3·0 or less (Rous, 1925); thus it is most probable that cathepsins D and E function in the cells near their optimum pH.

SUMMARY

1. Two proteolytic enzymes were found in relatively large amounts in aqueous extracts of rabbit bone marrow.

2. One of these enzymes was shown to be the same as the enzyme obtained from rabbit spleen by Lapresle & Webb (1960), and has been named rabbit cathepsin D. A further step in the purification of cathepsin D is described. Its electrophoretic mobility in agar gel at pH 8·2 was $-1{\cdot}7\times10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$

3. The other proteolytic enzyme has been named rabbit cathepsin E. It has been purified by chromatography on diethylaminoethylcellulose and gel-filtration with Sephadex G-75. It is present in only small amounts in rabbit spleen.

4. With human serum albumin as substrate, cathepsin E has an optimum pH at 2·5, and is not affected by cysteine, iodoacetate or di-isopropyl phosphorofluoridate. It is heat-labile.

5. Cathepsin E does not hydrolyse the synthetic substrates for cathepsins A, B and C.

6. The electrophoretic mobility of cathepsin E in agar gel at pH 8·2 is $-7{\cdot}2\times10^{-5}$ cm^2.v^{-1} sec.$^{-1}$

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Conversion of the *N*-Terminal Serine Residue of Corticotrophin into Glycine

BY H. B. F. DIXON AND L. R. WEITKAMP*
Department of Biochemistry, University of Cambridge

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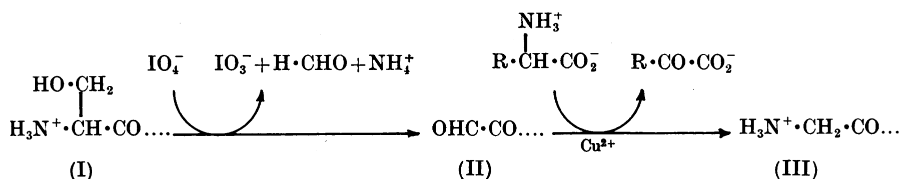
Periodate attacks the *N*-terminal serine residue of corticotrophin (I) (Geschwind & Li, 1954) to form a derivative of lower retention volume on chromatography on a cation-exchange resin (Dixon, 1956). This derivative was presumed to be a glyoxylyl compound (II) (Geschwind & Li, 1958; Dixon, 1962). Dr R. G. Shepherd suggested that it might be possible to aminate it reductively and thus convert the glyoxylyl group into a glycyl group. Attempts to reduce with tetrahydroborate (borohydride) in the presence of ammonia did not lead to the formation of a derivative more retarded on chromatography, but small yields of such a derivative were obtained by using hydrogen and palladium. Such a reductive amination had been accomplished for free glyoxylic acid and keto acids by transamination (Nakada & Weinhouse, 1953; Metzler, Olivard & Snell, 1954; Mix, 1959; Fleming & Crosbie, 1960). The glyoxylyl derivative was therefore incubated with L-glutamate in the presence of Cu^{2+} ions, and a good yield was achieved of material whose retention volume on a cation-exchange resin was restored to the value of the original corticotrophin. This material proves to have a *N*-terminal glycine residue (III). Following the convention of Rittel, Iselin, Kappeler, Riniker & Schwyzer (1957), Acher (1960), and others, we propose to call it Gly^1-corticotrophin A_1.

* Present address: University of Rochester School of Medicine, Rochester 20, New York, U.S.A.



When the terminal serine residue of corticotrophin is converted into a glycollyl residue, the action of the hormone on the adrenal gland *in vivo* is greatly diminished, but it still retains measurable activity on the adrenal cortex and adipose tissue *in vitro* and in producing hypoglycaemia and increasing liver fat in the mouse (Boright, Engel, Lebovitz, Kostyo & White, 1962). The Gly¹-corticotrophin is the first derivative of corticotrophin in which the terminal serine residue is modified, without loss of its amino group. It proves to have the same activity as the native corticotrophin on the adrenal gland *in vivo* and *in vitro* and has the same potency in mobilizing lipid from adipose tissue *in vitro* and in inducing hypoglycaemia in the mouse, but it differs from native corticotrophin by being less stable in solution (H. E. Lebovitz & F. L. Engel, personal communication).

A summary of this preparation has been published (Dixon & Weitkamp, 1961).

