L-serine or L-tyrosine specifically stimulated riboflavin synthesis; L-tyrosine was the least effective. D-Threonine was inactive. L-Glutamate, L-aspartate and L-asparagine stimulated both growth and flavinogenesis, whilst L-cysteine inhibited both. Other amino acids tested were without obvious effect on riboflavin synthesis, but some inhibited growth slightly.

3. Under the same conditions the purines xanthine and adenine, and adenosine, stimulated flavinogenesis whilst most pyrimidines and their derivatives were without effect, although alloxan inhibited both growth and riboflavin synthesis. At very high concentrations, uracil inhibited riboflavin synthesis, although it did not inhibit the adenine-stimulated production of riboflavin.

4. Neither 5:6-dimethylbenzimidazole nor 1:2-dimethyl-4-amino-5-(D-1'-ribitylamino)benzene stimulated riboflavin production under the same conditions.

5. Of the other nitrogen-containing compounds tested, urea in low concentrations occasionally stimulated and $(\text{NH}_4)_2\text{SO}_4$ was without effect. D-Glucosamine was a powerful inhibitor of both growth and riboflavin.

6. The effects of the stimulatory amino acids are not cumulative, whilst those of a purine and an amino acid are.

7. The significance of these results in relation to the problem of the biosynthesis of riboflavin is discussed.

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On the Significance of γ -Glutamyl Transpeptidation in Peptide Biosynthesis

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Transpeptidation reactions have been receiving increasing emphasis in connexion with the syntheses of peptide and amide bonds and hence of proteins (Hanes, Hird & Isherwood, 1950, 1952; Hanes, Connell & Dixon, 1953; Waelsch, 1953; Fruton, Johnston & Fried, 1951; Fruton, 1950). Particular interest attaches to the γ -glutamyl transpeptidation

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reaction of Hanes *et al.* (1950, 1952) because the hypothesis has been advanced that glutamine and glutathione serve to direct the energy of the energy-rich bonds of adenosine triphosphate into the formation of peptide bonds by means of the transpeptidation and transamidation reactions (Waelsch, 1953). The hypothesis further assumes that the γ -glutamyl peptides formed by these reactions would either rearrange to α -peptides or by further exchanges

between themselves or other amino acids form α -peptide linkages. The experimental basis for the theory is that glutathione incubated with a substrate amino acid in the presence of a dialysed extract from fresh sheep kidney or certain other tissues yielded the corresponding γ -glutamyl peptides, of which γ -glutamylphenylalanine and γ -glutamylglycine have been identified (Hanes *et al.* 1952).

The object of the present investigation was to test the γ -glutamyl transpeptidation hypothesis in several tissue systems known to be capable of incorporating amino acids into the cellular protein material. The work involved two approaches, one to study the effect of the addition of γ -glutamyl peptides to tissues undergoing active amino acid incorporation, the other an attempt to demonstrate the natural occurrence of γ -glutamyl peptides formed from ^{14}C -labelled substrates. It will be seen that the results of this study cannot be interpreted as being favourable to the γ -glutamyl transpeptidation hypothesis. A preliminary report of the results has been published (Hendler & Greenberg, 1952).

METHODS

^{14}C -compounds. The isotopic compounds were [1:2- ^{14}C]glycine, DL-[2- ^{14}C]valine, DL-[4:4'- ^{14}C]valine hydrochloride, DL-[3- ^{14}C]phenylalanine hydrochloride (obtained from Tracerlab Inc., Boston, Massachusetts), and γ -glutamyl-[1:2- ^{14}C]glycine, synthesized as described below.

Synthesis of γ -glutamylglycine

The steps of the reactions utilized are: glutamic acid $\rightarrow$ γ -ethyl L-glutamate $\rightarrow$ γ -ethyl benzyloxycarbonyl-L-glutamate (γ -ethyl BOC-L-glutamate) $\rightarrow$ BOC- γ -L-glutamylhydrazide $\rightarrow$ BOC- γ -L-glutamylazide $\rightarrow$ BOC- γ -L-glutamylglycine $\rightarrow$ γ -L-glutamylglycine.

γ -Ethyl L-glutamate (Hanby, Waley & Watson, 1950), γ -ethyl BOC-L-glutamate (Hegedus, 1948) and BOC- γ -L-glutamylhydrazide (LeQuesne & Young, 1950) were prepared by the standard procedures described in the references cited. The γ -ethyl BOC-L-glutamate, after being taken up in ether, was washed with water and dried *in vacuo* over P_2O_5 . This material, without further purification, was employed to prepare the BOC- γ -L-glutamylhydrazide, which, upon recrystallization from water, yielded white needles (m.p. 179–180°).

