Acid–Base Studies of Glutathione (L-γ-Glutamyl-L-cysteinyl-L-glycine) by One- and Two-dimensional Nuclear Magnetic Resonance Spectroscopy

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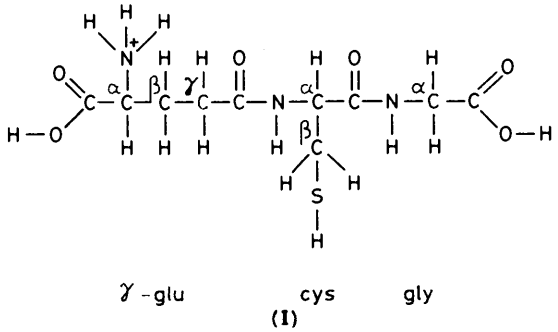
All the ^{13}C resonances for the tripeptide glutathione are assigned, and their behaviour in the pD range 0.7–12.3 explored, by means of ^{13}C n.m.r. titrations and *via* two-dimensional C–H correlation experiments involving both C–H one-bond relationships and long-range connections from C=O groups to remote protonated carbons.

The reduced form of glutathione is a tripeptide (I) with a free mercapto group [γ-Glu-Cys(SH)-Gly] which serves as a mercapto buffer that maintains the cysteine residues of haemoglobin in a reduced form.[1] It also appears to be essential for maintaining normal red-cell structure and for keeping haemoglobin in the iron(II) state. Reduced glutathione plays a role in detoxification by reacting with peroxides which may be produced by drugs or radiation. It is a biological reducing agent in thiol-dependent enzyme reactions. Related to glutathione are the tripeptides ophthalmic acid and norophthalmic acid, all three of which have been shown to be present in calf lenses,[2,3] and their relationship with cataract formation is of interest.[4] More is known about the biochemical role of glutathione, which is a common constituent of most cells, than of ophthalmic acid and norophthalmic acid.

In spite of its wide biochemical interest very few n.m.r. studies have been carried out on glutathione.[5–9] Amongst these studies, only one is concerned with the structure and conformation of glutathione in solutions of various pH as studied by ^1H n.m.r.[9] We decided, therefore, to carry out a similar study of the acid–base behaviour of glutathione using ^{13}C n.m.r. and, at the same time, attempt a full assignment of the ^{13}C signal using a two-dimensional heteronuclear correlated n.m.r. approach.

Titration shifts for ^1H sites in peptide systems have been widely used for pK_a determinations. The advent of very high field spectrometers has enabled the movement of individual resonances to be followed more accurately even for complex molecules as, for example, in the investigation of the pH behaviour for the active-site fragment of splenin.[10] Unless essentially first-order resonances can be observed, it may be difficult and time-consuming to explore titration behaviour *via* ^1H signals; for the 100 MHz data on glutathione already reported[9] it was necessary to perform iterative spin simulations in order to derive accurate shift positions for each pH value. Although ^{13}C n.m.r. spectroscopy suffers from a considerable sensitivity disadvantage relative to ^1H studies, the routine use of noise-decoupling does produce *single line resonances*, the chemcial shift values for which can readily be obtained in hard copy form by standard spectrometer peak listing procedures. Thus, ^{13}C shift data may, in practice, be acquired more rapidly, although at the expense of more spectrometer time than would be necessary for ^1H studies. Information is also gained concerning non-protonated carbon sites inaccessible from ^1H measurements, but data concerning ^1H–^1H couplings, which describe conformational variability as pH is changed, are lost. In principle it would be possible to design ^{13}C experiments in which data for the pH behaviour of long-range ^{13}C–^1H couplings were gathered, since these will also be conformationally sensitive, but this approach would be lengthy and difficult in practice.



