Acidic Peptides of the Lens

6. METABOLISM OF γ -GLUTAMYL PEPTIDES IN SUBCELLULAR FRACTIONS OF RABBIT LIVER*

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Ophthalmic acid (I; R = CH₃), an analogue of glutathione (I; R = SH), has been isolated from calf lens (Waley, 1958), and probably also occurs in rat liver.

The two tripeptides are similar in structure; they share some biological properties (Cliffe & Waley, 1958b), and compete in others (Cliffe & Waley, 1959a) and are synthesized by the same route in extracts of calf lens (Cliffe & Waley, 1958a). The comparison between ophthalmic acid and glutathione has now been extended by the choice of a tissue that not only contains enzymes that bring about the synthesis of the tripeptides but also enzymes that catalyse their breakdown. Such a tissue is rabbit liver.

Subcellular fractionation has been used to study these enzymes. This method enabled us to find out whether both glutathione and ophthalmic acid were synthesized, or broken down, in one fraction (i.e. we compared substrates) and whether the synthesis and breakdown took place in the same fraction.

The results of our comparisons are, in brief, as follows. There is a parallel distribution of the enzymes concerned in the metabolism of glutathione and ophthalmic acid; they may, indeed, be the same enzymes, and, where it has been tested, this idea is supported by the results of competition experiments. Synthesis and breakdown take place (mainly) in different fractions: the nuclear fraction contains the enzyme (γ -glutamyl transpeptidase) that catalyses the hydrolysis of the tripeptides; and the supernatant fraction contains the enzyme system that brings about their synthesis. A preliminary account of this work has already appeared (Cliffe & Waley, 1959b).

MATERIALS AND METHODS

Tissues. Lenses were removed from the eyes of calves at the slaughterhouse within 2 hr. after death of the animals. Livers of rabbits (3–5 months old), guinea pigs (adult),

pigeons (adult) and rats (90–150 g.) were used fresh from laboratory animals which had been starved for 24 hr. Rabbits and guinea pigs were killed by a sharp blow on the back of the neck, and pigeons and rats by decapitation. Calf liver and sheep kidney were obtained fresh from the slaughterhouse and brought to the laboratory packed in ice.

Dialysed calf-lens preparation. This was prepared as described by Cliffe & Waley (1958a) and was sometimes stored at -15° overnight before use.

Peptides and amino acids. Ophthalmic acid, norophthalmic acid, γ -L-glutamyl-L- α -amino-n-butyric acid and L- α -amino-n-butyrylglycine were prepared as described by Waley (1958); the glutathione was a gift from The Distillers Co. Ltd. (Biochemicals); other peptides and amino acids were commercial preparations.

Solutions of glutathione, and cysteine hydrochloride were prepared immediately before use, and the pH was brought to 6.6 with aqueous KOH. Other solutions of acidic amino acids and peptides were neutralized with aqueous KOH.

Cofactors. The barium salt of D-3-phosphoglyceric acid (L. Light and Co. Ltd., Colnbrook, Bucks.) was converted into the soluble potassium salt with Amberlite IR-120(H⁺) and KOH (Wade & Morgan, 1955).

Phosphate buffer-salt medium. This contained potassium phosphate buffer, pH 7.4, (70 mM), KCl (70 mM) and MgSO₄ (7 mM).

Liver preparations

All manipulations were done as quickly as possible at $0-2^\circ$. The homogenizer, Fischer mincer and all the reagents were chilled before use.

Whole liver. The liver was chilled in ice-cold 0.15M-KCl, blotted lightly and forced through a Fischer mincer ($\frac{1}{8}$ in. mesh grid). The mince was homogenized for 2 min. with phosphate buffer-salt medium (2 ml./g. wet wt. of liver) in a stainless-steel homogenizer of the Potter-Elvehjem type. Preparations of sheep-kidney cortex were made in the same way.

Subcellular particles. The method of fractionation was similar to that of Schneider (1948). Whole liver mince was homogenized for 2 min. with 0.25M-sucrose and made up in boiled water (4 ml./g. wet wt. of mince). Three fractions were prepared from the whole homogenate by differential centrifuging in a refrigerated centrifuge: nuclei and debris (which will be referred to as crude nuclei) were sedimented at 600 g for 10 min., mitochondria were sedimented at 8500 g for 10 min., and microsomes were sedimented at 19000 g for 60 min. The crude nuclei were washed twice by resuspension in 0.25M-sucrose (1 ml./g. wet wt. of mince) and resedimented at 600 g for 10 min., and the mitochondria were washed in a similar way with 0.25M-sucrose

* Part 5: Waley (1959).

(0.5 ml./g. wet wt. of mince) and resedimented at 8500 *g* for 10 min. At each stage the washings were added to the remaining unfractionated material. The 'fluffy layer', which sedimented immediately on top of the mitochondria, was kept with the mitochondrial fraction. All resuspension of particles was effected with a revolving Perspex pestle machined to fit the centrifuge tubes.

For experiments in which peptide breakdown was assayed, the particles were resuspended in phosphate buffer-salt medium, and the suspension was kept at 4° for up to 4 hr. while the other particles were separated. For experiments in which peptide synthesis was studied, the fractions were suspended in water (about 5 ml.) and an acetone-dried powder was prepared. This was extracted by the procedure of Snoke & Bloch (1955).

