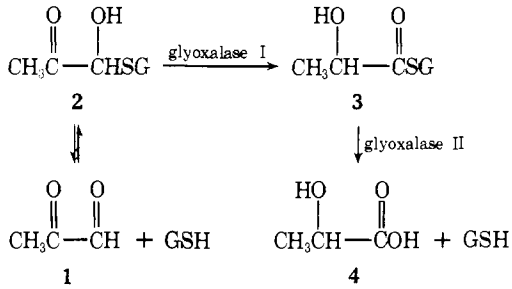
(10) J. I. Zink, *Inorg. Chem.*, **14**, 446 (1975).
(11) (a) F. Feigl and V. Anger, "Spot Tests in Inorganic Analysis", 6th ed, Elsevier, New York, N.Y., 1972, p 346; (b) M. Fradeiro, L. Solorzano, and J. D. H. Strickland, *Anal. Abstr.*, **15**, 7000 (1968).
(12) V. Balzani and V. Carassiti, "Photochemistry of Coordination Compounds", Academic Press, New York, N.Y., 1970, Chapter 7.

Micheal Katz, Harry D. Gafney*
City University of New York
Department of Chemistry, Queens College
Flushing, New York 11367
Received July 1, 1976

Glyoxalase I Enzyme Studies.[1] 2.[2] Nuclear Magnetic Resonance Evidence for an Enediol-Proton Transfer Mechanism

Sir:

In contrast to the generally accepted 1,2-hydride shift mechanism[3] for the action of glyoxalase I, we wish to present evidence that in the conversion of the α-ketohemithiol acetal **2** (methylglyoxalglutathionylhemithiol acetal) to the α-hydroxythiol ester **3** (*S*-lactoylglutathione) the mechanism involves an enediol-proton transfer rather than a hydride shift. The glyoxalase enzyme system composed of glyoxalase I [*S*-lactoylglutathione methylglyoxal lyase (isomerizing); E.C. 4.4.1.5], the coenzyme glutathione (GSH), and glyoxalase II (S-2-hydroxyacylglutathione hydrolase; E.C. 3.1.2.6) converts methylglyoxal (**1**) to lactic acid (**4**). The role of this enzyme system, which is found widely distributed in cells of all forms of life,[4,5] in metabolism has become a topic of controversy because of its suggested[5] involvement in the regulation of cell division.



Mechanistically, the most intriguing step in the reaction sequence is the rearrangement of the hemithiol acetal **2** to the thiolester **3** for which one might consider either an intramolecular 1,2-hydride shift or an enediol-proton transfer mechanism (originally proposed by Racker).[2,3b,6] Distinguishing the two mechanisms should be straightforward either by detecting the incorporation (or lack of incorporation) of solvent protons into the product or the retention (or loss to the medium) of an isotope of hydrogen when using a properly labeled substrate. Indeed, studies of the former have been reported in deuterium oxide and tritium-enriched water. In each case the 1,2-hydride shift mechanism was proposed because of the lack of detection of deuterium (3% maximum incorporation)[3a] or low incorporation of tritium (less than 4%)[3b] in the lactic acid product, **4**. These results, however, do not rule out the possibility of a fast proton transfer mechanism via an enediol taking place in a highly protected active site. As a matter of fact, the detection of *any* solvent proton incorporation suggests such a possibility. This can be tested by observing incorporation or increased incorporation of solvent protons as the temperature is raised.[7] We report here the results of such a study in which incorporation of solvent protons was detected using NMR spectroscopy. At 25 °C there is incorporation of deuterium (ca. 15%) when the enzyme reaction is run in deuterium oxide. The incorporation increases to ca. 22% when the temperature is raised to 35 °C. These observations clearly demonstrate that

