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Structure and Properties of a Homologue of Glutathione

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In the preceding paper (Carnegie, 1963*a*) the isolation of a new disulphide peptide from *Phaseolus aureus* was described. In this paper evidence will be presented to show that its structure is

γ-L-Glu-L-CyS-β-Ala
|
γ-L-Glu-L-CyS-β-Ala

This peptide in the thiol form is closely similar to glutathione, from which it differs by only one extra methylene group in the *C*-terminal amino acid, and it has the chromatographic properties expected of a higher homologue of glutathione and will substitute for glutathione in certain enzymic reactions. The name homoglutathione (homoGSSG) has been suggested as a short name for this peptide (Carnegie, 1963*b*).

This is the first isolation of a homologue of glutathione. A lower homologue, asparthione (β-aspartylcysteinylglycine), has been synthesized (Miller, Behrens & du Vigneaud, 1941), as has a higher homologue containing homocysteine in place of cysteine (Herrick & Todd, 1955; Gawron & Draus, 1959). Neither of these homologues has been found in Nature. A somewhat similar type of peptide, δ-(α-aminoadipyl)cyst(e)inylvaline, has been described in extracts of *Penicillium chrysogenum* (Arnstein & Morris, 1960). Two analogues of gluta-

thione, which are devoid of thiol groups, ophthalmic acid (γ-glutamylaminobutyrylglycine) and norophthalmic acid (γ-glutamyl-α-alanylglycine), have been found in the bovine lens (Waley, 1957, 1958). Dr W. J. P. Neish, Sheffield, has synthesized a mixture of the α- and γ-isomers of the cuprous mercaptide of homoglutathione (personal communication).

Price (1957) reported that the leaves of species of *Phaseolus* contained a thiol other than glutathione. He coupled *N*-(4-hydroxynaphth-1-yl)isomaleimide with the free thiol groups in leaf extracts and then separated them by paper chromatography. Apart from mentioning that it contained an amino group no evidence of its chemical structure was presented. Phaseothione, the name that he gave it, is rather inappropriate since his own results showed it to be present in *Glycine max* and *Trifolium repens*. The *N*-(4-hydroxynaphth-1-yl)isomaleimide adduct of phaseothione had a greater R_F in propan-2-ol–butan-1-ol–acetic acid (75:10:15) than the corresponding adduct of glutathione but was slower than the adduct of cysteine. The *N*-ethylmaleimide adduct of the reduced form of homoglutathione (homoGSH) has a similar relationship in butan-1-ol–acetic acid–water (4:1:5) to the *N*-ethylmaleimide adducts of glutathione and cysteine. From this chromatographic similarity and the finding that homoGSH was the only thiol that could be detected in freshly expressed juice of *Phaseolus aureus* seedlings (Carnegie, 1963*a*), it is thought that phaseothione is the same as homoGSH. HomoGSH is also probably identical with a peptide reported to be present in several varieties of bean seeds (Rinderknecht, Thomas & Aslin, 1958). This peptide, which was named peptide T, was not isolated but had similar behaviour to homoGSSG on paper chromatograms and on anion-exchange resin.

RESULTS AND DISCUSSION

Purity and properties

The isolated peptide was readily crystallized from water. When it was heated slowly in water it suddenly went into solution at 75–80°. The crystals were fine colourless needles, which melted with decomposition at 194–197°. In contrast there is no report in the literature on the crystallization of GSSG, which is more hygroscopic. The thiol form of the peptide appeared to be more hygroscopic than GSH and was not crystallized. Purity of the recrystallized peptide was checked by chromatography and electrophoresis by the methods described in the preceding paper (Carnegie, 1963*a*). Only one spot was detected with ninhydrin, iodoplatinate, nitroprusside–cyanide and chlorine–starch–potassium iodide. When it was oxidized with performic acid to the sulphonic acid derivative of homoGSSG (homoGSO_3H), and the product examined by paper electrophoresis, only one spot was detected. If the peptide had been isolated as a mixed disulphide with GSH or other thiols, then two or more spots would have been observed.

Because of the low solubility of the peptide and the limited amount of material available the molecular weight has not been determined directly. The equivalent weight was estimated from the titration curve (Fig. 1). Two groups were found to titrate between the iso-ionic point pH 3·2 and the region of no buffering around pH 7·5, which gave a value of 640 g. (required for homoGSSG: 640·7 g.) for the equivalent weight. From the titration curve the approximate pK values were estimated. The results, together with published data on GSSG, are shown in Table 1. The absorption bands in the infrared spectrum of the peptide were sharper than the bands with GSSG. The main bands are listed in Table 1. Elementary analyses of the peptide gave values reasonably close to the required values except for the nitrogen value. However, when the analyses were repeated the nitrogen value was closer to the required value for $C_{22}H_{36}N_6O_{12}S_2$.

