Incorporation of labelled glycine into reduced glutathione of intact human erythrocytes by enzyme-catalysed exchange

A nuclear-magnetic-resonance study

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^1H , ^2H and ^{15}N n.m.r. spectroscopy was used to monitor the incorporation of free glycine into the glycine residue of reduced glutathione (GSH) in suspensions of intact human erythrocytes. The following results were obtained. (i) By using ^1H spin-echo n.m.r. the exchange reaction between [$^2\text{H}_2$]glycine and the protonated glycine residue of GSH was studied at various [$^2\text{H}_2$]glycine concentrations, thus enabling the calculation of an apparent Michaelis constant (K_m) and maximal velocity ($V_{\max}$) for the process. (ii) The reaction is catalysed by glutathione synthetase and proceeds most rapidly in the absence of glucose, which is the main physiological energy source of the erythrocyte. (iii) ^{15}N n.m.r. spectroscopy, with a one-pulse sequence, and ^2H n.m.r. spectroscopy, with an inversion recovery method, enabled demonstration of the incorporation of labelled glycine into an intra-erythrocyte peptide, consistent with incorporation into GSH. (iv) The exchange reaction, although inhibited by glucose, appeared not to be dependent on low ATP or 2,3-bisphosphoglycerate concentrations.

The tripeptide GSH has a primary role in protecting the erythrocyte against oxidative damage. It is the most abundant intracellular thiol compound and is present normally in human erythrocytes at a concentration of 2–3 mmol/l of cells (Beutler, 1975, p. 149). GSH in human erythrocytes is constantly turned over, having a half-life, under normal physiological conditions, that is estimated at 4–5 days (Dimant *et al.*, 1955). Whereas many of the functions of the tripeptide are well characterized, the mechanisms by which GSH is turned over in the human erythrocyte are poorly understood (see, e.g., McIntyre & Curthoys, 1980).

N.m.r. has a unique advantage over most techniques used for assaying enzymes and metabolites in cells in that it allows non-invasive study of the intact tissue. In the case of the erythrocyte, the application of spin-echo techniques has made ^1H n.m.r. ideal for monitoring enzymic activity and metabolic changes *in situ* on a time scale extending from minutes to hours (Brown *et al.*, 1977; Isab & Rabenstein, 1979; Brindle *et al.*, 1979; Kuchel, 1981).

The first n.m.r.-based studies in GSH metabolism in human erythrocytes followed the changes in

oxidation state of GSH after the addition to them of *t*-butyl hydroperoxide (Brown *et al.*, 1977). More recently information on the turnover of GSH has been obtained by monitoring the incorporation of [$^2\text{H}_2$]glycine into GSH (Isab & Rabenstein, 1979).

Studies of GSH synthesis *de novo* from radioactively labelled precursor amino acids have shown that glycine is incorporated primarily into GSH by an exchange reaction catalysed by glutathione synthetase (EC 6.3.2.3) (Griffith, 1981). This suggests that the incorporation of [$^2\text{H}_2$]glycine into GSH inferred from ^1H n.m.r. data is principally a manifestation of this exchange reaction with only minimal participation of GSH synthesis from the three constituent amino acids.

Work reported in the present paper shows that this exchange process, followed by monitoring the protonated glycine resonance in the ^1H n.m.r. spectrum, occurs rapidly in erythrocytes that have been washed free of glucose. This is in spite of the requirement by erythrocytes for glucose to supply energy for the rephosphorylation of ADP to ATP, which in turn is necessary for GSH synthesis from its constituent amino acids (Snoke *et al.*, 1953).

Further experiments reported in the present paper employed erythrocytes washed free of glucose in order to determine the dependence of the exchange

Abbreviations used: GSH and GSSG, reduced and oxidized glutathione respectively.

rate on (i) the concentration of added [$^2\text{H}_5$]glycine and (ii) the intracellular concentration of GSH.

It has also been possible to calculate an apparent Michaelis constant (K_m) and maximal velocity (V_{max}) for glutathione synthetase in the intracellular environment.