(I)

Glutathione at pH < 2

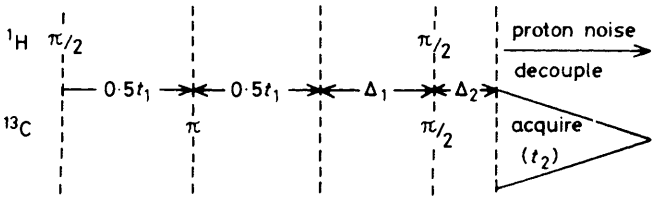


Figure 1. Pulse sequence for 2D C–H correlation spectroscopy

The use of heteronuclear two-dimensional shift correlation spectroscopy for resonance assignments is now well documented.[11] This powerful method is most widely applied to ^1H and ^{13}C nuclei where information about ^1H chemical shifts is transmitted to ^{13}C sites *via* C–H one-bond coupling interactions which are typically 130–180 Hz in magnitude. In the pulse sequence shown in Figure 1 the 'evolution time' delays are linearly incremented for measurements of successive sets of free-induction decays and serve to define the width, in Hz, of the $F_1(^1H)$ axis of the two-dimensional experiment. With the time development for polarisation transfer (Δ_1) set to $\simeq(^2J_{CH})^{-1}$, the final paired pulses then transfer magnetisation between protons and carbons, and Δ_2 can be chosen as $(^4J_{CH})^{-1}$ as a compromise to detect CH_n groups (n = 1–3), or alternatively as $(^2J_{CH})^{-1}$ selectively to observe correlations between H and C nuclei in methine groups.

It is well known[12] that this correlation pulse sequence can be used to detect connections between carbons and more remotely sited carbons *via* long-range $^nJ(CH)$, ($n \geqslant 2$), couplings which may be 10 Hz or even less in magnitude. Thus, quaternary carbons may be identified through their relationships to one or more distant protons. In this paper we make use of 'long-range' C–H correlations from the four peptide carbonyl carbons to specific proton sites in glutathione, in order to confirm their

assignments. Delay times, $\Delta_1 = 9.26$ ms and $\Delta_2 = 46.3$ ms, were employed, corresponding to assumed long-range couplings of $\simeq 4.5$ Hz, giving a non-selective correlation in order also to detect the glycine methylene protons. After the completion of this work a paper appeared[13] describing similar correlations for a tetrapeptide; the sequence as described, however, with $\Delta_1 = \Delta_2$, would not show connections to glycines.

The simple pulse sequence of Figure 1, whilst now becoming readily available for modern pulsed Fourier-transform spectrometers, is not efficient when correlations *via* long-range couplings are being examined. This is due to proton transverse relaxation in t_1 and the now relatively long Δ_1, and to carbon relaxation in Δ_2, which gives a resultant free induction decay (f.i.d.) of significantly reduced amplitude. The efficiency is further reduced by the need to use longer recycle times for quaternary carbons than for the more efficiently relaxing protonated carbons. For spectrometers on which new pulse sequences can easily be implemented, the COLOC experiment,[14] also recently described as a method for correlating carbonyls, offers significant advantages compared with that of Figure 1 for the t_1 evolution time is incorporated as part of the delay Δ_1.

Experimental

The glutathione was obtained from Sigma and was used as received. Solutions of glutathione containing 100 mg in 1.5 cm^3 of D_2O were prepared and the pD adjusted by adding small quantities of concentrated HCl or NaOH in D_2O. The pD was measured with a Pye model 79 pH-meter. The chemical shifts were measured relative to the ^{13}C signal 3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionic acid sodium salt (TSP). The spectra were recorded at ambient temperature with a JEOL FX-100 spectrometer operating at 25.05 MHz. One-dimensional n.m.r. measurements were performed using a tip-angle of 22° and a pulse repetition time of 1 s. Responses of 5 000 Hz spectral width were acquired into 8 K and zero-filled to 16 K data points and an exponential broadening 1 Hz was applied prior to Fourier transformation. The one-dimensional ^{13}C spectra of a total of 29 solutions of glutathione were measured covering the pD range 0.70—12.30.

The two-dimensional C–H correlation experiments were performed using JEOL 2D n.m.r. software with the standard quadrature phase-cycled pulse sequence modified to permit the introduction of different delays at Δ_1 and Δ_2. The 90° pulse-widths and approximate ^{13}C T_1 values were determined for the samples to be examined.