MATERIALS AND METHODS

Preparation of carboxymethylcellulose. This material was prepared from Whatman cellulose powder by the method of Ellis & Simpson (1956), with 4M-chloroacetic acid. Titration showed that it had a capacity of 0·7 m-equiv./g. Lumps were broken by plunging a moderately tightly fitting pestle up and down in a tube containing a suspension of the powder. Fine particles were removed by settling and decantation before the columns were prepared and equilibrated.

Preparation of corticotrophin A_1. The hormone was isolated by the method of Dixon & Stack-Dunne (1955) with the minor modifications cited by Waller & Dixon (1960). When, however, many batches of Amberlite CG-50 were found to give trailing of the peaks on ion-exchange chromatography, carboxymethylcellulose was used in later preparations (see Farmer, 1959). For chromatography on the latter adsorbent, the sample was dissolved in 0·3M-acetic acid and an equal volume of 0·24M-disodium succinate was added. The solution was centrifuged and the supernatant added to a column of carboxymethylcellulose equilibrated and later eluted with a solution containing acetic acid (0·15M), disodium succinate (0·12M) and toluene (0·03%).

Treatment of corticotrophin A_1 with periodate. Corticotrophin A_1 (39 mg.) was dissolved in water (8·6 ml.) and 395 mg. of NH_4HCO_3 was added. Then 1 ml. of 25 mM-$NaIO_4$ was rapidly blown in and stirred; 40 sec. later 1 ml. of 10% (v/v) ethanediol was similarly added. Water (85 ml.) and acetic acid (5 ml.) were then added, and the solution was subjected to reduced pressure and shaken to remove dissolved gas. The product was desalted by adsorption on Amberlite CG-50 (type II), the salts were washed out with 5% (v/v) acetic acid, and the product was displaced with 50% (v/v) acetic acid (Dixon, 1959). The same method was used for subsequent desaltings. The solution in acetic acid was concentrated by rotary evaporation, diluted with water, and dried while frozen. The product (33·2 mg.) was subjected to chromatography.

Chromatography of periodate-treated corticotrophin. A column of carboxymethylcellulose (0·87 cm.² × 40·7 cm.) was equilibrated with a buffer solution the strength of which was three-quarters that of the buffer used for the chromatography of corticotrophin A_1, i.e. it contained acetic acid (0·1125M), disodium succinate (0·9M) and toluene (about 0·02%). The sample was dissolved in 1·5 ml. of 0·3M-acetic acid. Then 1·3 ml. of water, 1·2 ml. of 0·3M-disodium succinate and 2·5 ml. of buffer were added. This solution was applied to the column and washed on with small samples of buffer. Elution with the buffer was continued at a rate of about 10 ml./hr., and fractions of effluent were collected. In earlier preparations chromatography was performed on the fraction of Amberlite CG-50 that settled (as the sodium salt) at 0·4–1·5 cm./min. in the fractionation method of Hamilton (1958); the buffer contained Na_2HPO_4 (75 mM) and NaH_2PO_4 (75 mM). The absorption at 280 mμ of the fractions was measured, and those comprising the peak (Fig. 1) were desalted. Their combined absorption suggested a yield of 23 mg., though the amount scraped from the flask after drying was 18·8 mg.

Treatment of periodate-treated corticotrophin with glutamate. The sample was washed into a flask with 2·5 ml. of water, and 7·5 ml. of copper glutamate solution was added. This solution had been made by heating monosodium L-glutamate, $H_2N\cdot CH(CO_2H)\cdot CH_2\cdot CH_2\cdot CO_2Na,H_2O$ (9·35 g.), and cupric oxide (1·59 g.) with water until dissolved, adding 25 ml. of 0·1M-Na_2HPO_4–0·1M-NaH_2PO_4 buffer, pH 6·7, and diluting to 50 ml. Its final concentration was 1M in glutamate, 0·4M in copper, and its pH was 6·8. The mixture was incubated for 2 hr. at 50°, diluted to about 200 ml. with 5% acetic acid and desalted. A pale-blue product (23·6 mg.) was obtained. In later preparations the sample was dissolved in 5 ml. of water, and 5 ml. of a modified copper glutamate solution was used. The change in its composition was made because a precipitate, presumably a copper phosphate, slowly appeared in the original one. The same quantities of sodium glutamate and cupric oxide were dissolved by heating in water and the solution was filtered. The residue, which contained iron, evidently an impurity in the cupric oxide used, was washed. Acetic acid (0·285 ml., final concn. 0·1M) was added to the filtrate and washings, which were diluted to 50 ml. with water. This solution gave equally good overall results (although the yields of the individual steps were not measured); its pH was 5·8, so that it was unnecessary to add sodium acetate with the acetic acid as a buffer.

