5. Evidence is provided for the presence in extracts of rat liver of a substance that can be bound to rat albumin. The specificity of this binding factor is examined. The results of the experiments on the synthesis of serum albumin are interpreted in the light of these findings.

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The Purification and Properties of a Proteolytic Enzyme, Rabbit Cathepsin E, and Further Studies on Rabbit Cathepsin D

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A proteolytic enzyme was prepared from rabbit spleen by Lapresle & Webb (1960) and was shown to be different from cathepsins A, B and C by Webb & Lapresle (1960). This enzyme will be called rabbit cathepsin D, since it is analogous to the cathepsin D isolated from bovine spleen by Press, Porter & Cebra (1960).

Further studies reported here demonstrated the existence in rabbit spleen of traces of another proteolytic enzyme. The investigation of the proteolytic activity of different rabbit-leucocyte preparations has shown that this second enzyme is present in larger amount in polymorphonuclear cells than in lymphocytes (J. Štefanovič, T. Webb & C. Lapresle, unpublished work). Bone marrow being

much richer in polymorphonuclear cells than the spleen, rabbit bone marrow was used as the starting material for the preparation of this second enzyme. This enzyme was purified and shown to be different from cathepsins A, B, C and D; it has been named cathepsin E.

The purification and properties of cathepsin E as well as further studies on rabbit cathepsin D are described in this paper.

MATERIALS

Diethylaminoethylcellulose and carboxymethylcellulose. Diethylaminoethylcellulose (DEAE-cellulose) and carboxymethylcellulose (Selectacel) were obtained from Brown

and Co., New York, U.S.A. They were used in columns provided with a cooling jacket kept at 4°.

Sephadex G-75 and carboxymethyl-Sephadex C-50. These were obtained from Pharmacia, Uppsala, Sweden. They were used in columns at room temperature.

Potassium phosphate buffers. The molarity of the buffers used is expressed with respect to phosphate, and the pH was measured with a pH meter.

Rabbit spleens and long bones. These were obtained from exsanguinated rabbits and stored frozen.

Human serum albumin. This was 'Squibb fraction V' prepared by ethanol precipitation and kindly given by the American Red Cross.

Synthetic peptides. Benzoyloxycarbonyl-L-glutamyl-L-tyrosine, benzoyl-L-arginine amide hydrochloride and glycyl-L-phenylalanine amide acetate were obtained from Mann Laboratories Inc., New York, U.S.A.

EXPERIMENTAL

Protein determination. The amount of protein in the enzyme preparations was determined with the biuret reagent prepared according to Weichselbaum (1946); the extinction of the colour developed with this reagent was measured at 555 m μ with reference to a standard curve made with various weights of a freeze-dried crude spleen extract.

Enzyme assays: (a) Cathepsin D. Cathepsin D was assayed with human serum albumin as substrate by the method described by Lapresle & Webb (1960), but without the addition of CaCl₂ and MgCl₂.

(b) Cathepsin E. A solution of human serum albumin, 10% (w/v) in water, was adjusted to pH 2.5 with HCl. The albumin solution (0.5 ml.) was added to 0.5 ml. of enzyme preparation (in 0.15M-NaCl) and the mixture incubated for 30 min. at 45°. Then 1 ml. of 4% (w/v) trichloroacetic acid was added, and the suspension was incubated for 10 min. at 45° and centrifuged. A portion (1.0 ml.) of the clear supernatant was added to 1.5 ml. of biuret reagent prepared according to Weichselbaum (1946) but containing twice the usual quantity of NaOH to neutralize the trichloroacetic acid. After 30 min. of incubation at 37°, the extinction was read at 555 m μ . Enzyme-substrate blanks were prepared by incubating the substrate solution for 30 min. at 45° and then adding the enzyme and trichloroacetic acid simultaneously. A curve was drawn relating extinction of the supernatant to milligrams of the cathepsin E preparation (from step II, see below) used. An arbitrary unit of proteolytic activity was defined from the initial part of the curve as that amount of enzyme which would cause an increase of 0.001 unit of extinction/min. of digestion. A further curve (Fig. 1) was then constructed relating the increases in extinction of the supernatants to the number of units employed.