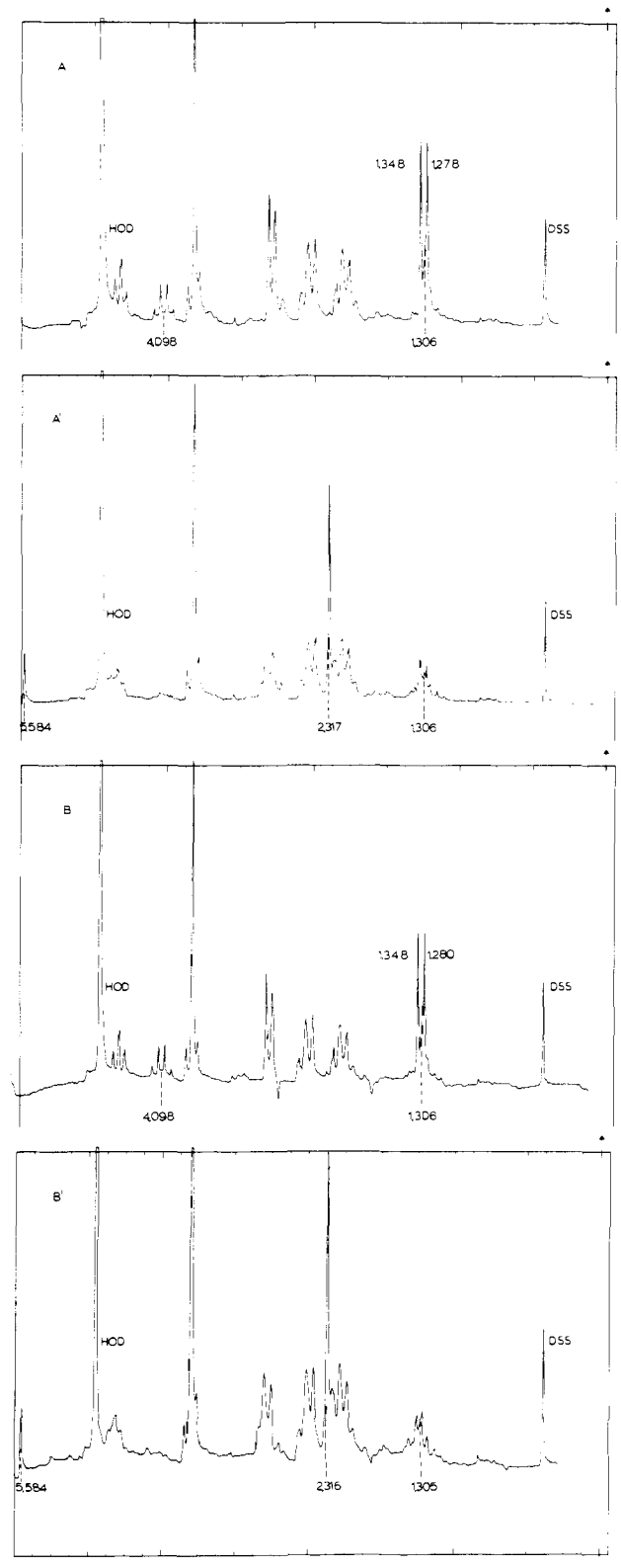


Figure 1. ^{1}H NMR spectra for the glyoxalase I catalyzed conversion of methylglyoxal to lactic acid. Spectra A and B represent the enzyme reaction at 25 and 35 °C, respectively; A′ and B′ are the corresponding control spectra. Chemical shifts are quoted in parts per million from DSS and the assignments are discussed in the text. The unassigned peaks belong to glutathione.

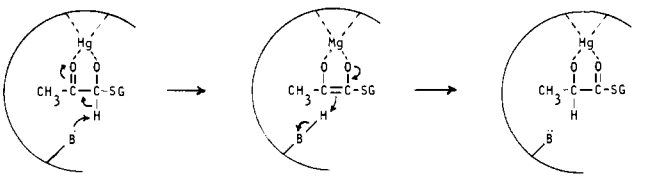
the mechanism proceeds via a proton transfer rather than a hydride shift.