Chromatography of the final product. The same column of carboxymethylcellulose was re-equilibrated with a buffer containing acetic acid (0·15M), disodium succinate (0·12M) and toluene (0·03%). The product of the incubation with glutamate was dissolved in 0·5 ml. of 0·3M-acetic acid, and to this solution were added 2 ml. of buffer and 0·5 ml. of 0·24M-disodium succinate. This solution was added to the column, washed on, and elution was continued with the buffer at a rate of about 10 ml./hr. while fractions of effluent were collected. The tubes containing the peak of absorption were desalted to yield a white product (17·2 mg., overall yield 44%). In earlier preparations this product was chromatographed on Amberlite CG-50 under the same conditions originally used for the corticotrophin A_1, but this was abandoned because of the trailing of peaks on some batches of resin. In neither system did the peak differ from corticotrophin A_1 in elution volume. In a later preparation, in view of the results on the state of methionine reported below, the sample was dissolved in 2 ml. of 0·3M-acetic acid containing 2% (v/v) of 2-mercaptoethanol (2 ml.), heated to 70° for 4 hr. and transferred to the column after the addition of 2 ml. of 0·24M-disodium succinate. The flask was washed with small samples of 0·3M-acetic acid to which equal volumes of 0·24M-disodium succinate were added before transfer to the column. This appeared equally satisfactory.

Amino acid analysis. Samples were hydrolysed by boiling under reflux with constant-boiling HCl for 24 hr. The hydrolysate was analysed by chromatography on a sulphonic acid resin (Moore, Spackman & Stein, 1958).

Analysis of methionine sulphoxide. Since Cu^{2+} ion, iodate and periodate are oxidizing agents, a check was made to discover if the methionine residue had been converted into its sulphoxide during the preparation. Iodate can oxidize methionine, at least under acid conditions (Lavine, 1947). Since methionine sulphoxide can largely revert to methionine on acid hydrolysis (Ray & Koshland, 1960) the method of Neumann (1960) was used for this analysis. He oxidized the methionine sulphoxide residue to its sulphone by treatment with performic acid after protecting methionine by prior reaction with iodoacetate. In the present work the detailed conditions were chosen on the basis of the work of Gundlach, Moore & Stein (1959) on the reaction of methionine with iodoacetate and that of Hirs (1956) on oxidation with performic acid.

To obtain oxidized corticotrophin to check the method, corticotrophin A_1 (7·7 mg.) was dissolved in 4·45 ml. of 0·1N-acetic acid, and H_2O_2 solution (0·545 ml., 9·17M) was added to give a final concentration of 1M. After $1\frac{1}{2}$ hr. at room temperature 5 ml. of water was added and the solution frozen and dried under reduced pressure. Although the bulk remained frozen it did appear to thaw partially as it dried. The product was redissolved in 0·1N-acetic acid and dried while frozen.

Samples of corticotrophin A_1, periodate–glutamate-treated corticotrophin A_1 and the oxidized sample were each dissolved in 5 ml. of a solution of 372 mg. of iodoacetic acid [recrystallized from light petroleum (b.p. 60–80°)] dissolved in 15 ml. of water, adjusted to pH 2·8 with NaOH solution and diluted to 20 ml. (0·1M). The samples were incubated at 40° for 22 hr., 5 ml. of 5% (v/v) acetic acid was added to each, and the solutions were desalted (Dixon, 1959) on columns (1 cm. diam. × 4 cm.) of Amberlite CG-50 (type II). The products in 50% acetic acid were evaporated to dryness on a rotary evaporator, dissolved in water and dried while frozen. Each was dissolved, after being cooled, in 3 ml. of a mixture at 0° of 0·5 ml. of 9M-H_2O_2 plus 9·5 ml. of 99% (v/v) formic acid which had previously stood for 1 hr. at room temperature to allow formation of performic acid (Toennies & Homiller, 1942). After 2 hr. at 0° the solutions were frozen and dried under reduced pressure. Since some thawing occurred on drying, the samples were redissolved in water and dried while frozen before being subjected to amino acid analysis.