Evidence for the structure

Amino acid analysis. Qualitative analysis of the hydrolysate of the crystalline peptide by chromatography and electrophoresis showed only three ninhydrin-positive spots with R_F values, mobilities and colours of glutamic acid, cystine and β-alanine. There was no trace of glycine or any other amino

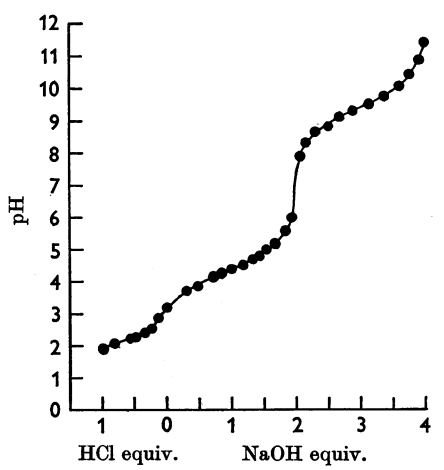

Fig. 1. Titration curve of the isolated peptide (640·7 g.) against equivalents of acid or alkali. Calculated from experiment with 13·45 mg. of peptide in 3 ml. of water titrated against 0·5N-NaOH and 0·5N-HCl; corrected for blank titration. For other details see text.

Table 1. *Comparison of approximate* p*K* *values and infrared spectra of the isolated peptide (homoGSSG) and oxidized glutathione*

Intensity: m, medium; s, strong.

	p*K*							
	p*I*	pK_1	pK_2	pK_3	pK_4	pK_5	pK_6	Infrared-absorption bands (wave no., cm.$^{-1}$)
HomoGSSG	3·2	—	2·3	3·9	4·9	8·9	9·9	3410m, 3040m, 2560, 1722m, 1659s, 1592s, 1537s, 1517s, 1442m, 1401m, 1377m, 1307m, 1278m, 1195, 1168, 1100, 1080, 1065, 1038
GSSG	2·85*	2·0	2·6	3·3	4·0	8·7	9·6	3290m, 3080m, 2600, 1727m, 1657s, 1537s, 1447m, 1410m, 1345m, 1235m, 1079, 1038
	—	—	—	3·15†	4·03	8·57	9·54	—

* Values from Pirie & Pinhey (1929).
† Values from Li, Gawron & Bascuas (1954).
Utzinger, Strait & Tuck (1963) have determined the infrared spectrum of GSSG.

acid when the papers were overloaded. Additional evidence for the identity of glutamic acid in the hydrolysate was obtained from experiments with L-glutamic acid decarboxylase, which caused the disappearance of glutamic acid, and instead γ-aminobutyric acid was found in the solution. β-Alanine gave a positive reaction with the specific reagent for ω-amino acids (Olesen-Larsen & Kjaer, 1960).

Quantitative paper chromatography of the hydrolysate of the peptide gave molar proportions cystine : glutamic acid : β-alanine, 1 : 2·05 : 2·05. Quantitative paper chromatography of the hydrolysate of the sulphonic acid derivative of the peptide gave the molar proportions cysteic acid : glutamic acid : β-alanine, 1 : 0·97 : 0·98, with cysteic acid representing 0·95 mole/369·4 g. (369·4 is the theoretical molecular weight of homoGSO_3H). Chromatography of a similar hydrolysate on a column of diethylaminoethyl-Sephadex A 25 gave recoveries of cysteic acid 0·92, glutamic acid 0·90 and β-alanine 0·94 mole/369·4 g.

These recoveries were rather low. The extent of destruction of the amino acids during oxidation and hydrolysis of the peptide bonds is not known. Schram, Moore & Bigwood (1954) used a somewhat similar procedure for the determination of cystine as cysteic acid. They could achieve a recovery of only 90 ± 2 %.

Additional quantitative information on the amino acid content of the peptide comes from experiments discussed more fully below. From the determination of carboxyl nitrogen on the peptide before hydrolysis the glutamic acid content was estimated to be 1·93 moles/mole of peptide (assuming molecular weight 640·7). From the determination of thiol groups in the reduced peptide the cystine content was estimated to be 1·03 moles/mole of peptide.

