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The Chemical Synthesis of Glutamic Acid Specifically Labelled with Deuterium or Tritium in the *Alpha* or 2 Position*

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SUMMARY

Glutamic acid specifically labelled with isotopic hydrogen in the 2 position was produced by exchange equilibration with labelled protons during the reaction of acetylglutamic acid with acetic acid and acetic anhydride. The position of label was established by n.m.r. and mass spectrometry of the deuterium-labelled compound, and by transaminase-catalyzed exchange with protons in the case of tritium-labelled compound. The specific and complete exchange obtained in our experiments contrasts with the previously reported virtual nonlabelling under somewhat different conditions in which the presumed azlactone enolized and hydrolysed in an aqueous medium.

The racemization and *alpha*-hydrogen exchange of most of the N-acetylamino acids in either glacial acetic acid and acetic anhydride, or in aqueous sodium acetate and acetic anhydride, is well known [1, 2, 3]. The racemization-exchange usually proceeds through enolization of an azlactone intermediate [4]. Exceptions include glutamate and aspartate, which react readily only in the nonaqueous medium, through enolization of true anhydrides [5, 6, 7].

Wenzel and coworkers showed that glutamic acid was not labelled in the 2 position with tritium by means of the exchange reaction of acetylglutamic acid in aqueous sodium acetate and acetic anhydride, under conditions

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where acetylglutamic acid might possibly have undergone slow racemization [8]. Shortly before their report, we had prepared specifically labelled glutamic acid by a similar exchange reaction in acetic acid-acetic anhydride. The reason for our success was undoubtedly due to the greater stability of the anhydride in nonaqueous solution; indeed du Vigneaud and Meyer reported some difficulty in racemizing glutamic acid in the aqueous sodium acetate-acetic anhydride system [2]. In view of the reported lack of success with the chemical exchange method with the sodium acetate medium, we wish to report our preparation of specifically labelled glutamic acid by the simple exchange in anhydrous acetic acid-acetic anhydride.

Experimental procedure.

Melting points were taken in sealed evacuated capillaries and are corrected. Nuclear magnetic resonance (n.m.r.), infrared, and mass spectra were taken with the Varian A60, Perkin Elmer 21, and CEC 11OB instruments, respectively. Radioactivity was assayed with the Beckman LS-110 scintillation counter.

DL-Glutamic Acid-2-^{3}H. — Tritium-labelled water (1.55×10^{5} to 3.0×10^{10} d.p.m./mmole in several experiments) 0.6 ml, was added to 3.75 ml acetic anhydride and allowed to reach equilibrium of labelled acetic acid and acetic anhydride. N-Acetylglutamic acid, 0.3 g., was added and allowed to react 10 min at 100° C. The solvent was recovered by lyophilization, and the tritium-labelled acetylglutamic acid was hydrolyzed in 3 ml of 5 M HCl for 3 hr at 110° C. Following lyophilization, the glutamic acid hydrochloride was twice recrystallized from ethanol-water. Yield 0.11 g (55 %). M.P. 191-192° C, specific activity was 71 % that of the tritiated water, or 1.1×10^{5} to 2.1×10^{10} d.p.m./mmole. Radiochemical purity was greater than 99 % by paper chromatography [9]

DL-Glutamic Acid-2-^{2}H. — The above procedure was used, with 99 % $^{2}H_2O$ in place of tritiated water. The melting point was identical with that of the tritiated product. The infrared spectrum was different in several peaks in the fingerprint region; it was otherwise identical with the nonlabelled compound.

DL-Glutamic Acid Dimethyl Ester Hydrochloride. — Glutamic acid was esterified by reaction with methanolic HCl-methyl acetate [10] for 3 hr at 75° C. Following lyophilization, the product was recrystallized from methanol-ethyl acetate. Yield 85 %. M.P. 153-154° C. (Literature [11] 149° C).

Results.

Position of deuterium label. — The n.m.r. spectra of normal and deuterium-labelled glutamate are shown in Table I. The resonance at δ 3.77 of the *alpha* proton is virtually absent in the deuterium-labelled compound, and the areas

TABLE I. Nuclear Magnetic Resonance of Disodium Glutamate

Protons	Chemical Shift, δ	Number of Protons	
		Normal	Glutamate-2-^{2}H
3 and 4	2.12, 2.33	4.1	4.6
2	3.77	1.0	<0.2

Spectra were taken of 1 M disodium glutamate in deuterium oxide. Chemical shifts were relative to hexamethyldisilane in another tube.

of the *beta* and *gamma* protons indicate 4 protons in both spectra. The glutamic acid -2-^{2}H thus is labelled in the 2 or *alpha* position exclusively as determined by n.m.r.

Corroborative evidence is afforded by the mass spectra of the dimethyl esters of normal and deuterium-labelled glutamic acid, as shown in Figure 1. The esters are seen to fragment in the same manner as the previously studied ethyl ester [12]. Thus, the molecular ions appear at m/e = 175 and 176 for the normal and ^{2}H compounds, respectively. The base peaks at m/e = 116 and 117 (loss of $COOCH_3$) contain all the hydrogen atoms originally in the glutamic acid, and indicate 0.87 atom ^{2}H per molecule of labelled material. The peaks at 88 and 89 (loss of $CH_2CH_2COOCH_3$) include only the *alpha* hydrogen, and provide unequivocal evidence for the position of label at the *alpha* carbon.

Position of tritium label. — The glutamic-pyruvic transaminase reaction was used to verify the position of tritium in DL-glutamic acid [13, 8]. The enzyme catalyzed an exchange of the *alpha* hydrogen of L-glutamate with water, which was subsequently lyophilized and assayed for tritium, as shown in Table II.

