BBA 4073

ADRENOCORTICOTROPIN

XXVII. ON THE PRESENCE OF PIG-TYPE ADRENOCORTICOTROPIN IN SHEEP PITUITARIES, AND A SIMPLE METHOD FOR THE ISOLATION OF α_S-ADRENOCORTICOTROPIN*

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SUMMARY

A simple, rapid method for the preparation of highly purified ACTH by means of chromatography on CM-cellulose is described. Pig-type ACTH has been isolated from sheep pituitaries and appears to represent about 10 % of the ACTH present. Evidence is adduced for the presence in sheep-pituitary extracts of a peptide having the sequence of ACTH but lacking the NH_2-terminal hexapeptide and the COOH-terminal amino acid.

INTRODUCTION

Highly purified ACTH has been prepared by several different methods. The preliminary purification steps have been outlined in an earlier review[1]; the final stages of purification have utilized chromatography on ion-exchange resins[2,3] and countercurrent distribution[3,4]. FARMER[5] used chromatography on CM-cellulose and DEAE-cellulose to prepare highly purified pig ACTH (α_P-ACTH, corticotropin-A). SCHALLY *et al.*[6] used gradient elution from columns of substituted celluloses to isolate the pig-type β-MSH (β_{Glu}-MSH). We have extended their procedure to provide a simple, rapid method for the preparation of highly purified ACTH from sheep pituitaries.

Chemical structures have been established for ACTH isolated from pigs[7], sheep[8], oxen[9] and humans[10]. The hormones differ from each other only in the sequence of amino acids in one certain area of the molecule. Indeed, beef, sheep and human ACTH have the same amino acid composition, whereas pig ACTH differs only by one more leucine and one less serine. Whereas it has been reported[11] that sheep pituitaries may contain β-MSH of both pig (β_{Glu}-MSH) and beef (β_{Ser}-MSH) types, there have been no reports hitherto of more than one type of ACTH in any given species. The present investigation provides evidence that sheep pituitaries contain pig-type ACTH.

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EXPERIMENTAL AND RESULTS

Fractionation of ACTH concentrates on CM-cellulose

ACTH concentrates were obtained from whole pituitaries of sheep by adsorption on oxycellulose followed by precipitation with dioxane as described previously[1]. The sample (~ 500 mg) was applied to a column (60 × 1 cm) of CM-cellulose which had been equilibrated with 0.01 M ammonium acetate buffer (pH 4.6). After 3–4 hold-up volumes had been collected, a gradient with respect to pH and concentration was started by introducing 0.1 M ammonium acetate buffer (pH 6.7) through a 500-ml mixing flask containing the starting buffer. Peptide concentration in the eluate was determined by reading the absorbancy at 278 mμ. After several peaks (Fig. 1) had been eluted, the absorbancy of the eluate began to return to the baseline value. When this occurred, the gradient was increased by substituting 0.2 M ammonium acetate (pH 6.7) as the solution flowing into the mixing flask.

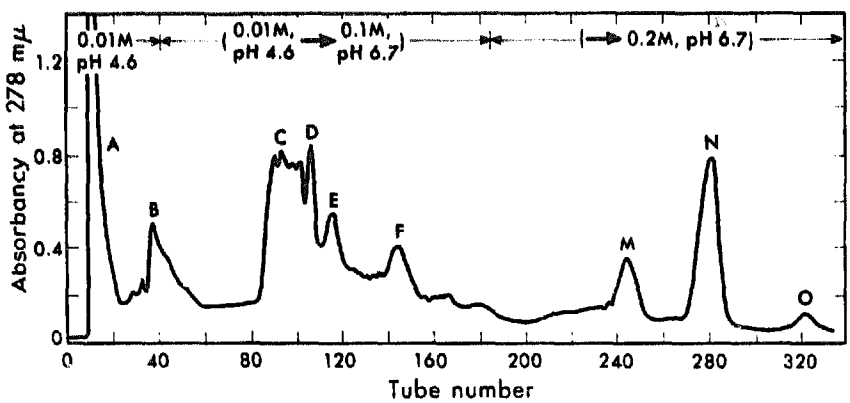

Fig. 1. Chromatography of an ACTH concentrate on CM-cellulose. 516 mg of concentrate was placed on a 50-ml column and the eluate was collected in 4-ml fractions.

