENHALL† AND CHOH HAO LI.

From the Department of Biochemistry, University of California, Berkeley.

In a recent paper(1), we described the purification of adrenocorticotropically active peptides (ACTH peptides) in paper partition chromatography, displacement development analysis and carrier displacement chromatography. The present paper concerns the results obtained from starch and diatomaceous earth chromatographic columns. A preliminary account on the purification of ACTH peptides in starch has appeared(2).

Experimental. The ACTH peptide mixture was prepared from the pepsin digest of the sheep protein hormone by the method previously described(2,3). The adrenocorticotropic activity was estimated by the ascorbic acid depletion in the adrenals of hypophysectomized male rats(4).

The potato starch and diatomaceous earth‡ were commercial products. The columns were prepared and operated according to the procedure described by Stein and Moore(5,6). The effluent fractions (0.5 cc) were analyzed by the ninhydrin colorimetric method(7); alternate tubes were selectively pooled and dried out and the nitrogen content was determined by the micro-Kjeldahl technic.§ The column size was 0.9 x 30 cm and the rate of flow was maintained by a pressure head of 15 cm Hg. Some of the developing solvents were those suggested by Stein and Moore (5,6) for the separation of amino acids.

Results. Three developing solvents were employed for the *diatomaceous earth* column: Solvent A, ethanol: 0.9% NaCl = 1:1; Solvent B, butanol: glacial acetic acid: water = 63:10:25, and Solvent C, benzyl alcohol: butanol: water = 1:1:0.288 with an addition of 0.5% thiodiglycol. It may be noted in Table I that a large amount of ninhydrin-reacting material appears in Fractions No. 42-62, comprising 70% of the material applied in the column when Solvent A was used as the eluent. There are some small peaks after Fraction No. 115, indicating that a large number of components is present in the original peptide mixture. A recovery of 97% of the nitrogen was achieved. Using Solvent B as the developing solvent, a large

* This work is supported in part by grants from U. S. Public Health Service, the Armour Laboratories and the Merck Co.

† Cutter Laboratories Predoctorate Fellow, 1950-51.

‡ The diatomaceous earth, Dicalite 223-T, was a special acid washed material and obtained through the courtesy of Dr. G. W. Breger, Great Lake Carbon Corp., Walteria, Calif.

§ We wish to thank Harold Papkoff for the nitrogen determinations.

TABLE I. Nitrogen Distribution of ACTH Peptides in Diatomaceous Earth and Starch Chromatography.

Chromatography	Solvent	Fraction No.	Nitrogen Content, μg	Nitrogen Recovery, %
Diatomaceous earth	A	42-62	441	97
		64-249	188	
	B	23-40	743	100
		42-141	340	
		155-200	304	
	C	26-42	202	100
		44-64	187	
		66-91	468	
		93-299	828	
Starch	D	26-44	416	91
		46-220	174	
	E	6-20	1298	100
		22-37	246	
	A	9-20 (Fraction A)	872	
		23-34 (Fraction B)	55	71

Solvent A: Ethanol: .9% NaCl = 1:1. B: Butanol: glacial acetic acid: water = 63:10:25. C: Benzyl alcohol: butanol: water = 1:1:.288. D: Butanol: propanol: .1 M HCl = 1:2:1. E: n-Propanol: .5 M HCl = 1:1.

amount of nitrogen was again recovered in the first peak (Table I). The second peak, appearing at the end of the run, is particularly interesting, as it contains 22% of the original nitrogen and must represent a distinct fraction in comparison with the peak in front. Solvent C resolves the peptide mixture into a number of small peaks and the pattern is very different from that obtained by the other two solvents. The fractionation here is only partial, due to the failure of any of the peaks to return completely to the base line. All the nitrogen was recovered in the effluent (Table I).

Three developing solvents were employed for the *starch* column: Solvent D, butanol: propanol: 0.1 N HCl = 1:2:1; Solvent E, n-propanol: 0.5 HCl = 1:1; and Solvent A. When Solvent D serves as the developing solvent, a large ninhydrin-reacting peak occurs in the region just before the appearance of leucine-isoleucine in the chromatogram of Stein and Moore(5). This large peak contains 70% of the nitrogen applied initially to the column (Table I). There are only 3 small peaks after Fraction No. 60.

