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Acidic Peptides of the Lens

8. *S*-(αβ-DICARBOXYETHYL)GLUTATHIONE*

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The presence of a number of unidentified acidic peptides in extracts of calf lens was recorded by Waley (1956). Electrophoresis separated these compounds into a 'slow acid' fraction and a 'fast acid' fraction. The main new constituents of the former are ophthalmic acid (γ-glutamyl-α-*n*-butyrylglycine) and norophthalmic acid (γ-glutamylalanylglycine; Waley, 1957, 1958). The fast acid fraction was further subdivided into fractions called 1α and 1β. The main constituent of 1α is *S*-sulphoglutathione (Waley, 1959). The present paper describes the identification of the main constituent of 1β as *S*-(αβ-dicarboxyethyl)glutathione, i.e. GS·CH(CO_2H)·CH_2·CO_2H, where GSH is glutathione. Lens extracts also contain the related amino acid, *S*-(αβ-dicarboxyethyl)cysteine, i.e. CyS·CH(CO_2H)·CH_2·CO_2H, where CySH is cysteine. Neither the amino acid nor the peptide had previously been recognized as occurring in tissue extracts, although they had been prepared by Morgan & Friedmann (1938). The amino acid was formed from cysteine and maleic acid and the peptide from GSH and maleic acid. The corresponding reactions proceed with fumaric acid, but are slower, and were not detected by Morgan & Friedmann (1938).

EXPERIMENTAL

Materials

S-(αβ-*Dicarboxyethyl*)*cysteine*. This was prepared by a method different from that used by Morgan & Friedmann (1938); the omission of inorganic salts facilitates the isolation of the amino acid. The same product was obtained from the reaction of either maleic acid (*a*) or fumaric acid (*b*) with cysteine, and was characterized as the *bisdicyclohexylamine salt*.

(*a*) Dicyclohexylamine (2 ml.) was added to maleic acid (582·5 mg.) and L-cysteine hydrochloride (394 mg.) in water (5 ml.). The amine hydrochloride was removed by filtration, more dicyclohexylamine (0·5 ml.) was added and the solution diluted with acetone. The bisdicyclohexylamine salt of *S*-(αβ-dicarboxyethyl)cysteine (962 mg.) crystallized in needles, and after recrystallization from aqueous acetone had m.p. 118° (Found: C, 57·6; H, 9·8; N, 6·4. $C_{31}H_{57}N_3O_6S,2{\cdot}5H_2O$ requires C, 57·7; H, 9·7; N, 6·5%). The presence of water of crystallization could not be confirmed by the value of the loss of weight (15·1%) after drying at 100° *in vacuo*, since this value, and analysis of the residue, showed that some of the dicyclohexylamine was also lost on drying.

(*b*) Fumaric acid (214·4 mg.) was added to cysteine (220·7 mg. prepared from the hydrochloride in ethanol by addition of triethylamine) in water (3 ml.), and the mixture was kept at room temperature overnight, warmed briefly so that the remaining fumaric acid dissolved and kept for a further 48 hr. Dicyclohexylamine (0·725 ml.) was added, the solution diluted with acetone and the solid (765 mg.) collected. After recrystallization from aqueous acetone it had m.p. 118–119°, undepressed on mixing with the product from the reaction with maleic acid.

The infrared spectra of the two bisdicyclohexylamine salts were indistinguishable; they are presumably both mixtures of diastereoisomers. The free amino acid was prepared by adding fumaric acid (213·8 mg.) to cysteine (220·2 mg.) in water (3 ml.); the mixture was stored at room temperature overnight, and then warmed briefly. The solution was diluted with acetone and the solid (406·2 mg.) collected, washed with acetone, dried and extracted with hot water (2 ml.). The filtered solution was diluted with acetone and the *S*-(αβ-dicarboxyethyl)cysteine (374·4 mg., 87% yield) collected. The solid had m.p. 125–126° (decomp.), and was indistinguishable (by m.p. and infrared spectrum) from the product similarly obtained from cysteine and maleic acid. The equivalent weight was 122; the value calculated on the assumption that two carboxyl groups were being titrated was 119. Satisfactory analytical figures for the amino acid were not obtained; it contained ash and also acetone (as shown by the isolation of the 2,4-dinitrophenylhydrazone).