(c) Cathepsins A, B and C. The appropriate synthetic substrates were incubated with an aqueous extract of bone marrow and a preparation of purified cathepsin E. Enzyme and substrate controls were incubated under the same conditions. The moles of carboxylic group liberated during the reaction were measured by the method of Grassman & Heyde (1929) and the percentage hydrolysis is given by the relation:

$$\frac{\text{moles of carboxylic group liberated} \times 100}{\text{moles of substrate employed}}$$

Detection of cathepsins D and E after electrophoresis in agar. Different enzyme preparations were subjected to electrophoresis in agar gel according to the technique of Grabar & Williams (1955) under the following conditions: volume of preparation used, 0.05 ml.; plate of 1% agar, 10.5 cm. long; veronal buffer, pH 8.2; 6–7 v/cm.; duration, 50 min. After electrophoresis, the proteolytic activity was studied by the technique described by Uriel (1960) under the following conditions: the agar plates were immersed in a substrate solution of 0.2% human serum albumin in either 0.5M-glycine-HCl buffer, pH 2.5, or 0.5M-sodium acetate containing cysteine (5 mM), pH 3.5. The plates were incubated at 37° in the substrate solution for 90 min. and kept at 37° for 3 hr. The plates were then fixed in 10% (v/v) acetic acid solution in 50% (v/v) ethanol for 1 hr., dried under filter paper at 37° and coloured with Amido Black.

This technique of direct detection of proteolytic activity was used to determine the electrophoretic mobility of cathepsins D and E as described by Uriel, Webb & Lapresle (1960); levan and human serum albumin were used as standards.

Preparation of cathepsin D. Cathepsin D was prepared from rabbit spleen by a modification of the method described by Lapresle & Webb (1960).

Steps I, II and III. These were carried out as described by Lapresle & Webb (1960).

Step IV. The crude preparation from step III was dialysed against 0.05M-potassium phosphate buffer, pH 8.0, and passed through a column of DEAE-cellulose equilibrated with the same buffer. Cathepsin D passed in the reddish 'break-through' fraction and the eluates containing appreciable amounts of cathepsin D were pooled.

Step V. The active fraction from step IV was dialysed against 15 mM-potassium phosphate buffer, pH 6.75, and

Fig. 1. Standard curve for the digestion of human serum albumin by cathepsin E (see text). The extinction figures refer to changes per 30 min.

the reddish inactive material removed by adsorption on a column of carboxymethylcellulose equilibrated with the same buffer. The active material passed through the column and the eluates containing cathepsin D were pooled. *Step VI.* The active material from step V was dialysed against 5 mM-potassium phosphate buffer, pH 8.0, and adsorbed on a column of DEAE-cellulose equilibrated with the same buffer. After the passage of an inactive 'break-through' fraction the enzyme was eluted in a small volume by 0.1 M-potassium phosphate buffer, pH 7.0.

Step VII. The concentrated enzyme solution was dialysed against 15 mM-potassium phosphate, pH 6.75, and then passed through a column of carboxymethyl-Sephadex. After the passage of an inactive 'break-through' fraction the cathepsin D was eluted by the same phosphate buffer containing NaCl (0.5 M).

For the preparation of cathepsin D from rabbit bone marrow steps II and III were omitted.

Gel-filtration of cathepsin D on Sephadex G-75. A column (4 cm. diam. $\times$ 40 cm.) of 50 g. of Sephadex G-75 suspended in 0.15 M-NaCl was prepared. A sample (5 ml.) of a preparation of cathepsin D (73 units/ml.) was filtered through the column by washing with 0.15 M-NaCl.

Preparation of cathepsin E. Step I. The bones were broken and the frozen marrow was removed. The marrow was broken up in a small volume of water in a Waring Blender and then dialysed against water for 2 hr. The suspension was centrifuged at 55 000g for 45 min. and the clear supernatant fluid (the aqueous extract) was siphoned off from between the solid cake of lipid which formed on top and the packed precipitate which sedimented to the bottom.