Configuration of the amino acids. The specific rotation of the peptide was $[\alpha]_D^{20.5} - 59.5 \pm 0.6°$ (*c* 0·5 in water, supersaturated). In comparison GSSG has a specific rotation of $[\alpha]_{5461} - 108°$ (Harington & Mead, 1935). On the other hand GSH has a specific rotation $[\alpha]_{5461} - 21°$ (Harington & Mead, 1935). Similarly, when the peptide was reduced by nascent hydrogen the specific rotation fell to $[\alpha]_D^{20} - 16·4°$ (*c* 1·3 in water). However, this cannot be considered as the true value for the thiol derivative since it was obtained on a solution which contained, in addition, approximately 10 % of the disulphide form, as judged by paper chromatography of the *N*-ethylmaleimide adduct, and a trace of glutamic acid. Additional evidence for the L-configurations was obtained by the use of L-glutamic acid decarboxylase, which decarboxylated all the glutamic acid to give γ-aminobutyric acid. Cystine might be expected to be partially racemized during the period of acid hydrolysis. However, L-amino acid oxidase oxidized all the cystine in the acid hydrolysate.

Sequence of the amino acids. For some of these studies the peptide was oxidized with performic acid to the sulphonic acid derivative. Glutamic acid was identified as the *N*-terminal amino acid by the fluorodinitrobenzene technique. From partial acid hydrolysates of the sulphonic acid derivative a peptide was identified as cysteinyl-β-alanine by the fluorodinitrobenzene technique, and from chromatographic and electrophoretic examination of its hydrolysates.

Carboxypeptidase did not hydrolyse the bond between β-alanine and cystine in the isolated peptide. This is in agreement with the published specificity of pancreatic carboxypeptidase. Hanson & Smith (1948) showed that this enzyme would not release β-alanine from benzyloxycarbonylglycyl-β-alanine.

Evidence for the presence of β-alanine in the *C*-terminal position was obtained from the titration curve (Fig. 1) and from a comparison of the electrophoretic mobilities of the peptide with γ-glutamyl-β-alanine (Fig. 2). From the titration curve two carboxyl groups were found to titrate in the pH 3–7 region. The values for pK_3 3·9 and pK_4

4·9 were substantially higher than the values pK_3 3·3 and pK_4 4·0 assigned to the glycine carboxyl groups in GSSG (Pirie & Pinhey, 1929), but similar to what would be expected for a peptide with β-alanine in the *C*-terminal position, because free glycine has pK_1 2·34 and free β-alanine has pK_1 3·60 (Greenstein & Winitz, 1961).

Waley (1958) showed that GSSG had similar electrophoretic mobility at pH 4 to γ-glutamyl dipeptides containing neutral amino acids. Hence, if β-alanine is in the *C*-terminal position and glutamic acid is linked to cystine by a γ-peptide bond, then the peptide should have approximately the same electrophoretic mobility at pH 4·0 as γ-glutamyl-β-alanine. This similarity is shown in Fig. 2.

Evidence for γ-glutamyl bond. If the amino group of glutamic acid is free, then γ-glutamyl peptide bonds are much more labile than α-peptide bonds (Wieland, 1954) and can be hydrolysed by dilute acid. Glutamic acid was liberated, from both the disulphide form and sulphonic acid derivative of the peptide, by heating them at 100° with N-hydrochloric acid for 2 hr.

Waley (1958) showed that γ-glutamyl peptides could be distinguished from α-glutamyl peptides by paper electrophoresis at pH 4·0. However, a glutamyl peptide with β-alanine in the *C*-terminal position would be expected to have a lower anionic mobility, owing to the weaker carboxyl group of β-alanine, than γ-glutamyl peptides with neutral α-amino acids in the *C*-terminal position. As already stated the peptide had similar mobilities to γ-glutamyl-β-alanine at pH 4. Electrometric titration showed a group with pK 2·3, which is close to the value pK_2 2·6 assigned to the α-carboxyl group of glutamic acid in GSSG (Pirie & Pinhey, 1929), and to the value 2·39 assigned to glutamic acid in ophthalmic acid (Calam & Waley, 1962).

Strong evidence for the γ-glutamyl peptide bond comes from determinations of the carboxyl nitrogen by the procedure of Van Slyke, Dillon, MacFadyen & Hamilton (1941). For carbon dioxide to be produced in the reaction with ninhydrin an amino group must be adjacent to a carboxyl group. Carboxyl nitrogen of the peptide was 32·2 % of the total theoretical nitrogen at pH 4·7. The required value for homoGSSG is 33·3 %. Van Slyke *et al.* (1941) found for glutathione 33·3 % at pH 2·5.