The number of intact cells in the crude nuclear fraction was determined. A suspension in 0.25 M-sucrose spotted on six separate microscope slides was examined under the phase-contrast microscope at a magnification of 500 times. The number of nuclei and intact cells were counted in five fields chosen at random on each slide; the concentration of the suspension was adjusted so that the total number of nuclei and intact cells in each microscope field was about 30. Assuming (a) that no nuclei were lost during homogenization or fractionation and (b) that about 30% of the liver cells were binucleate, the proportion of remaining intact cells was calculated. In the absence of a value for the number of binucleate cells in rabbit liver, the value for rat liver (Harrison, 1953) has been used. The proportion of intact cells in the crude nuclear fraction was consistently in the range 5–10% and was not lowered by homogenization for 4 min. instead of 2 min. On two occasions, the nuclear fraction was prepared from rat liver by the same procedure as described above for rabbit liver, and the percentage of intact cells was 2–5%. The cells of rabbit liver seem more difficult to disrupt than those of rat liver.

Alternative preparation of nuclei. Rabbit liver was perfused *in situ* (immediately after death) via the portal vein with 0.15 M-KCl (0.5–1 l.) followed by 0.25 M-sucrose solution containing 1.8 mM-CaCl₂ (40 ml.). The gall bladder was removed and the liver excised. The liver was blotted dry, forced through a Fischer mincer and homogenized for 2 min. in a medium of 0.25 M-sucrose containing 1.8 mM-CaCl₂ (9 ml./g. wet wt. of perfused mince). The homogenate was sieved through a layer of sterile surgical lint, and the filtrate was homogenized for a further 2 min. The number of intact cells was now 1–2%. The nuclei were separated from the whole homogenate as described by Hogeboom, Schneider & Striebig (1952). They were washed three times by resuspension in the medium and packed into a small pellet by centrifuging at 10 000 *g* for 10 min. A suspension of nuclei in phosphate buffer-salt medium was used for enzymic studies.

Supernatant fraction. As the enzymic activities that we were concerned with were low they could not be measured in the supernatant fraction unless it was concentrated. A whole liver homogenate was prepared as described above and centrifuged at 19 000 *g* for 60 min. to sediment the particles; the supernatant fraction was removed with a bent-tipped pipette. For experiments in which peptide breakdown was studied, the supernatant fraction was dialysed overnight at 4° against water to remove the sucrose, and an acetone-dried powder prepared. This was extracted at 4° by occasional grinding during 1 hr. with

phosphate buffer-salt medium; about 2.5 ml./0.4 g. of powder usually gave a convenient concentration of enzyme for assay. The mixture was centrifuged at 3000 *g* for 10 min. at 1°, and the supernatant was used. For experiments on the synthesis of peptides, the supernatant fraction was dialysed at 4°, first against 0.08 M-NaHCO₃, pH 8, for 4 hr., and then against water for a further 12 hr. An acetone-dried powder was prepared and extracted as described by Snoke & Bloch (1955).

Preparation of acetone-dried powders. In general, the tissue or subcellular fraction was macerated with acetone cooled to –10° (10 ml./g. wet wt. or /ml.). The precipitate was collected on a Büchner funnel with a Whatman no. 52 paper, and washed with more cold acetone. The pad of insoluble material was carefully scraped off the filter paper (a process which is easier when the hard no. 52 paper is used) and finally dried in a vacuum desiccator overnight.

Standard reaction mixture

System used for the synthesis of peptides. The reaction mixture contained adenosine triphosphate (ATP) (Sigma Chemical Co.) (4 mM), 3-phosphoglyceric acid (33 mM), KCl (100 mM), MgSO₄ (10 mM), KCN (15 mM), 2-amino-2-hydroxymethylpropane-1,3-diol buffer (100 mM), glycine (15 mM), L-glutamic acid (10 mM), L-cysteine (10 mM) or DL- α -amino-n-butyric acid (20 mM), and 1 ml. of an extract of acetone-dried whole liver or subcellular particles prepared by the method of Snoke & Bloch (1955), in a total volume of 2 ml. In some experiments 0.16 ml. of dialysed calf lens was included. The mixture was incubated for 6 hr. at 37° and the pH was 7.8. Samples (0.2 ml.) were withdrawn at hourly intervals for estimation.

System used for the breakdown of peptides. The reaction mixture contained ophthalmic acid (4 mM) or glutathione (4 mM) or γ -L-glutamylglycine (10 mM) and 0.45 ml. of liver preparation in phosphate buffer-salt medium in a total volume of 0.6 ml. It was incubated for 5 hr. at 37° and the pH was 7.4; 0.05 ml. samples were withdrawn at 30 min. intervals.

Following the course of the reactions

Removal of protein. Samples from the reaction mixtures were pipetted into centrifuge tubes containing 1 ml. of the appropriate concentration of acetic acid to give a final pH of 5.3 (Hunter, 1957). The tubes were heated in a boiling-water bath for 3 min. with glass 'bobbles' on top, cooled and centrifuged at 3000 *g* for 5 min. A measured portion of supernatant was dried in a vacuum desiccator. The residues were taken up in 10% (v/v) propan-2-ol and examined by paper electrophoresis or paper chromatography.

