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Isolation and Nature of Intracellular Peptides from a Cephalosporin C-Producing *Cephalosporium* sp.

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1. Three peptides containing α-aminoadipic acid and cysteine have been obtained in small amounts from the mycelium of a *Cephalosporium* sp. 2. The peptides were precipitated as cuprous mercaptides together with glutathione and resolved from the latter and from each other by preparative paper electrophoresis and chromatography either in the sulphonic acid form or as *S*-sulphonyl derivatives. From the *S*-sulphonyl derivatives they were obtained in the thiol form. 3. One peptide (P3) was shown by amino acid analysis and the mass spectrum of the *NS*-ethoxycarbonyl derivative of its methyl ester to be δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. A second peptide (P2) contained α-aminoadipic acid, cysteine, valine and glycine, and the third peptide (P1) contained α-aminoadipic acid, cysteine, β-hydroxyvaline and glycine.

A tripeptide, δ-(α-aminoadipyl)cysteicylvaline, was obtained from the mycelium of *Penicillium chrysogenum* by Arnstein & Morris (1960), but was reported by Arnstein, Artman, Morris & Toms (1960) to be contaminated with a peptide, or peptides, containing glutamic acid and glycine. It was isolated in too small an amount for the optical configuration of its constituent amino acids to be determined. Warren, Newton & Abraham (1967) reported that they had obtained peptide material in very small amounts from a *Cephalosporium* sp. which yielded α-aminoadipic acid, cysteic acid and valine on hydrolysis (after oxidation with performic acid) but also certain other amino acids. G. G. F. Newton & T. Takano (unpublished work) explored the use of column chromatography for resolving the apparent mixture of peptides in the sulphonic acid form, but a series of experiments indicated that this approach was liable to be time-consuming and inefficient. Moreover, the isolation of the peptides only in the sulphonic acid form was attended by certain disadvantages. In this form they were unsuitable for measurements of mass spectra and appeared to be resistant to the action of carboxypeptidase. Hence it was desirable to devise a new method for their isolation, taking into account the very low concentration in which they were present in the mycelium.

Glutathione can be readily isolated from cells as a crystalline cuprous mercaptide (Pirie, 1931). It was therefore decided to find out whether the α-aminoadipic acid and cysteine-containing peptides in the *Cephalosporium* sp. could be obtained, together with glutathione, in this form, and if so, whether the components of the resulting mixture could be resolved as their *S*-sulphonates, since the latter could be reconverted to the corresponding thiols by reduction.

In a preliminary experiment with the *Cephalosporium* sp. (C91) grown in a chemically defined medium in shake flasks, about 120mg of a crystalline cuprous mercaptide was obtained from 190g of damp-dry mycelium by a method based on that of Waelsch & Rittenberg (1941) for the isolation of glutathione from animal tissue. Further supplies of this material were obtained from 10 litre fermentations.

A sample (1mg) of the product obtained by decomposition of the mercaptide with hydrogen sulphide and subsequent oxidation with performic acid was subjected to electrophoresis on paper at pH 1.8. Coloration with ninhydrin revealed a major spot in the position of glutathionesulphonic acid and a very small spot (estimated visually to correspond to about 3–5% of the amount of glutathionesulphonic acid) in the position occupied by synthetic δ-(L-α-aminoadipyl)-L-cysteicyl-L-valine. The relative sizes and intensities of the two spots were similar to those found by Smith, Warren, Newton & Abraham (1967) in preparations from aqueous ethanol extracts of mycelium. This was consistent with the result of an earlier experiment which had shown that all-L-δ-(α-aminoadipyl)cysteinylvaline was co-precipitated with glutathione as a cuprous mercaptide (Loder, Abraham & Newton, 1969).

