[CONTRIBUTION FROM THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY]

Raman Spectra and Ultraviolet Absorption of Glutathione and Possible Thiazoline Derivatives Formed from It[1,2]

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The Raman and ultraviolet spectra of glutathione solutions were studied at various hydrogen ion concentrations. As in the previously studied case of cysteine, the ionization of the sulfhydryl group can be followed by observing the decrease in intensity of the Raman frequency at 2580 cm.$^{-1}$ with increasing pH. The sulfhydryl and the ammonium group lose their acidic protons in the same pH range in alkaline solution. In strong acid glutathione appears to undergo rearrangement into at least two and possibly more forms which contain double bonds, other than those of peptide linkages and absorb in the ultraviolet near 265 mμ and below. There is reason to believe that one of these forms is a thiazoline derivative, as previously suggested by Calvin. The amount formed and rate of formation increase with increasing H^+ concentration, as judged by the rate of increase of ultraviolet absorption with time.

In 1954 Calvin[4] presented evidence for the formation of a thiazoline derivative, with distinctive ultraviolet absorption, from glutathione in acid solution. This report describes a further investigation of this question, both by study of the kinetics of formation of this possible thiazoline compound and by examination of the Raman spectra of glutathione. Raman spectra in alkaline solutions, which give evidence concerning the ionization of the sulfhydryl group in glutathione, are also reported.

Experimental

The technique of obtaining the Raman spectra has been described.[5] An optical anomaly in the spectrograph used (corrected toward the end of this study) partially obscured the region 1600–1700 cm.$^{-1}$, effectively making the lines in this region fainter than they would be otherwise.

Glutathione (assayed at 98% pure) was obtained from Schwartz Laboratories, Mount Vernon, N. Y. For the measurement of Raman spectra a 14% (approximately saturated) solution of isoelectric glutathione was prepared, and the pH was adjusted with hydrochloric acid or potassium hydroxide. The latter contained 0.01 M potassium cyanide to complex heavy metal ions in order to reduce metal-catalyzed oxidation.[6,7] Neutral or acid solutions were filtered through Whatman No. 4 paper with charcoal. In preparing alkaline solutions an isoelectric solution was prepared and filtered. Alkali was then added to adjust the solution to the desired pH. It was then flushed with nitrogen and kept under nitrogen. Further adjustments of pH were sometimes made on these solutions for additional studies.

When a solution, prepared as described was made alkaline for the first time, a yellow-brown color appeared immediately. As with cysteine[7] this color faded on standing and was partially bleached by light. It was unaffected by bubbling nitrogen through the solution, but each exposure to air promptly made it stronger, until eventually it was a dark red. This color is not characteristic of known glutathione–ferric ion complexes.[8] The prompt appearance of the color suggests that it involved traces of impurities existing initially in the solutions, rather than oxidation products.

Ultraviolet spectral studies were carried out in a Beckman DU spectrophotometer with quartz cuvettes, using a photomultiplier attachment and a hydrogen arc source. Kinetic studies, unless indicated otherwise, were initiated by adding 6 to 20 mg. of solid glutathione to 3 ml. of HCl (or other solution) in the cuvette, after which the optical densities were followed. Any solutes other than glutathione were first dissolved separately and then added to the solution. Beer's law was assumed to hold throughout.

Results

The Raman spectra of the various forms of glutathione are shown in Table I.

The Raman spectra of substituted thiazolines are not available, but examination of some of their infrared spectra[9] indicates that Raman lines would be expected in the region 1450–1600 cm.$^{-1}$, in which no lines are found in the spectrum of isoelectric glutathione. Such lines should be expected in any double-bonded heterocyclic compound. A number of lines in this region do appear in the strongly acid glutathione spectra. It is impossible to deduce from the Raman data just what compounds of this type are present, or how many, but the number of lines observed may well indicate more than one compound, and the tentative conclusion that at least one such compound is present may be drawn.

It is noteworthy that alkaline solutions show weaker Raman spectra than acid solutions of the same concentration; no satisfactory explanation is as yet available. In any case, many lines appear or disappear on going from acid to alkali.

As the pH increases, the sulfhydryl group (the ionization of which can be followed by measuring the decrease in intensity of the 2580 cm.$^{-1}$ line) ionizes in a manner similar to that of cysteine.[7] The observed values of pK_3' and pK_4' of glutathione are 8.75 and 9.65, respectively.[10] These ionization constants must involve both the sulfhydryl and the ammonium group. We cannot specify that $pK_{SH} = 8.75$, because there would then be no un-ionized SH left at pH 9.97; likewise setting $pK_{SH} = 9.65$ contradicts the fact that the amount of un-ionized SH has greatly decreased at pH 9.1. Accordingly the ionization of the sulfhydryl and ammonium groups must be described by a set of microscopic constants, of which the observed pK's are composites.[11,12] The behavior of the line at 860 cm.$^{-1}$, which is probably a bending

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(4) M. Calvin in "Glutathione," ed. by S. Colowick, *et al.*, Academic Press, New York, N. Y., 1954, p. 3.