For the one-bond C–H correlations, an F_2 (^{13}C) spectral width of 1 000 Hz was employed and the free induction decays acquired into 1 K data points then exponentially broadened by 0.7 Hz to reduce noise prior to Fourier-transformation. The pulse repetition time was 2.5 s. The F_1 (^1H) axis was built up using t_1 increments of 2.5 ms corresponding to a spectral width of 400 Hz. In preparation for the second Fourier transformation, the 128 t_1 increments for each position across the F_2 axis were zero-filled to 256 then the time-dependent responses were exponentially broadened by 0.3 Hz. In the pulse sequence of Figure 1, Δ_1 3.9 ms and Δ_2 1.95 ms.

For the C=O–H correlations a similar experiment was performed, but with an F_2 (^{13}C) axis of 200 Hz, acquired using 256 data points and zero-filled to 1 K. The repetition time was 7.8 s. Delays of Δ_1 92.6 ms and Δ_2 46.3 ms were used.

Results and Discussion

The assignments of the signals in the ^1H n.m.r. spectrum of glutathione were checked by a series of decoupling experiments and the assignments given by Kozlowski[9] are confirmed, namely, in order of increasing δ values the bonds are assigned to glu-β, glu-γ, cys-β, glu-α, gly-α, cys-α [see structure (I)].

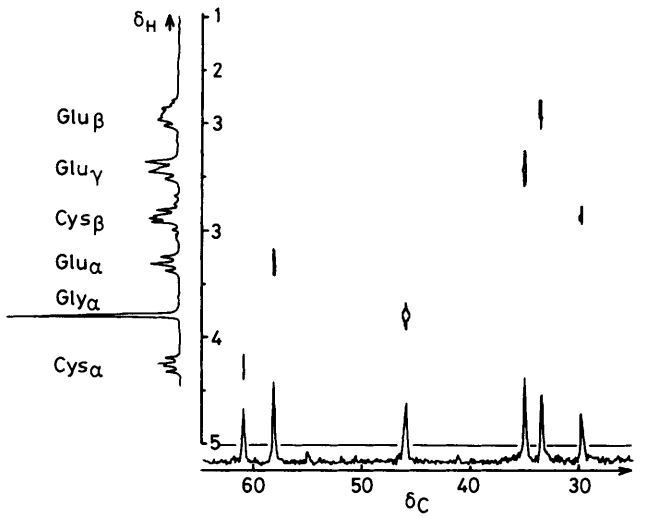


Figure 2. C–H Correlation single-contour plot for the six protonated carbons of glutathione determined at pD 10.57

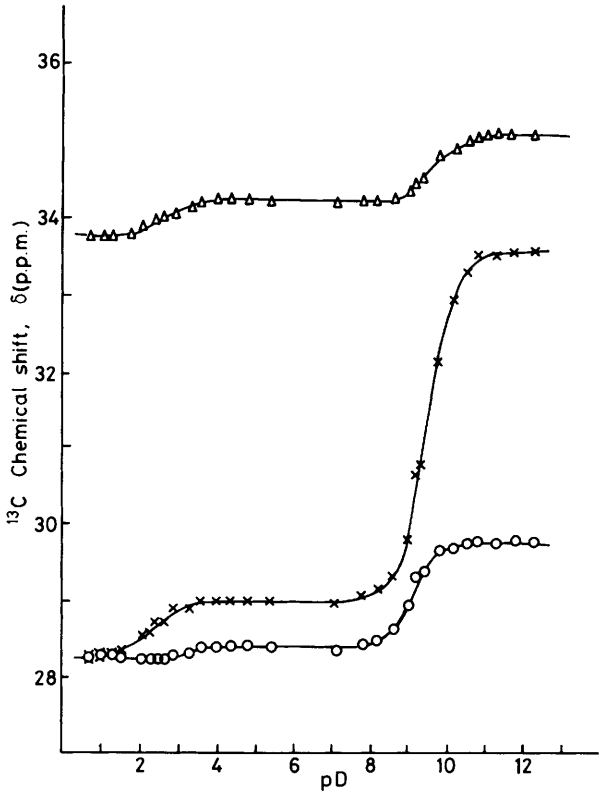


Figure 3. ^{13}C Chemical shifts of protonated carbons of glutathione as a function of pD: ○, cys-β; ×, glu-β; △, glu-γ

Once the ^1H assignments are established it is possible to assign unequivocally the ^{13}C signals for the six protonated C atoms using 2D-heteronuclear correlations. This was, in fact, carried out at two values of solution pD, namely 3.25 and 10.57, *i.e.* near the extremities of the pD range, in order to confirm the assignments. A single-level contour plot determined at pD 10.57 is shown in Figure 2 and the one-dimensional ^1H and ^{13}C spectra are also presented for comparison. By means of Figure 2 we now assign the ^{13}C signals in the region δ 25–65 p.p.m. (in order of increasing δ values) as being the proton-bearing carbons cys-β, glu-β, glu-γ, gly-α, glu-α, and cys-α.