The synthesis was carried out on a micro scale. To secure a maximum yield based on glycine it was found desirable to increase the molar ratio of BOC- γ -L-glutamylhydrazide to glycine over that used by LeQuesne & Young. On a 0.1 molar scale, by increasing the molar ratio of hydrazide to glycine from 0.64 to 1.0, the yield of recrystallized BOC- γ -L-glutamylglycine was increased from 20 to 61%, based on glycine. By this step it was possible to obtain 110 mg. of recrystallized BOC- γ -L-glutamylglycine, which yielded 26.2 mg. of twice-reprecipitated γ -L-glutamylglycine after catalytic hydrogenation. The yield of pure γ -L-glutamylglycine on the micro-scale synthesis was 24% (based on glycine) compared with the overall yield obtained by

LeQuesne & Young of the same product (not reprecipitated) of 16%.

Synthesis procedure. BOC- γ -L-glutamylhydrazide (157 mg.) was dissolved in 2.14 ml. water containing 0.2 ml. of 12N-HCl in a 10 ml. separating funnel having a side arm with a ground glass joint. After addition of ethyl acetate (1.35 ml.), a miniature vacuum-driven stirring motor with a 1 mm. bent glass rod stirrer was placed above the solution. The separating funnel was almost completely immersed in a beaker of ice-water in the cold room. With vigorous stirring a solution of NaNO_3 (61.8 mg.) in water (0.18 ml.) was added drop by drop through the side arm over 5 min. Rapid stirring was continued for a further 10 min. The ethyl acetate layer was separated and washed twice with cold water (about 0.2 ml. each). It was then added slowly with stirring from the 10 ml. separating funnel into the side arm of a 5 ml. separating funnel containing [1:2- ^{14}C]glycine (40 mg., 2.5 $\mu\text{C}/\text{mg.}$) and KHCO_3 (188 mg.) dissolved in water (1 ml.), which was cooled in ice-water as above. The 10 ml. funnel was rinsed with 0.2 ml. ethyl acetate which was added to the glycine-containing funnel. Stirring was continued for 15 min. in the cold and the separating funnel left for 2.5 hr. at room temp. and then for 10–15 min. in warm water (about 40°). (Sometimes about 0.3 ml. of fresh ethyl acetate was added to replace loss of ethyl acetate by evaporation at room temperature and at 40°.) The aqueous layer was acidified with 12N-HCl, and extracted 4 times with 1 ml. portions of ethyl acetate. The combined extracts were washed with water (about 0.3 ml.) and placed in a 10 ml. beaker. Concentration was effected by placing the beaker covered with Parafilm (Polythene) in a vacuum-filtering device (Fisher Filtrator) fitted with a long capillary (containing glass wool) through a rubber stopper at the top. The capillary tip was introduced through the Parafilm just above the liquid, and upon evacuation of the Filtrator a fine jet of air was played upon the solution. This apparatus, illuminated by i.r. lamps, gave a very effective means for easy concentration with minimum loss of material. The remaining oil was washed with ether and left in a vacuum desiccator over P_2O_5 overnight (yield 141.6 mg.). This was recrystallized by dissolving it in 7 drops of boiling water and allowing it to cool slowly while its container (a 5 ml. beaker) was immersed in a larger beaker of hot water. The ppt. was filtered and washed with copious quantities of cold water (yield 110 mg.). The BOC- γ -L-glutamylglycine (110 mg.) was dissolved in 0.5 ml. of methanol containing 0.02 ml. glacial acetic acid and 2 drops of water in a micro-bubbling apparatus, palladous oxide (12.5 mg.) was added and, with the tip of a steel pin encased in a glass capillary tube (1 $\times$ 5 mm.) to serve as a magnetic stirrer, a stream of hydrogen was passed through. After 37 min., methanol (0.5 ml.), glacial acetic acid (0.02 ml.) and water (2 drops) were added to the reaction flask to replace that lost by evaporation. After about 1 hr. no further CO_2 was evolved, showing that the reaction was complete. The weight of BaCO_3 collected from the effluent gas was 55 mg., indicating a maximum possible yield of 57 mg. of γ -glutamylglycine. The reaction tube was centrifuged, the supernatant fluid sucked into a drawn out capillary tube fitted with a rubber bulb and filtered through Whatman no. 1 paper into a small beaker. The remaining material in the reaction flask was washed with (0.5 ml.) water and this also was filtered into the beaker. The contents of the beaker were evaporated to dryness by placing the open end of a sealed capillary tube in the liquid and putting the beaker in a desiccator over P_2O_5 under

reduced pressure; yield of crude γ -glutamylglycine, 52.4 mg. The material was twice dissolved in 15 drops of water and precipitated by adding 68 drops of ethanol. It was filtered through Whatman no. 50 filter paper supported on an inverted filter stick. Yield 26.2 mg. (24%). (Found: C, 41.3; H, 6.0; Calc. for $C_7H_{12}O_2N_2$: C, 41.2; H, 5.9; N, 13.7%. Found for a sample of unlabelled peptide prepared by the identical procedure: C, 41.3; H, 5.9; N, 13.6%.)

