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Biochem. J. (1961) **78**, 348

Studies on Pituitary Adrenocorticotrophin

3. IDENTIFICATION OF THE OXIDATION-REDUCTION CENTRE*

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(Received 8 July 1960)

The oxidation-reduction behaviour of corticotrophin A_1 was studied in detail by Dedman, Farmer & Morris (1957). The hormone is oxidized by hydrogen peroxide under appropriate conditions to give a biologically inactive product which can be distinguished chromatographically from the untreated corticotrophin (Dixon & Stack-Dunne, 1955; Farmer & Morris, 1956). Reduction of the oxidized hormone with thiol compounds completely restores the biological activity, and the reduction product is chromatographically identical with the original untreated hormone. The oxidation-reduction process is thus readily reversible.

Dedman *et al.* (1957) examined the potentially oxidizable amino acid residues in corticotrophin A_1 and concluded that it was difficult to explain the experimental results on the basis of the known structure. In particular, methionine was excluded because Raney-nickel-treated corticotrophin, which was apparently sulphur-free, exhibited the characteristic oxidation-reduction behaviour. The subsequent inability to isolate α -aminobutyric acid from Raney-nickel-treated corticotrophin A_1 has prompted a re-examination of this material. Improved analytical methods with hydrolysis by leucine aminopeptidase show that it contains methionine, which was destroyed by the earlier method of acid hydrolysis. The evidence excluding this amino acid from participation in the oxidation-reduction centre is thus no longer valid. This paper presents evidence that the oxidation-reduction centre of corticotrophin is in fact the thioether grouping of methionine.

* Part 2: Dedman, Farmer & Morris (1957).

EXPERIMENTAL

Bioassay methods. The biological activities of all preparations were measured by the adrenal-ascorbic acid-depletion method of Sayers, Sayers & Woodbury (1948) with minor modifications (Morris, 1951).

Preparation of corticotrophin A_1 . The hormone was isolated from pig anterior-pituitary glands by the method of Dedman *et al.* (1957), or by the method of Farmer (1959). The oxidized hormone was prepared by H_2O_2 oxidation as described by the former authors.

Preparation of leucine aminopeptidase. The enzyme was prepared according to the procedure of Hill & Smith (1957). Enzymic activity was measured by the digestion of leucyltyrosine. The enzyme was stored at 2° as a solution in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 8.0, which was 0.05M with respect to $MgCl_2$. The enzyme was activated before use by heating for 30 min. at 40° at pH 8.5 (Hill & Smith, 1957).

Preparation of the tryptic-digestion products of corticotrophin A_1 and oxidized corticotrophin A_1 . (a) Digestion. Corticotrophin A_1 (9.8 mg.) was dissolved in an aqueous 0.025% solution of trypsin (L. Light and Co. Ltd., 50 Northrop units/mg.) in 0.01M-sodium phosphate buffer, pH 8.0 (1.0 ml.). The mixture was maintained at 30° for 30 min. 1M-Pyridine-acetate buffer (pyridine, 16 ml.; 2N-acetic acid, 100 ml.), pH 4.4 (0.11 ml.), was added and the mixture was heated at 90° for 2 min. to inactivate the trypsin. Oxidized corticotrophin A_1 (8.8 mg.) was also digested by this procedure.

(b) Chromatography. Carboxymethylcellulose (0.6 mequiv./g.; 2 g.) was suspended in aqueous 0.5% pyridine and gently dispersed in an all-glass Potter-Elvehjem homogenizer. After the greater part of the material had sedimented, the supernatant liquid and very fine material were removed. The carboxymethylcellulose was washed several times with 0.067M-pyridine-acetate buffer, pH 4.4 (re-distilled pyridine, 5.30 ml.; 2N-acetic acid, 66.7 ml.; diluted to 1 l. with water). A column 62 cm. $\times$ 0.5 cm. was packed

with the carboxymethylcellulose in approx. 10 cm. sections. The tryptic hydrolysate was applied to the top of the column and development of the chromatogram carried out with 0.067 M-pyridine-acetate buffer, pH 4.4, at a flow rate of 5 ml./hr. Usually 100 × 1.55 ml. fractions were collected. Fig. 1 (column 1) shows the separation of the tryptic-digest peptides from corticotrophin A_1 , and Fig. 1 (column 2) shows the corresponding separation from oxidized corticotrophin A_1 .