Evidence for the disulphide linkage. No pink colour was obtained when the peptide was treated with nitroprusside alone. Addition of cyanide caused the typical pink colour given by thiol groups to appear (Newton & Abraham, 1953). The peptide formed no adduct with *N*-ethylmaleimide before reduction with nascent hydrogen. After reduction an adduct was formed. Iodate titration showed no thiol groups before reduction of the peptide but after reduction there were 2·06 moles of thiol/mole of peptide (assuming molecular weight 640·7). When disulphide groups of GSSG, γ-glutamylcystine

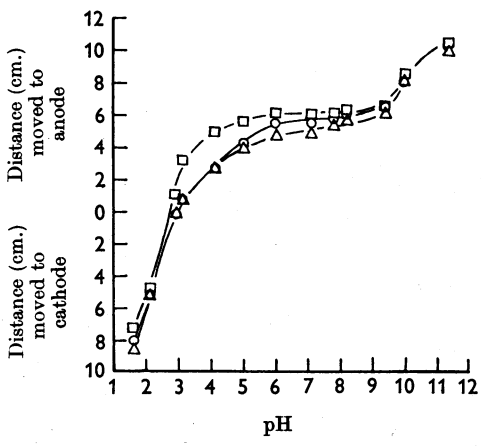

Fig. 2. Electrophoretic behaviour of the isolated peptide (○), GSSG (□), γ-glutamyl-β-alanine (△): distance moved (cm.) at different pH values on Whatman no. 3 MM paper at 4° with a field strength 23 v/cm. for 45 min.

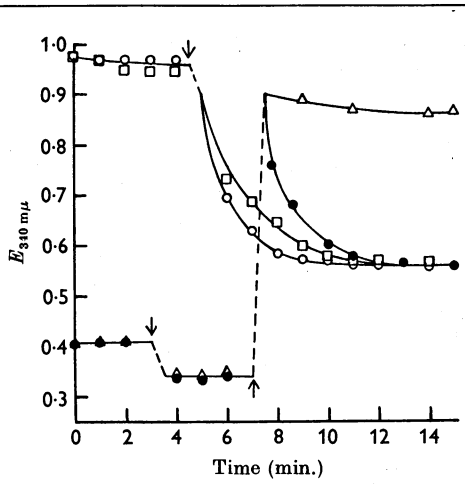

Fig. 3. Oxidation of NADPH by the isolated peptide (homoGSSG) and glutathione reductase prepared from seeds of *Phaseolus aureus*. Solutions used: NADPH, 0·3 mg./ml. of buffer; 0·78 mM-homoGSSG; 0·81 mM-GSSG; 0·1 M-phosphate buffer, pH 6·50. ○, Initially present: 1·5 ml. of buffer, 0·5 ml. of enzyme soln., 0·5 ml. of NADPH; 0·5 ml. of homoGSSG was added at the arrow. □, Initially as for ○; 0·5 ml. of GSSG was added at the arrow. ●, Initially present: 1·5 ml. of buffer, 0·5 ml. of NADPH; 0·5 ml. of homoGSSG was added at the first arrow, 0·5 ml. of enzyme soln. was added at the second arrow. △, Initially as for ●; 0·5 ml. of water was added at the first arrow; 0·5 ml. of enzyme soln. was added at the second arrow. - - -, Time taken for additions (0·5 min.). Extinction at 340 mμ was read against buffer in a Hilger Uvispek spectrophotometer at 25°.

Table 2. *Electrophoretic mobilities and chromatographic properties of oxidized glutathione, the isolated peptide (homoGSSG), their derivatives and some amino acids*

Electrophoresis: in pyridine–acetate, pH 4·0 (Grassmann, Hannig & Plöckl, 1955), Whatman no. 3MM paper on cooling plate at 4°, 23 v/cm. for 45 min. Approx. 10–20 μm-moles were applied. *N*-2,4-Dinitrophenylethanolamine was used as marker of electroendosmosis. Chromatography: on Whatman no. 1 paper, butan-1-ol–acetic acid–water (4:1:5) aged for 24 hr., descending development for 20 hr. at approx. 28°. Approx. 20 μm-moles were applied. Spots were located with ninhydrin. For abbreviations see text. $R_M = \log([1/R_F] - 1)$.

Compound	Anionic mobility $10^5\mu$ (cm.^{2}v^{-1} sec.$^{-1}$)	R_{Ala} (R_F for Ala 0·30)	R_M	ΔR_M
GSSG	6·3	0·22	1·124	−0·119
HomoGSSG	3·0	0·30	1·005	
GSH	4·8	0·80 streak	0·501	−0·070
HomoGSH	2·6	0·91 streak	0·431	
GSO_3H	13·0	0·30	1·005	−0·096
HomoGSO_3H	9·5	0·35	0·909	
GSH (*N*-ethylmaleimide adduct)	4·5	1·12	0·288	−0·112
HomoGSH (*N*-ethylmaleimide adduct)	2·3	1·33	0·176	
Aspartic acid	8·5	0·66	0·549	−0·094
Glutamic acid	4·6	0·85	0·455	−0·088
α-Aminoadipic acid	3·5	1·00	0·367	
Cystine	−1·3	0·25	—	—
Cysteic acid	15·0	0·36	—	—
Cysteine (*N*-ethylmaleimide adduct)	−1·3	1·50	—	—

(Harington & Mead, 1935) and L-cystine (Greenstein, Birnbaum & Otey, 1953) are reduced there is a change in the specific rotation from a high negative value to a more positive value. There was a similar change after reduction of the peptide. Pirie (1932) described how GSSG could be reduced with cuprous oxide to form the cuprous mercaptide. Cuprous oxide also reduced the peptide but the yield of mercaptide was poor.