Preparation of samples and counting of radioactivity

The isolation and treatment of protein and other materials for counting of radioactivity, treatment with mercapto-ethanol, and plating have been described in previous publications (Peterson & Greenberg, 1952; Lien & Greenberg, 1952). Briefly this consisted of precipitating the proteins with trichloroacetic acid (TCA) (final concentration about 7%, w/v) after incubation, washing with hot and room-temperature TCA solutions, and dehydrating and preparing the proteins for plating with washes of ethanol, ethanol-ether, ether, and ether-light petroleum-acetone. To remove disulphide-bound material, when desired, the protein was suspended for 20–30 hr. under N_2 at room temperature in a mercaptoethanol solution. It was then reprecipitated with TCA, washed and plated as above and recounted. Protein was plated on aluminium disks of large diameter (1.75 in.) and the fraction from Dowex or Amberlite columns were received and evaporated to dryness in polyethylene cups (Lien & Greenberg, 1952) and counted in windowless gas-flow counters. In the case of paper chromatograms, radioactive spots were detected by means of a scanning windowless gas-flow counter tube held over each 0.75 in. of the path of the spots for 30 sec., using a T-square to guide the counter in its path.

Enzyme preparations and incubation procedures

Cell particles-supernatant fluid (PS preparation). In a typical experiment, the washed liver (approx. 9 g.) of a 200–230 g. rat was homogenized cold in an equal weight of buffer (0.12M-KCl + 0.04M- $KHCO_3$ equilibrated with 95% O_2 -5% CO_2). The particulate fraction was isolated by differential centrifuging (Peterson & Greenberg, 1952) and suspended in an equal volume of the liver supernatant fluid obtained by centrifuging part of the above homogenate for 30 min. at 4000 rev./min. Portions (0.3 ml.) of this preparation were added to 12 ml. centrifuge tubes containing the radioactive amino acid and the other desired constituents, which had been evaporated to dryness in the tubes in vacuum at 40°, in 0.03 ml. of water (final vol. 0.33 ml.). Incubation was performed at 37° in a rotating circular rack immersed in a constant temperature bath.

Cell particles-synthetic medium (PM preparation). The same particulate fraction as above was suspended in a synthetic incubation medium, yielding final concentrations of 6 mM citrate, 10 mM Mg^{2+} and 3 mM ATP. In addition, the medium contained a mixture of amino acids shown to have an activating effect (Peterson & Greenberg, 1952), namely, 1.6 mM methionine, 2 mM aspartate, 3 mM glutamate, 1.2 mM arginine, 1 mM proline and 2.2 mM alanine.

Gardner mouse lymphosarcoma (6C3H-ED). The mouse tumours were pooled and forced through a 60-mesh monel metal screen (Farber, Kit & Greenberg, 1951) to yield a suspension of essentially free cells.

Mouse spleen. Suspensions of cells were prepared as described for the lymphosarcoma.

Mouse spleen homogenate. The cell suspension obtained as above was homogenized for 5 min. at 0° with 2 vol. of buffer in a tight-fitting all-glass homogenizer. Microscopic examination, after staining in methylene blue in 1% (v/v) acetic acid, showed that about 50% of the cells were ruptured.

Kidney homogenate. One g. of rat kidney was homogenized with 6 ml. of the buffer described by Hunter (1949), containing also glucose (11.4 mg.) and $KHCO_3$ (7.2 mg.).

Hanes et al. (1952) transpeptidase preparation. This was prepared as described by these authors from a sheep kidney obtained in the laboratory within 25 min. of the death of the animal. Various modifications of the above enzyme preparation were tried with rat kidney. The manner of dialysis was varied; the extract was employed in different dilutions from full strength to 1/4 strength, the incubations were carried out both at 32 and 37°, and the buffer concentration was varied between 0.017 and 0.05M.

The incubation volumes of the Peterson & Greenberg systems were 0.33 ml., of all others 1.0 ml. All incubations were carried out for 60 min. at 37° unless otherwise noted. When mM concentrations of substrates are mentioned in the text or tables they represent final concentrations.

Chromatographic procedures

Paper chromatography (Whatman no. 4 and/or no. 1 paper) was performed with the solvent systems: 80% (v/v) propanol-water (Hanes et al. 1950), butanol-acetic acid-water (100:22.5:50, v/v), and phenol-water (100 g. of reagent grade phenol, distilled over zinc at atmospheric pressure, dissolved in 38 ml. water).

Dowex column chromatography. The columns (55 × 1 cm.) were prepared by standard methods (Stein & Moore, 1950; Lien & Greenberg, 1952), using Dowex-50 (Micro-chemical Specialties Co., Berkeley, California) in the acid form and were eluted with 1.5N-HCl alone, except in the case of phenylalanine, when this was followed by 6N-HCl. The samples were applied in about 0.5 ml. vol. and represented the supernatant fluid from the different tissue preparations after addition of TCA to about 7% (w/v) concentration.