Zone A (Fig. 1, column 1) is a mixture of the C-terminal peptides of corticotrophin A_1 and corresponds mainly to the peptide T_1 obtained by Shepherd *et al.* (1956) from a tryptic digest of β -corticotrophin. Zone B is the N-terminal peptide (T 10 of Shepherd *et al.* 1956), containing residues 1–8 of the corticotrophin molecule, and zone C is the adjacent peptide (T 14 of Shepherd *et al.* 1956), comprising residues 9–15. The very basic peptide T 17 of Shepherd *et al.* (1956) remained on the column during the development and could only be recovered by alkaline elution. The chromatographic separation of the tryptic-digestion products of corticotrophin A_1 reported here with a 62 cm. column is markedly superior to that obtained after counter-current distribution through 1400 transfers by Shepherd *et al.* (1956). Zones A', B' and C' are the corresponding peptides from oxidized corticotrophin A_1 . In several experiments zone B' moved slightly faster than zone B.

The peptides could be recovered from the appropriate pooled fractions by direct freeze-drying.

Chromatographic comparison of the T 10 and T 14 peptides from corticotrophin A_1 and oxidized corticotrophin A_1 . A 127 cm. × 0.6 cm. column was prepared from 6 g. of carboxymethylcellulose in the 0.067 M-pyridine-acetate buffer. A mixture of peptide B (1.0 mg.) and peptide B' (1.25 mg.) was applied to the column and development continued with the pyridine-acetate buffer. Fig. 2 (column 3) shows the course of the separation.

A similar experiment was carried out with peptides C (0.75 mg.) and C' (0.75 mg.) and the corresponding chromatogram is shown in Fig. 2 (column 4).

Acid hydrolyses of peptides. Hydrolyses were carried out in a sealed tube with constant-boiling-point HCl (0.25 ml.) for periods of 24–48 hr.

Leucine aminopeptidase hydrolyses. In representative experiments, 0.1–1.0 mg. of the material to be hydrolysed was dissolved in 0.2–0.5 ml. of water and 0.2–0.5 ml. of a solution of activated leucine aminopeptidase was added. The mixture was kept at 40° for 18 hr., and the reaction was terminated by the addition of 2 N-acetic acid (0.2 ml.). The digestion mixture was evaporated to dryness *in vacuo*. For paper-electrophoretic analysis the residue was dissolved in 10 μ l. of formic acid-acetic acid solution (see below) and a 5 μ l. portion was taken for analysis. Paper-

Fig. 1. Chromatographic separation of tryptic-digestion peptides of corticotrophin A_1 (column 1) and oxidized corticotrophin A_1 (column 2) on carboxymethylcellulose in 0.067 M-pyridine-acetate buffer, pH 4.4. For designations of individual peptides see text.

Fig. 2. Chromatographic comparison of T 10 (column 3) and T 14 (column 4) peptides from the tryptic digestion of untreated (B and C) and of oxidized (B' and C') corticotrophin A_1 on carboxymethylcellulose in 0.067 M-pyridine-acetate buffer, pH 4.4.

chromatographic examination of the products of prolonged autolysis of the enzyme showed only a very small amount of glutamic acid.

Amino acid analyses. In the earlier part of this work amino acid separations were carried out by paper chromatography in the system butan-1-ol-acetic acid-water (60:15:25, by vol.). The amino acid spots were developed with ninhydrin according to the method of de Wael & Cadavieco (1954) and the pigment was eluted with aqueous 50% (v/v) propan-1-ol. Extinction values were determined at 570 m μ . Standard quantities of all amino acids measured were chromatographed and developed simultaneously.

Later analyses were carried out by high-voltage electrophoresis on paper by a simplified form of the method of Atfield & Morris (1960). The separation was carried out on a 65 cm. $\times$ 15 cm. strip of Whatman no. 3 MM paper in the apparatus of Gross (1956). A solution containing formic acid (40 g.) and acetic acid (155.4 g.) in water (2 l.), pH 1.9, was used as electrolyte for the majority of the separation of neutral and acidic amino acids. This system could be used for the determination of glycine, serine, tyrosine, methionine, methionine sulfoxide, glutamic acid and phenylalanine by electrophoresis at 25 v/cm. for 7 hr. Determination of the basic amino acids histidine, arginine and lysine was carried out in pyridine-acetate buffer, pH 6.1 (5% pyridine in aqueous 0.5% acetic acid, v/v), at 25 v/cm. for 3.5 hr. The amino acids were estimated after separation by the cadmium-ninhydrin method of Heilmann, Barollier & Watzke (1957), the extinction of the red complex being measured at 500 m μ .

RESULTS

Amino acid composition of T10 peptides from corticotrophin A₁ and oxidized corticotrophin A₁. The analyses carried out by quantitative paper chromatography on a HCl hydrolysate of the chromatographically separated peptides are given in Table 1. Methionine sulfoxide was detected qualitatively in a preliminary paper-electrophoretic

examination of the oxidized hormone. It was absent from the untreated material.