The eluates corresponding to the various peaks were lyophilized several times to remove ammonium acetate, and the resultant peptides were examined for biological activity. ACTH activity was determined *in vitro*[12,13], and melanocyte-stimulating activity was assayed according to the procedure of SHIZUME, LERNER AND FITZPATRICK[14]. Peaks M and N (Fig. 1) were found to contain the bulk of the ACTH activity whereas the most active peaks by the MSH assay were peak C and peak D*.

Preparation of highly purified ACTH

Peaks M and N were each submitted to further chromatography on CM-cellulose by a slightly different procedure from the initial chromatography. The step producing the gradient to 0.1 M ammonium acetate was omitted, and a gradient to 0.2 M ammonium acetate buffer (pH 6.7) was started as soon as the hold-up volume of 0.01 M buffer had been collected. Peaks M and N retained their chromatographic identities in this second chromatography, peak N having a slightly higher retention volume than peak M (Fig. 2).

When samples of peaks M and N were assayed for steroidogenic activity[12,13] *in vitro*, they were found to have potencies of from 100 to 150 units/mg, the same

* Peaks A, B, E and F are still being investigated.

potency as highly purified ACTH obtained by other methods[1]. The potencies of peaks M and N did not differ significantly one from the other, although peak N always tended to have a slightly higher potency than peak M. The melanocyte-stimulating activity *in vitro*[14] of both peaks M and N was found to be $8 \cdot 10^7$ units/g.

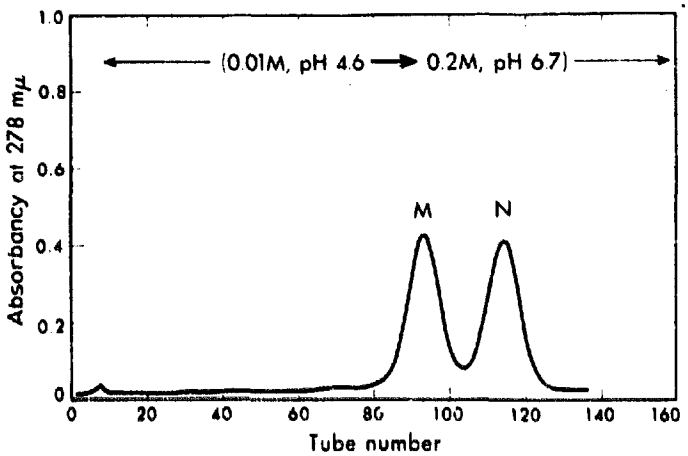

Fig. 2. Chromatographic identity of peaks M and N. Rechromatographed peak M (5.3 mg) and peak N (5.0 mg) were mixed and applied to a 10-ml column of CM-cellulose. The gradient to 0.2 M ammonium acetate of pH 6.7 was made through a 125-ml mixing flask and the eluate was collected in 1.5-ml fractions.

The nature of peptides of peaks M and N

Samples of peaks M and N were hydrolyzed with 5.7 N HCl for 22 h at 110° and the hydrolyzates were examined with the automatic amino acid analyzer[15]. Table I shows the amino acid compositions obtained in this manner. In addition to the amino

TABLE I

AMINO ACID COMPOSITION OF ACTH, PEAKS M AND N

Composition is given in molar ratios.

Composition	Preparation*		Theoretical values**	
	Peak M	Peak N	α_β-ACTH	α_p-ACTH
Lysine	4.0	4.0	4	4
Histidine	0.9	1.0	1	1
Arginine	2.8	3.0	3	3
Aspartic acid	2.0	2.0	2	2
Serine***	2.5	3.0	3	2
Glutamic acid	4.9	4.9	5	5
Proline	3.9	4.0	4	4
Glycine	3.1	3.0	3	3
Alanine	2.9	2.9	3	3
Valine	2.9	2.9	3	3
Methionine	1.0	0.9	1	1
Leucine	1.7	1.1	1	2
Tyrosine	1.9	1.9	2	2
Phenylalanine	2.7	2.8	3	3
Ammonia	2.3	2.5		

* See Figs. 1 and 2.
** See ref. 1.
*** Corrected for 15% destruction during hydrolysis.

acids shown in the table, the preparations contained very small amounts of isoleucine and threonine. The amino acid analysis of peak N was exactly that expected for α_S-ACTH, whose structure has been determined[8]. The molar ratios of the amino acids found in peak M are also in good agreement, except that serine was low and leucine correspondingly high. The amino acid composition of pig ACTH (α_P-ACTH) differs from that of sheep ACTH (α_S-ACTH) only by one extra leucine and one less serine. Thus the possibility arose that peak M was a mixture of two ACTH's, one of the sheep type and one of the pig type.