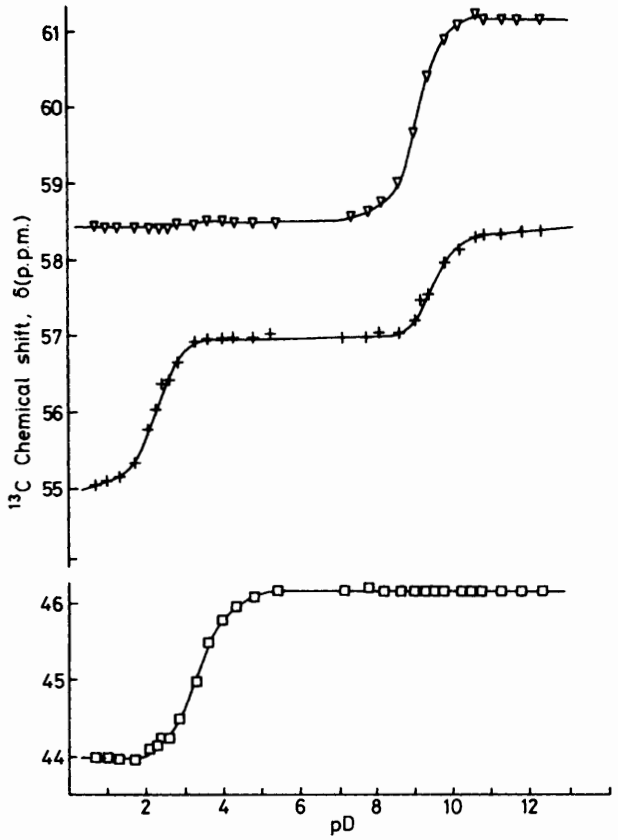


Figure 4. ^{13}C Chemical shifts of protonated carbons of glutathione as a function of pD: □, gly-α; +, glu-α; ▽, cys-α

Table 1. pK_a values of glutathione estimated from the n.m.r. titration curves for the six protonated C atoms

C atom	pK_a [and Δδ (p.p.m.)]	
glu-α	2.4 (1.95)	9.4$_5$ (1.40)
glu-β	2.3 (0.80)	9.5 (4.60)
glu-γ	3.2 (0.45)	9.5 (0.85)
cys-α	3.2 (0.05)	9.0 (2.70)
cys-β	3.4 (0.15)	9.0$_5$ (1.35)
gly-α	3.3$_5$ (2.15)	

Table 2. pK_a values of glutathione estimated from the n.m.r. titration curves for the four C=O groups

C=O group	pK_a [and Δδ (p.p.m.)]	
glu-α	2.3 (0.65)	9.5 (1.20)
glu-γ	2.2$_5$ (2.90)	9.5 (8.60)
cys	3.6 (0.90)	9.1 (1.85)
gly	3.4 (3.50)	9.1 (0.20)

In Figures 3 and 4 are plotted the variation of chemical shift of the protonated carbons as a function of pD where it can be seen that the changes are similar to those observed for simple amino acids[15] and also for the ^{1}H chemical shifts of glutathione.[9] The curves reflect the changes occurring as the glutathione molecule progressively dissociates. The pK_a values estimated from these curves are given in Table 1 along with the approximate changes in chemical shift (Δδ).

