The Enzymic Reaction of Amino Acids with Glutathione

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(Received 4 August 1953)

It has been shown that kidney extracts will bring about the exchange of L-amino acids for cysteinylglycine in reduced glutathione (GSH) to form the corresponding γ -glutamyl peptides (Hanes, Hird & Isherwood, 1950, 1952). The enzymic reaction of an amino acid, e.g. L-alanine with GSH, and the products formed in the system studied can be represented diagrammatically as follows:

In the previous investigations the evidence for a reaction was the appearance of a new peptide on chromatograms of digest samples and was of a qualitative nature. A quantitative comparison of the reactivities of the various amino acids with GSH in the system under investigation involves the estimation of either a peptide or an amino acid, and the estimation must be made in the presence of other peptides and amino acids.

Several possible methods for the estimation of the reactivity of an amino acid with GSH were considered. (1) The estimation of the appropriate γ -glutamyl peptide formed. Large variations in the physical and chemical properties of these peptides would make each estimation an individual problem, especially since such estimations would have to be made in the presence of amino acids and closely related peptides. However, in some cases roughly quantitative estimations can be made by comparing the colour intensity of peptide spots after separation by paper chromatography.

(2) The estimation of residual GSH by use of glyoxalase. In the system being studied, two molecules of GSH would react to form the tetrapeptide γ -glutamylglutathione which might not act as a coenzyme for glyoxalase. If this were so, one molecule of cysteinylglycine would be formed but two molecules of GSH would 'disappear'. Thus there would be a disparity between residual GSH and the exchange reaction. This has indeed been shown by Waelch (1952). Further, an amino acid reacting with γ -glutamylglutathione could set free a molecule of GSH and no cysteinylglycine.

(3) The estimation of cysteinylglycine and cysteine by the Lugg (1933) modification of the Sullivan method (1929). Initially this was thought to be suitable but it was found that during the estimation procedure GSH gave rise to significant amounts of cysteinylglycine and/or cysteine. The method was abandoned in favour of the following.

(4) The estimation of cysteine. The enzymic preparations contained an active peptidase which hydrolysed cysteinylglycine to cysteine and glycine. Preliminary experiments showed that the addition of certain amino acids to the enzyme-GSH system brings about the liberation of cysteine in excess of that liberated by the control without amino acid. The amount of cysteine produced in excess of that liberated by the control varies with the different amino acids and, with certain qualifications, can be taken as an index of their reactivity in the system.

EXPERIMENTAL

Estimation of cysteine

The method used is a modification of that developed by Nakamura & Binkley (1948). It depends on the blue colour produced specifically by cysteine in the presence of brucine and persulphate under acid conditions. Difficulties were encountered with the method. The colour intensity was found to be dependent not only on the concentration of cysteine but was also influenced by the concentrations of brucine, persulphate and hydrogen ions, as well as by time and temperature. However, these conditions can be standardized.

We found that tryptophan and methionine (cf. Nakamura & Binkley, 1948) seriously interfered with colour development and the method could not be used in their presence. In addition, ethionine was found to inhibit the colour production. The concentration of glycine was doubled to eliminate the small depression of colour production which was caused by amino acids. In the absence of cysteine the reagents produce a reddish colour, the absorption spectrum of which is reproduced in Fig. 1. This colour production falls off with increased cysteine concentration as shown. The absorption due to the red complex is negligible at 660 m μ , and optical densities have accordingly been measured at this wavelength. Absorption due to the red colour was not increased by addition of chloride which is known to react at a higher temperature to give a similar colour (Binkley, 1948). To enable low cysteine concentrations to be measured, this competing reaction, leading to the red complex, was prevented by adding 0.2 mg. cysteine to every estimation.

Fig. 1. Absorption curve of cysteine-brucine complex in the presence of varying amounts of cysteine. Recorded optical densities of solutions of cysteine-brucine complex were read against a water blank.

Fig. 2. Cysteine and cysteine-GSH calibration curves. Optical densities recorded were read against 0.2 mg. cysteine blank. In cysteine-GSH solutions the proportions of each were such as arise from GSH by hydrolysis. Optical densities were measured at 660 $m\mu$.