Amberlite ion-exchange. Amberlite IR-4B (Resinous Products Corp., Philadelphia, Pa.) in a column 3 × 1 cm. was generated into the NH_4 form by approximately 100 ml. of N-NaOH. Extensive washing with water brought the effluent pH to that of distilled water. 3 ml. of clear supernatant fluid from an incubation were passed through Amberlite IRC-50 in the acid form (column 2.5 × 1.3 cm.) and the first 10 ml. of effluent was applied to IR-4B. Water was passed through the basic resin so that 30 ml. were collected in a 50 ml. volumetric flask which had 20 ml. of water carefully pipetted into it. This fraction contained all materials not adsorbed to the column. A second fraction of 4 ml. was then taken to assure that the radioactivity was at the base line, after which elution was accomplished with N-HCl. The third fraction consisted of 20 ml. of this eluate collected in a 25 ml. volumetric flask containing 5 ml. water. 1 ml. portions from each of the three fractions (further diluted when necessary) were pipetted into polyethylene planchets in triplicate and counted. The HCl eluate from the IR-4B resin is referred to as the acidic peak.

RESULTS AND DISCUSSION

Amino acid incorporation

The incorporation and Dowex chromatographic experiments were performed with glycine, valine, and phenylalanine. These amino acids were chosen because Hanes *et al.* (1950, 1952) reported their corresponding glutamyl peptides, and because they would be expected to be involved in any general scheme for amino acid incorporation. The most thorough study was made with glycine for which both the radioactive and the unlabelled γ -glutamyl peptide were available. In every case the label was more efficiently incorporated into the cellular proteins when the incubation was carried out with free glycine than with an equimolar quantity of γ -glutamylglycine (Table 1).

In the PS system, the glycine of the γ -glutamyl peptide was utilized 73, 63 and 87% as efficiently as free glycine. To eliminate the possibility that the amount of γ -glutamyl peptide used was in excess of an optimum effective concentration, two lower concentrations were tried. The incorporation in these experiments was correspondingly lower.

Mercaptoethanol treatment, which has been shown by Peterson & Greenberg (1952) to remove an appreciable amount of radioactivity from the proteins of the PS system after incubation with glycine, did not alter the results. As much or more activity was removed by this treatment from the proteins after incubation with γ -glutamylglycine, than with glycine. The proteins of whole-cell suspensions were resistant to this treatment.

The spleen whole-cell suspension used the peptide glycine only 24 and 18% as efficiently as free glycine. Since the relative amount of glycine incorporated into TCA-insoluble protein of the spleen system was only about one-third that of the cell-free liver

system, the question of cell-membrane permeability arose. Tests with a spleen homogenate showed the same relative incorporation of the peptide glycine with respect to the free glycine as for the whole-cell suspension (although the actual uptake was much less).

A cell suspension of the Gardner lymphosarcoma (6C3H-ED) from mice used the peptide glycine approximately as effectively (13% compared with glycine) as its analogous tissue (the spleen).

Rat kidney homogenate showed very slight fixation of [^{14}C]glycine into the insoluble protein. Uptake was not improved by supplying the glycine peptide.

The addition of glutamic acid, glutamine, glutathione, or unlabelled γ -glutamylglycine had no significant effect on the uptake of glycine, except in the spleen and Gardner lymphosarcoma cell suspensions, in which stimulation by these compounds is indicated (Table 2).

The uptake of valine in the PS system was not significantly influenced by addition of the same glutamyl compounds listed above (Table 2). Since the PS system might already be saturated with γ -glutamyl compounds (this is extremely unlikely from the results to be described in the section on chromatography with Dowex) a PM system employing a synthetic medium was tried, and this too showed no activation. In another experiment, ATP and citrate were omitted from the synthetic medium since the tissue might have been able to synthesize adequate amounts of γ -glutamyl peptide with this energy supply. Uptake dropped and no significant activation was shown by addition of unlabelled γ -glutamylglycine.

The kidney homogenate system definitely was not activated towards the incorporation of phenylalanine by addition of the glutamic acid-containing compounds; in fact, an inhibition by these compounds was observed.

Table 1. *Incorporation of radioactive glycine into the proteins of suspensions of various tissues*

Tissue suspension incubated with [1:2- ^{14}C]glycine or γ -glutamyl-[1:2- ^{14}C]glycine (GG) for 60 min. at 37°. Proteins were precipitated and washed as described in the text. The figures in columns 3-5 are the mean percentage $\pm$ the mean deviations of the corresponding glycine values arbitrarily taken as 100. Unless otherwise indicated, the final concentration of the labelled compounds was 1.2 mM.