TABLE II

AMINO ACID COMPOSITIONS OF THE COOH-TERMINAL PEPTIDES OBTAINED BY DIGESTING PEAKS M AND N WITH TRYPSIN

Composition is given in molar ratios.

Amino acid	*Peptide from peak M*	*Peptide from peak N*
Aspartic acid	2.0	2.0
Serine	0.5	1.0
Glutamic acid	4.0	4.0
Proline	1.9	2.1
Glycine	1.1	1.0
Alanine	3.0	3.0
Valine	1.0	0.9
Leucine	1.6	1.0
Tyrosine	0.9	0.9
Phenylalanine	1.9	1.9

As a preliminary experiment to explore this possibility, samples of peaks M and N were submitted side-by-side to the action of trypsin, and the COOH-terminal peptides were isolated by countercurrent distribution in the system butanol–acetic acid–water (4:1:5) as described by LI, DIXON AND CHUNG[9]. The amino acid compositions of the COOH-terminal peptides obtained from peaks M and N respectively are shown in Table II. Again, the analysis of the peptide derived from peak N was exactly as expected, but the peptide from peak M showed the same differences with respect to serine and leucine as peak M itself.

Pig ACTH has been shown in the past to exhibit higher partition coefficients than sheep ACTH in several solvent systems used for countercurrent distribution[16]. Accordingly, samples of peaks M and N were distributed for 120 transfers in the solvent system 2-butanol–0.1 % trichloroacetic acid (1:1, v/v). In order to keep the conditions as identical as possible for the two samples, a 240-tube countercurrent machine was filled with both phases of the solvent system; peak N was distributed in the first 120 tubes and peak M in the last 120, with the machine connected for recycling. The distribution pattern is shown in Fig. 3.

This solvent system always gives rise to a complexity in the form of a slow-moving shoulder[16], a phenomenon which has not been satisfactorily explained; in this instance, the shoulders peak N_1 and peak M_1 were observed. Both peak N and peak M showed major peaks with partition coefficients of about 0.3 (peak N_2, 0.30; peak M_2, 0.35) but, although there was a peak (M_3) with $K = 0.67$ in the distribution pattern of peak M, no such material was present in peak N. The amino acid analyses of peaks

N_2, M_2 and M_3 are shown in Table III, and it can be seen that peak M_3 has the same amino acid composition as α_β-ACTH, while both peaks M_2 and N_2 have the same composition as α_α-ACTH. Once it was established that peak M is a mixture of ACTH's, it remained to explain the difference between peak N and the sheep component of

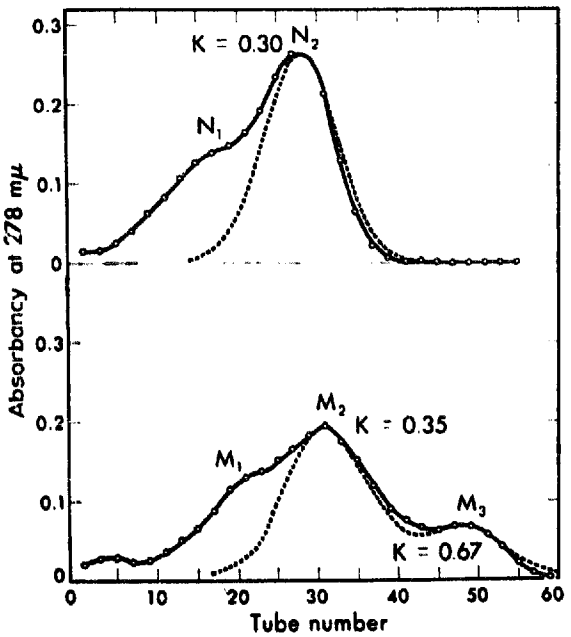

Fig. 3. Countercurrent distribution of peaks M and N. Peak M (26 mg) and peak N (21 mg) were distributed for 120 transfers in the system 2-butanol–0.1% trichloroacetic acid. Fractions were pooled as follows: peak N_1, 3–22; peak N_2, 23–39; peak M_1, 11–26; peak M_2, 27–37; peak M_3, 45–57. Experimental curve, O—O; theoretical curve, ------.