In a typical experiment a stock solution was prepared by dissolving a mixture of 48 mg (0.47 mmol) of methylglyoxal,[8] 146 mg (0.47 mmol) of glutathione, and 12 mg of DSS (NMR internal chemical shift and integration reference, sodium 4,4-dimethyl-4-silapentanesulfonate) in 6 ml of ^2H_2O (99.7%). The pH of this stock solution was immediately adjusted to 7.01 (p^2H 7.41) by the addition of 203 mg of anhydrous K_2HPO_4 (buffer component) and equilibrated at 21 °C for 30 min before 0.68-ml aliquots were transferred to 5-mm NMR tubes. Then each tube was equilibrated at its designated experimental temperature (25 or 35 °C) for 5 min before 50 units of glyoxalase I (5 μl, ^2H_2O solution)[9] was added and the solution incubated for 5 min in its respective bath. All NMR tubes were then placed in the 25 °C bath and 4 units of glyoxalase II (20 μl, ^2H_2O solution)[10] was added to hydrolyze the thiol ester **3**. After 20 min, the NMR tubes were cooled to 0 °C. As soon as possible (within 5–20 min) the NMR spectrum of each sample was recorded (probe at 28 °C) at 100 MHz using a Jeol Model JNM-PS-FT-100 fast Fourier transform NMR spectrometer (25 transients using a 90° pulse, 8K data points, 625 Hz spectral width). The NMR chemical shifts are reproducible to ±0.003 ppm. In all cases identical control reactions were carried out side-by-side with the above reaction tubes except that glyoxalase I was omitted.

Figure 1 displays the NMR spectrum of each reaction (spectra A and B) along with its control (spectra A′ and B′). To facilitate unequivocal assignments, after the above spectra were obtained various amounts of authentic lactic acid (1.315 ppm (3 H, d, J = 6.9 Hz, peaks at 1.279 and 1.348 ppm), 4.098 ppm (1 H, q, J = 6.9 Hz)) and [2-^{2}H]lactic acid (1.306 ppm (3 H, apparent s)) were added to each NMR tube and the spectra (not shown) determined again.[11] By this procedure, it was clearly demonstrated that the spectra in Figure 1 contained mixtures of lactic acid and [2-^{2}H]lactic acid (as well as glutathione). The percent incorporation (ca. 15% at 25 °C and 22% at 35 °C) was estimated by measuring (planimetry) the area of the methyl doublet and singlet due to the lactic acid mixture and correcting for any possible lactic acid formation in the control. Using the DSS in each spectrum as an integration reference, it is also clear that the relative area of the methyl singlet at 1.306 ppm due to [2-^{2}H]lactic acid increased as the temperature of the enzyme reaction was raised, and the relative areas of the methyl doublet centered at 1.315 ppm and the tertiary hydrogen quartet centered at 4.098 ppm of lactic acid decreased. It should also be noted that sufficient enzyme was used since the methyl singlet at 2.317 ppm and the tertiary hydrogen singlet at 5.584 ppm from the α-ketohemithiol acetal **2** present in each control spectrum is absent in the corresponding reaction spectrum. Other important control experiments established that neither *S*-lactoylglutathione (**3**) nor lactic acid (**4**) incorporates deuterium when exposed to these reaction conditions.

In addition, the fast enediol-proton transfer mechanism for the mode of action of glyoxalase I was unequivocally confirmed by the following experiments. When either phenylglyoxal, also a substrate for this enzyme system, in ^2H_2O or [1-^{2}H]-phenylglyoxal[12] in H_2O was exposed to the experimental enzyme conditions previously discussed, mixtures of mandelic acid and [2-^{2}H]mandelic acid (determined by NMR and MS) were obtained. Finally, all model systems that we have examined, as would be expected, result in nearly quantitative incorporation.[13]

Clearly, the mechanism of glyoxalase I proceeds via an enediol-proton transfer rather than a 1,2-hydride shift. The low incorporation of solvent protons indicates a fast shielded proton transfer that is occurring at a highly protected active site. Such a mechanism is depicted where a substrate is shown chelated to a Mg^{2+} ion at the active site[2,15] and the B group represents a basic amino acid residue that is part of the active site of glyoxalase I.[16]