Enzyme system	Glycine		GG (% of glycine)	GG (% of glycine)
	(Counts/min./ mg. protein)	(%)		
PS	26	100 $\pm$ 11	73 $\pm$ 2	42 $\pm$ 9 (0.4 mM)
PS	33	100 $\pm$ 14	63 $\pm$ 1	16 $\pm$ 1 (0.24 mM)
PS	27	100 $\pm$ 3	87 $\pm$ 2	—
Spleen whole cells	200	100 $\pm$ 10	24 $\pm$ 1	8 $\pm$ 0 (0.24 mM)
Spleen whole cells	175	100 $\pm$ 10	18 $\pm$ 0	—
Spleen homogenate	53	100 $\pm$ 15	18 $\pm$ 5	—
Lymphosarcoma	32	100 $\pm$ 5	13 $\pm$ 4	—
Kidney homogenate	3.2	100 $\pm$ 19	67 $\pm$ 34	—

Table 2. *Incorporation of radioactive amino acids in presence and absence of glutamyl compounds*

Incubations were for 60 min. at 37°. Proteins were precipitated and washed as described in the text.

Enzyme system	Labelled substrate†	None		Cosubstrates*							
		(Counts/min./mg. protein)	(%)	Glutamic acid		Glutamine		Glutathione		γ -Glutamylglycine	
				(%)	(Rt†)	(%)	(Rt†)	(%)	(Rt†)	(%)	(Rt†)
PS	Glycine	26	100 $\pm$ 11	78 $\pm$ 2	1	87§	1	—	—	—	—
PS	Glycine	33	100 $\pm$ 14	100 $\pm$ 14	1	82§	1	92 $\pm$ 15	0.1	97 $\pm$ 2	0.1
PS	Valine	31	100 $\pm$ 5	100 $\pm$ 4	0.1	97 $\pm$ 1	0.1	104 $\pm$ 3	0.1	—	—
PS	Valine	26	100 $\pm$ 9	111 $\pm$ 5	0.1	108 $\pm$ 8	0.1	107 $\pm$ 11	0.01	—	—
PS	Valine¶	18	100 $\pm$ 3	96 $\pm$ 6	0.33	—	—	100 $\pm$ 1	0.06	88 $\pm$ 8	0.06
PM	Valine¶	93	100 $\pm$ 4	90 $\pm$ 4	0.33	—	—	—	—	95 $\pm$ 6	—
PM (citrate and ATP absent)	Valine¶	51	55 $\pm$ 1	—	—	—	—	—	—	37 $\pm$ 5	0.03
PM	Valine¶	31	100 $\pm$ 4	93 $\pm$ 2	0.33	—	—	—	—	131 $\pm$ 6	0.03
PM (citrate and ATP absent)	Valine¶	22	63 $\pm$ 6	—	—	—	—	—	—	77 $\pm$ 0	0.03
Spleen whole cells	Glycine	200	100 $\pm$ 10	125 $\pm$ 2	1	123 $\pm$ 1	1	—	—	127 $\pm$ 2	0.1
Spleen homogenate	Glycine	53	100 $\pm$ 15	95 $\pm$ 5	1	117	1	—	—	—	—
Lymphosarcoma	Glycine	32	100 $\pm$ 5	95§	1	140 $\pm$ 29	1	—	—	147 $\pm$ 4	0.1
Kidney homogenate	Glycine	3.2	100 $\pm$ 19	67 $\pm$ 13	1	—	—	—	—	78 $\pm$ 28	0.1
Kidney homogenate	Phenylalanine	29	100 $\pm$ 2	87 $\pm$ 1	0.44	84 $\pm$ 6	0.44	104 $\pm$ 0	0.44	77 $\pm$ 11	0.44
Kidney homogenate	Phenylalanine	38	100 $\pm$ 6	—	—	—	—	107 $\pm$ 3	0.44	—	—
Kidney homogenate	Phenylalanine	46	100 $\pm$ 9	39 $\pm$ 1	0.13	21 $\pm$ 3	0.13	56 $\pm$ 1	0.13	42 $\pm$ 1	0.05
								59 $\pm$ 3	0.26	—	—
Kidney homogenate	Phenylalanine	12	100 $\pm$ 14	95 $\pm$ 7	0.13	41 $\pm$ 1	0.13	79 $\pm$ 3	0.13	91 $\pm$ 18	0.05
Kidney homogenate	Phenylalanine	103	100 $\pm$ 9	44 $\pm$ 4	0.13	—	—	—	—	43 $\pm$ 1	0.13
										36 $\pm$ 1	0.26

* Uptake of the free amino acid in absence of cosubstrates but in otherwise complete systems (col. 3) is arbitrarily taken as 100 (col. 4). Figures (mean percentage $\pm$ deviation) in cols. 5, 7, 9 and 11 are relative to this arbitrary standard. The figures (col. 4) referring to the PM system without citrate or ATP are also relative to this standard.

† Final concentrations of labelled amino acids were: glycine, 1.2 mM; valine, 4 mM; phenylalanine, 0.9 mM in upper three and 1.5 mM in last two experiments.