Cuprous mercaptide prepared in a similar manner

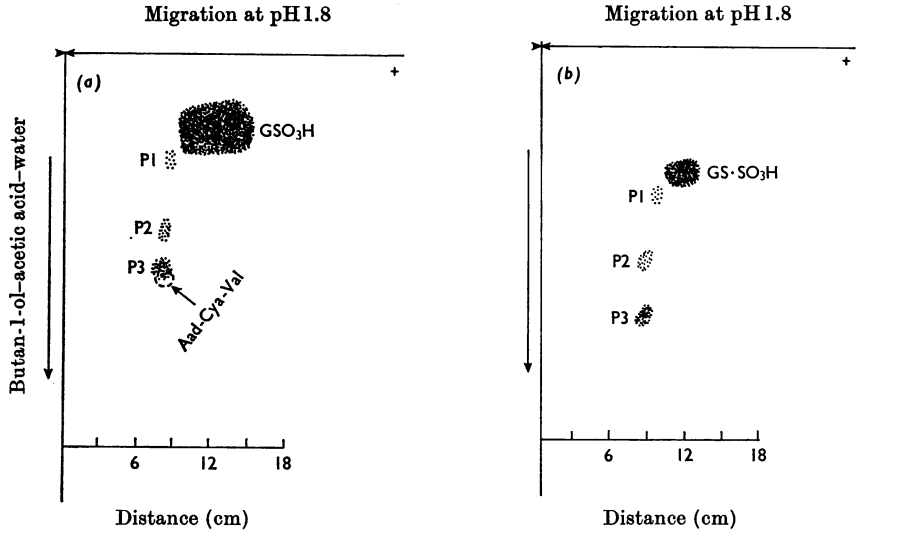


Fig. 1. Two-dimensional electrophoresis and chromatography on paper of: (*a*) peptidesulphonic acids obtained from cuprous mercaptide after oxidation with performic acid. (*b*) peptide-*S*-sulphonates obtained after incomplete resolution from glutathione-*S*-sulphonate ($GS \cdot SO_3H$) by preparative paper electrophoresis at pH 1.8. Other abbreviations: GSO_3H, γ-glutamylcysteicylglycine; Aad-Cya-Val, δ-(α-aminoadipyl)cysteicyl-valine.

from a high cephalosporin C-yielding strain of the *Cephalosporium* sp. was kindly provided by Glaxo Laboratories Ltd., Ulverston, Lancs., U.K. The material obtained after decomposition and oxidation of a sample of this preparation showed a spot on electrophoresis at pH 1.8 which occupied the same position as δ-(α-aminoadipyl)cysteicylvaline and corresponded in size and intensity to about 10% of that given by the glutathionesulphonic acid which was also present.

Further analysis of this material was carried out by two-dimensional paper electrophoresis at pH 1.8 and chromatography in butan-1-ol–acetic acid–water. Coloration with ninhydrin revealed a large spot corresponding to glutathionesulphonic acid, and three minor spots corresponding to the sulphonic acids of peptides designated P1, P2 and P3 (Fig. 1). The latter three peptides were present in relative proportions of about 1:2:10 respectively. The spot due to the sulphonic acid of P3 overlapped, but did not coincide precisely with, that given by synthetic all-L δ-(α-aminoadipyl)cysteicylvaline.

After separation of the sulphonic acids of peptides P1, P2 and P3 by paper electrophoresis and chromatography on a preparative scale, each peptide was hydrolysed with 6M-hydrochloric acid for 18h at 105°C. The hydrolysates were analysed by paper electrophoresis at pH 4.5 and paper chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.) followed by coloration with ninhydrin. Visual comparison indicated that approximately equal amounts of the constituent amino acids were present in each case. The results are summarized in Table 1. Hydrolysates of eluates of corresponding paper blanks were analysed in the same manner and confirmed that no amino acids were eluted in detectable quantity from the paper alone.

Table 1. *Properties of peptides from the* Cephalosporium *mycelium*

Peptides were isolated and analysed as described in the text. Abbreviation: R_{GSO_3H} is the *R* value relative to glutathionesulphonic acid in butan-1-ol–acetic acid–water (4:1:4, by vol.). The size and intensity of spots given by the amino acids on paper compared with those from standard mixtures indicated that the different amino acids in each hydrolysate were present in approximately equimolar amounts.

