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Treatment of Corticotrophin with Periodate and Borohydride

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Periodate reacts with the terminal serine residue of corticotrophin (Geschwind & Li, 1954). Dixon (1956a) showed that it destroys the corticotropic action of the hormone and claimed that the melanocyte-stimulating activity was unchanged. In later unpublished assays he and A. B. Lerner and M. Ruth Wright found that the melanocyte-stimulating action of corticotrophin was increased by periodate treatment. Geschwind & Li (1958) demonstrated that treatment of corticotrophin with periodate destroyed not only its property of evoking depletion of adrenal ascorbic acid, which Dixon (1956a) had used in his study, but also its property of maintaining adrenal weight.

M. S. Raben in preliminary unpublished experiments (personal communication) found that periodate-treated samples of corticotrophin still retained some fat-mobilizing activity in the mouse. In view of this, when Engel & Engel (1958) reported that the ketogenic action of corticotrophin, which is mediated by an extra-adrenal mechanism, resembled its corticotropic action in that it could be reversibly abolished by the same oxidation-reduction procedure, it became interesting to prepare, characterize and test periodate-treated corticotrophin. The present paper concerns the preparation and characterization of such a material, and the accompanying paper (Boright, Engel, Lebovitz, Kostyo & White, 1962) describes some of its biological properties.

METHODS

Treatment of corticotrophin with periodate and borohydride. Pig corticotrophin A_1 (20.7 mg.), prepared by the method of Dixon & Stack-Dunne (1955) with the minor modifications cited by Waller & Dixon (1960), was dissolved in 8.6 ml. of water. Ammonium hydrogen carbonate (395 mg.) was added. Into this solution there was rapidly introduced 1 ml. of 25 mM- $NaIO_4$ (final concentration 2.5 mM), followed 30 sec. later by 54 mg. of KBH_4 , which was quickly stirred in. After 2 min. 2-mercaptoethanol (0.1 ml.) was added; the solution was acidified with acetic acid (3 ml.) and diluted to about 100 ml.

A reducing agent such as mercaptoethanol was added because, once the borohydride was destroyed by acidification, the iodide that had been produced was otherwise oxidized by air to iodine which might then have attacked the product. Mercaptoethanol is preferred to thioglycollic acid, which was used in much of the work, because it avoids the danger of acylation by thio esters which are generated during storage of thioglycollic acid (cf. White, 1960).

The product was desalted by adsorbing it on a carboxylic resin (Amberlite CG-50, type II), washing away the salts and un-ionized substances with dilute acetic acid and displacing the product with 50% acetic acid as described by Dixon (1959). The solution in acetic acid was dried while frozen and the product (14.9 mg.) submitted to chromatography.

Ion-exchange chromatography on Amberlite IRC-50 (Amberlite CG-50, 200-mesh). Chromatography was carried out according to the methods of Hirs, Moore & Stein (1953) and Dixon & Stack-Dunne (1955). The stock solution of

buffer used for the isolation of corticotrophin A_1 contained 0.1M- Na_2HPO_4 , 0.1M- NaH_2PO_4 and 0.25% (w/v) chlorobutanol (1,1,1-trichloro-2-methylpropan-2-ol). The buffer used for chromatography of periodate-treated samples was a dilution of the stock buffer to three-quarters of its strength ($[\text{Na}^+]$, 0.225M). All columns were equilibrated before use so that the effluent possessed the same pH value as the influent to within 0.01 unit.

The product was detected in the effluent from the chromatographic columns by its absorption at $280\text{ m}\mu$ and was desalted and dried as described above by the method of Dixon (1959). The yield from 14.9 mg. was 9.6 mg. (overall yield 46%). This material is referred to as periodate-borohydride-treated corticotrophin.

RESULTS

Reaction conditions

Several buffer solutions were used with pH values from 7 to 10 without apparent difference. If the buffers did not contain ammonia, L-histidine hydrochloride (1 mg./ml.) was added to react with the formaldehyde produced.

When periodate was reduced to iodide with bisulphite instead of borohydride, the product gave a single peak when chromatographed at once, but several peaks after storage in the dry state for some days. Periodate-borohydride-treated corticotrophin, on the other hand, is stable. If the reaction with periodate was stopped by adding 0.1 ml. of ethane-1,2-diol, the corticotrophin derivative was much less soluble in the buffer used for chromatography than periodate-borohydride-treated corticotrophin. Finally, when the reaction was once carried out in borate buffer, pH 9, turbidity increased for several seconds after the dissolution of borohydride.