Removal of glutathione. Reduced glutathione (GSH) has the same electrophoretic mobility as ophthalmic acid and therefore interferes with the estimation of ophthalmic acid on electrophoresis papers. In experiments where both tripeptides were present in the reaction mixtures, glutathione was oxidized to the sulphonic acid (GSO₃H), which has a higher electrophoretic mobility towards the anode at pH 4.

The residues of samples after deproteinization were taken up in performic acid solution, which was freshly prepared on each occasion by mixing 0.1 ml. of H₂O₂ (A.R., 30%, w/v) and 0.9 ml. of formic acid (A.R., 98–100%). Twenty μ l. of performic acid/ μ mole of sulphydryl compound was used;

this is sufficient for the reaction to go to completion. (In calculating the amount of performic acid the cysteine, which was in the samples, was also taken into account.) The solutions were left for 1 hr. and evaporated to dryness in a vacuum desiccator. Excess of performic acid was decomposed by taking up the residue in water and evaporating to dryness again.

Separation and estimation of peptides and amino acids. In most cases the compounds were separated by paper electrophoresis at pH 4 (Cliffe & Waley, 1958a). On occasions they were separated by descending paper chromatography on Whatman no. 52 paper with butan-1-ol-acetic acid-water (40:9:20, by vol.) as the solvent system. Glutathione was separated (as GSSG) by descending paper chromatography on Whatman no. 52 paper with 72% (w/w) phenol-3% (w/v) NH_3 soln. as solvent. The electrophoresis and chromatography papers were dried and developed in ninhydrin- CoCl_2 reagent, and the intensities of the spots measured by direct photometry on the paper (Cliffe & Waley, 1958a).

RESULTS

γ -Glutamyl lactamase

The distribution of this enzyme in subcellular fractions of rabbit liver is described first, as the results are particularly clear-cut. The substrate chosen to assay the enzyme was γ -L-glutamylglycine (γ -Glu. Gly); the reaction catalysed by γ -glutamyl lactamase leads to 5-oxopyrrolidine-2-carboxylic acid (PCA) and glycine (Gly) (Connell & Hanes, 1956):

The reaction is detected and followed by paper electrophoresis; the spot due to the dipeptide (γ -glutamylglycine) disappears, and only one ninhydrin-positive spot (glycine) appears. The other

product of the reaction 5-oxopyrrolidine-2-carboxylic acid was detected with chlorine (Reindel & Hoppe, 1954). Glutamic acid is not formed. The formation of 5-oxopyrrolidine-2-carboxylic acid is characteristic of γ -glutamyl lactamase; this criterion will be used later in discussion of the breakdown of ophthalmic acid.

The breakdown of γ -glutamylglycine, catalysed by an extract of acetone-dried rabbit liver, was first-order (Fig. 1). The rate constant (k) from the plot, in units of min^{-1} , is converted into units of 1 g. wet wt. of liver mince/ml. of incubation mixture (k') in Table 1, which also shows the activities of the subcellular fractions.

The figures in Table 1 were arrived at as follows: the fifth row is taken as an example. Minced liver (3 g.) yielded an acetone powder (0.65 g.), a portion (0.37 g.) of which was extracted with buffer (2.5 ml.). A sample (0.45 ml.) of this extract was used for the reaction; the volume of the reaction mixture was 0.6 ml., and the rate constant (k)

Fig. 1. Breakdown of γ -glutamylglycine, catalysed by an extract of acetone-dried rabbit liver. The products are 5-oxopyrrolidine-2-carboxylic acid and glycine.

Table 1. Breakdown of γ -glutamylglycine in subcellular fractions of rabbit liver

The conditions for the assays, and the meaning of k' are given in the text. The relative specific activity of a fraction is derived from the figures in the third and fourth columns, e.g. $85/100 \div 118/217.5 = 1.6$, and takes into account the differing amounts of protein in each fraction.

	$10^3 k$ (min^{-1})	$10^3 k'$	Activity in fraction (% of activity in whole liver)	Amount of protein in each fraction (mg.)	Relative specific activity
Whole liver	11.52	46.1	(100)	217.5	(1.0)
Nuclear fraction	0.99	1.3	3	44.5	0.1
Mitochondria	3.00	1.1	2	23.3	0.2
Microsomes	5.07	2.3	5	30.6	0.4
Whole liver (acetone powder)	9.46	18.4	(100)	217.5	(1.0)
Supernatant fraction (acetone powder)	32.7	15.6	85	118	1.6

was found to be $9.46 \times 10^{-3} \text{ min.}^{-1}$. The value of k' is thus given by

$$9.46 \times 10^{-3} \times \frac{0.65}{0.37} \times \frac{2.5}{3} \times \frac{0.6}{0.45} = 18.4 \times 10^{-3}.$$

Similarly, a value of 15.6×10^{-3} for k' was found for the supernatant fraction, and so the percentage activity in this fraction is $15.6/18.4 = 85\%$.

Virtually all the γ -glutamyl lactamase activity is thus in the supernatant fraction of rabbit-liver cells. Moreover, as there are hydrolytic enzymes in the nuclear fraction (see below), it follows that γ -glutamylglycine is not hydrolysed at all rapidly by these enzymes.