RESULTS

Chromatographic patterns

Fig. 1 shows the result of the chromatogram of the periodate-treated corticotrophin. Another

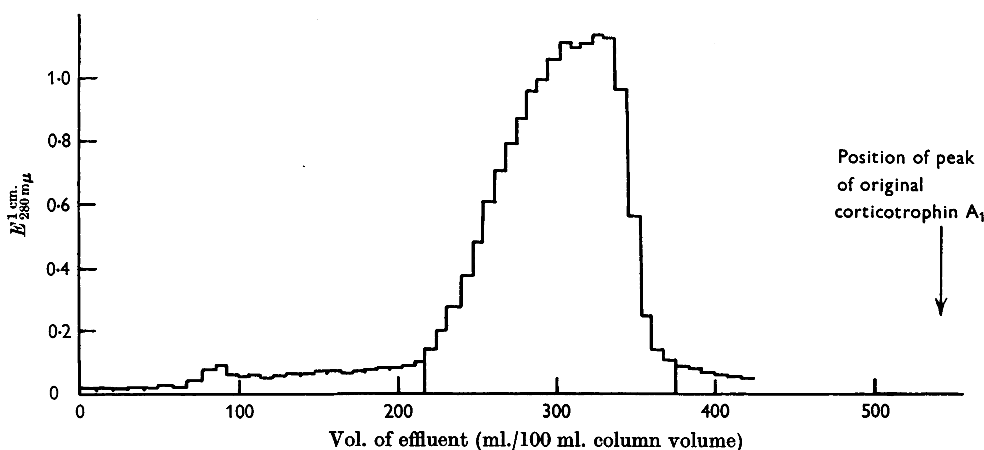


Fig. 1. Chromatography of periodate-treated corticotrophin A_1. A column (0·87 cm.^{2} × 40·7 cm.) of carboxymethylcellulose was equilibrated and developed with a solution containing acetic acid (0·1125M), disodium succinate (0·09M) and about 0·02% of toluene.

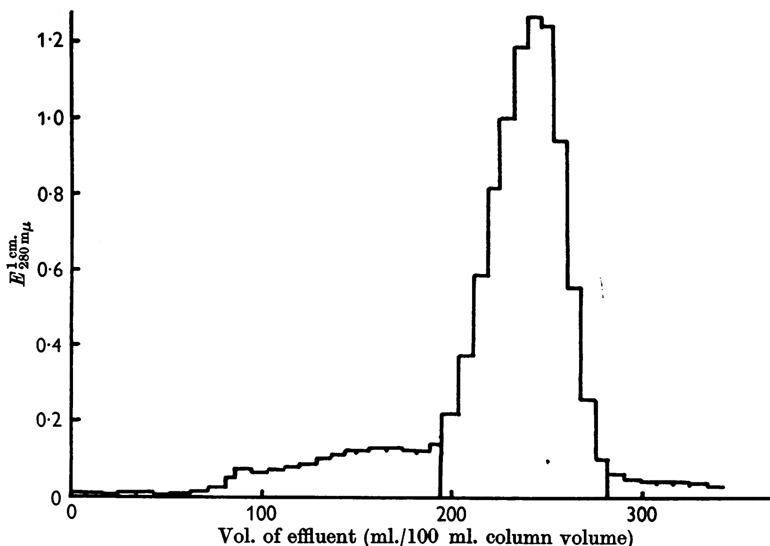


Fig. 2. Chromatography of periodate–glutamate-treated corticotrophin A_1. A column (0·87 cm.^{2} × 40·7 cm.) of carboxymethylcellulose was equilibrated and developed with a solution containing acetic acid (0·15M), disodium succinate (0·12M) and 0·03% of toluene.

chromatogram showed that corticotrophin A_1 ran in this system with an elution volume of 540 ml./100 ml. column volume (as shown by the arrow), so that the peak of periodate-treated material was free from unchanged corticotrophin.

Fig. 2 shows the elution diagram of the periodate–glutamate-treated corticotrophin in the system of higher buffer concentration. The rise above the base-line ahead of the peak is probably in part due to periodate-treated material which had not reacted further.