‡ R = molar concentration of cosubstrate.

§ Single determination.

|| Valine labelled in α -carbon.

¶ Valine labelled in methyl carbons.

Chromatography with Dowex-50

These fractionations were undertaken to determine the nature of the products formed from the various radioactive substrates employed in the experiments. A summary of representative results obtained is given in Table 3. The fractionation of the supernatant fluid from incubations with γ -glutamylglycine for 0.5, 20 and 60 min. on a Dowex-50 column revealed that glycine and serine appeared quite readily at the expense of the peptide in the PS system and more readily in the rat kidney homogenate system. Experiments with supernatant fluids from glycine incubations showed that serine is readily formed from glycine, and that no γ -glutamylglycine appeared in incubations carried out for 20 or 60 min. or for 20 min. when unlabelled γ -glutamylglycine was added as a 'trap' or as γ -glutamyl donor source. Of interest in the comparison of chromatograms from the spleen and kidney is the unknown peak which appeared when γ -glutamylglycine was incubated, but not when free glycine was used. This may well be α -keto- γ -glutarylglutamine (γ -carboxy- γ -oxobutyrylglutamine). The position of the spot on the autoradiograph is in keeping with a compound more acidic than γ -glutamylglycine. The increase with time of this peak in the spleen experiment, and the decrease with time in the kidney experiment would parallel the respective abilities of the two systems to cleave the γ -glutamyl bond.

The stability of the γ -glutamylglycine in mouse spleen is in striking contrast to its lability in the other two systems. Evidence that the γ -glutamyl peptide bond is stable to hydrolysis under the acidic conditions of chromatography on Dowex-50 can be seen in the experiment with spleen (Table 3). When the TCA supernatant from a 0.5 min. incubation was chromatographed on Dowex-50, over 99% of the radioactivity was in the discrete peak for γ -glutamylglycine. No activity was detected in glycine. Correspondingly, after incubation for 20 and 60 min., column development of the supernatant showed 97 and 93% of the radioactivity, respectively, in γ -glutamylglycine, whereas 2 and 5%, respectively, were in glycine. Radioactive γ -glutamylglycine also was chromatographed on Dowex-50 with no previous incubation, and no detectable counts appeared as glycine. The apparent ease of hydrolysis of the peptide in the liver and kidney is contrary to the proposed function of the γ -glutamyl link as a stable source of peptide bonds (by themselves or in adjacent α -peptide bonds, as in glutathione). That the disappearance of peptide and appearance of labelled glycine represents hydrolysis and not transpeptidation is supported by the fact that added unlabelled γ -glutamylglycine in the same systems was inert as a participant in trans-

peptidation with free labelled glycine, valine or phenylalanine (Table 3).

It is worth noting that the relative efficiency of utilization of the peptide glycine runs parallel to the ability of the system to hydrolyse the peptide. This suggests that prior hydrolysis is necessary for incorporation of glycine introduced from the γ -glutamylglycine.

In incubations with valine in the PS system at 20 and 60 min., and at 20 min. in the presence of unlabelled γ -glutamylglycine, no peak appeared which could be assigned to γ -glutamylvaline. If the compound was formed it would have to be in an amount less than 0.1% of the added valine. Methyl-labelled valine showed a significant conversion into aspartic acid, glutamic acid, and glycine and/or alanine. In addition, a prominent forepeak (or peaks) occurred (Table 3). This would indicate that the label readily enters the tricarboxylic acid cycle and that transamination is active in this system. The forepeak is indicative of labelled tricarboxylic acid cycle intermediates or of non-volatile fatty acids.

The supernatant fluid from an incubation with phenylalanine in rat kidney homogenate at 20 and 60 min., and at 20 min. in the presence of unlabelled γ -glutamylglycine showed that all the activity was retained in phenylalanine and in a broad forepeak which diminished markedly from 10% at 20 min. to 2% at 60 min. No evidence was obtained of the formation of γ -glutamylphenylalanine (Table 3). It could not have been present in the forepeak because this peptide would be expected to be retained more strongly than γ -glutamylglycine by the column.

γ -Glutamyl compounds may need concentrations corresponding to only a few hundredths of a per cent of the added labelled amino acid for an effective role in transpeptidation. However, the experiments with added labelled γ -glutamylglycine in diminishing amounts are not in accord with this possibility. It is also important to point out that the rather substantial conversions obtained by Hanes *et al.* (1950, 1952) do not occur in these systems which are incorporating amino acids into proteins.

Amberlite ion-exchange

If a neutral amino acid forms an acidic metabolite, an acid-adsorbing resin should be applicable to assay the extent of conversion. γ -Glutamyl peptides would be expected to have a degree of acidic character similar to glutathione which has a lower isoelectric point than glutamic acid. Amberlite IR-4B was used by Cannan (1944) to remove glutamic and aspartic acids quantitatively from protein hydrolysates. Chromatography with this resin appeared to us to be applicable to screen for further study the several different tissues for the

Table 3. *Radioactive fractions obtained by Dowex-50 chromatography from protein-free incubation fluids*

After incubation, a TCA extract (vol. below 1 ml.) of the homogenized tissue was introduced in the column and developed with 1.5N HCl.