Synthesis of ophthalmic acid

An enzyme preparation from rabbit liver, as used by Snoke & Bloch (1955) for the synthesis of glutathione, catalysed the synthesis of ophthalmic acid from the constituent amino acids. The product was identified by preparative paper electrophoresis; hydrolysis gave the three amino acids in equimolar proportions. The course of the synthesis is

illustrated in Fig. 2. Now the synthesis of ophthalmic acid in extracts from calf lens proceeds by the steps (Cliffe & Waley, 1958a):

where AB stands for α -amino-*n*-butyric acid, ADP adenosine diphosphate, and P phosphate. The shape of the lower curve in Fig. 2 suggests that there are also two steps, and that they proceed at comparable rates.

In the experiments with extracts of calf lens, the presence of cysteine depressed the rate of synthesis of ophthalmic acid (Cliffe & Waley, 1958a), and the same effect has now been found with liver extracts (Fig. 3). When cysteine is present, the synthesis of glutathione is competing with the synthesis of ophthalmic acid. This suggests that one (or, possibly, both) of the enzymes that catalyse the synthesis of glutathione also plays a part in the synthesis of ophthalmic acid.

In the synthesis of glutathione, it is important that the ADP formed should be reconverted into ATP, and the same may be expected to be true for the synthesis of ophthalmic acid. A relatively high concentration of 3-phosphoglyceric acid was therefore used; excess of 3-phosphoglyceric acid, however, depressed the extent of synthesis of ophthalmic acid, and the optimum concentration was about 33 mM. Variation of the concentration of

Fig. 2. Formation of ophthalmic acid from its constituent amino acids, catalysed by an extract of acetone-dried rabbit liver, in the presence (●) and absence (○) of dilute, dialysed, lens extract.

Fig. 3. Formation of ophthalmic acid from its constituent amino acids, catalysed by an extract of acetone-dried rabbit liver, in the presence (●) and absence (○) of cysteine.

ATP (in the range 1.5–8 mM) did not affect the rate of synthesis of ophthalmic acid. Lens extracts are active in the conversion of phosphoglyceric acid and ADP into ATP, and so we examined the effect of adding a dilute lens extract to the enzyme preparation, from rabbit liver, which was being used for the synthesis of ophthalmic acid. The synthesis of ophthalmic acid was, in fact, accelerated by the addition of the lens extract (Fig. 2, upper curve). The lens extract used was so dilute that, on its own, it did not catalyse the synthesis of detectable amounts of ophthalmic acid. The dilute lens extract was shown to convert 3-phosphoglyceric acid and ADP into ATP. The reaction was followed by ion-exchange chromatography (Cohn & Carter, 1950), and three-quarters of the added ADP was converted into ATP after 1 hr. at 37°. It is by no means certain that the effect of added lens extract on the synthesis of ophthalmic acid by the liver extract is due to the factors discussed above; in practice, however, the effect was useful.

A few experiments were carried out with extracts from the livers of other animals. Extracts of calf liver were considerably less active than those of rabbit liver; pigeon-liver extracts were comparable with rabbit-liver ones.

The intracellular distribution of the enzymes that catalyse the synthesis of ophthalmic acid (and of glutathione) have been studied. The results of the fractionation, from assays carried out with added (dilute) extracts of lens, were as follows. The activities in the nuclear fraction, the mitochondria and the microsomes were low and may be attributed to contamination by the supernatant fraction, which contains about 40 % of the total activity of the unfractionated tissue. The recovery is not high, but this is not surprising as the system is complex (some five enzymes must be present), and the manipulations prolonged. During the course of centrifuging, acetone precipitation and dialysis, there is some likelihood of loss in activity, which may be greater in one of the fractions than it is in the unfractionated tissue. The simplest conclusion is that the enzymes that catalyse the synthesis of ophthalmic acid are present only in the super-

natant fraction. Similarly, the only subcellular fraction in which the synthesis of glutathione could be demonstrated was the supernatant.

Breakdown of glutathione and of ophthalmic acid

Some conclusions reached later are anticipated here: there are two enzymes that catalyse the breakdown of ophthalmic acid; these are γ -glutamyl lactamase and γ -glutamyl transpeptidase. The results of the subcellular fractionation of the enzymes that catalyse the breakdown of the tripeptides is shown in Table 2. Two points are immediately apparent. The first is that the breakdown of glutathione occurs mainly in the nuclear fraction. The second is that the breakdown of ophthalmic acid occurs both in the nuclear fraction and the supernatant. The products of the breakdown of ophthalmic acid in the nuclear fraction and in the supernatant differ: glutamic acid is a product in the nuclear fraction, and 5-oxypyrrolidine-2-carboxylic acid in the supernatant fraction. The latter compound is characteristic of the action of γ -glutamyl lactamase, and this, as we have seen, is localized in the supernatant fraction. Hence the breakdown of ophthalmic acid in the supernatant fraction is ascribed to the action of γ -glutamyl lactamase. This conclusion was confirmed by the observation that the presence of γ -glutamylglycine depressed the rate of breakdown of ophthalmic acid in the supernatant fraction. Glutathione, since it is not broken down at an appreciable rate in the supernatant fraction, must be more resistant to γ -glutamyl lactamase.