Step II. The aqueous extract of rabbit bone marrow was dialysed against 0.05 M-potassium phosphate, pH 8.0, and passed through a column of DEAE-cellulose equilibrated with the same buffer. Cathepsin E was adsorbed on the cellulose under these conditions and, after the passage of a 'break-through' fraction containing cathepsin D, it was eluted with 0.05 M-potassium phosphate, pH 8.0, containing NaCl (0.5 M). The eluates containing appreciable amounts of cathepsin E were pooled.

Step III. The pool containing cathepsin E was dialysed against 5 mM-potassium phosphate, pH 5.78, and adsorbed on DEAE-cellulose equilibrated with the same buffer. After the passage of an inactive 'break-through' fraction further inactive material was eluted by the passage of the 5 mM-phosphate buffer containing NaCl (0.15 M). Cathepsin E was then eluted by the 5 mM-phosphate buffer containing NaCl (0.25 M). Some inactive material stayed on the column.

Step IV. For further purification and concentration of cathepsin E the eluates containing cathepsin E were pooled, dialysed against 5 mM-potassium phosphate buffer, pH 5.78, and re-adsorbed on DEAE-cellulose equilibrated with the same buffer. Some inactive material was again removed by the same phosphate buffer containing NaCl (0.15 M). Cathepsin E was eluted in a relatively small volume by the passage of the 5 mM-phosphate buffer containing NaCl (0.5 M).

Step V. The final step in the purification of cathepsin E was a filtration through a column of Sephadex G-75 suspended in 0.15 M-NaCl. The enzyme passed in the 'break-through' fraction and inactive material was held back on the Sephadex.

Chromatographic analysis of cathepsin E. Chromato-

graphic analysis was carried out on a preparation of cathepsin E obtained at step IV. The preparation was dialysed against 5 mM-potassium phosphate, pH 5.78, and put on a column of DEAE-cellulose equilibrated with the same buffer. After the passage of twice the volume of the column the enzyme was eluted by the passage of a NaCl gradient obtained by the cone-sphere technique of Sober & Peterson (1958). The molarity of the eluates was measured by conductivity.

Optimum pH of cathepsin E. The same conditions were used as in the assay of cathepsin E except that the albumin solutions were adjusted with HCl to various pH values as measured by a pH meter.

Influence of substrate concentration. The influence of substrate concentration on the amount of degradation products formed by cathepsin E was determined under the following conditions: pH 2.5; temperature, 45°; reaction time, 30 min. To increasing amounts of human serum albumin a constant quantity of cathepsin E (3.6 units/ml.) was added.

Effects of activators and inhibitors on cathepsin E. The activity of cathepsin E was measured in the presence of either 5 mM-cysteine or 1 mM-iodoacetate, or after the incubation at pH 7.0 with 10 mM-di-isopropyl phosphorofluoridate for 30 min. The activity of a preparation of cathepsin E was measured after heating at 60° for 60 min. and at 80° for 10 min.

Gel-filtration of human serum albumin degraded by either cathepsin D or E. Two samples of human serum albumin, 2% (w/v) final concentration, were degraded by 10 units of either cathepsin D or E/ml. With cathepsin D, the digestion medium contained 5 mM-cysteine and was adjusted to pH 3.5. With cathepsin E, the pH of the digestion medium was adjusted to 2.5. In both cases the digestion was carried out at 37° for 6 hr. and the reaction stopped by neutralization with NaOH. Both samples of degraded albumin were filtered through a column (4 cm. diam. $\times$ 40 cm.) of 50 g. of Sephadex G-75 suspended in 0.15 M-NaCl. The extinction of the 10 ml. eluates was measured at 280 m μ .

RESULTS

Detection of proteolytic activity after electrophoresis in agar of crude spleen extract, bone-marrow extract and purified cathepsins D and E. Fig. 2 shows the direct detection after electrophoresis in agar of the proteolytic activity at pH 3.5 and 2.5 of the following preparations: crude preparation from rabbit spleen, aqueous extract of rabbit bone marrow, purified cathepsin D and purified cathepsin E.