Leucine aminopeptidase hydrolyses of T10 peptides from corticotrophin A₁ and oxidized corticotrophin A₁. Preliminary qualitative experiments in which the preparations were digested with leucine aminopeptidase followed by high-voltage electrophoretic separation of the liberated amino acids showed that the T10 peptide (B) from corticotrophin A₁ contained serine, tyrosine, methionine,

Table 1. *Amino acid composition of T10 peptides from untreated and oxidized corticotrophins*

	Hydrolysis was with HCl.	
	Corticotrophin A ₁	Oxidized corticotrophin A ₁
	(moles of amino acid/mole of peptide)	
Serine	1.56	1.60
Tyrosine	0.42	0.43
Methionine	0.58	0.09
Glutamic acid	1.33	0.97
Histidine	0.82	0.72
Phenylalanine	0.89	0.81
Arginine	0.97	0.97

Table 2. *Amino acid contents of corticotrophin A₁ and its Raney-nickel reaction product*

Hydrolysis was with leucine aminopeptidase.

	Cortico- trophin A ₁	Reaction product (mean of two determinations)
	(moles of amino acid/mole of corticotrophin)	
Serine	2.00	2.00
Methionine	0.51	0.82
Methionine sulfoxide	0.0	0.0
Glutamic acid	0.98	0.89
α -Aminobutyric acid	—	0.00

Table 3. *Biological activities and amino acid analysis of oxidized and untreated corticotrophin*

Potencies are means with fiducial limits.

Preparation	Biological activity (relative to untreated hormone as unity)	Amino acid content (moles/mole of peptide)			
		Serine	Methionine	Methionine sulfoxide	Glutamic acid
A ₁ (1)	1	2.00	0.51	0.0	0.98
A ₁ (2)	1	2.00	0.93	0.08	1.03
A ₁ (3) (rechromatographed)	1	2.00	0.94	0.00	1.04
A ₁ (4)	1	2.00	0.94	0.07	0.93
Oxidized A ₁ (1)	0.02	2.00	0.0	0.89	0.80
Oxidized A ₁ (2)	0.024 (0.019-0.032)	2.00	0.05	0.82	1.00
Partially oxidized A ₁ (3)	0.60 (0.47-0.77)	2.00	0.58	0.40	0.86
Partially oxidized A ₁ (4)	0.93 (0.72-1.21)	2.00	1.00	0.16	0.74

glutamic acid, phenylalanine, histidine and arginine, in accordance with its established structure. The corresponding peptide (B') from oxidized corticotrophin A₁ contained serine, tyrosine, methionine sulphoxide, glutamic acid, phenylalanine, histidine, arginine.

Amino acid analysis on untreated and oxidized corticotrophin A₁ and the Raney-nickel reaction products after hydrolysis with leucine aminopeptidase. The materials were hydrolysed with leucine aminopeptidase and the products subjected to paper-electrophoretic separation and quantitative colorimetry as described in the Experimental section. Results are given as mole fractions of amino acid referred to 2 moles of serine as standard.

The amounts of certain amino acids in the *N*-terminal sequence of Raney-nickel-treated corticotrophin A₁ are compared with those in the starting material in Table 2. The analytical method was capable of detecting α -aminobutyric acid down to a mole fraction of 0.1 on the amounts hydrolysed.

A HCl hydrolysate of the Raney-nickel-treated material had a mole fraction of methionine of 0.28.

Values for untreated, partially and completely oxidized preparations of corticotrophin are given in Table 3.

DISCUSSION

The chromatographic separation of the T10 peptides from tryptic digests of oxidized and untreated corticotrophins and the apparent identity of the T14 peptides makes it probable that the oxidation-reduction centre is located in the *N*-terminal sequence of eight amino acids. The T1 and T17 peptides from this digest were not compared directly but the absence of oxidizable residues in this portion of the molecule makes the location of the oxidation-reduction centre there unlikely. The complete hydrolysis of the T10 peptide from oxidized corticotrophin by leucine aminopeptidase makes it improbable that this sequence contains any unusual residue, and also establishes the optical configuration of the amino acids in this sequence.

The only significant difference in compositions between the T10 peptides of the corticotrophins in the two cases lies in the conversion of methionine into methionine sulphoxide in the oxidized form (Tables 1 and 3).