TABLE III

AMINO ACID COMPOSITIONS OF PEPTIDES FROM COUNTERCURRENT DISTRIBUTION OF PEAKS M AND N

Composition is given in molar ratios.

Amino acid	*Preparations**			*Theoretical values***	
	Peak N_2	*Peak M_2*	*Peak M_3*	*α_α-ACTH*	*α_β-ACTH*
Lysine	3.9	3.8	4.0	4	4
Histidine	1.0	1.0	1.1	1	1
Arginine	3.0	2.8	3.0	3	3
Aspartic acid	2.0	2.0	2.0	2	2
Serine***	3.1	2.9	2.2	3	2
Glutamic acid	5.0	4.8	4.9	5	5
Proline	3.9	3.8	4.0	4	4
Glycine	3.0	3.0	3.0	3	3
Alanine	3.0	3.0	3.0	3	3
Valine	2.8	2.7	2.8	3	3
Methionine	0.8	0.9	0.8	1	1
Leucine	1.0	1.1	2.0	1	2
Tyrosine	1.8	1.7	1.8	2	2
Phenylalanine	2.8	2.7	2.9	3	3

* See Fig. 3.
** See ref. 1.
*** Corrected for 15% destruction during hydrolysis.

peak M. The lower retention volume of peak M on CM-cellulose suggested a difference in charge but, since such behavior could also be due to a difference in the degree of adsorption, it was decided to examine the electrophoretic mobilities of peaks M and N. Fig. 4 shows the results obtained when 2 mg of peak M and 2.6 mg of peak N were subjected to zone electrophoresis on starch[17] for 38 h at pH 9.0 (0.09 M in $NaHCO_3$ and 0.009 M in Na_2CO_3) in a potential gradient of 5 V/cm at 5°. The result agrees with the behavior on CM-cellulose in that peak N is slightly more basic than peak M.

In their studies on the purification of ACTH-A (α_p-ACTH) by means of ion-exchange chromatography on Amberlite IRC-50, DIXON AND STACK-DUNNE[2] also obtained two active components: ACTH-A_1 and ACTH-A_2. These workers found that if ACTH-A_1 were allowed to stand at pH 11.3 it was converted into ACTH-A_2 with the

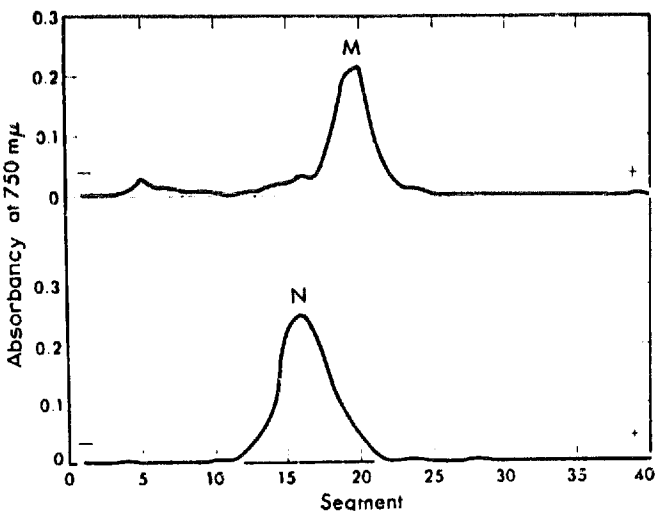

Fig. 4. Zone electrophoresis of peak M (2 mg) and peak N (2.6 mg) on starch. A potential gradient of 5 V/cm was applied for 38 h at 5°. Folin-Lowry color was developed in eluates from 1-cm segments of starch.