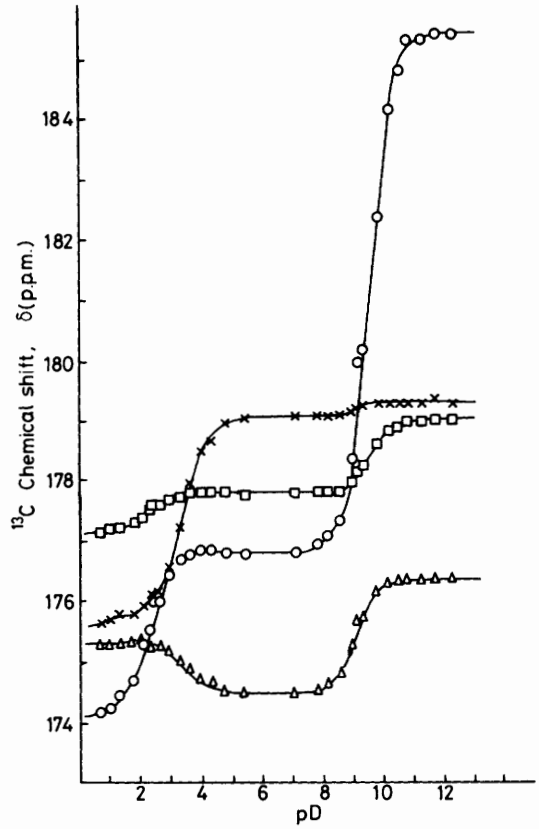


Figure 5. ^{13}C Chemical shifts of C=O groups of glutathione as a function of pD: △, glu-α; ○, glu-γ; ×, cys; □, gly

The pD–chemical shift titration curves for the four C=O groups are shown in Figure 5. Their behaviour is more complex in that the positions of the signals cross over as the pD of the solution is increased. In order to help to decide the identity of the signals after they had 'crossed-over', graphs of Δδ/ΔpD against pD were plotted. Since such plots need to be symmetrical about the end-points, it was possible to decide the direction the signals had taken after they had crossed. Furthermore, the pK_a values obtained from the curves for the four C=O groups were then consistent with those evaluated from the data for the six protonated carbons. From the values of pK_a the titration curves for the carbonyl groups can be assigned (Figure 4 and Table 2). One interesting feature is that both the C=O group and the β-carbon atom of the cysteine residue can sense the acidity of the glycine COOH group (pK_a 3.4), while the C=O of the glycine can just sense the acidity of the third group in the cysteine residue (pK_a 9.05).

The various acid–base equilibria for glutathione in D_2O and the corresponding values of pK_a as observed *via* ^{13}C n.m.r. are shown in Figure 6. The pK_a values are in good agreement with those obtained from ^{1}H n.m.r. studies.[9] In Table 3 are presented values of ^{13}C chemical shifts, at three values of pD, for the various carbon atoms of the three amino acid residues of glutathione.

A 2D C–H heteronuclear correlation *via* long-range couplings was carried out for the four C=O groups with glutathione at its natural pD in D_2O (3.25 at the concentration used). At this concentration of ^{13}C signals appear in the order of increasing δ value as cys, glu-γ, gly, and glu-α. The 2D single-contour plot resulting from this experiment is given in Figure 7, and the corresponding C–H correlations are given in Table 4. These observed connectivities, together with the titration data, allow definitive assignments to be made for all the carbonyl ^{13}C