The weakly- or non-coloured zones on the plates indicate the presence of proteolytic activity. With the crude spleen extract acting at pH 3.5 there exists one zone of activity on the cathodic side of the starting reservoir. However, at pH 2.5 there is, in addition, a second very faint zone of activity on the anodic side of the starting reservoir. With the bone-marrow extract both these zones of activity exist. The first zone, on the cathodic side, is partially masked by the presence of a high concentration of haemoglobin at the same

place. The second zone, on the anodic side, is much more evident at pH 2.5 than with the spleen extract.

The position of the zone of activity on the cathodic side of the reservoir corresponds to that of purified cathepsin D. The position of the zone of

activity on the anodic side of the reservoir corresponds to that of purified cathepsin E.

Preparation of cathepsin D. Steps I–VI in the preparation of cathepsin D from rabbit spleen gave approximately the same recovery and purification of activity as described by Lapresle & Webb (1960). The analysis, by gel-filtration on Sephadex G-75, of the preparation of cathepsin D taken after step VI showed (Fig. 3) that at this stage some inactive protein passed through the Sephadex and that the enzymic activity was held back by the gel and passed through only in later filtrates.

For the preparation of purified cathepsin D, inactive material was removed by using carboxymethyl-Sephadex under the conditions described in step VII. This step gave a recovery of 80% and a further purification from 50 to 130 units/mg. Purified cathepsin D can also be prepared in large amounts from rabbit bone marrow.

Electrophoretic mobility of cathepsin D. The electrophoretic mobility in agar gel at pH 8.2 of purified cathepsin D from both spleen and bone marrow was found to be -1.7×10^{-5} cm.²v⁻¹ sec.⁻¹ This mobility is slightly different from that reported by Uriel *et al.* (1960), but with the method used it is within the limits of error for proteins having such low mobilities (Uriel, 1958).

Preparation of cathepsin E. Table 1 gives the results of the different steps in the purification of cathepsin E from rabbit bone marrow. By using the same procedure on crude spleen extracts, only small amounts of cathepsin E could be prepared.

Properties of cathepsin E. (a) *Optimum pH.*

Fig. 2. Direct detection of proteolytic activity after electrophoresis in agar gel at pH 8.2 (see text). 1 and 2, Crude spleen extract (step III) incubated at pH 3.5 and 2.5 respectively; 3 and 4, aqueous extract of bone marrow (step I) incubated at pH 3.5 and 2.5 respectively; 5, purified cathepsin D incubated at pH 3.5; 6, purified cathepsin E incubated at pH 2.5. The starting reservoir is in the middle as indicated by the vertical lines in (6).

Fig. 3. Gel-filtration on Sephadex G-75 of a preparation of cathepsin D from step VI (see text). ----, Extinction; —, proteolytic activity.

Table 1. Preparation of cathepsin E from rabbit bone marrow

Experimental details are given in the Experimental section.

Step	Procedure	Enzyme activity	
		Total units	Units/mg. of protein
I	Aqueous extract	—	—
II	Elution from DEAE-cellulose by 0.05 M-phosphate, pH 8.0, containing NaCl (0.5 M)	1840	6.5
III	Elution from DEAE-cellulose by 5 mM-phosphate, pH 5.78, containing NaCl (0.25 M)	1800	57.0
IV	Elution from DEAE-cellulose by 5 mM-phosphate, pH 5.78, containing NaCl (0.5 M)	1750	103
V	Gel-filtration on Sephadex G-75	1750	145

Fig. 4. pH optimum of cathepsin E (see text). The reaction mixture contained human serum albumin (5 g./100 ml.) as substrate and cathepsin E (3.2 units/ml.).

Fig. 5. Influence of substrate concentration on the activity of cathepsin E (see text). The reaction mixture contained human serum albumin as substrate and purified cathepsin E (3.6 units/ml.).

Fig. 4 shows that the enzyme degraded human serum albumin in the pH range 1.5–4.5, with the maximum activity at pH 2.5.