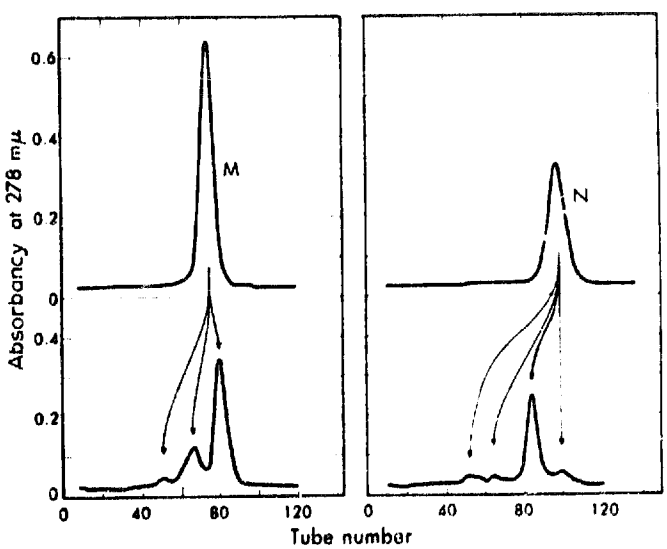

Fig. 5. Effect of mild treatment with alkali on retention volumes of peaks M and N on CM-cellulose. The upper chromatograms are of peak M (8 mg) and peak N (5.7 mg), and the peptides recovered from these peaks were allowed to stand overnight at pH 11.3. The lower chromatograms show the effects of this treatment on the retention volumes. Details of chromatography as in Fig. 2.

evolution of 1 mole of volatile base/mole of peptide. This observation suggests that ACTH-A_1 may differ from ACTH-A_2 by 1 amide per molecule. This experiment was repeated with peaks M and N. The peptide being studied (~ 5 mg) was dissolved in 10 ml of pH 11.3 buffer[2] and left overnight at room temperature. Salts were removed by dialysis against dilute ammonia[18] and the peptide was recovered by lyophilization. Fig. 5 shows the effect of this treatment on the retention volumes of peaks M and N on CM-cellulose. Peak N was largely converted into peak M with smaller amounts of two slower moving components, while peak M was partially converted into the two slower moving components.

Samples of peaks M and N were analyzed for amide content by hydrolyzing them with 2 N H_2SO_4 for 3 h at 100° and estimating the ammonia produced by the colorimetric method of STONE[19]. After correction had been made for ammonium salt content, peak M regularly gave 1.5 amides/mole and peak N 1.9 amides/mole. Moreover, the ammonia yield from total hydrolyzates of peak N, while consistently higher, never exceeded those from peak M by more than 0.3 mole/mole (Table I).

The nature of the peptide of peak O

Peak O emerged from the column shortly after the ACTH peaks (Fig. 1). The material had a low steroidogenic activity, the absolute potency varying from batch to batch (10–25 units/mg). When several batches of peak O were combined and rechromatographed, a single peak was obtained with the expected retention volume.

Amino acid analysis of peak O presented quite a problem at first. There were small, equivalent amounts of histidine and methionine, and, if peak O were a polypeptide containing one each of these residues, it would have to be a very large molecule. In view of the variability in the ACTH activity of peak O and the fact that α_8-ACTH has been found with different degrees of amidation[1], it was suggested that peak O might contain small amounts of ACTH with a higher amide content than peak M or peak N. It was therefore assumed that all the histidine present in the hydrolyzate of peak O was due to an ACTH contaminant and the relevant corrections were applied to the amounts of the other amino acids found with the amino acid analyzer. This procedure gave the analysis of the major component of peak O as $Lys_{4.0}$, $Arg_{3.0}$, $Asp_{2.0}$, $Ser_{0.9}$, $Glu_{3.8}$, $Pro_{3.8}$, $Gly_{3.1}$, $Ala_{3.0}$, $Val_{2.8}$, $Leu_{0.9}$, $Tyr_{0.8}$, $Phe_{1.8}$. When this analysis is compared with the composition of α_8-ACTH given in Table III, it is seen that peak O is deficient by two serines and one each of histidine, glutamic acid, methionine, tyrosine and phenylalanine.

The NH_2-terminal sequence of peak O was investigated by means of the paper-strip modification[20] of the EDMAN[21] procedure and found to be Phe–Arg–Try... In ad-

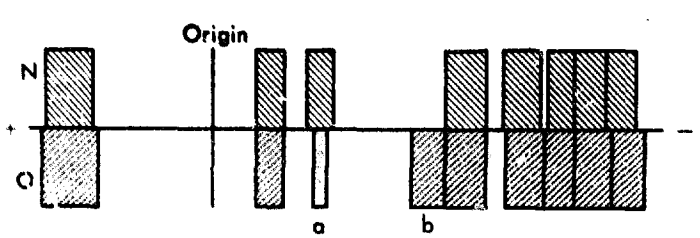

Fig. 6. Paper electrophoretic patterns obtained from tryptic digests of peaks N and O in collidine acetate (pH 6.9) for 5 h at 5° Note that the pattern of peak O differs from that of peak N by the presence of a faint band (a) and an extra band (b).

dition, small amounts of serine were encountered in Steps 1 and 3, and of tyrosine in Step 2, as would be expected if peak O contained some ACTH.