GSH does not itself react with brucine to give a blue colour, but it does markedly reduce the colour intensity produced by cysteine (Fig. 2). To estimate cysteine in the presence of GSH, a separate calibration curve is therefore necessary. On the cysteine-GSH calibration curve each point

represents cysteine and GSH concentrations corresponding to the amounts resulting from the progressive breakdown of GSH (1.012 mg.). This curve, in the range in which we are interested, is nearly linear, unlike the cysteine calibration curve. Because of the depression of colour production by GSH, the background cysteine enables smaller amounts of cysteine to be measured when GSH is present. Nakamura & Binkley (1948) do not record any interference by GSH. Their finding that in enzymic experiments GSH inhibits at higher concentrations has an alternative explanation in terms of the colour depression described.

Reagents

The reagents used were all of analytical quality.

Glycine-sulphuric acid. Glycine (5 g.) in 100 ml. of 12.5% (v/v) sulphuric acid. This reagent is stable for months.

Solution for background cysteine. Cysteine (0.2 mg., or equivalent amount of cysteine HCl) in 1 ml. water. This reagent is freshly made up daily.

Brucine solution. Brucine (1 g.) in 100 ml. 5% (v/v) sulphuric acid. This reagent is made up weekly.

Potassium persulphate solution. Potassium persulphate (0.5 g.) in 100 ml. water. This reagent is stable for at least 2 weeks.

Trichloroacetic acid (TCA). 50% (w/v).

Procedure

The colour was developed in test tubes which had a small process on the inside, just above the 8 ml. mark, to facilitate pipette drainage. The reagents were added in the following order: 2 ml. glycine/sulphuric acid reagent; 1 ml. cysteine reagent; 2 ml. brucine reagent; 2 ml. TCA filtrate containing from 0.0 to 0.4 mg. cysteine, or 2 ml. water in the control (the TCA and phosphate from the digest did not affect colour development); 1 ml. potassium persulphate reagent.

Optical densities were measured at 660 $m\mu$ on a Beckman spectrophotometer, Model DU.

The reaction resulting in development of colour begins when the persulphate is added. The persulphate was therefore added to successive tubes at 1 min. intervals. After mixing of the contents, the tubes were immersed and the colour developed in a water bath at $30 \pm 0.1^\circ$ for 45 min. The tubes were then removed and placed in an ice bath for 2 min. The blue solutions were transferred to the spectrophotometer cells and 4 min. after removal from the constant temperature bath the cysteine blank was read off against water (as a check on the reagents). For 0.2 mg. cysteine, $\log I_0/I$ at 660 $m\mu$ was about 0.170. The background cysteine solution was then set at zero (5 min. after removal from bath) and the unknowns were read at minute intervals, all 45 min. after the persulphate additions. The amount of cysteine present or the GSH broken down was then read from the graph (Fig. 2). The method is reproducible to $\pm 3\%$.

Estimation of cysteine produced from GSH by kidney extracts in the presence of amino acids

To enable the reactivities of a wide range of amino acids with GSH to be tested, it was convenient in most cases to use an enzyme concentration that brought about 35–40% GSH breakdown during the incubation period of 30 min. at 30° , i.e. in the absence of any added amino acid. Extracts

prepared in the following manner were suitable: 3 g. of frozen sheep kidney cortex were disintegrated in 20 ml. of ice-cold 0.2M potassium phosphate buffer, pH 7.4, in a glass homogenizer for 30 sec. at 1400 rev. min.⁻¹. After centrifugation for 15 min. at 1500 g to remove the coarse fraction, the supernatant was stored at -10° in 2 ml. portions until needed. Under these conditions the enzyme was stable for months, and it was therefore possible to compare a large range of amino acids using a single enzymic preparation. A small amount of cysteine and of GSH was present in the enzymic preparations. After incubation, cysteine (0.010-0.015 mg./2 ml. sample) was found in the six preparations tested. In any one enzymic preparation the endogenous cysteine was constant and therefore the order of reactivities of the amino acids was not altered.

