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Glyoxalase 2 Deficiency in the Erythrocytes of a Horse: ^{1}H NMR Studies of Enzyme Kinetics and Transport of *S*-Lactoylglutathione[1]

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In mammalian red blood cells the metabolism of methylglyoxal, and some α-ketoaldehydes, takes place via two, generally, highly active enzymes, glyoxalase 1 and 2. The ^{1}H NMR spin–echo spectra of horse erythrocytes, and the various reactants in the glyoxalase system, were characterized as a prelude to obtaining series of spectra in time courses of methylglyoxal metabolism. We characterized the kinetics of the enzyme system in red cells from a normal horse and also from one which had very low activity of glyoxylase 2. The kinetics of the reaction scheme, with methylglyoxal as the starting substrate, were obtained from ^{1}H NMR spectra and analyzed with a computer model of the scheme. The most salient feature of the normal system was the very high feed-forward inhibition (K_i^{HTA} = 0.1 μM) of glyoxalase 2 by the hemithioacetal which is the substrate of glyoxalase 1. The glyoxalase-2-deficient red cells were used to test whether *S*-lactoylglutathione is transported from red cells via the glutathione-*S*-conjugate transporter; this transport appeared not to occur. Because methylglyoxal is extremely rapidly removed (half-life, ~5 min) from normal red cells, it is difficult to assess the effect of this compound on glycolysis but the slow decline evident in the deficient cells allowed a study of the effects on L-lactate production; no effects were apparent.

The glyoxalase enzyme system is one of biochemistry's enigmas; although its existence has been known since 1913 (1) its overall role in metabolism remains unclear. The basic function of the system involves the conversion of reactive and potentially toxic α-ketoaldehydes to the less reactive corresponding α-D-hydroxyacid. Glyoxalase 1 (E.C. 4.4.1.5) catalyzes the conversion of the hemithioacetal, formed spontaneously between an α-ketoaldehyde and reduced glutathione (GSH),[3] an essential cofactor in the reaction, to *S*-D-hydroxyacylglutathione. Glyoxalase 2 (E.C. 3.1.2.6) then catalyzes the hydrolysis of this species, regenerating GSH and releasing the D-hydroxyacid (Fig. 1).

Glyoxalase 1 is as ubiquitous as GSH itself; indeed a species or tissue has yet to be found that does not contain glyoxalase 1 (2). Glyoxalase 2, on the other hand, has been demonstrated to be absent in human spleen, mouse heart, and rat skeletal muscle (3), although the result seems to depend on the technique used for assay. In the majority of neoplastic tissues, however, the activity of glyoxalase 1 has been found to be lower than in normal tissues, while the activity of glyoxalase 2 is not detectable, or very small (3). Additionally, in differentiating tissue, it has been found that changes in the activities of the glyoxalase enzymes occur with growth and depend on the stage of development (4, 5).

Methylglyoxal has been reported to inactivate some enzymes, by interacting with essential arginine residues (6). A half-inactivation time was reported for fructose-1,6-bisphosphate aldolase of 2.7 min in the presence of 2.5 mM methylglyoxal, with at least four other glycolytic enzymes being affected to a lesser extent. Methylglyoxal is purported to affect glucose use by platelets at the level

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[3] Abbreviations used: DQFCOSY, double-quantum filtered correlation spectroscopy; GSH, reduced glutathione; GSR, glutathione conjugate; HTA, hemithioacetal; IgE, immunoglobulin E; Mops, 4-morpholinepropanesulfonic acid; SCoP, Simulation Control Program; TSP, 2,2,3,3,-tetradeutero-3-trimethylsilylpropanoic acid.

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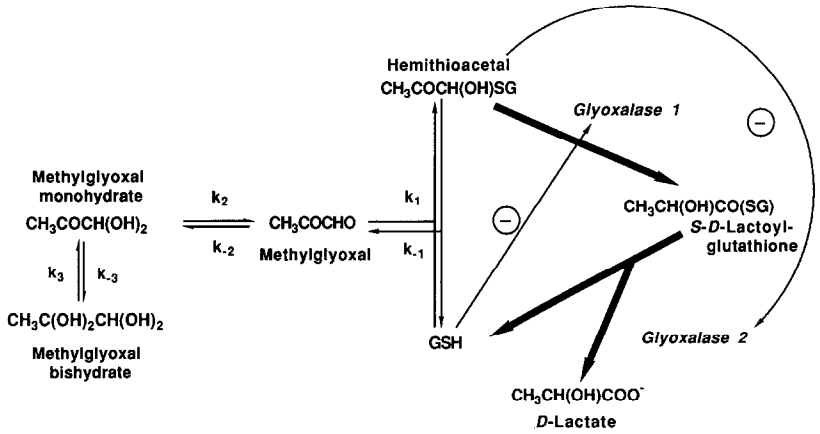


FIG. 1. The glyoxalase reaction scheme. The parameters $k_{\pm 1}$ to $k_{\pm 3}$ are first-order rate constants describing the chemical reactions between the hydrates of methylglyoxal and GSH. The enzyme-catalyzed reactions (bold arrows) are considered to follow Michaelis–Menten kinetics, with competitive inhibition (−) by the species indicated.

of glycolysis with a resultant decrease in pyruvate and lactate formation and an accumulation of some phosphorylated glycolytic intermediates (7).