These three observations could be explained by supposing that the product of the reaction with periodate reacts further with borohydride. Since the reaction of corticotrophin with periodate is likely to produce a glyoxylyl ($\text{CHO}\cdot\text{CO}\cdot$) group in place of the terminal serine residue, and since aldehydes are easily reduced to alcohols by aqueous borohydride (Chaikin & Brown, 1949), a further reaction to a glycollyl [$\text{CH}_2(\text{OH})\cdot\text{CO}\cdot$] group would be expected.

Chromatographic results

In the system used for the isolation of corticotrophin A_1 (carboxylic resin; pH 6.7; Na^+ , 0.3M) the periodate-borohydride-treated corticotrophin emerged with a retention volume (E , ml. of effluent/100 ml. of resin bed; cf. Dixon & Stack-Dunne, 1955) of 100 in contrast with untreated corticotrophin A_1 , for which $E = 200$. Since such a peak is not well separated from unretarded materials (for which $E = 50 = F$, the free volume of the column), the buffer was diluted to three-

quarters of its previous strength. This increased E for periodate-borohydride-treated corticotrophin to about 240 and for corticotrophin A_1 to about 500.

Fig. 1 shows the result of chromatography of the reaction product. Fig. 2 shows the result of running the two halves of the peak again. The slight

Fig. 1. Chromatography of the product obtained by treating corticotrophin A_1 with periodate and borohydride. A column (39.8 cm. $\times$ 1.0 cm. diam.) of carboxylic resin (Amberlite CG-50, 200-mesh) was equilibrated and developed with a buffer of 0.15M-phosphate and 0.225M- Na^+ ion concentrations. The sample of 14.9 mg. was dissolved in 0.3 ml. of 0.4M- H_3PO_4 , 0.3 ml. of buffer solution and 0.2 ml. of water. There were added 2.0 ml. of buffer and 0.3 ml. of 0.6N- NaOH , and the sample was transferred to the column with two washes of 0.6 ml. of buffer. The yield from desalting the tubes of the main peak was 9.6 mg. The amount in the peak, as calculated from the ultra-violet absorption and the value of $E_{1\text{ cm.}}^{1\%}$ at $280\text{ m}\mu$ of 16.0 for corticotrophin A_1 (Waller & Dixon, 1960), was 10.6 mg.

Fig. 2. Rechromatography of the halves of the peak desalted from the column shown in Fig. 1. The same column was used and the samples were similarly applied. Results with the material obtained from the effluent of the front half (a) of the original peak and from the back half (b) are shown.

Table 1. *Amino acid analysis of corticotrophin and periodate-borohydride-treated corticotrophin*

Amounts (moles) of amino acids were obtained by adjusting the total of the amino acids marked with asterisks to 24, the known number of residues of these amino acids in corticotrophin (White, 1953; Shepherd *et al.* 1956).

	α -Amino N in sample (μ g.)		Amino acid (moles)	
	Corticotrophin A ₁	Treated corticotrophin A ₁	Corticotrophin A ₁	Treated corticotrophin A ₁
Serine	21.2	10.6	1.77	0.90
Alanine*	32.3	33.0	2.70	2.81
Aspartic acid*	23.5	23.4	1.96	1.98
Glutamic acid*	56.9	59.0	4.74	5.01
Glycine*	32.7	36.2	2.73	3.08
Leucine*	26.6	24.7	2.22	2.10
Methionine*	12.8	11.6	1.07	0.99
Phenylalanine*	38.8	37.0	3.24	3.15
Proline	65.4	52.6	5.47	4.47
Tyrosine*	24.0	24.5	2.00	2.08
Valine*	39.7	33.5	3.31	2.85

difference in position, which was observed on other occasions but not invariably, suggests that the substance is not homogeneous. Although changes were made in the pH of reaction with periodate, and the products were allowed to stand overnight at pH 8 or treated again with borohydride, the slight difference in position of the halves persisted.

Heterogeneity could be due to a side reaction of the hormone with periodate. One known oxidation of corticotrophin is that of its methionine residue brought about by peroxide (Dedman, Farmer & Morris, 1961), and it is conceivable that periodate could also oxidize in the same way. Such an oxidation leads to a fall in chromatographic retention volume (Dixon & Stack-Dunne, 1955), which can be reversed by heating with a thiol (Dedman, Farmer & Morris, 1955, 1957; Dixon, 1955, 1956*b*). The front and back halves of a periodate-borohydride-treated corticotrophin peak which had differed when first run again were treated at pH 4.5 at 75° with 2% (v/v) 2-mercaptoethanol for 4 hr. and chromatographed again. No difference could be seen between them in retention volume, but in view of the small magnitude of the difference when it had been noted, it was not possible to determine whether they chromatographed as a first half or a second half. The experiment was repeated with identical results. It is therefore not possible to conclude whether or not there is heterogeneity due to partial oxidation by periodate, of the reversible type brought about by peroxide.