The systems and samples for cysteine analysis were prepared as follows. To 2 ml. enzymic preparation in 0.2M potassium phosphate buffer, pH 7.4, were added 2 ml. amino acid solution (neutralized when necessary) and 1 ml. GSH solution (6.32 mg./ml., 20.6 μ moles) in this order. This quantity of GSH corresponds on complete hydrolysis to 0.4 mg. cysteine in the final 2 ml. TCA supernatant. The GSH solution was prepared afresh before each incubation and dissolved in a vessel in which the air had been displaced by N₂. The reaction was carried out in stoppered test tubes under N₂ in a water bath at 30° for 30 min. At the end of this time, 4 ml. were transferred to a glass-stoppered 10 ml. centrifuge tube containing 5 ml. of water and 1 ml. of 50% TCA. The tube was stoppered, the contents were mixed, allowed to stand for 5 min. and then centrifuged lightly; 2 ml. of the supernatant was then used for analysis. Cysteine, when added to the enzymic preparations and incubated under the conditions used, could be recovered within experimental error, showing the absence of significant cysteine-desulphurase activity.

Chromatographic procedure

The system was on a scale of one-tenth of that used for cysteine estimations. To 0.2 ml. enzymic preparation in 0.02M potassium phosphate buffer, pH 7.4, were added 2 μ moles neutralized GSH in 0.1 ml., and 0.2 ml. amino acid solution (neutralized when necessary). Incubation was carried out in stoppered 9 x 75 mm. test tubes in an atmosphere of N₂. The enzymic preparation was dialysed in a moving bag against 0.02M potassium phosphate buffer for 4 hr. before use to remove amino acids present in the enzymic preparation. After 30 min. incubation at 30°, 1.0 ml. of warm ethanol containing 2.4 μ moles *N*-ethylmaleimide was added to precipitate proteins and block -SH groups (Hanes *et al.* 1950). Cysteinylglycine when coupled with *N*-ethylmaleimide in this manner is capable of being detected on chromatograms at a concentration of 0.001M. Under the conditions used, this represents a figure of 5% GSH breakdown. After centrifugation, the supernatant was collected, the precipitate washed once with 0.2 ml. 66% ethanol (v/v) and the combined supernatant liquids evaporated *in vacuo* over NaOH in a small evaporating cup. The residue was then taken up in 0.05 ml. water and 0.002 ml. portions were used for chromatography. Solvents used were phenol saturated with water, butanol: water: glacial acetic acid (50:40:10, v/v), propanol: water (80:20, v/v) as such, and with 0.5 ml. NH₃ (sp.gr. 0.880) added to the chromatography jar. Time of development varied according to separation required: 15 hr. for short

runs; up to 100 hr. for long runs. In the longer runs, papers had a thick pad of filter paper clipped to the bottom of the paper strip.

RESULTS

Trial experiments showed that with the concentration of GSH chosen, 20.6 μ moles/5 ml., and an amino acid concentration of 132 μ moles/5 ml., increases in cysteine liberated over a 30 min. incubation period could be observed. At this stage of the work the effect of the concentration of amino acids on the liberation of cysteine had not been determined. This aspect is discussed in the following section. Table 1 gives the excess of cysteine liberated from GSH by a wide range of amino acids and some of their derivatives. Estimations of the liberated cysteine were carried out in duplicate and always with a GSH control without added amino acid.

The results given in Table 1 show that L- α -amino acids differ markedly in their abilities to liberate cysteine from GSH. The DL-amino acids, where tested, gave approximately half the values of the

Table 1. *Comparative reactivity of various amino acids with GSH*

(System: as described in Experimental with 2 ml. enzyme preparation, 2 ml. amino acid solution containing 132 μ moles (control: 2 ml. water in place of amino acids) and 1 ml. solution containing 20.6 μ moles GSH. Incubation: 30 min. under N₂ at 30°. Figures represent additional cysteine liberated in excess of control by amino acids and are expressed in terms of % GSH breakdown. Figures in brackets calculated for L- acids from DL- acids $\times 2$. Reactivities measured with a single preparation have been listed separately to enable more accurate comparisons to be made.)