Peptide	R_{GSO_3H}	Amino acids obtained on hydrolysis
P1	1.4	α-Aminoadipic acid, cysteic acid, β-hydroxyvaline, glycine
P2	2.35	α-Aminoadipic acid, cysteic acid, valine, glycine
P3	2.75	α-Aminoadipic acid, cysteic acid, valine

The conclusion that β-hydroxyvaline was present in the hydrolysate of peptide P1 was based on the results obtained by two-dimensional paper electrophoresis (pH 4.5) and chromatography (butan-1-ol–acetic acid–water) of the hydrolysate alone and mixed with an authentic sample of DL-β-hydroxyvaline. No resolution was detected. Earlier, G. G. F. Newton & T. Takano (unpublished work) had found that a hydrolysate of crude peptide material containing peptide P1 gave an amino acid which showed no net charge at pH 4.5, but ran in the same place as aspartic acid in the amino acid analyser. Authentic DL-β-hydroxyvaline was then found to run in this position.

The peptides glutathione, P1, P2 and P3, which were present in the material obtained by decomposition of the cuprous mercaptide, were converted to their *S*-sulphonates by the method of Bailey & Cole (1959) which involves treatment with sodium sulphite and iodosobenzoate.

The *S*-sulphonates so obtained behaved in a manner similar to that of the corresponding sulphonic acids when subjected to electrophoresis on paper at pH 1.8 and chromatography in butan-1-ol–acetic acid–water (Fig. 1). However, the *S*-sulphonates of glutathione and peptide P1 were resolved less well in the chromatographic system than were their sulphonic acids. In consequence the *S*-sulphonates of peptides P2 and P3 were isolated without difficulty by preparative paper electrophoresis followed by preparative paper chromatography, but that of peptide P1 remained contaminated by some *S*-sulphonylglutathione.

A model experiment with *S*-sulphonylglutathione showed that reduction of the latter to the thiol form by Cleland's reagent (dithiothreitol) proceeded quantitatively (mol/mol). The *S*-sulphonate of peptide P3 was then reduced in a similar manner.

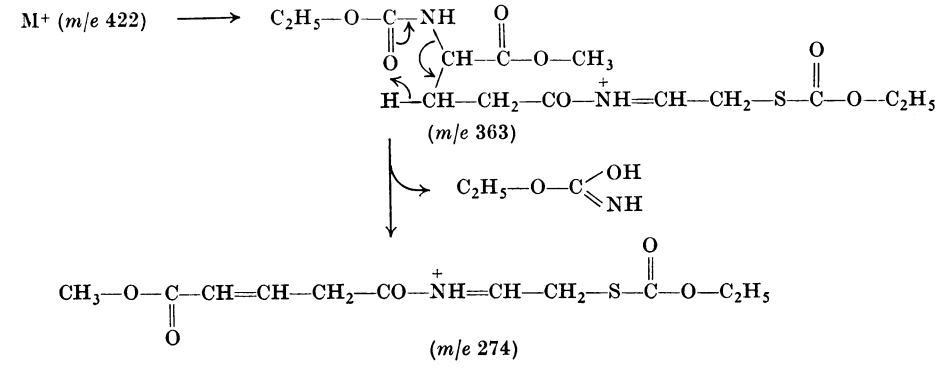
In view of the very small amount of peptide P3 which was available, it appeared that mass spectrographic analysis would provide the most feasible method for the determination of its structure. The relatively volatile ethoxycarbonyl derivatives of a number of peptide methyl esters had been used successfully for mass spectrometry, and the fragmentation pattern of *NS*-ethoxycarbonylglutathione dimethyl ester had already been studied (Kamerling, Heerma and Vliegenthart, 1968).