Glutathione reductase was thought to be absolutely specific for GSSG. Even asparthione and γ-glutamylcystine are not reduced (Conn & Vennesland, 1951). However, as shown in Fig. 3, the peptide was readily reduced by a dialysed acetone-dried powder from the seeds of *Phaseolus aureus* with NADPH as a coenzyme.

The action of yeast glutathione reductase, of high specific activity, on GSSG and the peptide was compared. The rate of fall in extinction at 340 mμ for the two disulphides was the same and the rate for a mixture of the two was only slightly slower. These experiments suggest that the structure of the peptide is closely similar to that of GSSG.

Chromatographic evidence for homologous structure. Chemical structure can be correlated with chromatographic behaviour. Howe (1960) showed that for a homologous series of dicarboxylic amino acids there was a linear relationship between a plot of R_M values, where $R_M = \log([1/R_F] - 1)$, against the number of CH_2 groups in the molecules. Thus the R_M values of homoGSSG, homoGSO_3H and the *N*-ethylmaleimide adduct of homoGSH should each be less than that of the corresponding form or derivative of glutathione. As shown in Table 2, the isolated peptide and its derivatives did have this expected relationship to glutathione. However, as conditions for chromatography were not ideal the R_M increment (ΔR_M) for each CH_2 group was not constant.

Comparison of glutathione and homoglutathione

HomoGSH is an activator of glyoxalase but on a molar basis it is only 55 % as active as GSH. The lower homologue of GSH, asparthione, and isoglutathione (α-glutamylcysteinylglycine) are respectively 67 % and 34 % as active as GSH (calculated from Behrens, 1941).

The chief differences between homoGSSG and GSSG are in their solubilities and in the higher pK_3 and pK_4 values of homoGSSG. One difference, which may be of physiological importance in people who eat food prepared from the genus *Phaseolus*, is the failure of pancreatic carboxypeptidase to hydrolyse the cystinyl-β-alanine bond in homoGSSG.

In species of *Phaseolus* homoGSH may fulfil the role GSH has in other tissues as a cofactor of several enzymic reactions. A comparison of the behaviour of these two thiols as cofactors may help to solve the problem of what the main role of GSH is in metabolism.

EXPERIMENTAL

Abbreviations. HomoGSSH and homoGSH, oxidized and reduced forms of homoglutathione; homoGSO_3H, sulphonic acid derivative of homoglutathione.

Materials. In the experiments described below the procedures were checked against commercial samples of GSH (99 % pure by iodate titration) from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and GSSG (Found: C

39·0; H, 6·3; N, 12·8. Calc. for $C_{20}H_{32}N_6O_{12}S_2$: C, 39·2; H, 5·3; N, 13·7%) from Mann Laboratories, New York. L-Amino acid oxidase type I, L-glutamic acid decarboxylase type I, carboxypeptidase A (treated with di-isopropyl phosphorofluoridate), NADPH type I and purified glutathione reductase type III (nominal activity 1 mg. reduces 250 μ-moles of GSSG/min.), were from Sigma Chemical Co., St Louis, Mo., U.S.A. Diethylaminoethyl-Sephadex A25 was from AB Pharmacia, Uppsala. γ-L-Glutamyl-β-alanine was kindly supplied by Dr C. J. Morris.

General methods

Except where indicated experiments were done at room temperature (28–31°).

Crystallization and properties of homoGSSG. Fractions 112–119 inclusive from the diethylaminoethyl(DEAE)-Sephadex column discussed in the preceding paper (Carnegie, 1963*a*) were evaporated to dryness and the dry matter (390 mg.) was dissolved in 11 ml. of water at 70–80°. When the solution was cooled to room temperature the peptide crystallized. The crystals were collected and washed with water, yield 184 mg. Crystallization, followed by three washings with water, was twice repeated; the crystals were dried to constant weight (126 mg.) over conc. H_2SO_4 and NaOH *in vacuo* at room temperature; they were stored over silica gel at 18°, and dried again as described above before any quantitative analysis. As the crystals were slightly hygroscopic, precautions were taken during weighing to exclude the humid Singapore air (relative humidity 75–90%). When heated at 1°/min. on a micro-hot-stage, preheated to 180°, bis-γ-L-glutamyl-L-cystinyl-bis-β-alanine melted with decomposition at 194–197° (uncorr.). Optical rotation was determined in a Hilger polarimeter with a 0·5 dm. micro-tube. HomoGSSG was dissolved in water at 80° and the solution quickly cooled to 20·5°: $[\alpha]_D^{20.5} - 59.5 \pm 0.6°$ (*c* 0·5 in water, supersaturated). The infrared spectra of homoGSSG and GSSG were obtained by using the KBr-disk technique on a Nikon Bunko IRS-5 spectrophotometer. The main bands are listed in Table 1.