Little is known about the glyoxalase system in the horse, although glyoxalase 1 has been found to be three times as active in horse erythrocytes as that in humans (8). Horse erythrocytes also have higher cellular levels of GSH (8.0 c.f. 6.6 μmol g^{-1} Hb) (9, 10).

Glyoxalase 2 deficiency in the erythrocytes of a family of humans, who also suffered from hereditary elliptocytosis, has been previously documented, although the two states were thought not to be connected. The enzyme deficiency did not extend to the leukocytes and was transmitted as an autosomal recessive trait. No discernable clinical or hematological abnormalities were found to be associated with it (11).

Because of the relatively high activity of the glyoxalase enzymes, it is experimentally difficult to study the effects of ketoaldehydes, such as methylglyoxal, on the metabolism and structure of cells. Also, the substrates, products, and intermediates of the glyoxalase reactions are chemically similar, making the reactions hard to monitor using techniques such as spectrophotometry. Similarly difficult to study is the fate of *S*-lactoylglutathione, the product of glyoxalase 2, which may be involved in the lactoylation of proteins; or it may be transported from the cell by the glutathione-conjugate (GSR) transporter, thus eliminating any deleterious effects of the molecule (12). Cellular levels of *S*-D-lactoylglutathione have also been shown to modulate anti-IgE-induced histamine release (13) and microtubule assembly (14) in neutrophils. Transport of GSR is comparatively slow, 496 μmol h^{-1} (liter red cells)$^{-1}$ in humans (15) and is ATP dependent and strictly unidirectional, occurring against a concentration gradient (16).

^{1}H NMR has been used to great advantage to study *in vivo* reactions in erythrocytes (e.g., (17)) and has recently been used to study the glyoxalase system in human erythrocytes, where it enabled individual and simultaneous quantification of each metabolite involved in the reaction (18). In this work, ^{1}H NMR was used to verify the deficiency of glyoxalase 2 in the erythrocytes of a particular thoroughbred horse and to study some of its metabolic effects, as well as to characterize kinetically the horse erythrocyte glyoxalase system. The horse in question is 12 years old and currently at stud. The enzyme deficiency does not seem to have affected his racing performance in any measurable way.

For the purposes of studying the glyoxalase system and its role in metabolism, however, the glyoxalase 2 deficiency is useful. Any ketoaldehyde added to the cell is not immediately metabolized, as is the situation normally, due to the reduction in the cellular concentration of GSH caused by formation of hemithioacetal and *S*-lactoylglutathione. It therefore became possible to study the effects of ketoaldehydes and *S*-acylglutathione on cellular metabolism at higher than normal concentrations, and over a longer time span, than would be the case in normal cells.

MATERIALS AND METHODS

Materials

Methylglyoxal was purified as described previously (18) from the dimethylacetal, which was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, and stored at −14°C until required. GSH was obtained from Sigma Chemical Co., St. Louis, Missouri. All other reagents used were of AR grade.

Preparation of Erythrocytes

Blood was collected by venipuncture into heparinized vacuum tubes and kept on ice during transport, which normally took 4 or 5 h. Upon receipt, it was washed immediately three times in isotonic saline by centrifugation at 1500*g* for 5 min, after which the buffy coat and supernatant were aspirated. Prior to the third wash, the cell suspension was bubbled for 5 min with CO to minimize the paramagnetic effects

of deoxyhemoglobin (19). The hematocrit of the cell suspension was typically 0.8. Cells were lysed by freeze–thawing in small aliquots (0.5 ml) at −14°C, since moderate sonication as used for human erythrocytes (20) was found to be ineffective in causing lysis. Lysates were stored at −14°C until required.

NMR Spectra

All spectra were acquired on a Varian XL/VXR 400, a Bruker AMX 400 or AMX 600 NMR spectrometer, operating in the Fourier transform mode with the sample temperature controlled at 22 or 37°C.

Spin–echo spectra. Washed red cells (0.65 ml) were dispensed into 5-mm NMR tubes and an internal capillary, which contained the chemical shift reference compound TSP (2,2,3,3-tetradeutero-3-trimethylsilylpropanoic acid) in D_2O, inserted into the tube to enable optimization of shims, and field/frequency locking. The Hahn spin–echo pulse sequence (90°-τ-180°-τ-acquire) was used to suppress broad protein resonances from within the cells (21). A refocusing delay (τ) of 68 ms was routinely used to allow complete inversion of the lactate methyl resonance. The large water resonance was minimized by selective presaturation. The spectral sweep width was 5000 Hz, with 32 K data points stored per free induction decay. The data were Fourier transformed with a factor of 2 of zero filling and 0.5 Hz line-broadening. Methylglyoxal was added to the tube following acquisition of an initial spectrum and a series of spectra was then recorded, with each spectrum derived from the sum of 128 transients.