Preliminary studies with the *NS*-ethoxycarbonyl derivatives of α- and γ-glutamylcysteine methyl esters showed that the two peptides could be clearly distinguished by their fragmentation patterns (Table 2). In both peptides rupture of the amide bond afforded a peak with *m/e* 216 due to the *N*-terminal sequence ion, which, in the case of the α-linked peptide, lost a carbonyl group to give the ion of highest abundance with *m/e* 188. The sequence ion from the γ-linked peptide did not lose a carbonyl group, presumably because this would have given an ion in which the positive charge could not be delocalised by transfer to the nitrogen atom. With the γ-linked peptide, the ion of highest abundance could be accounted for by the loss of the CO_2Me group from the molecular ion M^+ (*m/e* 422) followed by the loss of 89 mass units [C_2H_5O–C(OH)=NH] by means of a McLafferty rearrangement (Hill, 1966) as shown in Scheme 1. This rearrangement did not occur with the α-linked peptide. The mass spectrum of the derivative of δ-(α-aminoadipyl)cysteine became virtually identical

Table 2. *Mass spectra of* NS-*ethoxycarbonyl derivatives of the dimethyl esters of α- and γ-glutamylcysteine and of δ-(α-aminoadipyl)cysteine*

The sample temperature for the glutamylpeptides was 200°C and that for the α-aminoadipylpeptide was 175°C.

	Relative abundance (%) of ions from derivatives of			
Peak (*m/e*)	α-Glutamylcysteine	γ-Glutamylcysteine	Peak (*m/e*)	Relative abundance (%) of ions from derivative of δ-(α-aminoadipyl)cysteine
422 (M^+)	5	15	436 (M^+)	14
391	4	4	405	3
377	1	8	391	3
363	1	33	377	16
349	7	54	363	18
274		100	288	47
262		18	276	4
216	5	88	230	55
188	100	6	202	4
156	27	10	170	19
144		29	158	47
142	9	25	156	37
128	23	13	128	27
84	35	59	98	100



Scheme 1. McLafferty rearrangement of γ-linked peptide.

Table 3. *Mass spectrum of the* NS-*ethoxycarbonyl derivative of peptide P3 dimethyl ester*

The sample temperature was 165°C.

Peak (*m/e*)	Relative ion abundance (%)
535 (M^+)	8
476	4
462	4
405	9
387	8
377	52
230	100
170	13
158	23
156	20
98	70

with that of the γ-glutamyl peptide when fragmentation peaks involving the *N*-terminus were shifted by +14 mass units.

The mass spectrum of the *NS*-ethoxycarbonyl derivative of the methyl ester of peptide P3 (Table 3) showed the presence of a molecular ion with *m/e* 535, indicating that the molecular weight of the parent substance was that of a peptide containing one residue each of α-aminoadipic acid, cysteine and valine. The sequence ions *m/e* 405 and 230 showed that the peptide was an α-aminoadipylcysteinylvaline. The absence of a peak with *m/e* 202 (corresponding to *m/e* 188 in the α-glutamyl peptide) and the presence of a peak with *m/e* 387 (corresponding to *m/e* 274 from the γ-glutamyl peptide) indicated that the α-aminoadipic acid residue was δ-linked. Thus peptide P3 was δ-(α-aminoadipyl)cysteinylvaline (Scheme 2).

Optical-rotatory-dispersion curves confirmed the results of two-dimensional paper electrophoresis and chromatography in showing that the sulphonic acid forms of peptide P3 and the synthetic all-L tripeptide were not identical. The latter showed a positive Cotton effect in the carboxyl region with $[\Phi](\alpha \times \text{mol.wt.}/100) = +2210$ at 223nm. The former gave a curve which was different in sign and shape ($\Phi = -2000$ at 222nm). Comparison of the circular-dichroism curves for the amino acids isolated from an acid hydrolysate of peptide P3 in its sulphonic acid form with those for authentic L-α-aminoadipic acid, L-cysteic acid and L-valine showed that peptide P3 was δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine.

It appears possible that peptide P2 is δ-(α-aminoadipyl)cysteinylvalylglycine and peptide P1 is δ-(α-aminoadipyl)cysteinyl-β-hydroxyvalylglycine, but further work is required to establish the structures of these substances.