We are now in a position to compare the distribution of the enzyme (γ -glutamyl transpeptidase) that catalyses the hydrolysis of glutathione and of ophthalmic acid. From Table 2, the γ -glutamyl lactamase in the supernatant accounts for about half the activity in the breakdown of ophthalmic acid. The distribution of the other (hydrolytic) enzyme may therefore be obtained by doubling the values for the activities of the nuclear fraction, mitochondria and microsomes given in the second column of Table 2. Comparison with the values for the breakdown of glutathione shows that the

Table 2. *Distribution of enzymes that catalyse the breakdown of glutathione and of ophthalmic acid in subcellular fractions of rabbit liver*

The activity of each fraction is expressed as a percentage of the activity of the unfractionated extract.

Fraction	Breakdown of		Calculated hydrolysis of ophthalmic acid
	Glutathione	Ophthalmic acid	
Nuclei + unbroken cells	75	37	74
Mitochondria	12	4	8
Microsomes	14	9	18
Supernatant	0	47	—
Total	101	97	—

distribution of the two activities is now very similar. This suggests that the same enzyme may catalyse the breakdown of both tripeptides. This conclusion was confirmed by the observation that the presence of glutathione depressed the rate of breakdown of ophthalmic acid in the nuclear fraction.

Transpeptidations catalysed by the nuclear fraction

On incubation of ophthalmic acid and glycylglycine with rabbit-liver extracts, a new ninhydrin-positive, acidic, species was formed (Fig. 4). This product is probably γ -glutamylglycylglycine (γ -Glu.Gly.Gly), formed by transpeptidation:

Transpeptidation takes place more rapidly when the nuclear fraction from rabbit liver is used (Fig. 5). The acidic product was isolated by preparative paper electrophoresis; it was chromatographically homogeneous, and after acid hydrolysis the molar ratio of the amino acids (estimated by quantitative paper chromatography) was glutamic acid 1.0, glycine 1.9. This is consistent with our formulation of the product as γ -glutamylglycylglycine.

The rate of breakdown of ophthalmic acid (in the nuclear fraction) was about 30 times as great in the presence of glycylglycine (20 mM) as in its absence.

Fig. 4. Electrophoresis paper showing the course of the reaction between ophthalmic acid and glycylglycine, catalysed by an extract of rabbit liver. The overall reaction is

Electrophoresis was carried out at pH 4, and travel towards the anode is downwards in the illustration.

Fig. 5. Electrophoresis paper showing the course of the reaction between ophthalmic acid and glycylglycine, catalysed by the nuclear fraction from rabbit liver. The reaction and procedure were as in Fig. 4.

Thus with a relatively high concentration of an 'acceptor', transpeptidation becomes the main route of breakdown. On comparison of Figs. 4 and 5, the glycylglycine may be seen to be stable in the nuclear fraction, but broken down quite rapidly in the extract from whole liver. In rat liver, the enzyme that catalyses the hydrolysis of glycylglycine is in the supernatant fraction (Rademaker & Soons, 1957), and the same may well be true for rabbit liver. Hence transpeptidation proceeds especially rapidly in the nuclear fraction, both because the enzyme (γ -glutamyl transpeptidase) is present in this fraction, and because the glycylglycine is stable in this fraction. The instability of the 'acceptor' for transpeptidation in whole liver precludes any reliable measurement of the intracellular distribution of the enzyme that catalyses the transpeptidation.

The transpeptidation that has just been described leads to the breakdown of ophthalmic acid. Another transpeptidation that has been carried out leads to the formation of ophthalmic acid. When glutathione and α -amino-*n*-butyrylglycine (AB-Gly) are incubated with the nuclear fraction, ophthalmic acid is formed:

The course of this reaction is shown in Fig. 6. Here the samples from the reaction mixture were oxidized by performic acid to convert glutathione (GSH) into the sulphonic acid (GSO_3H), which has

a relatively high mobility (towards the anode) on electrophoresis at pH 4, whereas GSH and ophthalmic acid have nearly the same mobility. Ophthalmic acid is seen to be formed rapidly; it is also broken down, so that its concentration does not change greatly after the reaction has proceeded for several minutes.

The substance with the highest mobility towards the anode in Fig. 6 is cysteic acid, which is formed from cysteine (or cystine) in the reaction mixture by the oxidation with performic acid. Although cysteinylglycine (CySH. Gly) was written as a product in the transpeptidation between glutathione and α -amino-*n*-butyrylglycine, the cysteinylglycine seems to be hydrolysed to the amino acids, presumably by cysteinylglycine dipeptidase, which is commonly present in tissues.

Comparison of tissues

Table 3 shows some results obtained with tissues other than rabbit liver. The variation in the activity of the enzyme that catalyses the breakdown of glutathione is quite marked, and for the liver, the activity depends on the species (Neubeck & Smythe, 1944). The results for ophthalmic acid are similar to those for glutathione, and presumably it is generally true that the same enzyme (γ -glutamyl transpeptidase) attacks both the tripeptides. The dipeptide, γ -glutamylglycine, gives 5-oxopyrrolidine 2-carboxylic acid (and glycine) with rat-liver, or rabbit-lens, extracts; hence here the enzyme is γ -glutamyl lactamase. With sheep-kidney extracts, however, the products are glutamic acid and glycine, so that here the enzyme responsible for the breakdown is γ -glutamyl transpeptidase.