Elementary analyses. Analyses were by Pascher and Pascher, Bonn. On drying at 60° *in vacuo* the peptide lost weight (5·7%). Analyses were made on this dried product (Found: C, 41·6; H, 5·8; N, 12·5; O, 30·5; S, 9·9; ash, 0·0. $C_{22}H_{36}N_6O_{12}S_2$ requires C, 41·2; H, 5·7; N, 13·1; O, 30·0; S, 10·0%). On drying at 100° *in vacuo* it lost an additional 0·16% of its weight (Found: C, 41·5; H, 6·1; N, 13·2%).

Preparation of homoGSH. This was prepared from homoGSSG by electrolytic reduction. In principle the method of Dohan & Woodward (1939) was used, but, to avoid contamination by KCl, a new type of diaphragm reduction cell with a stirred mercury cathode was used (P. R. Carnegie, unpublished work). A solution of homoGSSG (18·54 mg. in 9 ml. of water) containing 0·05 ml. of N-HCl was treated in the cell with a current density of 2·5 mA/cm.^{2} at 4°. Portions (0·2 ml.) were removed at intervals and titrated with potassium iodate (Patterson & Lazarow, 1955). Initially there was 0·0 mole of SH/mole of homoGSSG (assuming molecular weight 640·7); after 2 min. there were 1·06, 7 min. 1·92, 12 min. 2·08 and after 26 min. 2·06 moles of SH/mole of homoGSSG. A portion of the solution was mixed with excess of *N*-ethylmaleimide solution (0·5%, w/v) and examined by chromatography in butan-1-ol–acetic acid–water (4:1:5, by vol.). All the peptide in the treated sample was now present as the *N*-ethylmaleimide adduct. There was a faint trace of glutamic acid. The solution of the thiol was freeze-dried. HomoGSH was very hygroscopic and was not crystallized. A solution of the freeze-dried homoGSH in water was titrated with iodate to determine the concentration. This solution had $[\alpha]_D^{20}$ −16·4° (*c* 1·3 in water). However, this value must not be taken as the true rotation of the thiol since an examination of a portion of the solution treated with excess of *N*-ethylmaleimide showed that roughly 10% of the ninhydrin colour was now given by the disulphide form. There was also a trace of glutamic acid. The remainder of the solution was freeze-dried.

Preparation of sulphonic acid derivative (homoGSO$_3$H). For some of the analyses it was advisable to oxidize the disulphide bond of cystine to the more stable sulphonic acid derivative. Performic acid (Sanger, 1949), 0·5 ml., was added to 5 mg. of the peptide and kept at room temp. for 1 hr. Water (1·5 ml.) was then added and the solution freeze-dried. Addition of water and freeze-drying was repeated twice. Chromatographic and electrophoretic examination showed one spot (see Table 2 for R_{Ala} value and mobility). HomoGSO$_3$H was not crystallized.

Structural studies

Amino acid composition. Hydrolysis was carried out with constant-boiling HCl at 100–105° for 16 hr. *in vacuo*. When the hydrolysate of homoGSSG was examined by two-dimensional chromatography and electrophoresis followed by chromatography (Carnegie, 1963*a*) there were spots with the R_F values and mobilities of glutamic acid, cystine and β-alanine. The colours given with the ninhydrin reagent (McEvoy-Bowe & Lugg, 1961) were typical of these amino acids. The hydrolysate of homoGSO$_3$H, when mixed with an equal amount of the authentic amino acids, gave by both procedures three spots with the R_F values and mobilities of glutamic acid, cysteic acid and β-alanine. When a chromatogram of the hydrolysate of homoGSO$_3$H was treated with copper nitrate (Olesen-Larsen & Kjaer, 1960) before ninhydrin β-alanine reacted to give a characteristic brown spot.

The hydrolysates of homoGSSG and homoGSO$_3$H were examined enzymically. Manometric procedures were used to ascertain completion of the reactions. L-Amino acid oxidase (Bonetti & Dent, 1954) caused the disappearance of cystine but not cysteic acid, glutamic acid or β-alanine. L-Glutamic acid decarboxylase (Najjar, 1957) converted glutamic acid in both hydrolysates into γ-aminobutyric acid. In these experiments electrophoresis and chromatography were used to identify the products.