Characterization of product

Amino acid analysis. Table 1 shows the results of analysis of corticotrophin A_1 and the product. They are consistent with the replacement of one serine residue by glycine. Although the value for tyrosine is low, the absorption spectrum (240–340 mμ) was indistinguishable from that of corticotrophin A_1 [Fig. 5 of Waller & Dixon (1960)], suggesting that tyrosine and tryptophan are unchanged.

N-*Terminal sequence.* The end group was determined by the phenylisothiocyanate method (Edman, 1950), according to the paper-strip method of Harris & Li (1954), on samples of 2–2·5 mg. The product gave glycine as *N*-terminal residue (0·56 mole/mole; corticotrophin A_1: serine 0·60 mole/mole). The degradation was repeated for a further step, and tyrosine proved to be the penultimate residue in both cases (product: 0·41 mole/mole; corticotrophin A_1: 0·61 mole/mole). The low values may be due in part to moisture as air-dried preparations were weighed out. The low yield of amino acids on acid hydrolysis (Table 1) is consistent with this.

Table 1. *Amino acid analysis of corticotrophin A_1 and the product*

Experimental details are given in the text. The values were obtained by dividing the values for μg. of α-amino N by (1/21) × the sum of the values for Ala, Asp, Glu, Leu, Met, Phe, Tyr and Val since the number of residues of these amino acids is 21. This value was 12·0 μg. of α-amino N/residue for corticotrophin A_1 and 5·4 μg./residue for the product. Errors will therefore be greater in the case of the product. The value '5·4 μg.' relates to half the sample of 4·9 mg. Some mistake must have occurred in the proline analysis of the native corticotrophin.

	Residues		
	Corticotrophin A_1		
	Expected	Found	Product
Serine	2	1·77	0·98
Glycine	3	2·73	4·19
Ala	3	2·70	3·31
Arg	3	3·01	2·83
Asp	2	1·96	1·87
Glu	5	4·74	4·89
His	1	1·23	0·89
Leu	2	2·22	2·26
Lys	4	4·39	4·02
Met	1	1·07	1·33
Phe	3	3·24	2·89
Pro	4	5·47	4·15
Tyr	2	2·00	1·54
Val	3	3·31	2·93

State of methionine. The results of analysis for methionine by the method of Neumann (1960) are given in Table 2. Fig. 3 shows the part of the chromatogram of the amino acids that contain the peaks of aspartic acid, methionine sulphone (representing methionine sulphoxide originally present) and serine. Fig. 3 (*c*) shows that there is perhaps a larger shoulder on the aspartic acid peak than its normal small tail and suggests that the product may contain about 10 % of the methionine sulphoxide derivative. A fresh preparation was therefore made, and the sample for the final chromatogram was heated for 3 hr. at 70–90° with 2 % (v/v) of mercaptoethanol (cf. Dedman, Farmer &

Table 2. *Amino acid analysis of samples treated with iodoacetate and performic acid*

Experimental details are given in the text. Iodoacetate was added to block methionine while performic acid oxidized methionine sulphoxide to its sulphone. The values for residues were obtained by dividing the values for μg. of α-amino N by (1/19) × the sum of the values for Ala, Asp, Glu, Leu, Pro and Val. This value was (*a*) 15·2, (*b*) 14·8, (*c*) 9·5 and (*d*) 11·4 μg. of α-amino N/residue.

	Residues			
	(*a*) Oxidized corticotrophin	(*b*) Corticotrophin	(*c*) Product	(*d*) Reduced product
Methionine sulphone	0·90	—	0·09	—
Glycine	3·05	2·92	3·41	3·74
Serine	1·69	1·56	0·85	0·86
Ala	2·99	2·93	2·94	3·06
Asp	2·07	2·19	2·03	2·12
Glu	4·76	4·79	4·67	4·71
Leu	1·97	2·09	2·00	2·00
Phe	2·15	2·62	2·48	2·69
Pro	4·43	4·09	4·18	4·06
Tyr	1·23	1·16	1·01	1·12
Val	2·87	2·95	3·21	3·05