Amino acid	GSH breakdown in excess of control (%)	
	In a single preparation	Results from other preparations
Glycine	33	26, 27, 28, 32
L-Alanine	24 (28)	17 (10)
DL- α -Amino-isobutyric acid	—	0
DL- α -Amino-n-butyric acid	12	6
L-Valine	—	2 (-1)
DL-Norvaline	—	22
L-Isoleucine	—	2 (0)
L-Leucine	21	16 (16)
DL-Norleucine	21	20
L-Phenylalanine	—	21, 25, 26
DL-Serine	20	17
DL-Threonine	3	3, 6
L-Aspartic acid	2	-1, -4
L-Asparagine	27	23
L-Glutamic acid	21	15, 16
L-Glutamine	37	42, 43, 44
L-Histidine	36	19, 21, 23, 24
L-Ornithine	17	—
L-Citrulline	37	—
L-Arginine	33	18
L-Lysine	22 (26)	20, 21, 24
L-Methionine sulfoxide	43	—

Table 2. *Comparative reactivity of the more reactive amino acids with GSH*

(System: as described in Experimental with 2 ml. enzymic preparation, 2 ml. amino acid solution containing 132 or 264 μ moles (control: 2 ml. water in place of amino acids) and 1 ml. solution containing 20.6 μ moles GSH. Incubation: for varying periods under N_2 at 30°. Figures represent additional cysteine liberated, in excess of control, by amino acids and are expressed in terms of % GSH breakdown. The controls corresponded to 12, 24 and 43% GSH breakdown for 5, 10 and 20 min. incubations respectively.)

spectively.)		Time of incubation (min.)		
		5	10	20
	Concentration	GSH breakdown in		
	(μ moles/5 ml.)	excess of control (%)		
Amino acid				
L-Methionine sulphoxide	132	36	62	41
L-Glutamine	132	28	53	45
L-Citrulline	132	28	42	35
DL-Norleucine	264	11	26	—
DL-Norvaline	264	9	26	—

L-form; there is thus no pronounced inhibition by the D-form. Amino acids substituted on the amino group such as sarcosine, L-proline and L-hydroxyproline were not reactive. Tryptophan, methionine and ethionine interfered with the colour production by cysteine, and so quantitative data for these amino acids cannot be given. However, chromatographic evidence has been obtained to show that these amino acids react with GSH in the system. Tyrosine was too insoluble for comparisons with other amino acids to be made. Urea, ammonium chloride, 8-hydroxyquinoline and chloramphenicol had no effect on the system.

With the more reactive amino acids, over 80% of the GSH was broken down by the end of the reaction period. This resulted in a reduction of the amount of GSH available as a substrate and in progressive slowing of the reaction; thus, these amino acids are more reactive than indicated in Table 1. As the range of reactivities of the amino acids was too great for them to be compared on the same time scale, the most reactive ones were tested over shorter periods of time. The results are given in Table 2.

Effect of amino acid concentration on the liberation of cysteine

Figs. 3 and 4 show the effect on the liberation of cysteine from GSH of increasing concentrations of various amino acids. It can be seen that as the concentrations of L-aspartic acid, L-valine and L-isoleucine were increased, the liberation of cysteine decreased. In contrast, the other L- α -amino acids, up to a certain concentration depending on the amino acid concerned, caused an increase in the amount of cysteine liberated. At higher concentrations, however, there was a decline in the cysteine

liberated, and in some cases it fell below the control with GSH alone, e.g. DL-threonine. The increase in cysteine liberation with increasing substrate concentration was probably due to increased saturation of the active centre of the enzyme. This increase in cysteine liberation was usually paralleled by an increase in corresponding γ -glutamyl peptide formation as judged from the chromatograms. Fig. 6 shows that at higher concentrations of glycine where cysteine liberation fell off, γ -glutamylglycine formation fell off also. At these high concentrations the residual GSH was high. This means that GSH was not being acted upon by the enzyme system. It is possible that the amino acids compete with GSH for the active centre of one enzyme, as seems to be the case with L-valine (Fig. 4). A decrease in cysteine liberation was also observed when the

Fig. 3.

Fig. 4.

Figs. 3, 4. The effect of varying concentrations of amino acids on release of cysteine from GSH expressed in terms of % GSH breakdown. Conditions as in Table 1 but with varying concentrations of amino acids.

reacting amino acid was replaced by similar concentrations of sodium sulphate and glucose. The effect of high concentrations of amino acids could therefore be a non-specific one. As any point on the amino acid concentration curves must represent a combination of reaction and inhibition, comparison of the relative reactivities of amino acids in the system is only possible on the ascending slope of the concentration curve.

Paper chromatography as the index of transpeptidation

Hanes *et al.* (1950, 1952) have shown that L-glutamic acid, L-glutamine, L-phenylalanine, L-leucine, L-valine, L-tryptophan, L-cysteine and glycine would react with certain γ -L-glutamyl peptides, including GSH, to form new spots running in the expected positions for the γ -glutamyl peptide corresponding to the added amino acid. In many cases these spots were eluted, hydrolysed and re-chromatographed to establish their composition.