(5) D. Garfinkel and J. T. Edsall, THIS JOURNAL, **80**, 3807 (1958).

(6) O. Warburg and S. Sakuma, *Pflüger's Arch. ges. Physiol.*, **200**, 203 (1923).

(7) D. Garfinkel and J. T. Edsall, THIS JOURNAL, **80**, 3818 (1958).

(8) T. Wieland in "Glutathione," ed. by S. Colowick, *et al.*, Academic Press, New York, N. Y., 1954.

(9) H. M. Randall, R. G. Fowler, N. Fuson and J. K. Dangl, "Infrared Determination of Organic Structures," D. Van Nostrand Co., New York, N. Y., 1949.

(10) N. C. Li, O. Gawron and G. Bascuas, THIS JOURNAL, **76**, 225 (1954).

(11) R. Benesch and R. E. Benesch, *ibid.*, **77**, 5877 (1955).

(12) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press, Inc., New York, N. Y., 1958, Chap. 9.

TABLE I[a]

RAMAN SPECTRA OF GLUTATHIONE (γ-GLUTAMYL-CYSTEINYL-GLYCINE) AT VARIOUS *p*H VALUES

Structure in neutral solution: $^{-}OOCCH(NH_3^{+})(CH_2)_2$-$CONHCH(CH_2SH)CONHCH_2COO^{-}$

At *p*H 1.61:

418(vw), 536(vw), 578(vw), 642(vw), 682(w), 705(vw), 746(w), 815(1vb), 867(1b), 893(2b), 947(w), 1012(vw), 1037(vw), 1080(vw), 1168(vw), 1248(1vb), 1277(w), 1309(w), 1347(w), 1421(3b), 2583(4b), 2889(vw), 2927(1), 2953(5b), 2997(3b)

At *p*H 0.9:

The lines at 418, 578, 705, 867, 1012, 1168, 1309 and 2889 have disappeared and additional lines have appeared at 790(vw), 998(vw), 1119(vw), 1293(2vb), 1388(1), 1590-(vw), 1568(vw), 1605(w) and 2875(w)

At *p*H 0.0:

The lines at 642, 790, 1293 and 1590 have disappeared, and lines have appeared at 503(vw), 873(w), 899(w), 1184(w), 1309(w), 1442(1b), 1468(w), 1527(w), 1546(w), 1677(w) and 2968(1)

In 3 *N* HCl:

The lines at 503, 608, 873, 899, 941, 1277, 1527 and 2927 cm.$^{-1}$ have disappeared, and lines have appeared at 397(vw), 463(vw), 1106(vw), 1219(w), 1510(w), 1538(w), 1593(w), 1663(2b), 1734(2) and 2911(1b)

In 7.5 *N* HCl:

A very weak spectrum was obtained, which shows lines at 934, 1274, 2528 (a tail to the 2575 cm.$^{-1}$ line, indicating H bonding) in addition to the stronger lines seen in 3 *N* HCl. Many lines have disappeared due to the general faintness.

In alkaline solution:

At *p*H 8.20:

447(vw), 688(w), 728(vw), 747(vw), 780(vw), 819(w), 880(vw), 917(1b), 936(vw), 995(vw), 1027(1b), 1112-(w), 1185(vw), 1240(w), 1269(w), 1301(w), 1339(w), 1412(2b), 1448(1), 2588(2), 2887(vw), 2945(4b), 2998-(1b).

At *p*H 9.1:

Lines have disappeared at 447, 728, 936, 1027, 1112, 1185, 1448, 2887 and 2998, the line at 2581 has decreased in intensity to 1b, and lines have appeared at 482(vw), 545(vw), 1041(w), 1070(vw), 1124(vw), 1200(vw), 1427(2), 1459(vw) and 2877(vw).

At *p*H 9.97:

The intensity of the 2586 cm.$^{-1}$ line is vw; and the line at 860 has disappeared. This spectrum is otherwise nearly the same as at *p*H 9.1.

[a] Intensities of Raman lines are indicated by 1, 2, 3, . . ., for lines of increasing intensity except that the weakest are designated very weak (vw) or weak (w); broad lines are designated b, and vb.

frequency of the sulfhydryl group, is similar to that of the stretching frequency at 2580 cm.$^{-1}$. A similar interpretation of the ionization constants has been reached by Stricks, Kolthoff and Kapoor[13] on the basis of oxidation-reduction studies and by Benesch and Benesch[11] by measurements of ultraviolet absorption as a function of *p*H.