Experimental

Materials

ADP, 2,3-bisphosphoglyceric acid, 5,5'-dithiobis-(2-nitrobenzoic acid), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), [$^2\text{H}_5$]glycine, hexokinase (EC 2.7.1.1), 3-phosphoglyceric acid, sodium penicillin G and streptomycin sulphate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ATP, enolase (EC 4.2.1.11), GSH, NADH, NADP $^+$, lactate dehydrogenase (EC 1.1.1.27), phosphoglycerate mutase (EC 2.7.5.3) and pyruvate kinase (EC 2.7.1.40) were obtained from Boehringer Mannheim (North Ryde, N.S.W., Australia). Hexafluorobenzene and t-butyl hydroperoxide were obtained from Merck (Darmstadt, West Germany). [2,3,4,6,6- ^2H]-Glucose and tetramethylsilane were obtained from Merck, Sharp and Dohme (Point Claire-Dorval, Que., Canada). Fungizone (amphotericin B) was obtained from E. R. Squibb and Sons (Princeton, NJ, U.S.A.). $^2\text{H}_2\text{O}$ (99.75%) was obtained from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, Sydney, N.S.W., Australia). All other reagents were of A.R. grade.

Preparation of erythrocytes for ^1H n.m.r. studies

Human erythrocytes were prepared from freshly drawn venous blood by washing once in iso-osmotic saline (0.9% NaCl) in $^2\text{H}_2\text{O}$ and three times with Krebs bicarbonate buffer (Krebs & Henseleit, 1932) in $^2\text{H}_2\text{O}$. The Krebs bicarbonate solution contained antibiotics [sodium penicillin G (75 $\mu\text{mol/l}$), streptomycin sulphate (75 $\mu\text{mol/l}$) and amphotericin B (1.1 mg/l)] and was gassed with O_2/CO_2 (19:1). This washing procedure led to complete elimination of glucose from the erythrocyte suspension. Where necessary, for control experiments, [2,3,4,6,6- ^2H]-glucose was added immediately after washing. The haematocrit of samples was adjusted to 0.8. Just before n.m.r. measurements, 0.5 ml of cells was placed in a 5 mm n.m.r. tube and the samples were thermally equilibrated at 37°C in a heating block. Various reagents were then added (see the Results section) and n.m.r. measurements begun. Spectral accumulation was commenced 1 h after the washing of the erythrocytes.

^1H n.m.r. spectra were measured at 400 MHz in the Fourier mode with a Bruker WM-400 spectrometer at 37°C. The spin-echo sequence (Campbell *et al.*, 1975) $90^\circ-\tau-180^\circ-\tau$, with suppression of the water signal, or the inversion-recovery spin-echo

sequence (Rabenstein, 1978) $180^\circ-\tau-90^\circ-\tau-180^\circ-\tau$, was used with $\tau = 60$ ms and a total repetition time of 0.94 s (spin-echo) or 1.00 s (inversion-recovery spin-echo). A total of 256 or 512 transients were averaged, with a sweep width of 5000 Hz and 8192 data points. Chemical shifts (in p.p.m.) are quoted relative to tetramethylsilane (in CCl_4) present in a small capillary inside the n.m.r. tube. The areas and amplitudes of time-dependent peaks were normalized with respect to (i) the external reference tetramethylsilane signal and to (ii) the combined quaternary amine methyl signals (2.9–3.1 p.p.m.), which have been shown previously (Isab & Rabenstein, 1979) to be time-invariant in any one preparation of whole cells. A calibration curve of glycine concentration (0–4 mmol/l of cells) plotted versus the intensity ratio glycine signal/ergothioneine quaternary amine methyl signal was prepared; a similar curve was prepared for GSH in lysate (haematocrit 0.8).

Preparation of erythrocytes for ^2H and ^{15}N n.m.r. studies

Erythrocytes were prepared as described above except that Krebs bicarbonate buffer in $^1\text{H}_2\text{O}$ was used for erythrocytes in ^2H n.m.r. studies and that for ^{15}N n.m.r. studies only the final wash was with Krebs bicarbonate buffer in $^2\text{H}_2\text{O}$. For time-course studies with ^{15}N n.m.r. erythrocytes (30 ml) were incubated with [^{15}N]glycine (0.610 M; 500 μl) at 37°C. At specified times (see the Results section) 5 ml samples were withdrawn, snap-frozen in liquid N_2 and stored at -12°C for subsequent analysis.

^2H n.m.r. spectra were measured at 61.4 MHz at 37°C. The inversion recovery sequence $180^\circ-\tau-90^\circ$, with τ varied, was used. Samples were measured in an 8 mm n.m.r. tube placed coaxially in a 10 mm n.m.r. tube containing hexafluorobenzene for field-frequency lock. A total of 256 transients were averaged with sweep width 1000 Hz and 2048 data points. ^{15}N n.m.r. spectra were recorded at 27.37 MHz, in 15 mm tubes, in the Fourier mode with a Bruker HFX-270 spectrometer at 4°C. The NO_3^- resonance of 5 M- $^{15}\text{NH}_4^{15}\text{NO}_3$ in 2 M-nitric acid/ $^2\text{H}_2\text{O}$, in a 5 mm (outside diameter) coaxial tube, was used to provide an external chemical-shift standard, and the data were then converted to the NH_3 standard.