On heating the amino acid to 120–125° for 30 min. gas was evolved, and the main product was a ninhydrin-negative acid, presumably the lactam, 2-carboxymethyl-3-oxo-1,4-thiazane-5-carboxylic acid. The same product, together with a little alanine, was obtained after heating the amino acid in aqueous solution for 40 hr. at 110°. After heating for 16 hr. at 110° in 6N-HCl, the amino acid was largely unchanged, but some cystine was formed.

S-(αβ-*Dicarboxyethyl*)*glutathione*. Glutathione (151·7 mg.) was added to maleic acid (57·9 mg.) in water (1·5 ml.) and the mixture was warmed to 50° for 5 min. and then stored at room temperature for 25 hr. A sample (5 μl.), on paper electrophoresis at pH 4, gave one strong ninhydrin-positive spot (initially purple), attributed to *S*-(αβ-dicarboxyethyl)glutathione, and very faint spots of GSH and of GSSG. The solution was treated with 0·3M-cupric acetate (1·65 ml.) and diluted with ethanol; the precipitate (161·9 mg.) was collected, washed with 50% (v/v) ethanol

* Part 7: Cliffe & Waley (1961).

and then with ethanol. The copper complex (151·7 mg.) was decomposed by suspension in water (7·5 ml.) and extracted with 0·1% (w/v) 8-hydroxyquinoline in $CHCl_3$ (20 × 5 ml.). Each portion of $CHCl_3$ was extracted successively with water (0·5 ml.), and the combined aqueous solutions were extracted with $CHCl_3$ (5 × 5 ml.) and then dried *in vacuo*. The residue (31·3 mg.) in water (0·1 ml.) was treated with acetone (1·1 ml.); the oil, which separated slowly, solidified and was collected (yield 24 mg.). The substance was homogeneous on electrophoresis at pH 4, but from its infrared spectrum, which showed only broad and featureless bands, it was still impure.

Hydrolysis of the peptide in 6N-HCl for 16 hr. at 110° gave glutamic acid, glycine, S-(αβ-dicarboxyethyl)cysteine and a little cystine; these products were identified by electrophoresis in 10% (v/v) acetic acid.

The reactions of GSH with maleate and fumarate at pH 7 were compared; the concentration of each component was 50 mM. The extent of reaction after 22·5 hr. with maleate was about 75%, but with fumarate only about 20% (as estimated by intensities of the spots obtained after paper electrophoresis at pH 4). Oxidation of the GSH to GSSG is a competing reaction at pH 7, and there was about twice as much GSSG present (after 22·5 hr.) in the reaction with fumarate as there was in the reaction with maleate. Both maleate and fumarate gave the same product, as judged by behaviour in chromatography and electrophoresis, and by the products after acid hydrolysis. The isomerization of some of the maleate to fumarate (Morgan & Friedmann, 1938) at pH 7 in the presence of GSH was also confirmed by paper chromatography.

Methods

Paper electrophoresis. This was carried out in a ridge-pole apparatus (Cliffe & Waley, 1958) at 8v/cm., usually on Whatman no. 52 paper. The pH 4 buffer was pyridine–acetate (Grassmann, Hannig & Plöckl, 1955); the pH 2·3 buffer was 10% (v/v) acetic acid. The ninhydrin reagent of Wiggins & Williams (1952), which gives red colours with most amino acids, was used; carboxylic acids were detected with acridine (Smith, 1960). Continuous electrophoresis was carried out with the pH 4 buffer in a Beckman apparatus (model CP) at constant current.

Anion-exchange chromatography. This was carried out on a column (1·2 cm. diam. × 29 cm. long) of Dowex 1 (acetate form; 200–400 mesh; X4). The buffers used (molarity with respect to the formic acid) were: 0·5M-pyridine–formate, pH 5·3, containing redistilled pyridine (81 ml.) and 90% (w/v) formic acid (21·2 ml.) and water to 1 l.; M-pyridine–formate, in which the concentrations of pyridine and formic acid were doubled. The pyridine was purified by addition of Br_2 (2 ml./l.) and distilling.

Paper chromatography. The solvents used were butan-1-ol–acetic acid–water (40:9:20, by vol.), butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.) and ethanol–aq. NH_3 (sp.gr. 0·88)–water (16:1:3, by vol.).