(13) W. Stricks, I. M. Kolthoff and R. C. Kapoor, THIS JOURNAL, **77**, 2057 (1955).

A number of changes with ionization occur which are not directly due to vibrations of the atoms in ionizing groups. In particular the CH stretching lines, above 2600 cm.$^{-1}$, and the deformation lines, in the region 1400-1440 cm.$^{-1}$, seem to be affected by the state of ionization of the $-NH_3^{+}$ group, in agreement with the results for amino acids and other peptides.[14] As with other peptides, vibrations characteristic of the side-chain groups of the constituent amino acids are observed. The CH stretching lines of cysteine at 2961 and 3008 in acid, and 2907 in alkali, are apparently present in glutathione, displaced downward by about 10 cm.$^{-1}$. Likewise, in cysteine the C–C stretching frequency at 680 is nearly as intense as the SH frequency at 2580, but in glutathione it is far weaker. No ready explanation can be suggested for the difference in behavior between glutathione and other peptides.

Ultraviolet Spectra. Additional evidence as to the number and possible nature of the compounds formed in HCl may be obtained by studying their kinetics of formation. Figure 1 shows the rate of change of ultraviolet absorption at 265 mμ. Obviously the observed rate is strongly dependent on HCl concentration. The fact that the rate of increase of optical density does not increase smoothly with acid concentration suggests that more than one acid-catalyzed pathway is involved.

An attempt was made to determine whether a true equilibrium could be approached, starting from either side. Such experiments were performed by starting with a suitable concentration of both glutathione and acid, letting the reaction proceed and then removing aliquots and diluting them to the conditions defined by one of the curves of Fig. 1, at various times such that the optical densities will lie either above or below the equilibrium value expected from the curve. The result of one such experiment is shown in Fig. 2. The curves do indeed converge initially, as expected for a single equilibrium, but the behavior after the first few hours shows that at least a second compound is formed and suggests that there may even be a third. It is known that there is a slow hydrolysis of glutathione in such solutions, which can involve either the γ-glutamyl-cysteinyl bond[8] or the cysteinyl-glycine bond.[15] However, none of the breakdown products so obtained would be expected to give rise to ultraviolet absorption at 265 mμ. An artificial hydrolysate is nearly transparent at this wave length. There is therefore reason to suspect the formation of a compound containing double bonds not present in glutathione itself or its hydrolysis products. Figure 3 shows the absorption spectra in 3 *N* HCl at several time intervals. It is seen that the absorption spectrum changes in a manner indicating the formation of a new compound. Absorption at 265 mμ appears rapidly and changes relatively little with time in this particular system, while absorption continues to rise progressively as time passes, at wave lengths from 260 down to 240 and below. It would appear that the first compound formed (with absorption maxi-

(14) M. Takeda, *et al.*, *ibid.*, **80**, 3813 (1958).

(15) E. Preaux and R. Lontie, *Biochem. J.*, **66**, 26P (1957).

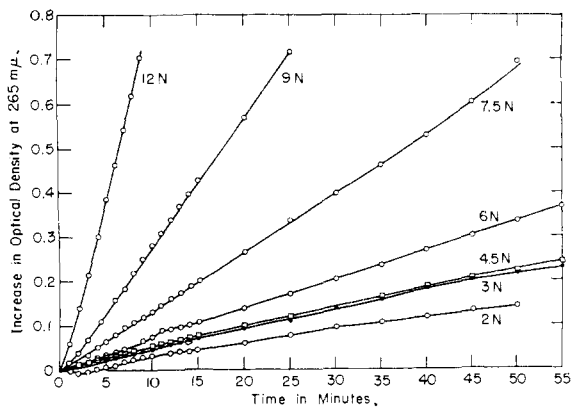

Fig. 1.—Increase in optical density with time: glutathione (3.3 g./l.) in the presence of various concentrations of hydrochloric acid, as indicated.

mum at 265 mμ) is the one observed by Calvin and considered by him to be the thiazoline form.

When glutathione in acid solution, after being allowed to stand long enough to form some of the ultraviolet absorbing material, is exposed to alkaline conditions (by addition of either NaOH or Na_2CO_3) and then returned to acid, it is found that most, if not all, of the ultraviolet absorbing material has disappeared.