Samples of peaks N and O were digested with trypsin for 24 h at pH 9 and 37°. The digests were lyophilized and examined by paper electrophoresis; the results are shown in Fig. 6. The digest of peak O gave a similar pattern to that of peak N with

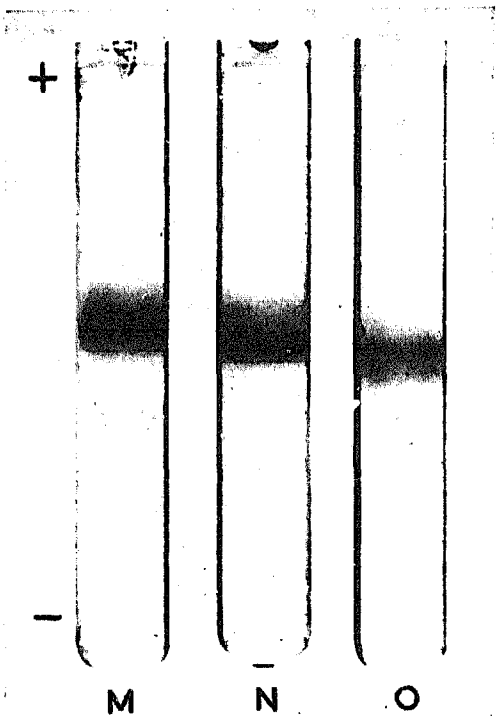

Fig. 7. Disc electrophoresis of peaks M, N and O. 50-μg samples were subjected to a potential gradient of 200 V/tube for 35 min. Electrophoresis occurred in 12.5% acrylamide gel at pH 4.5.

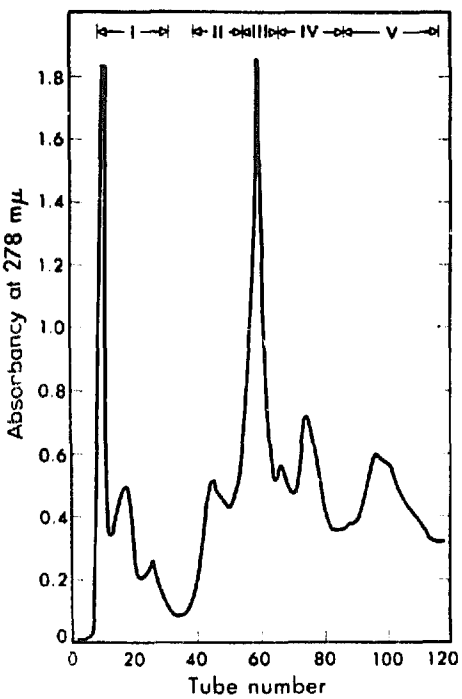

Fig. 8. Chromatography of peak C on DEAE-cellulose. 300 mg of peak C was applied to a 50-ml column of DEAE-cellulose equilibrated with 0.0075 M ammonium acetate (pH 7.0). At tube 24 a concentration gradient was begun by introducing 0.175 M ammonium acetate (pH 7.0) through a 500-ml mixing flask. 4-ml fractions were collected.

two striking differences. First, Band a which is known to be Ser–Tyr–Ser–Met–Glu–His–Phe–Arg is very weak in the digest of peak O, and second, peak O shows an extra band (Band b). This band was found to react with the Sakaguchi reagent and thus to contain arginine, but was negative in tests for tyrosine, histidine and tyrosine. Thus Band b is probably Phe–Arg.

Peaks O and N were digested with carboxypeptidase[22] for 24 h at pH 8.5 and 37° as described by LI, DIXON AND CHUNG[9]. Whereas 2 mg of peak N yielded 0.27 μmole phenylalanine, 2 mg peak O gave rise to only 0.02 μmole phenylalanine. Less than 0.01 μmole glutamic acid was recovered from either preparation.

Electrophoresis of peak M, N, and O in acrylamide gel

Samples of peaks M, N and O were subjected to disk electrophoresis in polyacrylamide gels[23,24]. 0.35 M β-alanine acetate (pH 4.5) was used as the buffer and electrophoresis was carried out for 35 min at 200 V with a current of 10 mA per tube (7 × 0.5 cm). Fig. 7 shows a photograph of the gels after staining with 'Amidoschwarz'.