In the present work other amino acids have been tested for their reaction with GSH by the presence or absence of new spots on chromatograms after concentration of digest samples. Positive evidence has been obtained for the reactivity of L-arginine, L-asparagine, L-lysine, L-histidine, L-methionine, DL-ethionine, DL-norleucine, DL-norvaline, L-alanine, DL-serine and DL-threonine. γ -L-Glutamyltyrosine has been shown to serve as a donor peptide (Hanes *et al.* 1952), and it is probable that tyrosine will react with other γ -glutamyl peptides in the system. With the exception of L-isoleucine and L-valine the reactivity of amino acids as followed by the appearance of a new spot on a chromatogram was always paralleled by a positive figure for cysteine liberation by the amino acid concerned. Similarly, failure to detect a reaction by chromatography was paralleled by a zero figure for cysteine liberation in the case of L-aspartic acid, L-proline and L-hydroxyproline.

Further investigations on the transfer reaction

The positive results given by chromatography are not consistent with the negative results given by estimation of cysteine liberated from GSH by L-isoleucine and L-valine. The figures given for excess of cysteine liberated (Table 1) in these cases do not measure the relative reactivities of the amino acids with GSH. Accordingly, we have examined the enzymic reaction further in an attempt to determine the relationship between extra cysteine liberated by the amino acids and their relative reactivities.

The liberation of cysteine from GSH is a two-step process. First, cysteinylglycine is liberated as a result of a transfer reaction involving the γ -glutamyl linkage. Secondly, the cysteinylglycine so formed is hydrolysed to cysteine and glycine. The enzymic

preparations, as well as catalysing the transfer of γ -glutamyl groups to amino acids, also form glutamic acid from GSH, presumably by hydrolysis. Fig. 6 shows that as the glycine concentration was increased up to a concentration of $67 \mu\text{moles}/0.5 \text{ ml.}$ digest, γ -glutamylglycine formation increased and glutamic acid formation decreased. This competition between water and glycine for the γ -glutamyl group in GSH makes it likely that the two effects, hydrolysis and aminolysis (transpeptidation) of the γ -glutamyl peptide bond, are properties of one enzyme. If this were so, the initial transfer reaction would involve water or the amino group of GSH as an acceptor. In the case of water as an acceptor, all three amino acids in GSH are liberated. In the case of an amino acid (including GSH) as an acceptor, cysteine and glycine only are liberated (the γ -glutamyl linkage being preserved in the new γ -glutamyl peptide formed). As the reaction proceeds there is a progressive increase in glutamic acid, cysteine and glycine and these amino acids can then participate in further transpeptidation reactions. The addition of other amino acids, with few exceptions, brings about the release of extra cysteine (due to extra γ -glutamyl peptide formation) at the expense of GSH. This is shown in the time/progress curve in Fig. 5 with glycine as the added amino acid. It is probable that each γ -glutamyl peptide formed serves as a donor peptide in further transpeptidation reactions.

For liberated cysteine to be taken as an index of transpeptidation activity, it is necessary that a relationship be established between cysteine liberation and γ -glutamyl peptide formation. Four aspects of the enzymic reactions that are concerned in such a relationship have been examined.

Fig. 5. Time/progress curve for % GSH breakdown with and without added glycine. System: 2 ml. enzyme preparation, 2 ml. glycine solution containing $660 \mu\text{moles}$, or 2 ml. water and 1 ml. solution containing $20.6 \mu\text{moles}$ GSH. Incubation: varying intervals of time at 30° under N_2 .