Spectra for kinetic analysis. A solution of methylglyoxal (~2 mM) and GSH (15 mM) was constituted in 20 mM phosphate buffer in D_2O, pH 7.2* (where pH* denotes the uncorrected pH meter reading). Following collection of a fully relaxed, initial spectrum, the equivalent of 1 μl hemolysate was added and spectra were recorded continuously for 2 h. Each spectrum took ~4 min to acquire and was derived from the sum of eight transients. Spectra were analyzed numerically as previously specified (18) in order to obtain estimates for the kinetic parameters describing the enzyme-catalyzed reactions. The kinetic parameters describing glyoxalase 2 in the deficient horse were determined from time

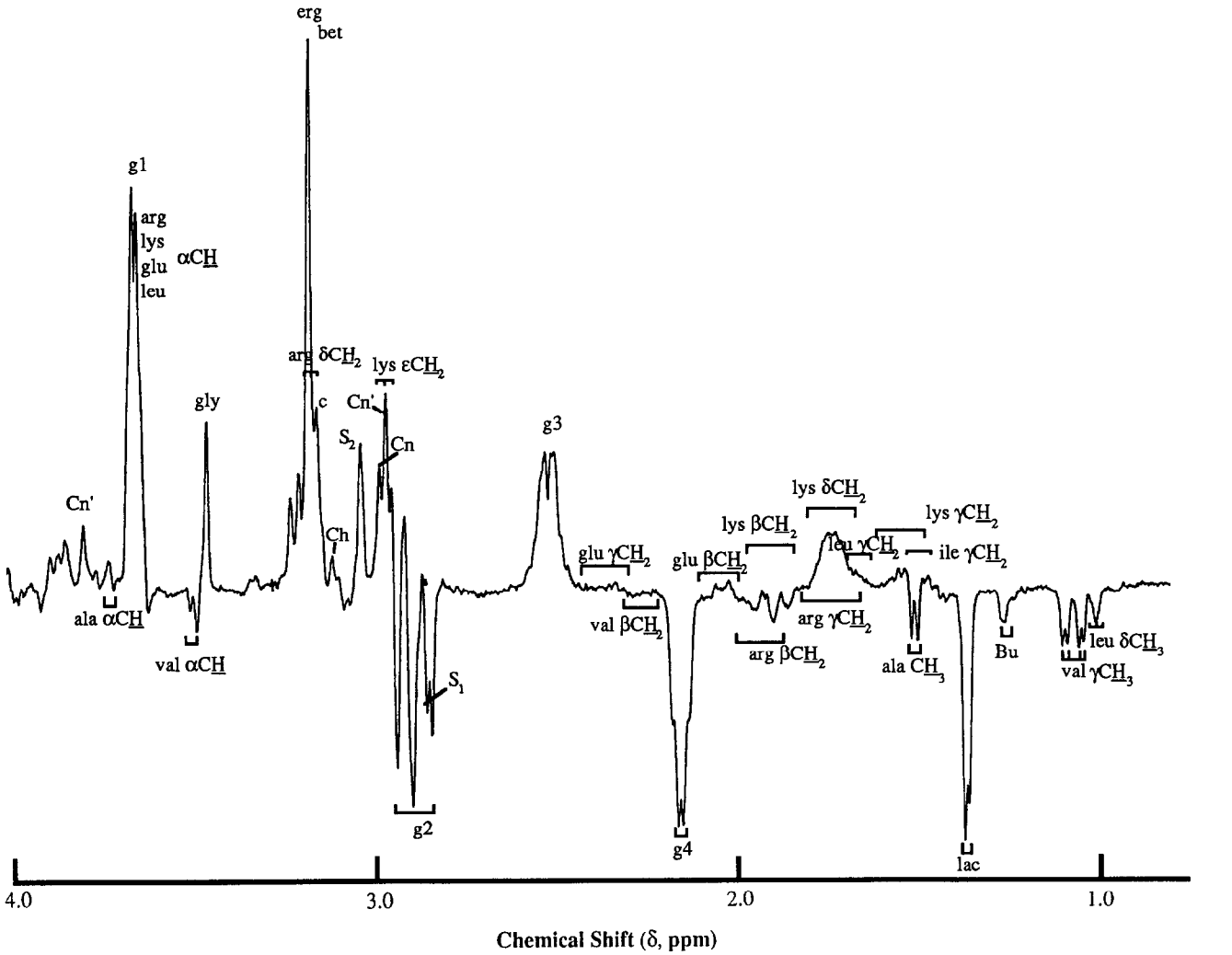
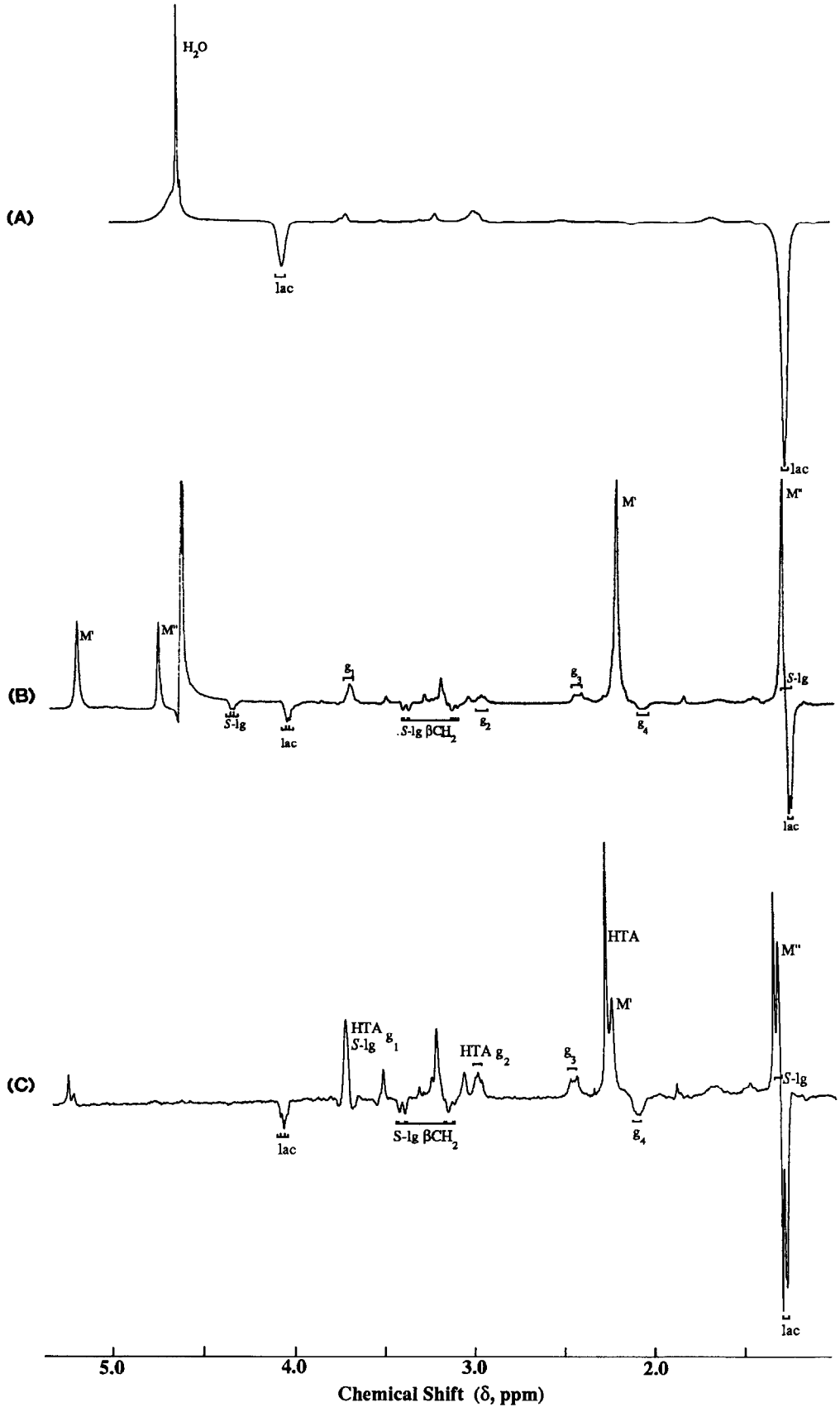