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References and Notes

(1) This investigation was supported primarily by PHS Research Grant No. CA 12984 from the National Cancer Institute.
(2) Part 1: S. S. Hall and A. Poet, *Tetrahedron Lett.*, 2867 (1970).
(3) (a) V. Franzen, *Chem. Ber.*, **89**, 1020 (1956); (b) I. A. Rose, *Biochim. Biophys. Acta*, **25**, 214 (1957).
(4) A. Szent-Györgyi, *Science*, **161**, 988 (1968).
(5) (a) A. Szent-Györgyi, L. G. Egyud, and J. A. McLaughlin, *Science*, **155**, 539 (1967), (b) L. G. Egyud, J. McLaughlin, and A. Szent-Györgyi, *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 1422 (1967); (c) R. Vince and W. B. Wadd, *Biochem. Biophys. Res. Commun.*, **35**, 593 (1969); (d) R. Vince and S. Daulge, *J. Med. Chem.*, **14**, 35 (1971).
(6) E. Racker, *J. Biol. Chem.*, **190**, 685 (1951).
(7) I. A. Rose and E. L. O'Connell, *J. Biol. Chem.*, **236**, 3086 (1961).
(8) The methylglyoxal was prepared by diluting a 40% aqueous solution with saturated $NaHCO_3$–NaCl and extracting with Et_2O. The organic layer was dried (Na_2SO_4) and concentrated in vacuo, and the residue distilled at 55–63 °C (95 Torr). This fraction was lyophilized for 20 h, deuterium oxide added, and, after a few hours, lyophilized again for 20 h. The NMR (100 MHz, D_2O) spectrum of this sample (singlets at −2.478 and −3.406 ppm relative to HOD) suggests a 1:1 mixture of the mono- and dideuterate of methylglyoxal.
(9) Glyoxalase I (grade X from yeast, lyophilized powder containing 95% protein and 5% citrate buffer salts) is available from Sigma Chemical Co. Just prior to the experiment, 0.244 mg (209 units) was dissolved in 21 μl of D_2O. One unit will convert 1.0 μmol of substrate to product per minute at pH 6.6 at 25 °C.
(10) Glyoxalase II (from beef liver, lyophilized powder containing 80% protein and 20% phosphate–citrate buffer salts) is available from Sigma Chemical Co. Just prior to use, 2.41 mg (20 units) was dissolved in 100 μl of D_2O. One unit will hydrolyze 1.0 μmol of substrate to product per minute at pH 7.4 at 25 °C.
(11) The authentic samples were prepared by the $NaBH_4$ and NaB^2H_4 reduction of pyruvic acid, respectively.
(12) Prepared by selenous acid oxidation of [^{2}H$_3$]acetophenone by the method of (a) D. L. Vander Jagt and L.-P.B. Han, *Biochemistry*, **12**, 5161 (1973); (b) H. L. Riley, J. F. Morley, and N. A. C. Friend, *J. Chem. Soc.*, 1875 (1932).
(13) Using these techniques we have found that all model catalysts, general bases[2,14] as well as Franzen's (V. Franzen, *Chem. Ber.*, **88**, 1361 (1955)), that mimic this enzyme reaction result in the incorporation of deuterium into the α-positions of lactic and mandelic acid. In all of these reactions the extent of incorporation is ca. 100% (within the limits of detection, purity of ^2H_2O, and possible isotope effects).
(14) S. S. Hall, A. M. Doweyko, and F. Jordan, unpublished results.
(15) K. A. Davis and G. R. Williams, *Biochem. Biophys. Acta*, **113**, 393 (1966).
(16) S. S. Hall, A. M. Doweyko, and F. Jordan, unpublished results. Characterized as a histidine residue by photooxidation techniques.
(17) Taken in part from the Ph.D. Thesis of A.M.D. that was submitted to the Graduate School, Rutgers University, Oct 1975.

Stan S. Hall,* Arthur M. Doweyko,[17] Frank Jordan*
Carl A. Olson Chemistry Laboratories
Department of Chemistry, Rutgers University
Newark, New Jersey 07102
Received October 29, 1975

Oligomerization and Two-Center Oxidative Addition Reactions of a Dimeric Rhodium(I) Complex

Sir:

We have shown previously that cationic arylisocyanide complexes of rhodium(I) aggregate in solution through for-