(b) *Influence of substrate concentration.* The results obtained in the study of the enzymic reaction with a constant amount of cathepsin E and increasing amounts of human serum albumin are shown in Fig. 5. The amount of degradation products formed increases with the amount of substrate used. However, the slope of the curve approaches zero with increasing amounts of substrate.

(c) *Activation and inhibition.* The activity of cathepsin E was not changed at pH 2.5 in the presence of cysteine and slightly increased at pH 3.5–4.5. Cathepsin E was not inhibited by iodoacetate or di-isopropyl phosphorofluoridate. After heating for 60 min. at 60°, only 20 % of the original activity was still present; after heating at 80° for 10 min., cathepsin E was completely inactivated.

(d) *Electrophoretic mobility.* The electrophoretic mobility in agar gel at pH 8.2 of cathepsin E was -7.2×10^{-6} cm.² v⁻¹ sec.⁻¹

(e) *Chromatographic analysis.* The chromatographic analysis (Fig. 6) of a preparation of cathepsin E taken at step V showed that cathepsin E was eluted from DEAE-cellulose as a single peak. All the activity was eluted by 15 mM-potassium phosphate buffer, pH 5.78, containing between 0.2M- and 0.3M-sodium chloride.

Fig. 6. Chromatography of cathepsin E. A preparation (step IV, 800 units) was put on a column of 5 g. of DEAE-cellulose (see text). - - -, Extinction; —, proteolytic activity.

(f) *Activity on synthetic substrates.* Table 2 shows that the synthetic substrates for cathepsins A, B and C were hydrolysed by the aqueous extract of rabbit bone marrow, thus demonstrating the existence in this tissue of enzymic activities analogous to those of cathepsin A, B and C. However, purified cathepsin E does not hydrolyse these substrates, showing that this enzyme is not cathepsins A, B or C.

Gel-filtration of human serum albumin degraded by either cathepsin D or E. Fig. 7 shows the filtration pattern on Sephadex G-75 given by samples of

Fig. 7. Gel-filtration on Sephadex G-75 of degraded human serum albumin (see text). —, Human serum albumin (30% non-precipitable by 2% trichloroacetic acid) degraded by cathepsin D; - - -, human serum albumin (38% non-precipitable by 2% trichloroacetic acid) degraded by cathepsin E;, undegraded human serum albumin; - · - · tryptophan.

Table 2. *Percentage hydrolysis of different synthetic substrates*

Hydrolysis was carried out at pH 5.0 and 37° for 24 hr. Where the substrates for cathepsin B and C were used, the hydrolysis was carried out in the presence of 4 mM-cysteine. The units are those of cathepsin E. The units of cathepsin E in the aqueous extract could not be measured directly owing to the presence of cathepsin D. The concentration of cathepsin E was therefore estimated from the amount of cathepsin E recovered in step II of its preparation.

Enzyme	Hydrolysis of synthetic substrate (0.05M in 0.1M-citrate buffer) (%)		
	Benzoyloxycarbonyl-L-glutamyl-L-tyrosine (substrate for cathepsin A)	Benzoyl-L-arginine amide hydrochloride (substrate for cathepsin B)	Glycyl-L-phenyl-alanine amide acetate (substrate for cathepsin C)
Aqueous extract of rabbit bone marrow (7.1 mg./ml.; 8.0 units/ml.)	38	40	100
Purified cathepsin E (0.19 mg./ml.; 28 units/ml.)	0	0	0

human serum albumin degraded by an equal amount of either cathepsin D or E, and as a control the pattern given by a solution containing undegraded albumin and tryptophan. The degradation products of both degraded albumins are filtered through after undegraded albumin and before tryptophan. The filtration patterns of the two samples are different. In addition albumin degraded by cathepsin E at pH 3.5 in the presence of cysteine gave the same gel-filtration pattern as albumin degraded by cathepsin E at pH 2.5 without cysteine.

DISCUSSION

The two proteinases studied in this work are called 'cathepsins' because the term is now generally used for intracellular proteinases obtained from animal tissues (Fruton, 1960).