FIG. 2. ^1H NMR spin–echo spectra of packed horse erythrocytes. This spectrum was acquired at 37°C and obtained from the sum of 256 transients. Assignments: The following six are from the N-CH_3 protons of Cn′, creatine; erg, ergothioneine; bet, betaine; c, carnitine; Ch, choline; Cn, creatinine; g_1, gly αCH_2, glu αCH of GSH; g_2, GSH cys βCH_2; g_3, GSH glu βCH_2; g_4, GSH glu βCH_2; lac, lactate; Bu, β-hydroxybutyrate CH_3; S_1, S_2, unassigned singlet resonances. The GSH cys βCH_2 resonance is altered from its "normal" appearance by the use of 68 ms mixing delay instead of the more usual 60 ms (21).


(A)
H_2O
lac
lac
(B)
M'
M''
M'
M''
S-lg
lac
g_1
S-lg βCH$_2$
g_2
g_3
g_4
S-lg
lac
(C)
HTA
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HTA S-lg g_1
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g_3
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S-lg βCH$_2$
g_4
lac
5.0
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Chemical Shift (δ, ppm)

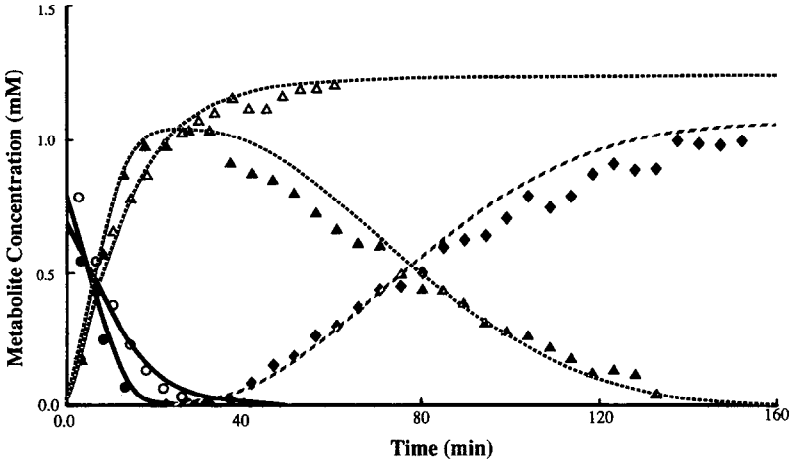

FIG. 4. Computer-generated time courses fitted to NMR-derived data. Symbols represent NMR-derived data from time courses with hemolysate from normal horses (closed symbols) and glyoxalase-2-deficient horses (open symbols): circles, integral of the $C\underline{H}_3$ resonance of HTA; triangles, integral of the $C\underline{H}_3$ resonance of *S*-lactoylglutathione; and diamonds, integral of the $C\underline{H}_3$ resonance of lactate. Integrals obtained from fully relaxed spectra were converted to concentrations by comparison with the $C\underline{H}_3$ resonance of a known amount of acetate, added as an internal standard. The curves represent: bold line, HTA; small-dotted line, *S*-lactoylglutathione; and large-dotted line, lactate. Curves corresponding to the normal and deficient horse glyoxalase systems are the results of nonlinear regression of the kinetic equations onto the data (method as in (18)).

courses monitoring the conversion of *S*-lactoylglutathione to lactate by 10 μl of hemolysate from the deficient horse in a series of ^{1}H NMR spectra acquired over a longer period of time (8–12 h). The spontaneous breakdown of *S*-lactoylglutathione to lactate in the buffer was also measured.