Peaks C and D

As was mentioned earlier, peaks C and D (Fig. 1) were very active in the assay for MSH. Peak C was chromatographed on DEAE-cellulose according to SCHALLY *et al.*[6]; Fig. 8 shows the chromatographic pattern. The melanocyte-stimulating activity was confined to peaks I and III.

Peak III (Fig. 8) was again chromatographed on DEAE-cellulose and then subjected to paper electrophoresis for 20 h in collidine acetate (pH 6.9) at 12.5 V/cm and 5°. Two fractions were obtained, a minor inactive one and a major active fraction which had a melanocyte-stimulating activity *in vitro*[14] of $3.8 \cdot 10^9$ units/g, and the amino acid composition of β_{Glu}-MSH (pig type).

When peak D (Fig. 1) was chromatographed on DEAE-cellulose it was largely unretarded and was therefore pooled with the similarly unretarded peak I (Fig. 8). This material was returned to CM-cellulose and then further purified by electrophoresis on starch[17] at pH 4.9 (pyridine acetate) for 24 h at 5 V/cm and 5°. In this manner a sample of β_{Ser}-MSH (beef type) which assayed at $1.2 \cdot 10^9$ units/g was obtained.

DISCUSSION

A peptide having the amino acid composition of pig ACTH has been isolated from sheep glands. It would appear that approx. 10% of the ACTH in sheep-pituitary extracts is of the pig type. The question arises as to whether all sheep have 10% pig ACTH in their pituitaries, or whether 10% of all sheep pituitaries contain only pig ACTH. No answer can be provided at present, but it may be mentioned that BURGERS[25] demonstrated the presence of melanocyte-stimulating agents with electrophoretic characteristics of both β_{Glu}- and β_{Ser}-MSH in single sheep pituitaries.

The pig-type ACTH was associated with peak M, but the bulk of this peak was composed of a peptide which gave the same amino acid analysis as sheep ACTH. Thus there are present in sheep pituitary extracts at least two fully active ACTH's, peaks M and N, with the same amino acid composition as α_s-ACTH. That peak N is more basic than peak M is evidenced both by its behavior on CM-cellulose and by its electrophoretic mobility. Thus one would suggest that the difference between the two peptides lies in the number of carboxyl groups that are present as amides, and, indeed, mild treatment with alkali converts peak N into peak M. The difficulty arises in deciding the actual number of amide groups in each preparation. From amide determination peak N was found to contain 1.9 amide/mole and peak M 1.5 amide/mole, and this lack of stoichiometric difference is borne out by the amount of ammonia obtained during amino acid analyses of hydrolyzates of the two peptides; specifically, 2.5 moles/mole for peak N, and 2.3 moles/mole for peak M. When allowed to stand in alkali, peak N was converted into peak M and two more acidic peptides (Fig. 5); from this, one could assume that peak N has 3 amide groups per mole and peak M 2 amide groups per mole. There is the possibility, however, that one or more of these minor components is due to oxidation of methionine to methionine sulfoxide[28], which would also decrease the retention volume[2]. In the past, α_s-ACTH has been assigned 2 amide groups per mole[27,28] with a suggestion[29] that this peptide arose, during purification, from a molecule having 4 amide groups per mole. With the seemingly conflicting data, no firm conclusion can be reached about the number of amide groups in peak M and peak N.

During the chromatography of sheep ACTH concentrates, a peak was obtained with a higher retention volume than the ACTH's from peaks M and N, and was designated peak O (see Fig. 1). This peak apparently contains 10–20 % of an ACTH which presumably has a higher amide content than peak N. Preliminary investigations suggest that the peptide making up the bulk of peak O is a molecule with the sequence of ACTH but lacking the NH_2-terminal hexapeptide (Ser–Tyr–Ser–Met–Glu–His) and the COOH-terminal amino acid phenylalanine. Such a conclusion can be only tentative, of course, since a full structural investigation of the pure peptide was not possible. It does, however, introduce some interesting possibilities.

There have been several reports[30–32] of the presence of proteolytic enzymes in pituitary extracts. The presence of peak O suggests that one of these enzymes may have a specificity allowing it to cleave the His–Phe bond in ACTH, although the peptide could equally well arise from an attack by an aminopeptidase step-by-step from the NH_2-terminus. Still another, though less likely, possibility is that the peptide does indeed occur in the pituitary. Whatever its origin, however, peak O suggests that a study of the "non-hormonal" peptides of the pituitary could be very fruitful for the understanding of pituitary biochemistry.