Fig. 6. Electrophoresis paper showing the course of the reaction between glutathione and α -amino-*n*-butyrylglycine, catalysed by the nuclear fraction from rabbit liver. The overall reaction is:

The thiols (GSH and CySH) have been oxidized to the corresponding sulphonic acids (GSO_3H and CySO_3H) before electrophoresis. Procedure as in Fig. 4.

Table 3. Breakdown of γ -glutamyl peptides in tissues

The reaction mixtures (total volume, 0.6 ml.) were 5 mm with respect to glutathione, or ophthalmic acid, or 10 mm with respect to γ -glutamylglycine, and contained 0.45 ml. of extract of the tissue. The tissues were minced, and the mince was ground with phosphate buffer (see Methods) (2 ml. of buffer/g. of mince). The units of the rates are $10^3k'$ (see Table 1). The reaction mixtures were incubated at 37° and pH 7.4 for 5 hr.

Tissue	Glutathione	Ophthalmic acid	γ -Glutamylglycine
Guinea-pig liver	109	—	—
Rabbit liver	10–30	10–33	29–49*
Rat liver	<1	<1	Active*
Sheep kidney	> 200	> 200	80†
Rabbit lens	<1	<1	8*

* The product is 5-oxopyrrolidine-2-carboxylic acid.

† The product is glutamic acid.

Occurrence of ophthalmic acid in rat liver

Rat liver (35 g.) was macerated with cooled 8% (w/v) trichloroacetic acid (120 ml.) and the protein removed by centrifuging. The supernatant solution was freed from trichloroacetic acid by extraction with *N*-methyldioctylamine (20 ml.) in CHCl_3 (400 ml.), and excess of base removed by two further extractions with CHCl_3 . The solution was concentrated to a volume of about 17 ml., and the pH raised to 7.6 with *N*-NaOH, before fractionation on a column (1.2 cm. $\times$ 30 cm. long) of Dowex 1 (acetate). The resin had been treated as previously described (Waley, 1959), converted into the acetate form, and washed with water. After the extract had been added to the column, the column was washed with water (50 ml.), and then eluted with pyridine-formate, pH 5.5 (4.2 ml. of 90% formic acid and 16.2 ml. of pyridine to 1 l.), at a rate of 12 ml./hr.; fractions of 3 ml. were collected. Fractions 35–47 were ninhydrin-positive, and were united and evaporated. Examination by paper chromatography and paper electrophoresis showed that, besides glutamic acid and aspartic acid, there was a spot which had the same mobility as that of ophthalmic acid. After hydrolysis in 6*N*-HCl at 110° for 16 hr., this spot was absent, but glycine and α -amino-*n*-butyric acid were now present. These results suggest the presence of ophthalmic acid in rat liver; the concentration, gauged by the intensity of the spot, is about $1 \mu\text{mole}/100 \text{ g. of liver}$.

DISCUSSION

γ -Glutamyl lactamase

The application of the technique of subcellular fractionation to the enzymes that catalyse the synthesis and breakdown of γ -glutamyl peptides has helped us to unravel these reactions, and also suggests a well-defined intracellular distribution of these enzymes. Centrifugal fractionation was used by Connell & Hanes (1956) in their purification of γ -glutamyl lactamase. They found that lactamase

from pig liver was in the supernatant fraction, whereas the γ -glutamyl transpeptidase was in the precipitated ('particulate') fraction. Our results on the enzymes from rabbit liver confirm, and extend, those of Connell & Hanes (1956). The results of the quantitative study of the distribution of γ -glutamyl lactamase (Table 1) show that, within the limits of the procedure, the enzyme is solely present in the supernatant fraction. One result of this distribution is that the enzyme may affect the course of the synthesis of the γ -glutamyl tripeptides *in vivo* (see Cliffe & Waley, 1958a).

The range of activity of γ -glutamyl lactamase has not been widely investigated. Connell & Hanes (1956) record that γ -glutamylglycine, γ -glutamylglutamic acid, γ -glutamylphenylalanine and glutathione are all attacked, and that γ -glutamylglycine is attacked more rapidly than glutathione. We also find that γ -glutamylglycine is readily cleaved by the enzyme from rabbit liver, and that ophthalmic acid is broken down, too; glutathione is considerably more resistant. Calf lens contains a γ -glutamyl lactamase (Cliffe & Waley, 1958a), but neither here, nor with rabbit lens, is ophthalmic acid attacked at a detectable rate. Thus it seems to be generally true that γ -glutamyl lactamases attack γ -glutamylglycine more rapidly than they attack the tripeptide, ophthalmic acid, but this difference is much more marked with the enzyme from the lens than with the enzyme from liver.