Two-dimensional spectra. The 2D spectrum of ultrafiltered hemolysate was acquired using double-quantum filtered correlation spectroscopy (DQFCOSY) (25). Each spectrum consisted of 128 transients, acquired after presaturation at the water frequency, with a total of 360 spectra being collected.

Transport of S-Lactoylglutathione from Cells

Previously washed red cells were washed twice in a "rejuvenation" medium consisting of 10 mM glucose, 10 mM inosine, 5 mM pyruvate, and 5 mM inorganic phosphate in isotonic saline (23) and then bubbled with CO as before. Packed cells (4 ml) were added to 4 ml of rejuvenation medium (23) in a conical flask. Methylglyoxal, sufficient to give a final concentration of 6 mM, was added to separate flasks containing blood from the enzyme-deficient horse and blood from a normal horse. A total of 1.3 ml cells was removed immediately and spun for 1 min in an Eppendorf microcentrifuge (Eppendorf Geratebau, Hamburg, Germany). The supernatant was removed, as was 650 μl of packed cells. Both samples were immediately frozen and stored at −14°C. Sampling was repeated at 2, 4, 8, and 24 h. Samples of cells and supernatants were subsequently examined by ^{1}H NMR for the presence of *S*-lactoylglutathione. The experiment was repeated by incubating freshly collected, washed cells in saline containing 15 mM glucose, rather than the rejuvenation medium, with 10 mM methylglyoxal.

Effect of Methylglyoxal on Lactate Production

Washed red cells from a normal horse and the glyoxalase-2-deficient horse were incubated at 37°C in conical flasks in 200 mM 4-morpholinepropanesulfonic acid (Mops), (pH 7.2, adjusted to physiological ionic strength with NaCl) and glucose (~7 mM). Methylglyoxal (17 mM) was added to the incubation flasks and 0.8 ml of cells was immediately removed and an equal volume of perchloric acid (8% w/v) added. The solution was spun in a bench centrifuge and the supernatant removed. Sampling was repeated at 15, 30, 60, 90, 120, 150, 180, 210, and 240 min. Samples were stored on ice and neutralized with K_2CO_3 and the supernatant was assayed for L-lactate using a Rapid Lactate assay (Behring Diagnostics, Inc., Somerville, NJ) and a Cobas-Fara centrifugal analyzer (Roche Products, Pty. Ltd, Dee Why, NSW, Australia).

Assignment of Red Cell Spectra

Hemolysate samples were diluted 1:10 and ultrafiltered under nitrogen (~200 kPa) through a YM10 (<10 kDa cutoff) membrane (Amicon Scientific Australia, Faulkner, Vic), which had been washed thoroughly in deionized water to remove the glycerol which would appear in the ^{1}H NMR spectrum. The filtrate was lyophilized overnight and then redissolved in D_2O. Spectra of the ultrafiltrates were compared with those of red cells and further assignments were made by addition of authentic compounds to the sample or by using DQFCOSY to establish molecular connectivities (22).

Assay of Glyoxalase 2 in Leukocytes

Peripheral blood leukocytes were isolated by centrifugation through Ficoll-Paque and were lysed by freezing at −70°C and thawing at 37°C. The cell extracts were subjected to nondenaturing electrophoresis and the gels were specifically stained for glyoxalase 2 activity by the methods described previously (24).

FIG. 3. ^{1}H NMR spin–echo spectra showing the effect of adding methylglyoxal to normal and glyoxalase-2-deficient horse erythrocytes. (A) Normal horse red cells 5 min after addition of 50 mM methylglyoxal. (B) Glyoxalase-2-deficient red cells ~8 min after addition of 50 mM methylglyoxal. Assignments: M″, methylglyoxal bishydrate methyl and aldehydic protons; M′, methylglyoxal monohydrate; HTA, hemithioacetal; and *S*-lg, *S*-lactoylglutathione methyl and methylene protons. (C) Glyoxalase-2-deficient horse red cells 20 min after addition of 20 mM methylglyoxal.

TABLE I

Estimates of Horse Glyoxalase Enzyme Kinetic Parameters

	Normal	Glyoxalase 2 deficient
Glyoxalase 1		
V_{max} (mmol liter^{-1} min^{-1})	126 ± 9	134 ± 24
K_m (mM)	0.093 ± 0.022	0.32 ± 0.11
K_i^{GSH} (mM)	7.80 ± 0.001	7.76 ± 0.001
Glyoxalase 2		
V_{max} (mmol liter^{-1} min^{-1})	24.0 ± 0.7	0.8
K_m (mM)	0.17 ± 0.02	0.35
K_i^{HTA} (μM)	0.100 ± 0.001	Not determined

RESULTS

NMR Spectra

A spin–echo spectrum of horse red cells with assignments is shown in Fig. 2. The assignment of resonances arising from amino acids was aided by the concomitant use of the DQFCOSY spectra of ultrafiltered hemolysate. That arginine, in particular, is present is difficult to visualize in 1D spectra without connectivities obtained from 2D spectra, as the major resonances are coresonant with those of other molecules. For example, the arginine $\epsilon C\underline{H}_2$ is coresonant with ergothioneine $N(C\underline{H}_3)_3$ and consequently increases the apparent amount of ergothioneine present in a sample. Assignments were also verified by addition of authentic compounds to the sample.