The hydrolysate of homoGSSG was examined by paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (4:1:5). Ninhydrin colours of the amino acids from the hydrolysate and from a control solution run on the same paper were estimated by the sensitive procedure of McEvoy-Bowe & Lugg (1961). The average of 12 determinations gave molar proportions cystine:glutamic acid:β-alanine, 1·0:2·05:2·05. By the same procedure the hydrolysate of homoGSO$_3$H was found to contain 0·95 mole of cysteic acid, 0·91 mole of glutamic acid and 0·93 mole of β-alanine/mole of homoGSO$_3$H (assuming molecular weight 369·4; this value is assumed in the calculations below). A portion (2 ml.) of a hydrolysate of homoGSO$_3$H (9·6 mM) was applied to a column (20 cm. × 0·5 cm. diam.) of DEAE-Sephadex A25 in the acetate form. β-Alanine was washed

off with water and made to 20 ml. Glutamic acid was eluted with approx. 18 ml. of N-acetic acid and after being approximately neutralized with 15N-NaOH the solution was made to 20 ml. Cysteic acid was eluted with 0·5N-HCl and after neutralizing was made to 20 ml. A portion (1 ml.) of the solutions was heated for 15 min. in a boiling-water bath, with 1 ml. of Moore & Stein's (1954) ninhydrin reagent and, after dilution with 50% (v/v) ethanol, extinctions were read at 570 mμ. Standard solutions of the component amino acids were used as a comparison. From two determinations the average values found were: 0·92 mole of cysteic acid, 0·90 mole of glutamic acid and 0·94 mole of β-alanine/mole of homoGSO_3H.

Partial acid hydrolysis. HomoGSO_3H (9·1 μmoles) was heated for 2 hr. at 100° with 1 ml. of N-HCl *in vacuo*. The hydrolysate was examined by quantitative paper chromatography as above. With ninhydrin there was a strong spot of glutamic acid (62% of theoretical total), a weak spot of β-alanine and a spot in the cysteic acid position. There was a spot not present in the strong acid hydrolysate with R_{Ala} 0·64. There was also a spot, detected with Cl_2–starch–KI (Rydon & Smith, 1952), with R_{Ala} 2·0, which was the same as that quoted by Smith (1960) for pyrrolid-2-one-5-carboxylic acid. The remainder of the hydrolysate was fractionated by chromatography on Whatman no. 3MM paper and the zone with R_{Ala} 0·64 eluted. Strong acid hydrolysates of this zone showed strong spots of cysteic acid and β-alanine in approximately equimolar amounts. There was also a weak spot of glutamic acid. Treatment of the material from the zone with 1-fluoro-2,4-dinitrobenzene (as below) and hydrolysis of the DNP derivative gave a spot with the chromatographic properties and solubility of DNP-cysteic acid. The only amino acid now reacting with ninhydrin was β-alanine.

N-Terminal and C-terminal amino acids. HomoGSO_3H (2·2 μmoles) was treated with excess of 1-fluoro-2,4-dinitrobenzene (Waley, 1956) and the ether-soluble fraction of the hydrolysate was examined by chromatography with the 'toluene' and 1·5M-phosphate buffer (Fraenkel-Conrat, Harris & Levy, 1955). DNP-glutamic acid was the only DNP-amino acid present. For a quantitative estimation a portion was chromatographed in the 'toluene' solvent with standard amounts of DNP-glutamic acid prepared by treating glutamic acid in the same manner as the peptide. The yield of DNP-glutamic acid was 94% of that expected for homoGSO_3H. In the aqueous fraction only cysteic acid and β-alanine were detected.

Attempts were made with carboxypeptidase (Fraenkel-Conrat *et al.* 1955) to determine the *C*-terminal amino acid of homoGSSG. Ratios of 50:1 and 20:1 moles of substrate: mole of enzyme were tried, and even with incubation at 37° for 24 hr. no β-alanine was liberated and the peptide had unchanged chromatographic and electrophoretic properties. The same enzyme preparation readily liberated glycine from GSSG.

Electrometric titration. HomoGSSG (13·45 mg.) was dissolved at 80° in 3 ml. of water, free from CO_2, and cooled to the operating temperature of 20°. A Beckman Zeromatic pH-meter with a combination glass electrode, no. 39183, was standardized against 0·05M-potassium hydrogen phthalate buffer, pH 4·0, and 0·01M-sodium tetraborate buffer, pH 9·22. The peptide was titrated against 0·5N-NaOH while the solution was stirred with N_2. The back titration, with 0·5N-HCl, coincided with the NaOH titration curve. The results, corrected for a blank titration with water, are shown in Fig. 1 and Table 1. Because of the high titration value for the water blank below pH 2, pK_1 of homoGSSG was not determined.