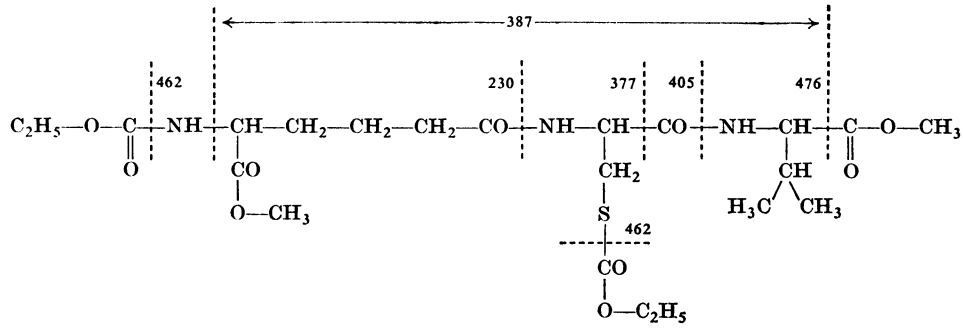
EXPERIMENTAL

Materials. Diethyl pyrocarbonate was obtained from Kodak Ltd., Speke, U.K.; *o*-iodosobenzoic acid was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and was recrystallized from hot water before use. Cleland's reagent (dithiothreitol) was from Calbiochem Ltd., London W.1, U.K. Diazomethane was prepared by the method of De Boer & Backer (1956). δ-(L-α-Aminoadipyl)-L-cysteine and γ- and α-L-glutamyl-L-cysteine were prepared as described by Loder *et al.* (1969). The disulphide of δ-(L-α-aminoadipyl)-L-cysteinyl-L-valine was kindly supplied by Professor Rudinger. Carboxypeptidase A treated with di-isopropyl phosphorofluoridate was from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

General methods. Paper chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.), paper electrophoresis at pH1.8 and pH4.5 at 60V/cm, and oxidation of thiol compounds to the corresponding sulphonic acid form were as described by Smith *et al.* (1967). Whatman no. 1 paper was used in analytical experiments and 3MM paper in preparative experiments. Elution of compounds from 3MM paper was as described by Walker & Abraham (1970).

Mass spectra. These were kindly recorded by Dr R. T. Aplin of the Dyson Perrins Laboratory, University of

M^+ has m/e 535



Scheme 2. Structure of *NS*-ethoxycarbonyl-δ-(α-aminoadipyl)cysteinylvaline dimethyl ester and its fragmentation pattern.

Oxford, U.K., with an AEI MS9 Mass Spectrometer operating at 70eV.

Optical-rotatory-dispersion and circular-dichroism curves. Optical-rotatory-dispersion measurements were kindly made by Dr P. M. Scopes, Westfield College, London N.W.3, U.K. Samples (about 2mg) were dissolved in water to give a concentration of about 2mg/ml and the measurements were made at room temperature in a 0.1cm cell with a Bellingham and Stanley/Bendix–Ericsson automatic recording spectropolarimeter. Circular-dichroism measurements were kindly made by Dr D. G. Dalgleish, Department of Biochemistry, University of Oxford, U.K. The amino acids from peptide P3 were dissolved in 6M-HCl (240μg/ml) and the circular-dichroism spectra between 200 and 240nm measured in 1mm and 2mm cuvettes in a Jouan Dichrographe II. The circular-dichroism spectra of authentic samples of L-α-aminoadipic acid, L-cysteic acid and L-valine in 6M-HCl (1mg/ml) were also measured. Comparison of the curves enabled the optical configurations of the amino acids from peptide P3 to be estimated to within ±10%.