Figure 3 contains spectra showing the effects of adding methylglyoxal to normal horse cells and to cells deficient in glyoxalase 2. In normal cells (Fig. 3A), 5 min after addition of methylglyoxal, no resonances were seen which were attributable to methylglyoxal, or to *S*-lactoylglutathione. Two large resonances appeared which arose from the $C\underline{H}_3$ (inverted doublet at 1.30 ppm) and $C\underline{H}OH$ (quartet at 4.10 ppm) protons of lactate, indicating that the methylglyoxal was completely metabolized to lactate via the glyoxalase enzymes.

In glyoxalase-2-deficient cells (Figs. 3B and 3C) the resonances of glyoxalase metabolites can be discerned. Figure 3B shows the cells ~8 min after addition of 50 mM methylglyoxal: singlet resonances attributable to the methyl protons of the monohydrate (M′) and bishydrate (M″) of methylglyoxal can be seen at 2.23 and 1.32 ppm, respectively, with the singlet resonances from the aldehydic proton at 5.22 and 4.77 ppm, respectively. The presence of hemithioacetal may be inferred from the cys $\beta C\underline{H}_2$ resonance of GSH (2.9–3.0 ppm), which has changed, indicating reaction of GSH with methylglyoxal. Hemithioacetal concentrations are obviously limited by the amount of GSH in the cell.

The *S*-lactoylglutathione methyl resonance (~1.32 ppm) is distorted by the methyl resonance of the bishydrate. Evidence that *S*-lactoylglutathione is present may be seen from its cys $\beta C\underline{H}_2$ resonance (3.15 and 3.41 ppm) and $C\underline{H}OH$ multiplet (4.36 ppm).

Figure 3C, from a different timecourse, shows a spin–echo spectrum of glyoxalase-2-deficient red cells (hematocrit, ~0.75) approximately 20 min after the addition of 20 mM methylglyoxal. Resonances from all the glyoxalase intermediates may be seen, including that of the hemithioacetal methyl protons (2.306 ppm). The *S*-lactoylglutathione $C\underline{H}OH$ multiplet and the methylglyoxal bishydrate aldehydic proton singlet are not seen due to "spillover" of radio frequency power from the water suppression.

Time Courses of Methylglyoxal Metabolism

Time courses, from NMR-derived data, showing the metabolism of methylglyoxal to lactate by horse glyox-

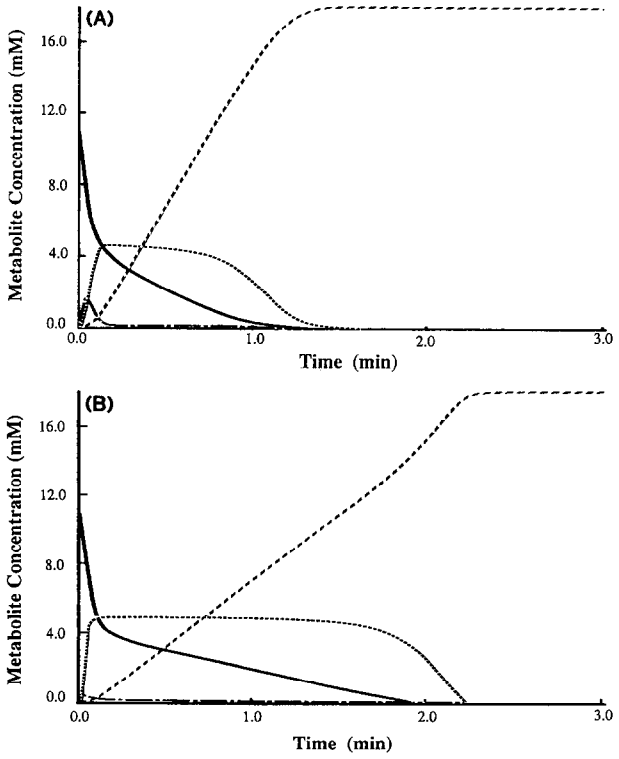

FIG. 5. Computer simulation of the erythrocyte glyoxalase system. First-order rate constants describing the initial chemical equilibria were not altered from the values shown below. Values used: k_{-1} = 19.2 min^{-1}, $k_1 = 3.0 \times 10^{-2}$ mM^{-1} min^{-1}, k_2 = 18.48 min^{-1}, k_{-2} = 9.6 min^{-1}, k_3 = 306 min^{-1}, and k_3 = 126 min^{-1} (21). The curves represent: bold line, methylglyoxal monohydrate; broken line, HTA; short dashes, *S*-lactoylglutathione; and long dashes, lactate. (A) Time course showing the effect of adding 18 mM methylglyoxal to packed human red cells. Values used (21): V^1_{max} = 70.4 mmol l^{-1} min^{-1}, K^1_M = 0.46 mM, K_i^{GSH} = 7.88 mM, V^2_{max} = 24 mmol liter^{-1} min^{-1}, K^2_M = 1.5 mM, and K_i^{HTA} = 0.29 mM. Assumed [GSH] = 4.1 mM. (B) Same as in A, but the model is altered to contain horse red cell enzymic parameters. Values used: V^1_{max} = 125.5 mmol liter^{-1} min^{-1}, K^1_M = 0.093 mM, K_i^{GSH} = 7.88 mM, V^2_{max} = 23.95 mmol liter^{-1} min^{-1}, K^2_M = 0.17 mM, and K_i^{HTA} = 0.1 μM. Assumed [GSH] = 5.0 mM.