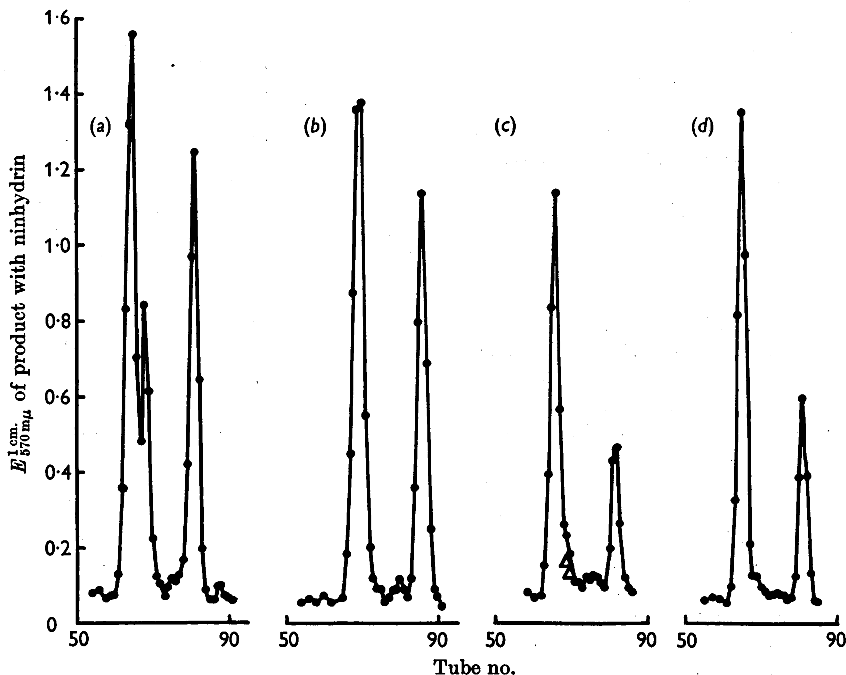


Fig. 3. Part of the amino acid analysis of hydrolysates of samples treated with iodoacetate and performic acid. The peak or shoulder of methionine sulphone between the peaks of aspartic acid and serine represents methionine sulphoxide originally present. ●, Experimental points; △, points taken for aspartic acid in making a rough estimate of methionine sulphone. Samples: (*a*) oxidized corticotrophin, (*b*) corticotrophin, (*c*) product, (*d*) reduced product.

Morris, 1957, 1961). Its analysis by the same method is shown in Fig. 3 (*d*). The size of the tail is again small, suggesting that it was virtually free from methionine sulphoxide. A precipitate formed during the thiol treatment. Therefore in a later preparation the heating with mercaptoethanol was in 0·3M-acetic acid before the addition of an equal volume of 0·24M-disodium succinate to make up the sample for chromatography. This appeared more satisfactory.

Conclusion. These experiments on the characterization and the chromatographic behaviour of the product suggest that replacement of the terminal serine residue by glycine is the only change the product has undergone with the exception of slight contamination with its sulphoxide derivative. Reduction with a thiol can eliminate this, and the product is therefore Gly[1]-corticotrophin A_1.

DISCUSSION

The loss of material during periodate treatment is unexplained. By contrast the yield on transamination is high. It might be expected that excess of glutamate would inhibit the reaction [cf. Mix (1959), especially Fig. 3, with scale corrected by a footnote of Mix & Wilcke (1960)] by diminishing the concentration of the 1:1 glutamate–copper complex which can combine with the glyoxylyl derivative. In fact, however, this complex is fairly insoluble, so it may not be very inefficient to maintain its low concentration by allowing it to be formed from an overwhelming excess of the 2:1 glutamate–copper complex (IV) and some free glutamate. There is presumably more of the 1:1 complex in the more recently used copper glutamate solution because of its lower pH (5·9).

The pH is easily controlled by making the solution from cupric oxide and excess of monosodium glutamate, since the product will not alter the pH of a buffer solution near neutrality. The fact that the pH is as high as 5·8 in the presence of acetic acid shows that another group is buffering at this pH. It may be that the pK of the γ-carboxyl group is raised by discharge of the amino group on combination with copper, or the pH may be low enough to evoke significant release of amino group from the complex.