Exposure to alkali by dialysis against ammonia solution resulted in partial conversion of periodate-borohydride-treated corticotrophin to a product which possessed 0.67 of its retention volume (0.55 of its partition coefficient between buffer and resin) in the chromatographic system of 0.225 M-Na⁺ ion concentration. This change seems analogous to the conversion of corticotrophin A₁ into A₂, which possesses 0.70 of the retention volume (0.60 of the

partition coefficient) of corticotrophin A₁ (Dixon & Stack-Dunne, 1955) in the similar system of 0.3 M-Na⁺ ion concentration.

Characterization of the product

Amino acid analysis. Table 1 shows the results of analysis of samples of corticotrophin A₁ and periodate-borohydride-treated corticotrophin by the method of Moore, Spackman & Stein (1958) after hydrolysis for 24 hr. with constant-boiling HCl under reflux. They are consistent with the destruction of one out of two serine residues.

N-Terminal group. The end group was determined by the phenylisothiocyanate method (Edman, 1950), according to the paper-strip procedure described by Harris & Li (1954). Corticotrophin A₁ (5.3 mg.) gave 0.6 μ M-phenylthiohydantoin, and 5.0 mg. of periodate-borohydride-treated corticotrophin A₁ less than 0.1 μ M. This is consistent with the destruction of the N-terminal serine residue.

DISCUSSION

The biological properties of periodate-borohydride-treated corticotrophin, which are its main interest, are discussed in the accompanying paper by Boright *et al.* (1962). For assessing their significance the characterization of the treated corticotrophin is of importance, as are also the criteria of purity of the original corticotrophin A₁.

Farmer (1959) succeeded in chromatographing corticotrophins on carboxymethylcellulose under equilibrium conditions (without any changes in eluent, whether stepwise or gradual), and he did not find heterogeneity of corticotrophin A₁ as isolated by chromatography on a ground form of Amberlite IRC-50, although it is obvious that his chromatograms showed great resolving power. This further criterion may be added to those of Dixon & Stack-Dunne (1955).

An unrelated contaminant is less likely to be present in the treated corticotrophin than in the corticotrophin A₁ because it is isolated after its preparation by chromatography in a system in which the original corticotrophin A₁ runs in a very different position. Any contaminant possessed equally by both of them could only be present by virtue of forming a complex with both that was stable under the conditions of chromatography, unless its chromatographic properties were altered to precisely the same extent by the periodate and borohydride treatments.

Some evidence of heterogeneity of periodate-borohydride-treated corticotrophin has been obtained, even though it is not clear-cut. The heterogeneity is likely to be due to a very small difference in the molecules, since the difference in chromatographic position is so small.

The fact that the treated corticotrophin is much less retarded than its parent corticotrophin A₁ by the carboxylic resin is presumably due to the loss of the positively charged amino group of the terminal serine residue. The fact that the apparently different product obtained by treating corticotrophin with periodate alone (and stopping the reaction by adding ethanediol) runs in the same position as corticotrophin treated with both periodate and borohydride suggests that small changes in the uncharged part of the molecule make little difference in retention volumes.

Nothing has been done to demonstrate that a glycolyl group is actually present. The fact that the periodate is in low concentration (2.5 mM) and is allowed to act for only 30 sec. renders side reactions unlikely (cf. Fujii, Arakawa & Aoyagi, 1957). However, quantitative yields have not been obtained in the stages of the method up to chromatography. Further evidence that a glyoxylyl group is in the terminal locus of the periodate-treated corticotrophin is given by the conversion of this group into a terminal glycine residue by transamination (Dixon & Weitkamp, 1961).

SUMMARY

1. A method is described for treating corticotrophin with periodate and subsequently with borohydride. This procedure would be expected to replace the *N*-terminal serine residue with a glycolyl residue.

2. The product has been characterized by chromatography, low serine content in comparison

with corticotrophin and absence of a *N*-terminal group. Its biological activities are reported in the accompanying paper (Boright *et al.* 1962).

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