(1) *Hydrolysis of GSH*. If the two transfer reactions, hydrolysis and transpeptidation, are properties of a single enzyme, then there is likely to be competition between water and amino acids for activated γ -glutamyl linkages. Therefore, the higher the amino acid concentration the less should be the hydrolysis. The depression of hydrolysis by added glycine as gauged by glutamic acid formation is shown in Fig. 6. The cysteine produced in the control therefore includes a greater proportion of cysteine which has its origin in GSH hydrolysis than the cysteine produced from GSH in the presence of added amino acid. This means that amino acids are more reactive in transpeptidation than is shown by the figures for extra cysteine liberated (Table 1). It is likely, however, that the amino acids most reactive in transpeptidation are also the best competitors with water. For instance, Fig. 7 shows that glycine depresses glutamic acid formation more than does L-valine at the same concentration. It is also a stronger reagent in transpeptidation, as measured by γ -glutamyl peptide formation (Fig. 7) and by the liberation of extra cysteine (Table 1). As long as the two effects, transpeptidation and depression of hydrolysis, work proportionally and in

the same direction with each amino acid, a comparison of reactivities can be made. The effect of amino acids on enzymic hydrolysis of GSH is at present being investigated further.

(2) *Hydrolysis of cysteinylglycine*. For cysteine to be an accurate measure of reactivity of amino acids with GSH, the cysteinylglycine formed must be hydrolysed completely and as rapidly as it is formed. The enzymic preparations used, however, did not carry the hydrolysis to completion. On concentrating the breakdown products ten times, it was possible to detect traces of cysteinylglycine on chromatograms. The addition of large amounts of cysteinylglycinase (prepared by the method of Binkley, 1952) to the digests did not result in either an increase in cysteine (measured spectrophotometrically) or a decrease in the cysteinylglycine present on chromatograms. It is possible, therefore, that the traces of this peptide which were present in the digests represented a final equilibrium mixture of cysteinylglycine, cysteine and glycine. Further, the trace amount of cysteinylglycine present in digests in the presence of various amino acids ($132 \mu\text{moles}/5 \text{ ml.}$) was constant as judged from chromatograms.

Fig. 6. Chromatogram showing effect of glycine concentration on the enzymic reaction with GSH. System: 0.2 ml. enzymic preparation (dialysed), $2 \mu\text{moles}$ GSH and 0, 13.2, 67 and $1330 \mu\text{moles}$ glycine, respectively. Solvent: propanol:water (80:20). Development: 60 hr. The highest concentration of glycine was obtained by weighing this amino acid into the incubation vessel.

Fig. 7. Chromatogram showing effect of glycine and L-valine at two concentrations on the enzymic reaction with GSH. System: 0.2 ml. enzyme preparation (dialysed), $2 \mu\text{moles}$ GSH and 0, 13.2 and $100 \mu\text{moles}$ of glycine or L-valine, respectively. Incubation: 30 min. at 30° under N_2 . Solvent: propanol:water (80:20). Development: 60 hr.

(3) *γ -Glutamyl peptide formation.* Some of the liberated cysteine reacted with GSH to form γ -glutamylcysteine and as such was not estimated. This might be the reason for the curve flattening off at about 80 % GSH breakdown (Fig. 5). The amount of cysteine liberated would therefore be greater than the amount estimated, and the latter cannot be a precise index of the reactivity of an amino acid. The error will be greatest with the most reactive amino acids, but would allow comparisons of reactivity to be made.

We have observed on chromatograms that an increase in the concentration of a reactive amino acid brought about the formation of additional γ -glutamyl peptide of the amino acid concerned and, by cysteine estimation, the release of extra cysteine. However, this is not true of L-valine and L-isoleucine. An increase in concentration of these amino acids led to a decrease in cysteine liberation (Fig. 4) but, as measured from chromatograms, corresponding γ -glutamyl peptide formation increased (Fig. 7). At a low concentration (132 μ moles/5 ml.), L-valine was less active than DL-norvaline, as measured by the formation of the corresponding γ -glutamyl peptide (Fig. 8). Similarly, L-isoleucine was less reactive than L-leucine and DL-norleucine in γ -glutamyl peptide formation. Qualitatively, the colour reactions of different γ -glutamyl peptides

Fig. 8. Chromatogram showing effect of L-valine and DL-norvaline on the enzymic reaction with GSH. System: 0.2 ml. enzyme preparation (dialysed), 2 μ moles GSH and 13.2 and 26.4 μ moles L-valine and DL-norvaline, respectively. Incubation: 30 min. at 30° under N_2 . Solvent: propanol:water (80:20) + 0.5 ml. ammonia (sp.gr. 0.880) in the chromatography jar. Development: 60 hr.