Radioactive substrate	Enzyme system	Incubation time (min.)	Total radioactivity of fluid† (counts/30 sec.)	Percentage of total radioactivity*							Forepeak or unknown compounds
				γ-Glutamyl-glycine	Glycine	Serine	Alanine	Phenyl-alanine			
γ-Glutamyl-[1,2- ¹⁴ C]glycine	Liver-PS	0.5	36 200	97	3	—	—	—	—	—	
		20	41 250	13	73	13	0.6	—	—	—	
		60	24 900	0.8	80	17	2.0	—	—	—	
	Intact spleen cells	0.5	42 150	99	—	—	—	—	—	0.3†	
		20	47 000	97	2	—	—	—	—	1	
	60	39 700	93	5	—	—	—	—	2		
	Kidney	0.5	34 400	54	44	—	—	—	—	3	
		20	46 150	—	98	2	—	—	—	0.3	
		60	31 000	—	97	3	—	—	—	—	
	Liver-PS	20	24 300	—	78	21	—	—	—	1§	
20		27 800	—	79	20	—	—	—	1		
60		21 800	—	71	23	—	—	—	6		
Intact spleen cells	20	38 800	—	99	1	—	—	—	—		
	20	35 400	—	99	1	—	—	—	0.3		
	60	35 650	—	98	2	—	—	—	—		
Kidney	20	25 500	—	87	13	—	—	—	—		
	20	30 500	—	90	10	—	—	—	—		
	60	31 500	—	89	11	—	—	—	—		
DL-[3- ¹⁴ C]Phenylalanine	Kidney	20	61 000	—	—	—	—	—	—	8†	
		20	65 100	—	—	—	—	—	—	10	
		60	67 100	—	—	—	—	—	—	2	
DL-[4,4'- ¹⁴ C]Valine	Liver-PS	20	259 000	93	—	—	—	—	—	Forepeak or unknown compounds	
		20*	259 800	93	0.3	—	—	—	—	2†	
		60	217 600	90	1	1.5	2.0	0.2	6		

* Compounds named were identified by paper chromatography against known compounds; solvents employed, phenol-water and butanol-acetic acid (see text).

† Obtained from summation of radioactivity in all peaks.

‡ Radioactivity in this group present in unknown compound or compounds.

§ Radioactivity in this group present in forepeak.

|| Unlabelled γ -glutamylglycine added.

system most abundant in γ -glutamyl peptides and thus, in all probability, most active in γ -glutamyl synthetic activity and utilization.

Control experiments showed that Amberlite IR-4B retained practically no valine (less than 0.1%), but had a strong affinity for acidic substances, such as glutamic acid, propionic acid, glutathione and γ -glutamylglycine. This resin therefore appeared to be ideal for studying the order of magnitude of the formation of γ -glutamyl peptides.

The technique was applied to a study of several different enzyme systems involving different amino acids, different methods of preparing the cell extract, and the reduction of permeability barriers of intact cells. The acids retained should then be indicative of the upper levels of formation of γ -glutamylpeptides, glutamic and aspartic acids, and any other acidic products which would also be retained. The results indicated minute upper levels for the formation of acidic peptides (a few tenths of 1%).

Unfortunately it was found later that the salts in the concentrations present in the buffers of the incubation media seriously interfered with the adsorptive ability of the resin. γ -Glutamylglycine, in the absence of buffer, was retained to the extent of 67%. In the presence of buffer only 12% was retained. The correction necessary was too great to attribute any quantitative significance to these experiments, but as an order of magnitude, it can probably be safely said that in all the incubation systems employed, the extent of conversion, if any, was less than 5%.

Hanes, Hird & Isherwood systems

Attempts to reproduce the observations by Hanes *et al.* (1950, 1952) were conducted with radioactive amino acids on rat kidney and in one series of experiments on sheep kidney. All of the other details in the procedure as described by these authors were very carefully followed, and in addition many modifications were tried with rat tissue. Radioactive valine was used in all experiments and radioactive phenylalanine was included in the experiment with sheep kidney. In addition to the 50 mM concentrations employed by Hanes *et al.*, experiments were performed with one-tenth of these concentrations, which are undoubtedly closer to average physiological concentrations. Evidence of enzymic activity in these preparations was given by the appearance of cysteine and cysteinylglycine (identified by its R_F value) at the expense of glutathione. At the 5 mM concentration the glutathione spot completely disappeared and was replaced by the hydrolysis products. At the amino acid concentrations of Hanes *et al.*, the radioactivity introduced as valine or phenylalanine travelled only with the corresponding amino acid. There were

a few sporadic cases, however, in which a slight peak of radioactivity appeared in an area that might be expected for γ -glutamylvaline. No ninhydrin colour was evident in these areas and the amount of radioactivity relative to valine was less than 1%.