Lens extracts. Lenses were removed from the eyes of calves within 2 hr. of the death of the animal. In experiments with single lenses, these were deproteinized at once. For the large-scale experiments, the lenses had been stored at −15° for about 5 weeks (Expt. 2), or for about 5 years (Expts. 1 and 3). The influence of the time of storage is discussed below. The lenses were ground or disintegrated in a macerator with 7% (w/v) trichloroacetic acid (4 ml./g. of lens) and the mixture was stored at 4° for 30 min. and then centrifuged at 10 000*g* for 15 min. at 0°. If the extract was turbid, Celite 535 (L. Light and Co. Ltd.) was added, and the extract was clarified by filtration. Trichloroacetic acid was removed by extraction with *N*-methyldioctylamine (British Hydrological Corp., Merton, Surrey) in $CHCl_3$ (5%, v/v), and the aqueous layer (pH about 6) was then extracted with $CHCl_3$ (to remove excess of base) and concentrated under reduced pressure to a volume equal to the weight of calf lenses used. The extract was used directly or stored at −15°.

RESULTS

Expt. 1: fractionation by electrophoresis

Lens extract (100 ml.) was fractionated by continuous paper electrophoresis at pH 4 at constant current (94 mA). The potential fell from 390 to 360v during the fractionation. The apparatus was equilibrated for 5 hr.; the lens extract (100 ml.) was then applied to the centre of the paper at about 1·4 ml./hr., and six sets of 32 fractions were collected; they were numbered from the cathode (1) to the anode (32). The fractions were concentrated and examined by paper electrophoresis. The unknown acidic compound was in tubes 27–29; as it was contaminated by some GSSG, it was refractionated in fourfold-diluted pH 4 buffer. The sample (12 ml.) was applied to the cathode side of the paper at 1·1 ml./hr.; fuchsin (0·9 mg. in 0·3 ml.) was added as a marker and indicated the approximate position of the peptide. Electrophoresis was carried out at a constant current of 40 mA. The peptide, contaminated with a little aspartic acid, was present in fractions 24–29. The presence of the new amino acid, S-(αβ-dicarboxyethyl)cysteine, in these fractions, which was not realized at the time, complicated interpretation of the results. Hydrolysis in 6N-hydrochloric acid for 16 hr. at 110° gave glutamic acid, glycine and an unidentified amino acid, which gave a characteristic colour with ninhydrin. Dinitrophenylation of the peptide, followed by hydrolysis, gave some dinitrophenylglutamic acid.

Expt. 2: fractionation by ion-exchange chromatography with gradient elution

In this experiment the lens extract was freed from strongly adsorbed acidic compounds by passage through Dowex 1 (formate form) and elution with M-pyridine–formate. The eluate (50 ml.) was concentrated and water added and removed by distillation four times to remove the buffer. The solution was brought to pH 7 with N-sodium hydroxide and adsorbed on the Dowex 1 (acetate form) column. Elution was carried out initially with 0·1M-pyridine–formate, then with a linear gradient to 0·5M-buffer and finally with a linear gradient to M-buffer (Fig. 1). Appropriate fractions were pooled, concentrated and examined

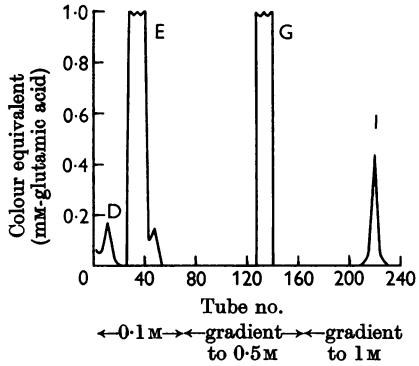

Fig. 1. Anion-exchange chromatography of extract from 44 g. of calf lens on a column (1·2 cm. × 30 cm.) of Dowex 1 (acetate form). Pyridine–formate buffers, pH 5·3, were used for elution; the molarities (with respect to formic acid) are shown. Fractions (vol. about 3·5 ml.) were collected at a rate of four/hr. Portions of the fractions were used for assay with ninhydrin.

by paper electrophoresis at pH 4. Fraction D contained taurine, aminoethyl phosphate and several unidentified compounds; fraction E contained mainly glutamic acid, ophthalmic acid and norophthalmic acid; fraction G contained GSSG; the last fraction (I) contained the new peptide. This experiment showed that it was easier to obtain a homogeneous sample of the new peptide by ion-exchange chromatography than by continuous paper electrophoresis and, since the peptide (I) was eluted when the concentration of buffer was between 0·5 and 1·0 M, these two concentrations were used to convert the procedure into a simple stepwise elution.