Isolation of cuprous mercaptides from Cephalosporium *sp. mutant C*91. The *Cephalosporium* sp. was grown as described by Smith *et al.* (1967). The mycelium from 20 shake-flasks (each 500ml) was harvested 72h after inoculation, when antibiotic production had reached about half its maximum. The mycelium was separated by filtration through hardened filter paper in a Buchner funnel, and washed by three successive resuspensions in the funnel in boiled water and refiltration. The mycelial pad was removed and blotted between layers of filter paper. The damp-dry mycelium (190g) was transferred to a Waring blender; 7.5% (w/v) trichloroacetic acid (330ml) was added and the mixture blended for 30s. The mixture was left at room temperature for 30min. The mycelium was filtered off and washed with 5% trichloroacetic acid (100ml). To the combined extracts (450ml), 6% (w/v) $CdCl_2,2.5H_2O$ (112ml) was added, followed by 23ml of 5M-NaOH to adjust the mixture to pH6. The mixture was then brought to pH7.0 with 90ml of 1M-$NaHCO_3$, when a voluminous grey–green precipitate formed.

The precipitate was allowed to settle for 2h at 4°C, after which time the clear supernatant was decanted and the remainder of the material centrifuged. The sediment was washed twice with water and centrifuged, and then M-H_2SO_4 (30ml) was added slowly until virtually all the precipitate dissolved. The solution was cleared by centrifugation, brought to pH2 with 2.5M-H_2SO_4, and warmed to 40°C. A suspension of red Cu_2O in water (du Vigneaud & Miller, 1952) was added dropwise until no more precipitate appeared. The crystalline cuprous mercaptide was washed by centrifugation with boiled water until the washings were free of sulphate, then with ethanol, and finally dried *in vacuo* (123mg).

Decomposition of cuprous mercaptides. The preparation was suspended in boiled water (100mg mercaptide/15ml) and decomposed with H_2S. The precipitated sulphide was removed by centrifugation and washed at the centrifuge with boiled water (0.5vol./vol. of original suspension). Supernatant and washings were combined, freed of H_2S *in vacuo*, and freeze-dried.

Preparation of S-*sulphonates of the intracellular peptides.* A freeze-dried powder (90mg) obtained after decomposition of the cuprous mercaptides from the *Cephalosporium* mycelium was dissolved in water (3ml). A freshly prepared solution of 0.5M-potassium *o*-iodosobenzoate (5ml) was added, followed immediately by 0.5M-Na_2SO_3 in 0.1mM-EDTA (5ml). After 3h at room temperature the solution was adjusted to pH3 with 0.5M-H_2SO_4 and sulphate was then precipitated from the reaction mixture by the addition of M-barium acetate. The $BaSO_4$ was removed by centrifugation and the supernatant applied to a column (20cm × 2cm diam.) of Dowex 50 (X8; H^+ form; 200–400 mesh). Elution was carried out with water. The ninhydrin-positive fractions were pooled and freeze-dried (28mg). Paper electrophoresis at pH1.8, followed by chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.), revealed the presence of *S*-sulphonylglutathione, a small amount of glutathionesulphonic acid, and three compounds apparently corresponding to the S-sulphonates of peptides which had previously been detected in the sulphonic acid form and designated P1, P2 and P3.

Separation of S-*sulphonates of peptides P*1, *P*2 *and P*3. The mixture of *S*-sulphonates of peptides P1, P2 and P3 and glutathione, obtained by the above procedure (56mg), was separated by preparative electrophoresis on Whatman 3MM paper at pH1.8 for 2.5h into two components. The

larger component, which migrated slightly further than the remainder of the material towards the anode, was glutathione-*S*-sulphonate. The smaller component (10% approx. of the total) was eluted with water from the paper and the eluate freeze-dried (5.5mg). This was separated by preparative paper chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.) into three components, one of which, peptide P1, was not completely resolved from some remaining *S*-sulphonylglutathione. Each of these three components was eluted from the paper and freeze-dried. The amounts of peptides obtained were: P1, 0.3mg; P2, 0.5mg; P3, 2.2mg. However, analysis of the material corresponding to peptide P1 showed that it was still contaminated with a small proportion of *S*-sulphonylglutathione.