Electrophoretic properties. The electrophoretic properties of homoGSSG, GSSG and γ-glutamyl-β-alanine were compared in the apparatus of Kunkel & Tiselius (1951) on a cooling plate at 4°. Whatman no. 3MM papers (12 cm. × 46 cm.) were dipped in the buffers. Universal buffer mixtures (Long, 1961), diluted with an additional 1200 ml. of water, were used for pH values from 2·9 to 11·5, and sodium acetate–HCl mixture (2 mM-sodium acetate titrated with N-HCl to the required pH) for pH 1·6 and 2·05. Papers were equilibrated for 30 min. before the samples of the peptides (5 μl.; approx. 1 mM) were applied to the centre of the paper. A voltage of 23 v/cm. was applied for 45 min. Peptides were located with ninhydrin. *N*-2,4-Dinitrophenylethanolamine was used as a marker of electroendosmosis. The results are shown in Fig. 2.

Miscellaneous methods. Routine chromatography and electrophoresis were carried out as described in the preceding paper (Carnegie, 1963*a*). Carboxyl N content of homoGSSG (2–3 mg.) was determined with ninhydrin at pH 4·7 (Van Slyke *et al.* 1941, as modified by Synge, 1951). Carboxyl N represented 32·2 ± 0·2% of the total theoretical N. The sulphonic acid derivative of GSH was prepared by the method used for homoGSO_3H. The *N*-ethylmaleimide adducts of GSH and cysteine were prepared by mixing a solution of the thiol with excess of 0·5% (w/v) *N*-ethylmaleimide.

Behaviour with glyoxalase and glutathione reductase

Activator of glyoxalase. A solution prepared from freeze-dried homoGSH recovered from the optical rotation measurements was used. By iodate titration the concentration of SH groups was 0·529 mM. A solution of GSH of approximately the same molarity (0·508 mM) was used as a comparison. An acetone-dried powder of yeast was used as a source of glyoxalase. The method used was that of Behrens (1941). From five determinations with homoGSH (0·106–0·529 μmole) the average rate of liberation of CO_2 was 63 μl./μmole/hr., and from four with GSH (0·102–0·254 μmole) the rate was 114 μl./μmole/hr. Thus on a molar basis homoGSH was only 55% as active as GSH.

Reduction by glutathione reductase. The experiments described in Fig. 10 (*a*) and (*b*) of the paper by Mapson & Goddard (1951) were repeated with dialysed acetone-dried powder of seeds of *Phaseolus aureus* instead of pea seeds. Extinction was read at 340 mμ. Spacers were used to raise the 1 cm. cells. The temperature was 25°. Fig. 3 gives the details of the solution and the results obtained. At the end of the experiment the solutions were treated with excess of *N*-ethylmaleimide. *N*-Ethylmaleimide adducts of GSH and homoGSH were observed on a chromatogram of these solutions.

The action of purified glutathione reductase (from yeast) on homoGSSG, GSSG and a mixture of the two disulphides was compared. Potassium phosphate buffer, pH 7·4 (Hird, 1962), was used in this experiment. The concentrations and conditions were as above and in Fig. 3. The enzyme solution was diluted 200-fold with buffer. Initially the cell contained 1·5 ml. of buffer, 0·5 ml. of disulphide solution and

0·5 ml. of NADPH solution. After a few minutes 0·1 ml. of the diluted enzyme solution (0·5 μg.) was added within 5–8 sec. The average rate of change in extinction at 340 mμ was determined over the first 2 min. from the addition of the enzyme. Oxidation of NADPH was complete at 4·5 min. The rate of change in extinction with GSSG and homoGSSG was the same (0·11/min.) and for the mixture of equal volumes of the disulphide solutions (0·25 ml.) the rate was slightly slower (0·094/min.).

SUMMARY

1. Evidence for the structure of a new disulphide peptide isolated from etiolated seedlings of *Phaseolus aureus* is presented. The peptide is bis-L-γ-glutamyl-L-cystinyl-bis-β-alanine, which, in the thiol form, is a higher homologue of glutathione, and has thus been called homoglutathione (homoGSSG).

2. Oxidized glutathione is much more soluble than homoGSSG. The pK_3 and pK_4 values of homoGSSG are appreciably higher than the corresponding values of GSSG.

3. Some chromatographic and electrophoretic properties of homoGSSG, homoGSH, the sulphonic acid derivative of homoGSSG and the *N*-ethylmaleimide adduct of homoGSH are listed.

4. Carboxypeptidase A does not hydrolyse the cystinyl-β-alanine bond of homoGSSG.

5. HomoGSH is an activator of glyoxalase but has only 55 % of the activity of GSH. HomoGSSG will oxidize NADPH in the presence of glutathione reductase.

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