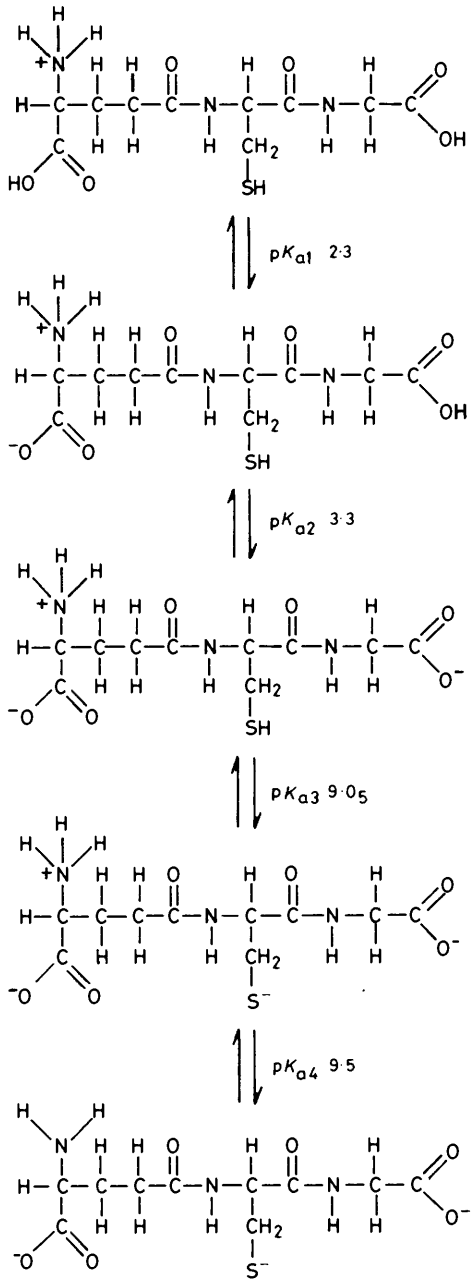


Figure 6. Acid–base equilibria of glutathione

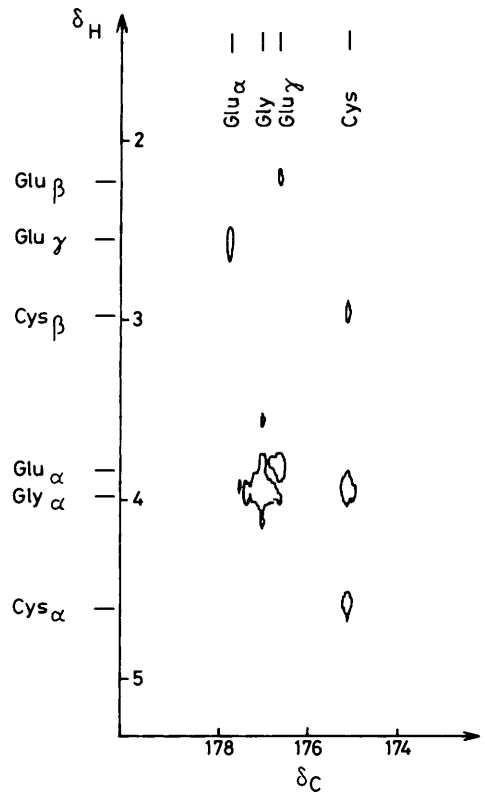


Figure 7. C–H Correlation single-contour plot for the four C=O groups of glutathione determined at pD 3.25

Table 3. ^{13}C Chemical shifts in glutathione

	^{13}C Chemical shift δ (p.p.m.)		
C-atom	pD 1.75	pD 7.10	pD 11.30
γ-Glutamyl			
C_α	55.35	56.95	58.30
C_β	28.35	28.95	33.50
C_γ	33.80	34.20	35.05
α-C=O	177.30	177.80	179.00
γ-C=O	174.70	176.80	185.30
Cysteinyl			
C_α	58.45	58.50	61.10
C_β	28.25	28.35	29.75
C=O	175.35	174.50	176.35
Glycyl			
C_α	44.00	46.15	46.15
C=O	175.80	179.15	179.30

Table 4. C–H correlations for the C=O groups of glutathione at pD 3.25

^{13}C=O		^{1}H
glu-α	sees	glu-γ
glu-γ	sees	glu-α and glu-β
cys	sees	cys-α, cys-β, gly-α, and weak correlation to glu-α
gly	sees	gly-α

resonances. For spectrometer systems where appropriate pulse sequences which optimise signal-to-noise ratios for these quaternary carbons can be implemented, C–H long-range correlations offer an attractive n.m.r. approach[14] to peptide sequencing.

At pD values above 9 the cysteine SH group and the glutamic acid NH_3 group dissociate and consequently one would expect these processes to affect the overall conformation of the glutathione molecule. At the physiological pH of 7.4, however, (pD *ca.* 7.8) the state of dissociation will be very similar to that at pD 3.25 where the C=O C–H correlations were made. Between pD 3.25 and 7.8 the largest chemical shift changes are for the glycine residue, whereas the other ^{13}C resonances change much less. It may be reasonable to assume, therefore, that the conformation of glutathione at physiological pH is similar to that at pD 3.25.

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