Table 3. The influence of L-valine on the liberation of cysteine from GSH by L-glutamine

(System: as described in Experimental with 2 ml. enzymic preparation, 1 ml. containing 132 μ moles L-glutamine, 1 ml. water and 1 ml. solution containing 20.6 μ moles GSH. The valine was weighed directly into the vessel. Control: 2 ml. water in place of amino acids. Incubation: 30 min. under N_2 at 30°. Figures represent additional cysteine liberated, in excess of control, and are expressed in terms of % GSH breakdown.)

L-Valine (μ moles/5 ml.)	GSH breakdown in excess of control (%)
0	31
132	30
500	17
1000	3

Fig. 9. Chromatogram showing effect of L-valine and DL-norvaline on the enzymic reaction with γ -L-glutamyl-L-glutamic acid. System: 0.2 ml. enzyme preparation (dialysed), 0.1 ml. solution containing 2 μ moles neutralized γ -L-glutamyl-L-glutamic acid, 0.2 ml. solution containing 13.2 and 26.4 μ moles L-valine and DL-norvaline, respectively. Incubation: 15 and 90 min. at 30° under N_2 . Solvent: phenol saturated with water. Development: 20 hr.

with ninhydrin resembled the colour given by glutamic acid and not by the amino acid linked to it. Further, it would be expected that the reaction of γ -glutamyl peptides with ninhydrin would involve the free α -amino group of glutamic acid. It is, therefore, likely that differences in colour intensity of γ -glutamyl peptides on chromatograms indicate differences in amounts of these peptides. It seems justifiable then to conclude that at low substrate concentration L-isoleucine and L-valine are less reactive than their structural isomers, and that the figures given for the liberation of cysteine by the amino acids tested indicate their comparative reactivities.

The effect of increasing the concentration of L-valine which was added to a digest containing GSH and glutamine is shown in Table 3. At low substrate concentration, L-valine brought about very little reduction in cysteine liberated by L-glutamine, and this indicates that the apparent inertness of L-valine in the system GSH + valine was not due to inhibition of cysteinylglycine by this amino acid. At high concentrations of L-valine, the liberation of cysteine by glutamine was reduced considerably. This reduction is consistent with: (i) L-valine inhibiting the attachment of the donor peptide, GSH, to the enzyme, (ii) L-valine, as a weak reactor, occupying the position of the acceptor amino acid on the enzyme and so reducing the number of transpeptidation reactions, (iii) L-valine, at high concentration, inhibiting cysteinylglycine.

In a further test of the reactivity of valine, γ -L-glutamyl-L-glutamic acid was used instead of GSH, as it gave a simpler chromatographic pattern. The chromatogram (Fig. 9) showed that residual γ -L-glutamyl-L-glutamic acid was present in greater amounts in the presence of L-valine than in the presence of DL-norvaline. This result suggests that L-valine at low substrate concentration inhibited cysteine liberation for one or both of the reasons outlined in (i) and (ii).

(4) *Effect of concentration of acceptor amino acids.* Above a certain concentration characteristic of each added amino acid, cysteine liberation decreased. For the amino acids tested, but with the exceptions of L-aspartic acid, L-isoleucine and L-valine, the concentration of 132 μ moles/5 ml. represented a point on the concentration curves (Figs. 3, 4) on or below the point at which reactivity began to fall off. Such a concentration was therefore suitable to compare reactivities.

DISCUSSION

The γ -glutamyl transfer reaction has been shown to be catalysed by extracts of kidney and pancreas but not by extracts of liver tissue (Hanes *et al.* 1952). The distribution of γ -glutamyl transferase coincides with

that of conjugase (Laskowski, 1950). Both enzymes are known to act on the γ -glutamyl linkage. Dakin & Dudley (1913) reported the presence of an antiglyoxalase in extracts of pancreas, and Woodward, Munro & Schroeder (1935) found that this enzyme was also present in extracts of kidney. The action of antiglyoxalase was found to be due to the destruction of GSH, the coenzyme in the glyoxalase system (Woodward *et al.* 1935; Salem & Crook, 1950). It is possible that these three enzymes are identical. Although there has been some tentative speculation about a possible role in protein synthesis (Fruton, 1950; Hanes *et al.* 1950, 1952) no function has been ascribed to the enzyme.

Unpublished observations by Hird, Neville & Springell have shown by use of differential centrifugation that γ -glutamyl transferase in sheep kidney and ox pancreas is particle bound and that by treatment with butanol (Morton, 1950) it can be liberated from the particles. Such preparations would still transfer the γ -glutamyl group to both water and amino acids.

