Studies on Pituitary Adrenocorticotrophin

3. IDENTIFICATION OF THE OXIDATION-REDUCTION CENTRE*

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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 $\alpha$-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 The evidence excluding this amino acid from participation in the oxidation-reduction centre is thus no longer valid.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.

with the carboxymethylcellulose in approx. 10 cm. sections. The trypic hydrolysat was applied to the top of the column and development of the chromatogram carried out with 0.067M-pyridine-acetate buffer, pH 4-4, at a flow rate of 5 ml./hr. Usually 100×1.56 ml. fractions were collected. 

Fig. 1 (column 1) shows the separation of the trypic-digest peptides from corticotrophin A₁, and Fig. 1 (column 2) shows the corresponding separation from oxidized corticotrophin A₁.

Zone A (Fig. 1, column 1) is a mixture of the C-terminal peptides of corticotrophin A₁ and corresponds mainly to the peptide T₁ obtained by Shepherd et al. (1956) from a trypic digest of β-corticotrophin. Zone B is the N-terminal peptide (T₁₀ of Shepherd et al. 1956), containing residues 1-8 of the corticotrophin molecule, and zone C is the adjacent peptide (T₁₄ of Shepherd et al. 1956), comprising residues 9-15. The very basic peptide T₁₇ 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 trypic-digestion products of corticotrophin A₁ reported here with a 62 cm. column is markedly superior to that obtained after countercurrent distribution through 1400 transfers by Shepherd et al. (1956). Zones A', B' and C' are the corresponding peptides from oxidized corticotrophin A₁. 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.

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**Chromatographic comparison of the T₁₀ and T₁₄ peptides from corticotrophin A₁ and oxidized corticotrophin A₁.**

A 127 cm. x 0.6 cm. column was prepared from 6 g. of carboxymethylcellulose in the 0.067M-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 2N-acetic acid (0.2 ml.). The digestion mixture was evaporated to dryness in vacuo. For paper-electrophoretic analysis the residue was dissolved in 10 µl. of formic acid-acetic acid solution (see below) and a 5 µl. portion was taken for analysis. Paper-

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**Fig. 1.** Chromatographic separation of trypic-digest peptides of corticotrophin A₁ (column 1) and oxidized corticotrophin A₂ (column 2) on carboxymethylcellulose in 0.067M-pyridine-acetate buffer, pH 4.4. For designations of individual peptides see text.

**Fig. 2.** Chromatographic comparison of T₁₀ (column 3) and T₁₄ (column 4) peptides from the trypic digestion of untreated (B and C) and of oxidized (B' and C') corticotrophin A₁ on carboxymethylcellulose in 0.067M-pyridine-acetate buffer, pH 4.4.
chromatographic examination of the products of prolonged autodigestion 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 & Cadaviec (1954) and the pigment was eluted with aqueous 50 % (v/v) propan-1-ol. Extinction values were determined at 570 m\(\mu\). 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. x 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 sulphoxide, 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 25v/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\(\mu\).

**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 sulphoxide 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>
<thead>
<tr>
<th>Oxidized</th>
<th>Corticotrophin A1</th>
<th>Reaction product (mean of two determinations)</th>
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</thead>
<tbody>
<tr>
<td>Serine</td>
<td>1.56</td>
<td>1.60</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.42</td>
<td>0.43</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.58</td>
<td>0.59</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.33</td>
<td>0.97</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.82</td>
<td>0.72</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.89</td>
<td>0.81</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.97</td>
<td>0.97</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Hydrolysis was with leucine aminopeptidase.</th>
</tr>
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<tbody>
<tr>
<td>Reaction product (mean of two determinations)</td>
</tr>
<tr>
<td>(moles of amino acid/mole of corticotrophin)</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Biological activity (relative to untreated hormone as unity)</th>
<th>Amino acid content (moles/mole of peptide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 (1)</td>
<td>1</td>
<td>Serine 2:00 Methionine 0:51 Methionine sulphoxide 0:09 Glutamic acid 0:98</td>
</tr>
<tr>
<td>A1 (2)</td>
<td>1</td>
<td>Serine 2:00 Methionine 0:93 Methionine sulphoxide 0:08 Glutamic acid 1:03</td>
</tr>
<tr>
<td>A1 (3) (rechromatographed)</td>
<td>1</td>
<td>Serine 2:00 Methionine 0:94 Methionine sulphoxide 0:00 Glutamic acid 1:04</td>
</tr>
<tr>
<td>A1 (4)</td>
<td>1</td>
<td>Serine 2:00 Methionine 0:94 Methionine sulphoxide 0:07 Glutamic acid 0:93</td>
</tr>
<tr>
<td>Oxidized A1 (1)</td>
<td>0:02</td>
<td>Serine 2:00 Methionine 0:0 Methionine sulphoxide 0:89 Glutamic acid 0:80</td>
</tr>
<tr>
<td>Oxidized A1 (2)</td>
<td>0:024 (0:019-0:032)</td>
<td>Serine 2:00 Methionine 0:05 Methionine sulphoxide 0:82 Glutamic acid 1:00</td>
</tr>
<tr>
<td>Partially oxidized A1 (3)</td>
<td>0:60 (0:47-0:77)</td>
<td>Serine 2:00 Methionine 0:58 Methionine sulphoxide 0:40 Glutamic acid 0:86</td>
</tr>
<tr>
<td>Partially oxidized A1 (4)</td>
<td>0:93 (0:72-1:21)</td>
<td>Serine 2:00 Methionine 1:00 Methionine sulphoxide 0:16 Glutamic acid 0:74</td>
</tr>
</tbody>
</table>

Potencies are means with fiducial limits.
glutamic acid, phenylalanine, histidine and arginine, in accordance with its established structure. The corresponding peptide (B') from oxidized corticotrophin A₁ contained serine, tyrosine, methionine sulfoxide, glutamic acid, phenylalanine, histidine, and 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 T₈ 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 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 sulfoxide 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 sulfoxide 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.

REFERENCES


Comparative Studies of 'Bile Salts'

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

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In the preliminary survey with which this work began the bile of ten species of snakes was examined. The Boidae (three species) gave cho- lonic lactone (3a:12a:16a-tribhydroxycholanic acid lactone), six other species yielded cholic acid (3a:7a:12a-tribhydroxycholanic 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 Pinnepeda. 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 Pinnepeda. The characteristic chemical feature of these acids is the C-23 hydroxyl group, found by Windaus & van Schoor (1928) in 'β-phocaecholic acid' (3a:7a:23-tribhydroxycholanic acid, I). This acid was now isolated from leopard-

![Chemical structure](I)

seal and Californian-sealion bile and conditions were found by which the -CH(OH)-CO₂H 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 [α]₀ + 48.5°, in contrast with [α]₀ + 11° for 'β-phocaecholic acid'. These facts, and the