Likewise with phenylalanine in the sheep kidney preparation at the lower concentration (4 mM), but not at the concentration of Hanes *et al.* (50 mM), a small peak of radioactivity appeared in an area that might be expected for γ -glutamylphenylalanine. This too yielded no ninhydrin colour. All the other ninhydrin spots described by these authors were observed.

We have no explanation for the failure to demonstrate the enzymic formation of γ -glutamyl peptides, although we carefully followed the instructions of Hanes *et al.*, employed radioactive tracers which should have greatly increased the ease of detection, and varied the experimental conditions in a variety of ways. It would appear that the γ -glutamyl transpeptidase enzyme system is subject to loss of activity by rather subtle differences in the preparation procedure.

In this connexion it is worthy of note that the formation of γ -glutamylarginine by ox-kidney extracts from glutathione or γ -glutamylglycine and arginine, reported by Kinoshita & Ball (1953) required greatly different experimental conditions from those of Hanes *et al.* The optimum amino acid and glutathione concentrations were 5 mM rather than 50 mM, and the optimum pH 9.0 instead of 7.4. Hanes and co-workers, found arginine to be a poor γ -glutamyl peptide former in their experiments.

More important than the demonstration of the accumulation of γ -glutamyl peptides is the question of their significance in a general scheme of peptide biosynthesis and protein formation. The work reported in this paper is not favourable to a hypothesis involving such a role for γ -glutamyl compounds.

SUMMARY

1. In a study of the significance of the γ -glutamyl transpeptidation hypothesis for the biosynthesis of peptides in various tissue preparations, it was found that free glycine was always incorporated into protein more efficiently than glycine from γ -glutamylglycine and no favourable influence of the γ -glutamyl peptide was observed on the incorporation of radioactive glycine.
2. The incorporation of glycine, valine or phenylalanine into protein was not increased by the addition of glutamic acid or γ -glutamyl compounds.
3. Chromatographic fractionation on Dowex-50 of the supernatant fluids of tissue preparations incubated with γ -glutamyl glycine showed that this peptide is rapidly hydrolysed by liver and kidney

homogenates, but is remarkably stable in the spleen. The chromatogram showed no evidence of the formation of the corresponding radioactive γ -glutamyl peptides from incubations with radioactive glycine, valine or phenylalanine in the presence or absence of unlabelled γ -glutamylglycine.

4. The formation of acidic metabolites, including γ -glutamyl peptides, from neutral substances was studied using chromatography with Amberlite IR-4B resin. An upper level of 5% was obtained as the possible extent of formation of γ -glutamyl peptides.

5. Attempts to confirm the formation of γ -glutamylvaline and γ -glutamylphenylalanine, employing radioactive amino acids, by the sheep kidney system of Hanes *et al.* (1950, 1952) and modifications of this system with rat kidney were unsuccessful.

6. A micro synthesis of γ -glutamyl-[1:2- ^{14}C]glycine is described.

[1:2- ^{14}C]Glycine and DL-[2- ^{14}C]valine were synthesized under the direction of Dr E. M. Gal of this Department, the DL-[4:4'- ^{14}C]valine was prepared by the Bio-organic Group of the Radiation Laboratory of the University by R. Ostwald, P. T. Adams and B. Tolbert (unpublished method). We are indebted to Mr G. Ellis of this Department for construction of the vacuum-driven stirring motor and the scanning windowless gas-flow counter. The paper represents work condensed from a thesis submitted by R. W. H. to the Graduate division of the University of California for the degree of Ph.D., June 1952, and was aided by research grants from the cancer research funds of the University of California and the Hobson Fund of the School of Medicine.

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Techniques in Tissue Metabolism

3. STUDY OF TISSUE FRAGMENTS WITH LITTLE OR NO ADDED AQUEOUS PHASE, AND IN OILS

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In conducting metabolic experiments with fragments of tissues, a common procedure during the past 30 years has been to suspend the tissue in some 30–200 times its volume of aqueous fluid. The artificial nature of this situation can be appreciated by noting that the volume of blood in an animal tissue at any one time is usually between one-thirtieth and one-third of its total volume. Even consideration of the rate of blood flow *in vivo* does not always justify the high fluid/tissue ratios which have been employed *in vitro*. Thus blood flows

through the human brain at about 50 ml./100 g./min.; in an experiment with cerebral tissue in 100 vol. of fluid, in which initial substrate level and utilization *in vitro* equalled those *in vivo*, over 3 hr. would be required *in vitro* to decrease the added substrate concentration to its level in venous blood *in vivo*. In metabolic studies with separated tissues the volume of added fluid is rarely considered in relation to the duration of metabolism or to the metabolic rate of a tissue. When substrate may become limiting, the expedient of increasing its