Precise interpretation of the results obtained in the reaction of amino acids with GSH is difficult. However, using the indices established in the present investigation, broad comparisons can be made, especially within the groups of amino acids. Within the aliphatic series the most reactive amino acids were those with long unbranched side chains, e.g. methionine sulphoxide, glutamine and citrulline had the greatest reactivity; norleucine and norvaline had greater activity than leucine, isoleucine and valine. Amidation of the distal carboxyl groups of L-aspartic acid and L-glutamic acid, respectively, markedly enhanced reactivity. This has also been noted by Binkley (1952) and Fodor, Miller & Waelsch (1952).

The inhibitory effect of the β -methyl group in L-valine and L-isoleucine has previously been shown by other workers. Fox & Winitz (1952) found that these amino acids were also unreactive in the enzymic synthesis of anilides. Fox, Pettinga, Halverson & Wax (1950) have shown that benzoyl-L-valine amide is resistant to enzymic hydrolysis, and Smith, Spackman & Polglase (1952) have shown this for the amides of isoleucine and valine. These results are in contrast to those given by the corresponding derivatives of leucine. Valyl peptides are also resistant to hydrolysis by acid (*cf.* Sanger, 1952).

The preliminary reports by Binkley (1952) and Fodor *et al.* (1952), working with γ -glutamyl transferase from pig kidney, gave figures for several amino acids and their results are in agreement with those reported in the present paper.

In a previous publication (Hanes *et al.* 1952), it was found that L-arginine did not react with γ -L-glutamyl-L-glutamic acid to form the corresponding γ -glutamyl peptide. In the present work, however,

it has been shown that L-arginine is quite reactive with GSH. The reaction of L-arginine with GSH has also been reported by Kinoshita & Ball (1953).

Binkley (1951) and Binkley & Olson (1951) have given a special role to L-glutamine in GSH hydrolysis and suggest the following reaction mechanism:

The evidence for this hypothesis is that L-glutamine increases the 'hydrolysis' of GSH without itself being hydrolysed in the process. The results of these workers can be explained by the formation of γ -glutamylglutamine with the liberation of cysteinylglycine followed by hydrolysis of the latter. The production of a new peptide (almost certainly γ -glutamylglutamine) when L-glutamine is incubated with GSH has been reported (Hanes *et al.* 1952). In the present work L-glutamine has also been shown to be very reactive. The range of amino acids tested by Binkley (1949) and Binkley & Olson (1951) was not extensive and the smaller effects of other amino acids was thought to be due to the removal of inhibitory ions. As can be seen from Tables 1 and 2 there are many amino acids which are as active as L-glutamine and some even more so. These do not have the amide configuration. This means that the hypothesis of L-glutamine as a coenzyme in the hydrolysis of GSH lacks unequivocal supporting evidence.

SUMMARY

1. A modification of the method of Nakamura & Binkley (1948) for the estimation of cysteine in the presence of glutathione (GSH) is described.

3. Sheep-kidney γ -glutamyl transferase, which catalyses peptide-bond transfers between GSH and amino acids, has been investigated. With certain qualifications, cysteine liberated by these transfer reactions can be taken as an index of the comparative reactivity of amino acids in the system.

3. The relative reactivities of L- α -amino acids has been determined. The most reactive amino acids in the aliphatic series were those with long, unbranched side chains. The presence of a β -methyl group, as in L-valine and L-isoleucine, interfered with the reactivity of the amino groups of these amino acids. The ability of the amino groups of L-

aspartic and L-glutamic acids to react with GSH was enhanced by amide formation as in L-asparagine and L-glutamine, respectively.

4. With the exceptions of L-aspartic acid, L-isoleucine and L-valine, an increase in amino acid concentration brought about an increase in the cysteine liberated from GSH. At higher concentrations reactivity fell off. Increases in the concentration of L-aspartic acid, L-isoleucine and L-valine caused a steady decrease in cysteine liberation.

5. Chromatographic evidence has now been obtained for transpeptidation with most of the common, naturally occurring amino acids, with the exceptions of L-aspartic acid, L-proline and L-hydroxyproline.

We wish to thank Mr A. M. Gallacher for preparing the glutathione.

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