Synthesis of the tripeptides

The enzyme system that catalyses the synthesis of ophthalmic acid, and of glutathione, is also confined to the supernatant fraction of rabbit liver. There are some indications (Holleman, 1954) that the same is true for rat liver. This enzyme system is quite complex. There are the two stages in the synthesis of the tripeptide, and three stages in glycolysis (catalysed by phosphoglyceric acid mutase, enolase and pyruvic kinase) by which ATP is 'regenerated' from ADP. The second stage in the synthesis of glutathione (in extracts of rat liver) has recently been shown to involve 'soluble'

ribonucleic acid (RNA) (i.e. the RNA from the supernatant fraction) (Bates & Lipmann, 1960); the same mechanism presumably obtains in extracts of rabbit liver. From the results of our 'competition' experiments it seems quite likely that soluble RNA also plays a part in the synthesis of ophthalmic acid. If, in fact, γ -glutamyl- α -amino-*n*-butyric acid can be bound to the RNA at the same site as γ -glutamylcysteine normally is, then this 'latitude' in the synthesis of tripeptides forms a contrast with the specificity in the synthesis of proteins. Loftfield, Grover & Stephenson (1953) have shown that labelled α -amino-*n*-butyric acid, on incubation with rat-liver slices, is not incorporated into the proteins at a significant rate, although it enters the cells. Moreover, α -amino-*n*-butyric acid has not been detected in the hydrolysates of proteins. Since we know that extracts of several mammalian livers can catalyse the synthesis of ophthalmic acid, and that rat liver probably contains it, the system that brings about the synthesis of the tripeptide thus seems less selective than that which is responsible for protein synthesis.

Breakdown of glutathione in the nucleus

The enzyme (γ -glutamyl transpeptidase) that catalyses the hydrolysis of glutathione, and of ophthalmic acid, is concentrated in the nuclear fraction (Table 2); the specific activity (i.e. the activity per unit weight of protein) of this fraction is about three times that of the whole tissue. We now have to consider whether, in fact, the enzyme is localized in the nuclei of the cells of rabbit liver. The nuclear fraction is contaminated with whole cells, erythrocytes, a few mitochondria and some debris. The high specific activity of the nuclear fraction, together with the fact that over 90 % of the cells were broken, makes it unlikely that all the activity in the nuclear fraction could be due to the few unbroken cells present. The activity cannot be due to erythrocytes, as the nuclear fraction from perfused liver was active, nor can it be due to mitochondria, as the specific activity of the mitochondrial fraction is less than that of the nuclear fraction. An enzyme might be adsorbed on a cell particle (rather than localized within it); this is how Rosenthal, Gottlieb, Gorry & Vars (1956) interpret their observation that arginase is no longer found in the nuclear and microsomal fractions when the sucrose medium contains metal ions. In our case, however, added metal ions did not lessen the activity of the nuclear fraction. A more homogeneous nuclear fraction was prepared by the procedure of Hogeboom *et al.* (1952), but this preparation did not catalyse the hydrolysis of

glutathione. This procedure involves, at one stage, sieving the extract through lint. We found that this sieving removed at least half the nuclei, and also left little activity in the homogenate, even before differential centrifuging. We therefore regard the negative results obtained by this procedure as inconclusive. The simplest explanation of the high activity of the nuclear fraction does seem to be that the enzyme, γ -glutamyl transpeptidase, is localized within the nucleus of the cell. If so, this is an unusual occurrence (Roodyn, 1959). The enzyme that catalyses the synthesis of diphosphopyridine nucleotide (DPN) is localized in the nucleus (Hogeboom & Schneider, 1952) and Morton (1958) has suggested that the synthesis of DPN in the nucleus, and its breakdown in the cytoplasm, may be a factor that determines cell-division. Glutathione, on the other hand, may be synthesized in the cytoplasm and broken down in the nucleus. This conclusion must be regarded as tentative, as the interpretation of the results of fractionation by differential centrifuging is fraught with pitfalls (Allfrey, 1959). There is also the possibility that, *in vivo*, glutathione might be mainly broken down in the cytoplasm by γ -glutamyl lactamase; this process could be more rapid than the diffusion of glutathione into the nucleus, and hydrolysis within it.

Although there are no other reports of a quantitative study of the subcellular fractionation of the enzyme that catalyses the hydrolysis of glutathione, Lang, Siebert & Müller (1951) noted the presence of this enzyme in the nuclear fraction from pig kidney, and Hird & Springell (1954*a*), in their purification of the enzyme, used a 'particulate' fraction. Bailie & Morton (1958) detected γ -glutamyl transpeptidase in the microsomes from the mammary gland, and Binkley, Davenport & Eastall (1959) also found the enzymes to be mainly present in the microsomes from kidney; in plant tissues, the distribution seems somewhat different (Webster, 1953).

Transpeptidation

In extracts of animal tissues, γ -glutamyl transpeptidase catalyses the hydrolysis of glutathione, and also catalyses transpeptidation reactions (Hird & Springell, 1954*b*). Indeed, there is some evidence that transpeptidation occurs even without an added amino acid either by the reaction of (hydrolytically liberated) cysteine with glutathione (Hird & Springell, 1954*b*):

or by the reaction between two molecules of glutathione (Revel & Ball, 1959):

Our experiments show that both transpeptidation and hydrolysis are catalysed by the nuclear fraction and thus support the idea that γ -glutamyl transpeptidase catalyses both hydrolysis and transpeptidation. There is a considerable advantage in the use of the nuclear fraction to study transpeptidation, as the best 'acceptors' are dipeptides, such as glycylglycine (Fodor, Miller & Waelsch, 1953), and the dipeptides are more stable in the nuclear fraction (compare Fig. 4 with Fig. 5), as the dipeptidases are mainly in the supernatant fraction. The absence of γ -glutamyl lactamase from the nuclear fraction is also an advantage in the study of transpeptidation.