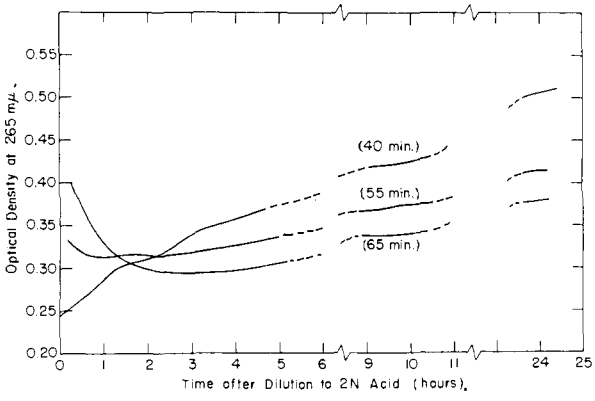

Fig. 2.—Glutathione dissolved in 6 *N* hydrochloric acid, then diluted with water to 2 *N* acid after the indicated times.

Discussion

The appearance, in acid solution, of Raman lines in the region between 1500 and 1650 cm.$^{-1}$ and the concomitant rise in ultraviolet absorption, favor the view that some molecular rearrangement of the glutathione molecule occurs in such solutions; the change appears to be in large measure reversible on adjusting the *p*H to neutral or alkaline values. There is evidence[4,16] that the structures formed in acid solution may contain a thiazoline ring, and our data appear to be in accord with this hypothesis.

Linderstrøm-Lang and Jacobsen[17] have shown that 2-methylthiazoline hydrolyzes in acid solution

(16) R. E. Basford and F. M. Huennekens, THIS JOURNAL, **77**, 3873, 3878 (1955).

(17) K. Linderstrøm-Lang and C. F. Jacobsen, *Compt. rend. trav. Lab. Carlsberg, (Ser. Chem.)*, **23**, No. 20 (1940).

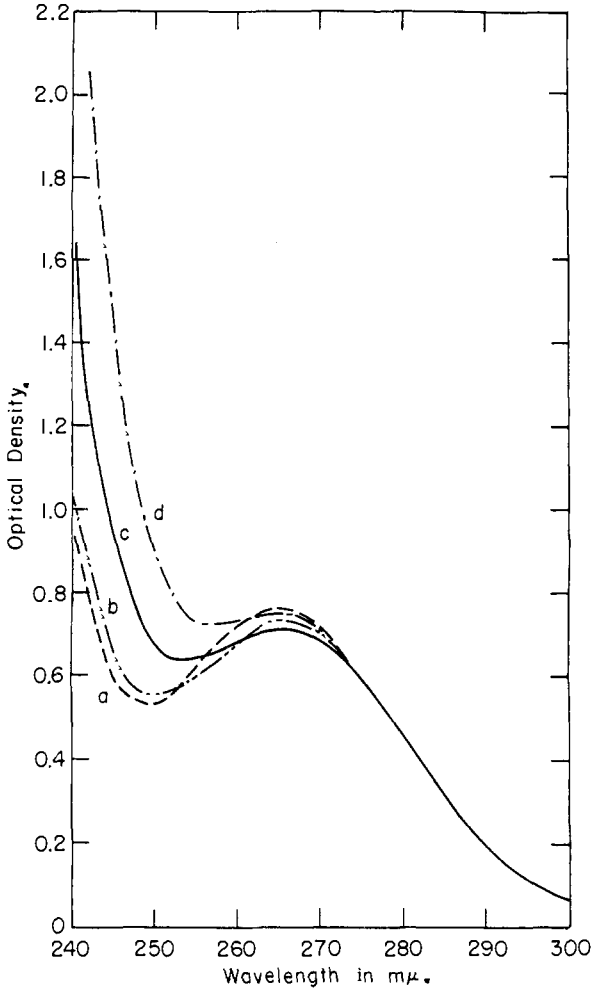

Fig. 3.—Glutathione (3.3 g./l.) in 3 *N* hydrochloric acid: absorption spectra after (a) 0.5 hour, (b) 1.5 hours, (c) 6 hours, (d) 20 hours.

to form a mixture of S-acetyl and N-acetyl aminoethylmercaptan; the reaction is apparently reversible. Thus in this case acidification favors the formation of a sulfhydryl compound from a thiazoline, whereas Calvin's studies have led him to conclude that a shift of the equilibrium in the opposite direction occurs when glutathione is exposed to acid. This appears somewhat puzzling; however, Preaux and Lontie[15] have shown that the hydrolysis of 2-methylthiazoline is inhibited in strong acid.

The glutamyl group of glutathione may undergo reaction to form a pyrrolidone ring,[4,8] and there can also be a spiran form containing both a pyrrolidone and a thiazolidine ring.[4] The latter, however, probably would lack the double bonds that would lead to ultraviolet absorption near 265 mμ. The number of possible structures present may be augmented by the presence of hydrolysis products[8,15] of glutathione, as already mentioned. Since the author is not now in a position to pursue the investigation further, the findings reported here are recorded to provide some new evidence concerning the behavior of glutathione.

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