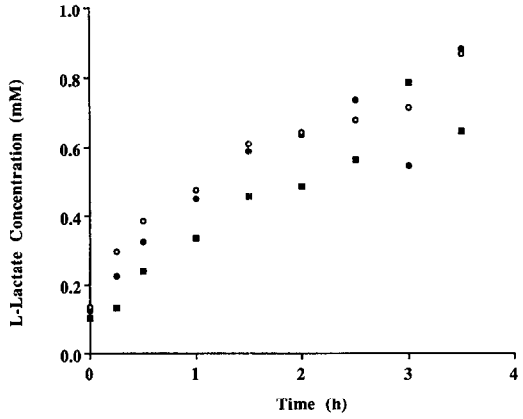

FIG. 6. Effect of methylglyoxal on glycolytic lactate production in glyoxalase-2-deficient cells. Data show the rate of production of L-lactate from horse red cells metabolizing glucose at 37°C. Symbols represent: closed squares, normal horse cells; closed circles, glyoxalase-2-deficient horse cells; and open circles, glyoxalase-2-deficient horse cells, with 17 mM methylglyoxal.

alase enzymes are shown in Fig. 4; metabolism by normal red cells and by deficient cells are compared. Values for the kinetic parameters describing the reaction in the normal and glyoxalase-2-deficient horses are shown in Table I. *S*-lactoylglutathione converted spontaneously to lactate with a rate constant of 3.9×10^{-7} s^{-1}. Furthermore, the enzyme-deficient cells do have the capacity to convert *S*-lactoylglutathione to lactate, although this capacity is very much reduced. Glyoxalase 1 in the deficient horse was assessed for competitive feedback inhibition by *S*-lactoylglutathione; solutions of equations describing the kinetic situation in which inhibition takes place were fitted to NMR-derived data from this horse. The results indicated that no inhibition took place under the conditions which were used in the experiments.

Computer Simulation of Time Courses

Figure 5 shows time courses generated by computer, from differential rate equations describing the reaction scheme (Fig. 1), illustrating the fate of methylglyoxal added to intact red cells (i.e., as illustrated in Fig. 3A). In this figure, the glyoxalase system in the horse (Fig. 5B) is compared with that in humans (Fig. 5A). The kinetic parameters that were used in the latter simulations were those reported previously (18).

S-Lactoylglutathione Transport from Cells

In the experiment designed to assess the transport of *S*-lactoylglutathione from cells, none was detected in the extracellular medium, even up to 24 h after addition of methylglyoxal to glyoxalase-2-deficient red cells. Methylglyoxal was metabolized slowly by the glyoxalase-2-deficient cells and was still present 8 h later. Resonances assignable to *S*-lactoylglutathione were seen in spin–echo spectra of enzyme-deficient cells, to which methylglyoxal had been added, up to 8 h later, and in normal cells, to which methylglyoxal had been added, up to 4 h later; the amount of *S*-lactoylglutathione in this latter case was much less. Gradual disappearance of glucose resonances from spectra of the extracellular medium, coupled with an increase in the size of the lactate resonances, showed that the cells were actively metabolizing glucose during the time course of the experiment. The rate of production of L-lactate by red cells was unaffected by the presence of methylglyoxal (Fig. 6), although an artifactual result, in which glycolysis was retarded, was obtained if the pH was not carefully controlled.

Leukocyte Glyoxalase 2

Glyoxalase 2 activity was detected in leukocytes from the horse that was deficient in the erythrocyte isoenzyme. The activity detected in the leukocyte extracts had an identical electrophorectic mobility to that of the isoenzyme found in normal horse erythrocytes.

DISCUSSION

The use of blood from the horse deficient in glyoxalase 2 has made it possible to assign resonances from glyoxalase intermediates in the ^1H NMR spin–echo spectrum due to the significant slowing of the glyoxalase 2 reaction. A particularly useful resonance for following the formation of GSH derivatives is that of the cys $\beta C\underline{H}_2$ moiety of glutathione. This moiety represents the most proximate ^1H NMR-visible resonance to the side group attached to the thiol of the cysteine residue of glutathione, whether it is a ketoaldehyde or hydroxy acid. The side group produces a change in chemical shift of the cys $\beta C\underline{H}_2$ resonance which is most marked in *S*-lactoylglutathione, producing resonances at 3.15 and 3.41 ppm (Fig. 3C). The methyl protons of *S*-lactoylglutathione are difficult to detect and quantify, since they are partially hidden by the methyl protons both of lactate and of the methylglyoxal bishydrate. Similarly useful for kinetic studies is the C<u>H</u>OH multiplet of *S*-lactoylglutathione (4.36 ppm, Fig. 3B), although the intensity of this resonance may depend to some extent on the selectivity of the saturation of the water resonance at 4.7 ppm.

One of the most interesting features of the horse glyoxalase system is the high level of feed-forward inhibition of glyoxalase 2 by the substrate of glyoxalase 1 (HTA; Fig. 1). The inhibition constant (K_i^{HTA}) is several orders of magnitude smaller than the K_M of the enzyme for its substrate, *S*-lactoylglutathione. This means that, given the initial concentrations of glutathione and methylglyoxal used in the time courses obtained with dilute hemolysate, glyoxalase 2 is effectively "switched off" while HTA is present.