Expt. 3: ion-exchange chromatography with stepwise elution

Lens extract (100 ml.) was concentrated to 25 ml., stored at 4° for 1 hr. and a solid (6·78 mg.) removed by centrifuging. A portion (3 ml.) of a 50 % (v/v) suspension of Dowex 1 (acetate form) was added to the solution, and, after 1 hr., gas was removed under reduced pressure for 30 min. A further portion of resin (2 ml.) was then added, and the mixture degassed for another 30 min. This procedure obviated the evolution of gas during chromatography, which probably arose from the decarboxylation of some remaining trichloroacetic acid in the extract, catalysed by contact with the resin. The combined portions of the resin were transferred to the top of the column of Dowex 1 (acetate form), and the extract passed through. The column was washed with water for 20 hr. at 11·2 ml./hr. to remove the neutral and basic amino acids, and eluted with 0·5 M-pyridine–formate; 64 fractions of about 2 ml. were collected, at 8·5 ml./hr. The buffer was then changed to 1 M-pyridine–formate, and 46 fractions were collected. Amino compounds were detected by putting drops on paper and developing with ninhydrin. Appropriate fractions were then pooled, concentrated and examined by electrophoresis at pH 4. Two series of pooled fractions gave ninhydrin-positive spots with the electrophoretic mobility of the peptide. One series from tubes 65–73 was eluted when the concentration of the buffer was changing; this proved to contain the new amino acid, *S*-(αβ-dicarboxyethyl)cysteine. The other series, from tubes 98–110, contained the peptide together with traces of *S*-sulphoglutathione. This separation of the amino acid and the peptide was the prelude to successful structural investigation.

Structural investigation

Neither the amino acid nor the peptide was homogeneous (although they were the major components of their fractions), and both were only available in small amounts. When it was observed that the main ninhydrin-positive spot in the earlier fraction (tubes 65–73) gave an atypical mauve colour with the ninhydrin reagent (containing cobaltous chloride), and was unchanged after acid hydrolysis, it became clear that this might be the unknown amino acid that was one of the hydrolysis products of the peptide. This was confirmed by electrophoresis at pH 4, followed by chromatography in butanol–acetic acid (Fig. 2). Both amino acid and the peptide were oxidized by performic acid; the products, which could be distinguished from cysteic acid ($CySO_3H$) and the sulphonic acid (GSO_3H) corresponding to glutathione (Fig. 2), were probably sulphones.

A provisional hypothesis at this stage was that the amino acid could be represented by CySX, and the peptide by γ-Glu·CySX·Gly, but the nature of the residue X was unknown. The migration towards the cathode on electrophoresis in 10 % acetic acid showed the absence of strongly acidic groups, and neither substance contained phosphorus. If the peptide was a thioether, as its oxidation suggested, then reduction with Raney nickel should cause fission to γ-glutamylalanylglycine (norophthalmic acid) (Waley, 1957). Losses, due to adsorption on the nickel, are minimized by the use of a small column of Raney nickel (Keil & Šorm, 1960), and this procedure was used for the peptide. The fine column (2·5 mm. × 6·5 mm.) tended to become clogged, and elution was slow, but it was possible to identify norophthalmic acid as one of the products. This confirmed that the peptide was a thioether derived from glutathione.

Some idea of the nature of the residue X in GS·X was gained from the electrophoretic mobility of the peptide at pH 4. It was greater than that of

S-(β-carboxypropyl)glutathione, a peptide that has been isolated from onion (Virtanen & Matikkala, 1960), which suggested that the residue X had two carboxyl groups. The simplest substituent is then $-CH(CO_2H)\cdot CH_2\cdot CO_2H$, with two carboxyl groups and two other carbon atoms. This compound was prepared and closely resembled the peptide from lens. This prompted a reinvestigation of the products of the reduction of the peptide with Raney nickel. One product was norophthalmic acid, as already mentioned. The ninhydrin-negative product was an acid, which was identified as succinic acid by paper chromatography in butanol–acetic acid and in ethanol–ammonia, and by paper electrophoresis at pH 4. This provides strong evidence for the structure of the new peptide as S-(αβ-dicarboxyethyl)glutathione (I) and hence the amino acid as S-(αβ-dicarboxyethyl)cysteine (II); the degradative reactions are summarized in Fig. 3.