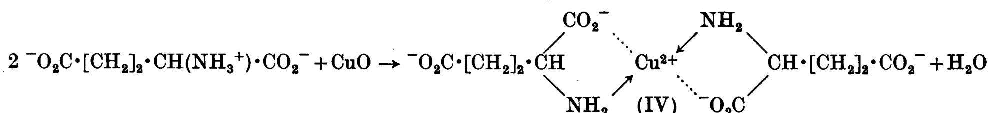
The biological activity of Gly[1]-corticotrophin has been assessed (H. E. Lebovitz & F. L. Engel, personal communication). Both the corticotrophic activity *in vivo* and *in vitro* and the extra-adrenal activities are undiminished in comparison with native corticotrophin A_1. The retention of the corticotrophic activity shows that its great loss on conversion of the terminal serine into a glycollyl group (Boright *et al.* 1962), and on other reactions (e.g. Waller & Dixon, 1960), must be due to the loss of the positively charged amino group rather than to the destruction of the hydroxymethyl side chain. The conclusion (Waller & Dixon, 1960; and others) that the terminal serine residue is essential must now be modified.

Dedman *et al.* (1961) have presented evidence that the reversible oxidation of corticotrophin by hydrogen peroxide which is associated with a reversible loss of biological activity is due to the oxidation of the methionine residue to its sulphoxide. The analysis by the method of Neumann (1960) here reported confirms this, since 1M-peroxide in dilute acetic acid appears to have changed the methionine residue into the sulphoxide. Dedman *et al.* (1957), however, claimed to stop the action of peroxide by acidification. Their results do not demonstrate that the action is stopped by acid, for though their Table 2 does show an increasing fraction of residual corticotrophin activity after oxidation under standard conditions when the pH is diminished from 8·5 to 5, the conditions may not have been comparable if the oxidation continued for different times after addition of acid, and, further, the fiducial limits on the estimates largely overlap. The oxidation of free methionine by hydrogen peroxide appears to be independent of pH over the range 5–1, but accelerated under still more acidic conditions (Toennies & Callan, 1939), so it would not be surprising if the reaction with corticotrophin also proceeded in dilute acetic acid. It is therefore likely that the reaction in acid is the same as that under neutral conditions.

Comparison of Tables 1 and 2 shows that iodoacetate and performic acid treatment as described destroy much tyrosine and also some phenylalanine.

SUMMARY

1. Corticotrophin A_1 has been treated with periodate to yield a derivative which has been isolated by ion-exchange chromatography. It has a lower retention volume than the corticotrophin in the chromatographic system and is presumed to have a glyoxylyl group in place of the *N*-terminal serine residue. Transamination to convert this group into a glycine residue was attempted by treating the derivative with copper glutamate, and a further product was obtained



which possesses the retention volume of the original corticotrophin.

2. The final product has been characterized and it appears to differ from the original corticotrophin only in possessing a N-terminal glycine residue in place of serine.

3. Preliminary observations that the product has the same corticotrophic activity as the native hormone are quoted.

We are grateful to Miss M. B. Thomas for her skilled assistance throughout the work, and to Dr R. G. Sheperd, Dr G. W. Crosbie, Dr J. I. Harris and Dr R. I. T. Cromartie for most helpful advice and discussion. We thank Mrs B. Brown for the amino acid analyses and Dr F. L. Engel for the gift of corticotrophin concentrates and him and Dr H. E. Lebovitz for permission to quote their results. We also thank the Medical Research Council for a grant for expenses.

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The Determination of the Tyrosine Content of Gelatins

By W. G. COBBETT, A. W. KENCHINGTON* and A. G. WARD†
The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

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The low and variable tyrosine contents of gelatins and collagens suggest that tyrosine may be present, at least in part, as an impurity. Orekhovitch, Tustanovskit, Orekhovitch & Plotnikova (1948) and Russell (1958) have claimed that samples of procollagen and gelatin respectively can be obtained substantially free from tyrosine. However, Harkness, Marko, Muir & Neuberger (1954) have found tyrosine in purified rabbit-skin collagen, and Schmitt, Gross & Highberger (1955) were unable, even with highly purified collagen, to decrease the tyrosine contents much below 0·41 %. Eastoe (1955) reported that fractionation of gelatin to remove impurities decreased the tyrosine content to about 0·2 %. Leach (1960*a*) could not confirm Russell's results, but was able to isolate 0·36 % of a minor component from gelatin, which, although considerably richer in tyrosine than the original gelatin, contributed only about 0·01 g. of tyrosine/100 g. of the parent material.

* Present address: National College of Food Technology, Weybridge, Surrey.

† Present address: Procter Department of Food and Leather Science, University of Leeds.