In this *in vitro* situation, the enzyme-catalyzed reactions, and not the spontaneous rate of formation of HTA, are the principle determinants of the rate of D-lactate production. (The experimental conditions were designed this way in order that the shape of the progress curves, defined by the equations describing the enzyme-catalyzed reactions, would be influenced mainly by the enzyme kinetic parameters and not by the potentially rate-limiting, spontaneous formation of HTA. Much better estimates of the kinetic parameters of the enzymes could be obtained this way.) *In vivo,* however, the situation is different, because the rate-limiting step of the reaction is the formation of HTA (or, strictly speaking, the rate of dehydration of methylglyoxal; k_2 in Fig. 1). Under these circumstances, any HTA produced is immediately metabolized by glyoxalase 1, resulting in very low *in vivo* levels of HTA (Fig. 5) and, consequently, much less inhibition of glyoxalase 2.

Figure 5 shows that, although horse erythrocyte glyoxalase 1 may have higher V_{max} and lower K_M values than its human counterpart (18), it is actually less efficient at removing methylglyoxal from the system, taking at least twice as long to metabolize >95% of it. This results from the higher level of inhibition of equine glyoxalase 2 by the HTA; this competitive inhibition of glyoxalase 2 is illustrated well by Fig. 5B, where, after ~2 min, the rate of formation of lactate increased when the HTA had been depleted in the system. The reason why such high inhibition has evolved in horses is unclear.

The high level of inhibition of glyoxalase 2 by the HTA also assisted in the study of *S*-lactoylglutathione transport. Addition of methylglyoxal in millimolar concentrations to glyoxalase-2-deficient horse cells resulted in the cells rapidly accumulating large amounts of *S*-lactoylglutathione; this was limited only by the amount of GSH in the cells. Any HTA present would further inhibit the already "disabled," or insufficient glyoxalase 2, effectively locking a large amount of *S*-lactoylglutathione inside the cell. Assuming *S*-lactoylglutathione is transported from cells at a similar rate to DNP-glutathione (157 μmol h^{-1} (liter red cells)$^{-1}$) (8), then 12–24 h of incubation with *S*-lactoylglutathione should have produced sufficient *S*-lactoylglutathione in the extracellular medium for it to be detected by ^{1}H NMR spectroscopy (lower limit of detection ~0.1 mM). Assuming a packed cell volume of ~50%, the concentration in the medium should be just over the lower limit of detection after 1 h.

The presence of glyoxalase 2 in cells would normally ensure that little transport of *S*-lactoylglutathione took place. However, glyoxalase-2-deficient cells may lose *S*-lactoylglutathione by transport from the cells via the relatively nonspecific GSR transporter (12). Our results showed a lack of *S*-lactoylglutathione transport from cells. This result suggests a lower size limit for the substrates of the *S*-conjugate transporter, which transports a wide range of xenobiotic glutathione *S*-conjugates (25) but not GSH itself (26).

Many metabolic roles have been proposed for the glyoxalase system, including the provision of *S*-lactoylglutathione (27), which has been implicated in microtubule assembly (14) and histamine release (13) in neutrophils. Hence cellular levels of this compound may be finely regulated.

High intracellular levels of *S*-lactoylglutathione did not appear to affect the production of lactate via glycolysis (Fig. 6), although other metabolic effects might be expected, due simply to the lowering of intracellular GSH concentration caused by the reaction of GSH with methylglyoxal and the subsequent glyoxalase-1-catalyzed formation of *S*-lactoylglutathione. *In vivo,* where methylglyoxal concentrations (0.023 ± 0.002 mM) (28) are much lower than those used in this study, *S*-lactoylglutathione concentrations would be expected to be correspondingly lower, resulting in less impact on cellular GSH concentrations (normally ~8 μmol (g Hb)$^{-1}$ in horses) (9).

A half-life of 2.7 min for deactivation of aldolase by methylglyoxal has been reported by Leoncini *et al.* (6) from an *in vitro* experiment. Four other glycolytic enzymes were also affected to a lesser extent. In the light of these observations we would expect glycolysis in the glyoxalase-2-deficient red cells to have been affected by methylglyoxal, even though fructose 1,6-bisphosphate and dihydroxyacetone phosphate have been reported to provide efficient protection against inactivation of aldolase by hydroxymethylglyoxal (29). However, in contrast to the expectation based on these earlier findings, the rate of lactate production in the glyoxalase-2-deficient cells, in which high methylglyoxal levels persisted for several hours, was not affected.

The presence of glyoxalase 2 in leukocytes from the horse deficient in the erythrocyte enzyme suggests that the deficiency may result from an unstable variant that degrades rapidly and is continually replaced in nucleated cells. Deficiencies of erythrocyte enzymes, which do not extend to other tissues, are not an uncommon finding. This appears also to have been the situation in the only documented case of human erythrocyte glyoxalase 2 deficiency (11). The relatively short (~120 days) lifetime of the red blood cell may circumvent effects of the enzyme deficiency which may be deleterious in cells which are longer lived.

In conclusion, there were obvious and readily characterizable changes in methylglyoxal metabolism in the glyoxalase-2-deficient red cells. ^{1}H spin–echo NMR spectroscopy proved to be a very useful means of monitoring the metabolic events. Computer simulation of the reaction pathway enabled a comparison with the corresponding system in humans. Exploitation of the enzyme deficiency enabled testing and rejection of the hypothesis that *S*-lactoylglutathione is transported from the red cell, as well

as illustrating the lack of effect of higher than normal *in vivo* concentrations of methylglyoxal.

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