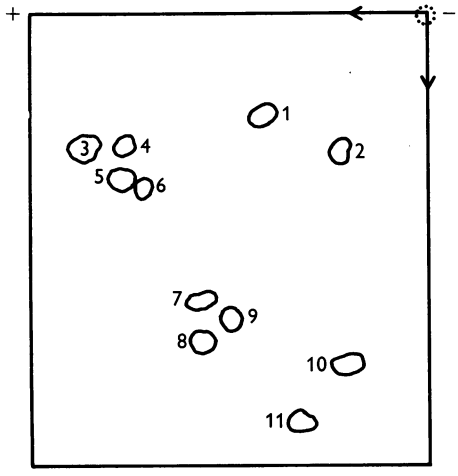

Fig. 2. Separation of new acidic compounds and related substances on Whatman no. 3 paper by electrophoresis at pH 4 for 4 hr. (horizontal direction) followed by chromatography in butanol–acetic acid (vertical direction) for 15 hr. The dotted circle shows the origin. (1) GSSG, (2) CySSCy, (3) $CySO_3H$, (4) GSO_3H, (5) S-(αβ-dicarboxyethyl)cysteine after oxidation with performic acid, (6) S-(αβ-dicarboxyethyl)glutathione after oxidation with performic acid, (7) S-(αβ-dicarboxyethyl)glutathione, (8) S-(αβ-dicarboxyethyl)cysteine, (9) aspartic acid, (10) glycine, (11) glutamic acid.

Comparison of synthesized compounds with those isolated from lens

Synthetic compound (II) was indistinguishable from the amino acid isolated from the lens when compared by paper electrophoresis at pH 4 and at pH 2·3 or by paper chromatography in butanol–acetic acid or butanol–acetic acid–water–pyridine. They both gave the characteristic mauve colour with ninhydrin. They were also compared by chromatography on a column (1·2 cm. × 96 cm. long). of Dowex 50 (X4), equilibrated and eluted with pyridine–formate buffer, pH 2·5 (59 ml. of 90%, w/v, formic acid and 16·2 ml. of pyridine diluted to 1 l.), at about 10 ml./hr.; both samples were present in fractions 58–66 (fractions were 2·2–2·5 ml.). The samples were also compared after they had undergone cyclization in aqueous solution at 100° for 40 hr.; the products were examined by paper chromatography in butanol–acetic acid; amino compounds were detected with ninhydrin and acidic compounds with acridine. Both samples gave the same pattern, arising from the conversion of the amino acid into the ninhydrin-negative acid (see the Experimental section). Both samples, on oxidation with performic acid, gave the same product (as judged by electrophoresis at pH 2·3).

The synthesized and isolated samples of the peptide were also found to be indistinguishable by electrophoresis and paper chromatography under the conditions described for the amino acid. Their oxidation and hydrolysis products were also the same.

The results of these comparisons leave little doubt that the synthetic and natural compounds have the same structure. It seems reasonable to assume that the amino acids have the L-configuration. The only uncertain point concerns the configuration at the asymmetric carbon atom of the αβ-dicarboxyethyl side chain

$$[-\overset{*}{C}H(CO_2H)\cdot CH_2\cdot CO_2H].$$

The synthetic materials may be mixtures of diastereoisomers; the same mixture was apparently

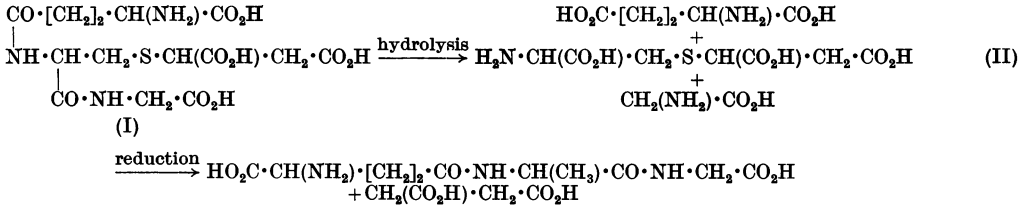

Fig. 3. Summary of degradative reactions of the peptide.

formed from both maleic acid and fumaric acid. Whether the natural products are also mixtures of diastereoisomers is unknown.

Is S-(αβ-dicarboxyethyl)glutathione an artifact?