Two distinct peaks were obtained in Solvent E. The data in Table I indicate that 84% of the nitrogen was found in the first fraction, in contrast to 16% in the second. The area of the 2 peaks is of interest, since it shows that the amount of ninhydrin-reacting material is almost the same for these two fractions. Hence, the average length of peptide chain in the two fractions must be different. As shown in Fig. 1, Solvent A resolved the peptide mixture into 2 large peaks. In contrast to Solvent E, the nitrogen recovery in this case was only 70%; no more nitrogen appeared after Fraction No. 50. Biological assay of the 2 peaks (Fractions A and B) showed that both fractions have adrenal-stimulating activity. Judging by assay data summarized in Table II, the adrenocorticotropic potency of Fractions A and B is similar.

Discussion. Starch columns chromatography has been employed by Borsook *et al.* (8,9) for the isolation of a peptide fraction

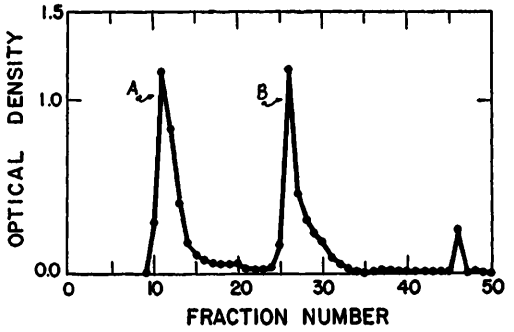

FIG. 1. Chromatogram of 10 mg ACTH peptides in starch column; solvent, ethanol: .9% NaCl = 1:1.

TABLE II. Biologic Activity Distribution of ACTH Peptides in Starch Chromatogram.*

Fraction	No. of animals	Ascorbic acid depletion/100 g adrenal,† mg	ACTH standard equivalent,‡ μg
A	21	126 ± 28§	6.4
B	10	129 ± 38§	7

* Ethanol: .9% NaCl = 1:1 used as the eluent, see Fig. 1.

† 1 μg N per 100 g body wt was inj. intrav. for assay.

‡ Estimated from a stand. assay curve, see (1).

§ Mean ± stand. dev.

from liver homogenate of various species. Their Peptide A fraction was eluted from the starch column by Solvent D a short time before the appearance of leucine. This result resembles the pattern obtained with the ACTH peptides. Since it is now clear that the peptic digest of ACTH protein is a very complex mixture(1), the occurrence of a single large fraction in the starch column cannot be taken to indicate that the fraction consists of a single peptide.

It is of interest to note that 2 distinct fractions appear in Solvents A and E in the starch column, both of which fractions are biologically active. This may suggest that more than one adrenocorticotropically active peptide occurs in the peptide mixture. Earlier studies(1) also support this view.

It should be noted that the chromatographic patterns of the ACTH peptides in starch and in diatomaceous earth columns obtained by Solvents A and D are similar. On the other hand, when the same eluting solvent is used, the behavior of the peptide mixture in these columns is quite different: One gives rise to only one large peak at the front, whereas the starch is capable of resolving into 2 fractions. It may also be noted that all nitrogen initially applied to the diatomaceous earth column was successfully removed by Solvent A, but only 70% of nitrogen recovery was achieved in the starch. If both starch and diatomaceous earth serve only as inert support for the true partition chromatography, one would expect to obtain the same chromatographic patterns in the 2 columns which have been developed by the same eluent. Hence, the results indicate that difference in degree of adsorption must play an important role in these chromatographic columns.

Summary. The ACTH peptides obtained from the pepsin digest of sheep protein hormone has been analysed chromatographically in starch and diatomaceous earth columns using various solvents as eluent. Results indicate the presence of many components in the peptide mixture. The presence of two ACTH active components in the peptide mixture has been suggested by starch column chromatography, using ethanol:0.9% NaCl as eluent.

1. Li, C. H., Tiselius, A., Pedersen, K. O., Hagdahl, L. and Carstensen, H., *J. Biol. Chem.*, 1951, v190, 317.

2. Li, C. H. and Pedersen, K. O., *Arkiv. Kemi*, 1950, v1, 333.

3. Li, C. H. Trans. Macy Conf. on Metabolic Aspects of Convalescence, 1948, v17, 114; *Nordisk Medicin*, 1950, v43, 363.

4. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1949, v42, 379.

5. Stein, W. H. and Moore, S., *J. Biol. Chem.*, 1948, v176, 337.

6. Moore, S. and Stein, W. H., *J. Biol. Chem.*, 1949, v178, 53.

7. Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1948, v176, 367.

8. Borsook, Henry, Heasy, C. L., Haagen-Smit, A. J., Neighly, A., and Lowy, P. H., *J. Biol. Chem.*, 1949, v179, 705.

9. Borsook, Henry, Heasy, C. L., Haagen-Smit, A. J., Neighly, A., and Lowy, P. H., *J. Biol. Chem.*, 1948, v174, 1041.

Received April 10, 1951. P.S.E.B.M., 1951, v78.