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REFERENCES

1 C. H. LI, *Advan. Protein Chem.*, 2 (1956) 101.
2 H. B. F. DIXON AND M. P. STACK-DUNNE, *Biochem. J.*, 61 (1955) 483.
3 C. H LI, I. I. GESCHWIND, A. L LEVY, J. I. HARRIS, J. S. DIXON, N. G. PON AND J. O. PORATH, *Nature*, 173 (1954) 251.
4 R. G. SHEPHERD, K. S. HOWARD, P. H. BELL, A. R. CACCIOLA, R. G. CHILD, M. C. DAVIES, J. P. ENGLISH, B. M. FINN, J. H. MEISENHELDER, A. W MOYER AND J. VAN DER SCHEER, *J. Am. Chem. Soc.*, 78 (1956) 5051.
5 T. H. FARMER, *Biochem. J.*, 73 (1959) 321.
6 A. V. SCHALLY, R. N. ANDERSEN, J. M. LONG AND R. GUILLEMIN, *Proc. Soc. Exptl. Biol. (Med.)*, 104 (1960) 290.
7 K. S. HOWARD, R. G. SHEPHERD, E. A. EIGNER, D. S. DAVIES, AND P. H. BELL, *J. Am. Chem. Soc.*, 77 (1955) 3419.
8 C. H. LI, I. I. GESCHWIND, R. D. COLE, I. D. RAACKE, J. I. HARRIS AND J. S. DIXON, *Nature*, 176 (1955) 687.
9 C. H. LI, J. S. DIXON AND D. CHUNG, *Biochim. Biophys. Acta*, 46 (1961) 324.
10 T. H. LEE, A. B. LERNER AND V. BUETTNER-JANUSCH, *J. Biol. Chem.*, 236 (1961) 2970.
11 I. I. GESCHWIND, in A. GORBMAN, *Comparative Endocrinology*, J. Wiley and Sons, New York, 1957, p. 421.
12 M. SAFFRAN AND A. V. SCHALLY, *Endocrinology*, 56 (1955) 523.
13 C. RERUP, *Acta Endocrinol.*, 29 (1958) 83.
14 K. SHIZUME, A. B. LERNER AND T. B. FITZPARTICK, *Endocrinology*, 54 (1954) 553.
15 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
16 J. S. DIXON, T.-B. LO AND C. H. LI, *Arch. Biochem. Biophys.*, 92 (1961) 296.
17 P. FØNSS-BECH AND C. H. LI, *J. Biol. Chem.*, 207 (1954) 175.
18 C. H. LI, *Bull. Soc. Chim. Biol.*, 40 (1958) 1757.

[19] W. E. STONE, *Proc. Soc. Exp. Biol. (Med.)*, 93 (1956) 589.
[20] H. FRAENKEL-CONRAT, *J. Am. Chem. Soc.*, 76 (1954) 3606.
[21] P. EDMAN, *Acta Chem. Scand.*, 4 (1950) 277, 283.
[22] J. I. HARRIS AND C. H. LI, *J. Biol. Chem.*, 213 (1955) 499.
[23] S. RAYMOND AND L. WEINTRAUB, *Science*, 130 (1959) 711.
[24] R. A. REISFELD, U. J. LEWIS AND D. E. WILLIAMS, *Nature*, 195 (1962) 281.
[25] A. C. J. BURGERS, *Endocrinology*, 68 (1961) 698.
[26] M. L. DEDMAN, T. H. FARMER AND C. J. O. R. MORRIS, *Biochem. J.*, 78 (1961) 348.
[27] J. LÉONIS AND C. H. LI, *J. Am. Chem. Soc.*, 81 (1959) 415.
[28] A. L. LEVY, I. I. GESCHWIND AND C. H. LI, *J. Biol. Chem.*, 213 (1955) 187.
[29] C. H. LI, I. I. GESCHWIND, J. S. DIXON, A. L. LEVY AND J. I. HARRIS, *J. Biol. Chem.*, 213 (1955) 171.
[30] E. ADAMS AND E. L. SMITH, *J. Biol. Chem.*, 191 (1951) 651.
[31] S. ELLIS, *Endocrinology*, 69 (1961) 554.
[32] U. J. LEWIS, *J. Biol. Chem.*, 237 (1962) 3141.