It was possible that in the lens extract GSH, present in high concentration, might react with fumarate, the concentration of which is unknown. That the peptide could be formed during the extraction was shown by adding 7% (w/v) trichloroacetic acid (40 ml.) to GSH (30·7 mg.) and fumaric acid (3·4 mg.) in water (10 ml.); these amounts (apart from that of the fumaric acid) are based on those found in the preparations of lens extracts. After being kept for 4·5 hr. at 4°, the solution was treated in the same way as the lens extracts and examined by paper electrophoresis at pH 4. A little *S*-(αβ-dicarboxyethyl)glutathione, probably about 2 μmoles, was formed. Whether as much would be formed in an extract of lens (in which the concentration of fumaric acid is probably less) is not known, but this result stressed the need for further experiments to show whether the peptide was an artifact.

The possibility of blocking the thiol group of GSH by reaction with *N*-ethylmaleimide was examined. For this to be a useful procedure, GSH must react more rapidly with *N*-ethylmaleimide than with fumarate. This was tested by adding GSH (0·15 ml. of a 150 mM soln. at pH 7) to a mixture of fumarate (0·5 ml. of a 150 mM soln. at pH 7) and *N*-ethylmaleimide (0·5 ml. of 150 mM). After 1 hr. at 20°, portions (5 μl.) were examined by paper electrophoresis at pH 4. No *S*-(αβ-dicarboxyethyl)glutathione was detected, although this was present in a parallel experiment in which the *N*-ethylmaleimide had been replaced by water. The GSH had reacted preferentially with the *N*-ethylmaleimide, which usually reacts rapidly with thiols of low molecular weight (Roberts & Rouser, 1958).

A similar experiment carried out with cysteine gave essentially the same results, except that here a trace of *S*-(αβ-dicarboxyethyl)cysteine was formed even in the presence of *N*-ethylmaleimide. However, about 90% of the cysteine reacted with *N*-ethylmaleimide rather than with fumarate. These results showed that the presence of *N*-ethylmaleimide during the preparation of extracts of lens would prevent the reaction of GSH (and cysteine) with any fumarate that might be present.

Another point (the effect of storage) was also checked in the experiment to be described. Fresh calf lenses were used, as opposed to the large-scale experiments with stored lenses. A calf lens (1·04 g.) and a little sand were ground under water (1 ml.) containing *N*-ethylmaleimide (3·75 mg.) for 10 min. Phenol (1 ml. of 90%, w/v) was added, and the solution clarified by centrifuging and extracted with chloroform. The aqueous layer (which is largely free from protein) was evaporated to dryness *in vacuo* and the residue (14·1 mg.) dissolved in water and a portion (equivalent to 35% of the total) examined by electrophoresis at pH 4 followed by chromatography in butanol–acetic acid–water–pyridine (Fig. 4). The *S*-(αβ-dicarboxyethyl)glutathione (which travels to a characteristic position well separated from other ninhydrin-positive spots in this procedure) was clearly seen. This provides good evidence for the existence of the peptide in calf lens; its concentration was estimated at about 30 μmoles/100 g. of lens.

S-(αβ-Dicarboxyethyl)cysteine was probably present, but at a lower concentration. As well as much *S*-(*N*-ethylsuccinimido)glutathione some GSSG was also detected. This, too, is probably present in the lens.

Experiments with calf liver and calf kidney

Portions of calf liver (or kidney) were ground under aqueous *N*-ethylmaleimide, and the extract was examined by electrophoresis followed by chromatography, as in the experiment with calf lens. There was no spot in the position of the peptide. A higher loading on the chromatogram (equivalent to about 0·75 g. of tissue) was possible when the extract was fractionated by adsorbing on Dowex 50 (H^+ form) and eluting with triethylamine