TABLE II. Transaminase-catalyzed Exchange of Tritium from Labelled Glutamate

Exp.	Changes in Experiment	Tritium in Water, % of Total
1.	None	52.0
2.	Enzyme and buffer boiled 10 min before remainder of reagents added.	2.0

The reaction system contained the following in a volume of 1 ml : glutamate-pyruvate transaminase, 0.01 mg/ml; potassium phosphate pH 6.8, 0.2 M; sodium ethylenediaminetetraacetate 1 mM; sodium pyruvate 10 mM; pyridoxal phosphate, 1 mM; and sodium DL-glutamate-2-^{3}H, 10 mM, 1.2×10^7 d.p.m./mmole. The system was incubated 1 hr at 37° C, followed by lyophilization and assay of the distillate for tritium.

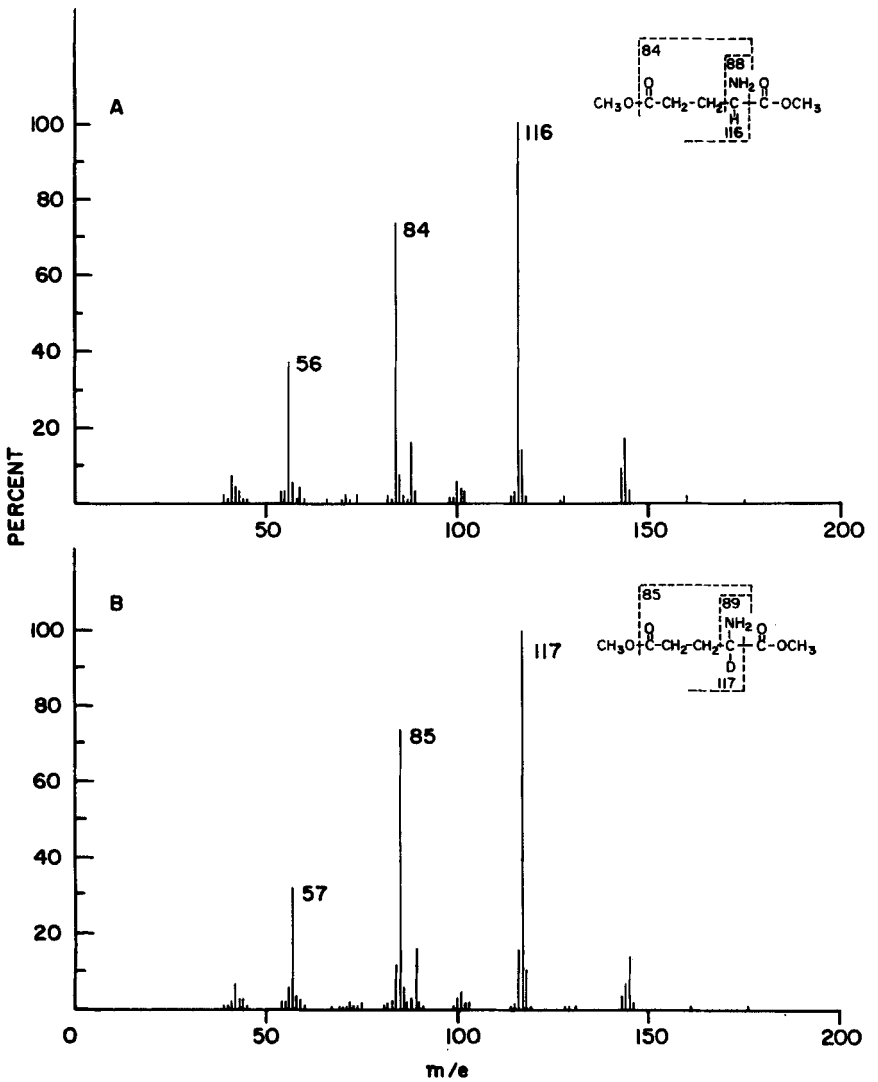

FIG. 1. Mass spectra of normal dimethylglutamate (A), and dimethylglutamate-2-^{2}H, (B). The ester hydrochlorides were injected, with source at 200° C, and probe at 160° C.

Although Oshima and Tamiya have shown the exchange of the *beta* hydrogens of alanine under the influence of transaminase catalysis [14], Wenzel and coworkers [8], and Grisolia and Burris [13] have shown that with glutamate as substrate, only the *alpha* hydrogens exchange with protons of water. Consequently the amount of tritium labilized during catalysis by enzyme can be taken as a good indication of the amount of labelling in the *alpha* position

DISCUSSION.

Racemization and *alpha* hydrogen exchange in acetic acid-acetic anhydride offers a convient route to glutamic acid labelled with hydrogen isotopes. Tritium-labelled valine has been prepared by Crawhall and Smyth using an essentially similar method [3], and *alpha* tritium-labelled phenylalanine has been previously prepared in our laboratory under the same conditions.

With regard to the amount of labelled glutamic acid that can be made from a given amount of tritium-labelled acetic acid-acetic anhydride, it was observed that when the ratio of acetylglutamic acid to solvent was greater than 1 : 10, the yield of product was low and tritium incorporation was low and inconstant; the reasons for this were not ascertained. The specific activity of tritium-labelled glutamic acid was consistently about 70 % that of the protons; presumably the difference in isotope content was due to an equilibrium isotope effect [15], or to a kinetic isotope effect if equilibrium was not attained [16].

The labelled glutamic acid is presently being utilized in studies of hydrogen transfer catalyzed by glutamate dehydrogenase; these studies will be reported at another time.

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