Dedman *et al.* (1957) had considered participation of the thioether group of methionine in the oxidation-reduction centre to be unlikely on account of the normal oxidation-reduction behaviour of corticotrophin in which this group had

apparently been removed by treatment with Raney nickel under vigorous conditions. Hydrolysis of this material by leucine aminopeptidase shows, however, that it still contains methionine (Table 2) but no α -aminobutyric acid. Acid hydrolysis, however, confirms the earlier experiments in showing a greatly diminished yield of methionine. The loss is presumably due to destruction in the presence of trace amounts of nickel in the preparations, despite ion-exchange purification. Hayes & White (1954) have previously observed the peculiar lability of methionine in the corticotrophin molecule.

The presence of methionine and the absence of α -aminobutyric acid in the Raney-nickel-treated preparation thus removes the objections to methionine as the oxidizable residue in the corticotrophin molecule, and the apparent correlation between the biological activity and the methionine content shown in Table 3 provides evidence that the thioether group is in fact the oxidation-reduction centre. The chemical reactivity of this centre described in the preceding paper of this series is also in agreement with this view.

SUMMARY

1. The products of tryptic digestion of corticotrophin A₁ and oxidized corticotrophin have been separated and compared by chromatography on carboxymethylcellulose. Only the *N*-terminal peptides differed significantly.

2. The amino acid composition of the *N*-terminal peptides has been determined. The oxidized hormone differs in the substitution of methionine sulphoxide for the methionine present in the untreated material.

3. The amino acid composition of Raney-nickel-treated corticotrophin has been investigated after leucine aminopeptidase hydrolysis. Contrary to the findings of earlier studies on acid hydrolysates, methionine is still present in the material.

4. On the basis of these studies and the correlation between biological activity and the extent of oxidation of methionine, it is concluded that the oxidation-reduction centre of corticotrophin A₁ is the thioether grouping of methionine.

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Biochem. J. (1961) **78**, 352

Comparative Studies of 'Bile Salts'

13. BILE ACIDS OF THE LEOPARD SEAL, *HYDRURGA LEPTONYX*, AND OF TWO SNAKES OF THE GENUS *BITIS**

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(Received 5 July 1960)

In the preliminary survey with which this work began the bile of ten species of snakes was examined. The Boidae (three species) gave pythocholic lactone (3 α :12 α :16 α -trihydroxycholanolic acid lactone), six other species yielded cholic acid (3 α :7 α :12 α -trihydroxycholanolic acid), but from the Gaboon viper, *Bitis gabonica*, no known bile acid was isolated (Haslewood & Wootton, 1950). An investigation of the bile salts of this species by paper chromatography (Haslewood & Sjövall, 1954) confirmed their unusual nature.

The work now reported included a detailed chemical examination of the bile salts of the Gaboon viper and also those of the puff adder, *Bitis arietans*, a closely related species. It was found that the bile salts of these snakes resembled those of Pinnepedia. For comparison, therefore, the leopard seal, *Hydrurga leptonyx*, was also investigated. Some knowledge was, incidentally, obtained of the bile acids of the Californian sealion, *Zalophus californianus*.

RESULTS

Paper chromatograms of the methyl or ethyl esters of Gaboon-viper bile acids showed faint spots corresponding to those given by methyl or ethyl cholate, together with two additional intense spots. In the solvent systems used, one of these intense spots (P) appeared just behind the methyl or ethyl cholate spot, and the other (Q) moved only a little way from the start-line. Fortuitously it was noticed that spots P and Q appeared at the same places on chromatograms as spots given by

the corresponding esters of leopard-seal and Californian-sealion bile acids. Chromatography on alumina of the ethyl esters of Gaboon-viper bile acids easily separated the fraction responsible for spot P from a 'spot Q-ethyl cholate' fraction, described later.

The ethyl ester giving spot P was hydrolysed and the (non-crystalline) acid was investigated, with the clue that it might be related to the bile acids of Pinnepedia. The characteristic chemical feature of these acids is the C-23 hydroxyl group, found by Windaus & van Schoor (1928) in ' β -phocaecholic acid' (3 α :7 α :23-trihydroxycholanolic acid, I). This acid was now isolated from leopard-

seal and Californian-sealion bile and conditions were found by which the $-\text{CH}(\text{OH})\cdot\text{CO}_2\text{H}$ grouping could be quantitatively estimated by lead tetraacetate oxidation. Application of this method to the 'spot P' of Gaboon-viper acid gave the same result, quantitatively, as had been obtained from ' β -phocaecholic acid' (I). However, the 'spot P' acid did not crystallize with (I), and its specific rotation was $[\alpha]_D + 48.5^\circ$, in contrast with $[\alpha]_D + 11^\circ$ for ' β -phocaecholic acid'. These facts, and the

* Part 12: Haslewood & Ogan 1959).