There is considerable variation in the γ -glutamyl transpeptidation activity of different organs. Hanes, Hird & Isherwood (1952) demonstrated transpeptidation with extracts from sheep kidney and pancreas, and Fodor *et al.* also used sheep brain and calf liver. Amongst a variety of tissues, Revel & Ball (1959) detected γ -glutamyl transpeptidase only in kidney and pancreas. Our results (Table 2) also show that sheep kidney was the most active of the tissues tested, although we were able to carry out transpeptidation quite readily with the nuclear fraction from rabbit liver. One such reaction (Fig. 6) led to the formation of ophthalmic acid from glutathione and α -amino-*n*-butyrylglycine:

This reaction does not seem likely to contribute towards the synthesis of ophthalmic acid *in vivo*.

Composition and metabolism of the lens

The amounts of protein in the subcellular fractions of calf lens have been determined; in Table 4 these amounts are compared with the corresponding amounts from rabbit liver. There is a striking difference between the 'composition' of rabbit liver and calf lens: in the calf lens nearly all (96%) of the protein is found in the supernatant fraction. Indeed, the lens appears unique in the

tissues so far studied, in the high proportion of protein in the supernatant fraction. Electron microscopy of lens fibres (Wanko & Gavin, 1959) shows the presence of nuclei, mitochondria and microsomes, but these become rather sparse in the fibres nearer the centre of the lens. The results of the differential centrifuging may be affected by proteins being extracted from the particles, but, whether or not this happens, a comparison of the results of subcellular fractionation of the lens and other tissues suggests that the lens would be expected to contain those enzymes that are commonly found in the supernatant fraction. On the other hand, those enzymes which are normally found in the nuclear, mitochondrial or microsomal fractions might be difficult to detect in lens extracts. This generalization does seem to hold quite well. Many of the enzymes that are concerned with glycolysis, for example, are quite active in the lens, and these enzymes are normally found in the supernatant fraction. Conversely, several of the enzymes of the tricarboxylic acid cycle are found in the mitochondrial fraction, and are barely detectable in lens extracts. Moreover, to revert to the metabolism of γ -glutamyl peptides, the enzymes that are present in the supernatant fraction from rabbit liver (γ -glutamyl lactamase, and the pair of enzymes responsible for the synthesis of glutathione, and of ophthalmic acid) are

present in the lens, but γ -glutamyl transpeptidase cannot be detected in lens extracts.

SUMMARY

1. The intracellular distributions of the enzymes that catalyse the synthesis and breakdown of glutathione and its analogue, ophthalmic acid, have been studied. The tissue chosen was rabbit liver.

2. The synthesis of both tripeptides follows the same course, and competition experiments suggest that the same enzymes may be involved. These enzymes are present in the supernatant fraction of rabbit liver; no appreciable activity was detected in the other fractions.

3. The hydrolysis of glutathione is catalysed by the nuclear fraction; the other fractions have little or no activity. The nuclear fraction also catalyses transpeptidation reactions. Hydrolysis and transpeptidation are probably brought about by the same enzyme, γ -glutamyl transpeptidase. The problem of whether γ -glutamyl transpeptidase is localized in the nucleus is discussed.

4. Ophthalmic acid is broken down in both the nuclear and supernatant fractions. In the nuclear fraction, ophthalmic acid is hydrolysed to the con-

Table 4. *Distribution of protein in the subcellular fractions of rabbit liver and of calf lens*

The amount of protein in each fraction is expressed as a percentage of the amount in the unfractionated extract. Protein was estimated by the method of Robinson & Hogden (1940).

Fraction	Rabbit liver	Calf lens
Nuclei + unbroken cells	21	0
Mitochondria	11	1
Microsomes	13	1
Supernatant	52	96
Total	97	98

stituent amino acids (glutamic acid, α -amino-n-butyric acid, and glycine); transpeptidation also occurred in the nuclear fraction, and γ -glutamyl transpeptidase is the enzyme which brings about hydrolysis and transpeptidation in the nuclear fraction. The breakdown of ophthalmic acid in the supernatant fraction gives 5-oxopyrrolidine-2-carboxylic acid rather than glutamic acid, and is ascribed to the action of γ -glutamyl lactamase. The localization of γ -glutamyl lactamase in the supernatant fraction was shown when γ -glutamylglycine was used as substrate.

5. In the subcellular fractionation of calf lens, nearly all the protein is in the supernatant fraction. The enzymes that are most readily detected in lens extracts are those which are present in the supernatant fraction of liver.

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Studies on the Flash Photolysis of Visual Pigments

1. PIGMENTS PRESENT IN FROG-RHODOPSIN SOLUTIONS AFTER FLASH-IRRADIATION

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Irradiation of visual-pigment solutions with brief intense bursts of light makes it possible to detect products of bleaching which have only a transient existence or which are themselves photo-reactive. Ideally, the requirement is for an in-

stantaneous flash of intensity sufficient to excite all the visual-pigment molecules: in practice, electronically-operated discharge tubes of the type used in photography provide the best available light sources. Such tubes are capable of producing