Reduction of S-*sulphonyl-peptide P*3. The *S*-sulphonate of peptide P3 (2.2mg) was dissolved in water (2.5ml). Cleland's reagent (4mg) was added and the solution brought to pH8 with 0.1M-NaOH. The solution was transferred to a spectrophotometer cuvette and N_2 bubbled through the solution for 2min. The cuvette was stoppered and the increase in E_{283} measured at 15min intervals. After 3h the reaction was complete. The reaction mixture was applied to a column (2cm×1cm diam.) of Dowex 50 (X4; H^+ form; 200–400 mesh) and the resin washed with boiled water (10ml). The column of resin was extruded into water (3ml) and 1M-pyridine was added with stirring until the supernatant had been brought to pH6. After removal of the resin by filtration, the filtrate was diluted with water (3vol.) evaporated *in vacuo* to approx. 3ml, and freeze-dried (0.5mg).

Preparation of ethoxycarbonyl derivatives of peptide methyl esters. The reduced peptide (0.3–0.5mg) was dissolved in water (2ml) and M-$NaHCO_3$ (5μl) and diethyl pyrocarbonate (2μl) was added to the solution. The mixture was shaken vigorously by hand for 45min, then acidified to pH2 with M-HCl and extracted twice with ethyl acetate (2ml). The ethyl acetate layers were combined, washed twice with water (2ml) and dried briefly over Na_2SO_4. Diazomethane in ether was added to the ethyl acetate solution until the latter remained pale yellow. After 15min the solution was evaporated to dryness. The residue (0.1–0.3mg) was redissolved in ethyl acetate for measurement of the mass spectrum.

Separation of the peptides as sulphonic acids. After oxidation of some preparations of the peptide mixture with performic acid, the sulphonic acids of peptides P1, P2, P3 and glutathione were separated by preparative paper electrophoresis and chromatography in the same manner as were the *S*-sulphonates. The sulphonic acid of peptide P1 was obtained uncontaminated by glutathionesulphonic acid.

*Isolation of amino acids from sulphonic acid of peptide P*3. The peptide (1.5mg) was hydrolysed in 6M-HCl at 105°C for 18h. The hydrolysate was evaporated to dryness, redissolved in 0.5M-acetic acid (0.5ml) and applied to a column (30cm×1cm diam.) of Dowex 1 (X8; acetate form; 200–400 mesh). The column was eluted with 0.5M-acetic acid and fractions (2ml) were collected every 15min. Analysis of samples (4×5μl) from each fraction by paper chromatography followed by coloration with ninhydrin indicated that valine was present in fractions 3–6, and α-aminoadipic acid in fractions 11–16. The column was then eluted with M-HCl. Analysis of samples (0.2ml) from each fraction (2ml) by the ninhydrin method of Moore & Stein (1954) indicated that cysteic acid was present in fractions 27–35. This was confirmed by paper electrophoresis and chromatography followed by coloration with ninhydrin. Fractions 3–6, 11–16 and 27–35 were each combined and freeze-dried. Yields: α-aminoadipic acid, 0.3mg; cysteic acid, 0.4mg; valine, 0.3mg.

Incubation of GSH, GSSG and glutathionesulphonic acid with carboxypeptidase A. GSH, GSSG and glutathionesulphonic acid (3.1mg) were each dissolved in 0.5ml of 0.1M-*N*-ethylmorpholine acetate, pH8.0, and 10μl of a solution of carboxypeptidase A (240μg/ml), pH9, added. The solutions were incubated at 27°C and samples (5μl) taken at 0, 24 and 48h for analysis by electrophoresis on paper at pH4.5 and paper chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.) followed by coloration with ninhydrin. Semi-quantitative estimations of the amount of glycine formed were made visually by comparison with glycine standards. GSH was hydrolysed more slowly than GSSG, but hydrolysis of both was virtually complete after 48h. Glutathionesulphonic acid showed only a trace of hydrolysis after 48h.

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