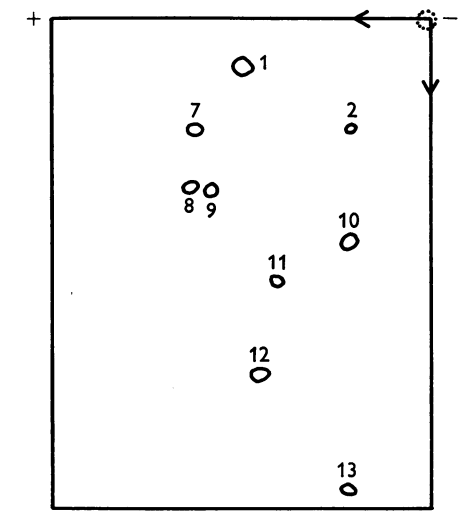

Fig. 4. Identification of new acidic compounds in lens extract prepared in the presence of *N*-ethylmaleimide. Electrophoresis at pH 4 for 4 hr. (horizontal direction) followed by chromatography in butanol–acetic acid–water–pyridine for 15 hr. (vertical direction). The spots are numbered as in Fig. 2; 12 is the adduct from *N*-ethylmaleimide and GSH, and 13 the adduct from *N*-ethylmaleimide and CySH.

(Harris, Tigane & Hanes, 1961). A very faint spot in about the correct position for the peptide was seen in the extract from liver, but not in the extract from kidney. The peptide, S-(αβ-dicarboxyethyl)glutathione, may thus be present in calf liver, at a concentration of about 2–5 μmoles/100 g. of liver. The amino acid, S-(αβ-dicarboxyethyl)-cysteine, was not detected, but unless present at a high concentration, it would be obscured by aspartic acid, which is present in greater amounts than in lens.

DISCUSSION

The isolation of S-(αβ-dicarboxyethyl)glutathione and S-(αβ-dicarboxyethyl)cysteine from lens extracts was accomplished most readily by ion-exchange chromatography. Continuous paper electrophoresis, although superior to the electrophoresis on cellulose powder used by Waley (1956), was not as convenient as ion-exchange chromatography for the isolation of the compounds just mentioned. The advantage of continuous paper electrophoresis lies in the fractionation of the complex mixture of substances in an extract of tissue into different classes; it is less satisfactory for separation of the members within one class.

The relative concentrations of the amino acid, S-(αβ-dicarboxyethyl)cysteine, and the peptide, S-(αβ-dicarboxyethyl)glutathione, depended on whether fresh or stored calf lenses were used. Lenses that had been stored (at −15°) for several years contained more of the amino acid, but fresh lenses contained more of the peptide. This suggests that the peptide was partially hydrolysed during storage of the frozen lenses. Similarly, some of the ophthalmic acid and glutathione were gradually hydrolysed as there was more α-amino-*n*-butyric acid, and cystine, in the stored lenses. The cystine separated from the neutral fraction (after concentration) obtained by continuous electrophoresis, and was identified from its infrared spectrum.

The experiment in which a fresh calf lens was ground with aqueous *N*-ethylmaleimide (Fig. 4) ruled out the possibility that the peptide was an artifact, and so the question arises of how the peptide is formed *in vivo*. A lens mush did not catalyse the reaction between fumarate and GSH, and so there is no evidence for enzymic catalysis of this reaction. The (non-enzymic) rate of this reaction is probably slow compared with the rate of hydration of fumarate (catalysed by fumarase), but the reaction should be borne in mind as a possible control mechanism for the operation of the citric acid cycle.

Other routes to the peptide are possible. It might be formed from malate and GSH (cf. the conversion of serine into cysteine; Schlossmann & Lynen, 1957) or be built up from the constituent amino acids.

S-(αβ-Dicarboxyethyl)glutathione seems to be the first thioether derived from glutathione to be detected in animal tissues. The most closely related peptide is S-(β-carboxy-*n*-propyl)glutathione, from onions (Virtanen & Matikkala, 1960). The related amino acid, S-(β-carboxy-*n*-propyl)cysteine, has been detected in urine (Mizuhara & Oomori, 1961), but presumably might be of dietary origin.

SUMMARY

1. The amino acid, S-(αβ-dicarboxyethyl)cysteine, and the peptide, S-(αβ-dicarboxyethyl)-glutathione, have been isolated from extracts of calf lens. The peptide, and probably the amino acid, are present in extracts of fresh calf lens prepared with *N*-ethylmaleimide, and are thought to be genuine constituents of the lens.

2. The structure of the peptide was deduced from its hydrolysis to glutamic acid, S-(αβ-dicarboxyethyl)cysteine and glycine, and its reduction to γ-glutamylalanylglycine and succinic acid.

3. The amino acid was prepared by the reaction of cysteine with either maleic acid or fumaric acid, and the peptide by the reaction of glutathione with either unsaturated acid. The synthesized compounds were indistinguishable from the substances isolated from the lens.

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