The enzyme obtained from rabbit spleen by Lapresle & Webb (1960) and shown not to be cathepsin A, B or C by Webb & Lapresle (1960) is called rabbit cathepsin D since this name was used by Press *et al.* (1960) to designate an analogous enzyme isolated from bovine spleen. Press *et al.* (1960) pointed out that cathepsin D is probably identical with the haemoglobin-splitting enzyme partially purified by Anson (1939).

As shown by gel-filtration on Sephadex G-75, the preparation of cathepsin D obtained previously still contained inactive protein. A further purification was obtained by elution of the enzyme from carboxymethyl-Sephadex.

Studies of aqueous extracts of rabbit bone marrow by direct detection of proteolytic activity after electrophoresis in agar gel showed that two enzymes were present in appreciable amounts. The exact amounts of these two enzymes could not be measured in these extracts because each is active at the optimum pH of the other.

One of these enzymes is identical with rabbit spleen cathepsin D as it can be purified in the same manner and has the same properties.

The second proteolytic enzyme, which has been named cathepsin E, does not seem to have been described previously. It was separated from cathepsin D by chromatography on DEAE-cellulose (step II). Its activity could then be measured at pH 2.5 with the same procedure as for cathepsin D (Lapresle & Webb, 1960). In this procedure, human serum albumin is used at a concentration of 5 g./100 ml. because the amount of degradation products formed is then only slightly dependent on changes in substrate concentration.

The purification of cathepsin E was relatively easy because under conditions in which most of the inactive proteins are eluted cathepsin E is retained on DEAE-cellulose. This fact is probably related to

the high negative-charge density of the enzyme shown by electrophoresis. Cathepsin E is very stable under the conditions used for its purification and is recovered in very high yield.

Cathepsin E is also present in spleen extracts, but in amounts small enough to explain why the proteolytic activity of crude spleen extracts at pH 3.5 was found to be attributable to cathepsin D (Lapresle & Webb, 1960).

Cathepsins D and E differ greatly in their electrophoretic mobility at pH 8.2, which facilitates their separation. From their behaviour on Sephadex G-75 cathepsin E has a higher molecular weight than cathepsin D.

Cysteine does not activate cathepsin E at pH 2.5 but does augment the activities of both cathepsins D and E at pH 3.5–4.5. It is possible that cysteine does not act on the enzymes themselves but acts, at certain pH, on the albumin molecule or its products.

Gel-filtration of albumin degraded by either cathepsin D or E showed that in both cases the degradation products were a mixture of components having molecular weights intermediate between that of albumin and those of amino acids. With cathepsin D this was shown by ultracentrifugal analysis (Lapresle & Slizewicz, 1958). On the other hand, the gel-filtration pattern of human serum albumin degraded by cathepsin E is different from that of albumin degraded by cathepsin D, which suggests that the two enzymes do not attack albumin in the same manner.

Experiments in progress have shown that human serum albumin degraded with cathepsin E gives an immunoelectrophoretic pattern roughly similar to that given by albumin degraded with cathepsin D. Further work is required to determine the immunological properties of the products of the degradation of human serum albumin by cathepsin E.

Studies of the distribution of cathepsins D and E in different types of rabbit leucocytes has shown that cathepsin D is present in polymorphonuclear cells, macrophages and lymphocytes. On the other hand, cathepsin E is present in large amounts in polymorphonuclear cells, in smaller amounts in macrophages and only in traces in lymphocytes (J. Štefanovič, T. Webb & C. Lapresle, unpublished work). This is probably the reason why the spleen, which is composed mainly of lymphocytes, contains large amounts of cathepsin D and only small amounts of cathepsin E. On the other hand, bone marrow, which is composed of many types of cells including a large proportion of polymorphonuclear cells, contains both cathepsins D and E in appreciable amounts.

When the intracellular pH of certain of these types of cells could be measured it was found to be

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 -1.7×10^{-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 -7.2×10^{-5} cm.² V⁻¹ sec.⁻¹

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

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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 Cu²⁺ 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 & Schwyzler (1957), Acher (1960), and others, we propose to call it Gly¹-corticotrophin A₁.

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