Assays of ATP, 2,3-bisphosphoglycerate and GSH

Washed erythrocytes $\pm$ glucose (10 mmol/l) were incubated at 37°C with glycine (10 mmol/l). At specified times 1 ml of the sample was removed, snap-frozen in liquid N_2 and stored at -12°C for subsequent analysis. Samples were thawed and divided to provide a metaphosphoric acid/EDTA/NaCl extract for GSH analysis, a perchloric acid extract for ATP analysis and a diluted sample of

lysate for 2,3-bisphosphoglycerate analysis. The metabolites were analysed by standard methods [ATP (Beutler, 1975, pp. 104–106); GSH (Beutler, 1975, pp. 112–114); 2,3-bisphosphoglycerate (Hickey *et al.*, 1979)] adapted to a Centrifichem 300 centrifugal analyser (Union Carbide Corp.). Metabolite concentrations were corrected for dilution and haematocrit and are expressed as mmol of metabolite/l of cells.

Results

Fig. 1 shows a typical sequence of ^1H n.m.r. spin-echo spectra obtained from intact erythrocytes incubated with $[\text{}^2\text{H}_5]\text{glycine}$ (10 mmol/l of suspension) in the absence of glucose. The assignments of the relevant peaks are given in the legend to Fig. 1 in accordance with those reported by Brown *et al.*

(1977) and Kuchel (1981). The resonance for glycine (peak b; 3.27 p.p.m.) was observed to increase rapidly in amplitude relative to the resonance of ergothioneine (peak c), which remained unaltered in any one preparation during incubation. The amplitude of the GSH glycyl- CH_2 resonance (peak a; 3.50 p.p.m.) was observed to decrease, but not to the same extent as the concomitant increase of the glycine resonance.

In erythrocytes washed free of glucose the relative increase in the time-dependent resonance of protonated glycine was greater than in erythrocytes supplied with glucose. This is demonstrated by the two time courses shown in Fig. 2. In the glucose-free erythrocytes the exchange between $[\text{}^2\text{H}_5]\text{glycine}$ and the glycine residue of GSH shows no apparent initial lag period; the exchange reaction proceeded at a linear rate, within the limits of experimental error, over the period of study.

Fig. 1. Exchange of $[\text{}^2\text{H}_5]\text{glycine}$ with the GSH glycine residue

Spin-echo spectra (400 MHz; 512 transients) of a suspension of human erythrocytes in Krebs bicarbonate buffer in $^2\text{H}_2\text{O}$ are shown. At $t = 0$ $[\text{}^2\text{H}_5]\text{glycine}$ (10 mmol/l) was added to the sample. Assignments: a, protons of $\alpha\text{-C}$ of GSH glycine residue; b, protons on $\alpha\text{-C}$ of glycine; c, methyl protons of ergothioneine. Other assignments are as given by Brown *et al.* (1977) and Kuchel (1981).

Fig. 2. Glycine CH_2 resonance in $[\text{}^2\text{H}_5]\text{glycine}$ -treated erythrocytes with and without glucose

The change in amplitude, as a function of time, of the glycine CH_2 resonance, relative to the ergothioneine resonance (internal reference), in human erythrocytes suspended in Krebs bicarbonate buffer in $^2\text{H}_2\text{O}$, is shown, with $[\text{}^2\text{H}_5]\text{glucose}$ (10 mmol/l) ($\square$), or free of glucose ($\circ$). At $t=0$, $[\text{}^2\text{H}_5]\text{glycine}$ (final concentration 10 mmol/l) was added to each suspension.

Similarly, the resonance of the GSH glycyl CH_2 was observed to decrease in amplitude more rapidly in erythrocytes washed free of glucose than in those with glucose. However, glycine exchange measured by monitoring the GSH glycyl- CH_2 resonance is complicated by oxidation of GSH. Assays for GSH showed that the concentration was maintained in erythrocytes supplied with glucose, but that GSH was oxidized at a rate of $4 \pm 1 \mu\text{mol/min}$ per l of packed cells in those washed free of glucose. Since the amplitude of the resonance of GSH glycyl CH_2 is known to decrease in the oxidized form relative to the reduced (Brown *et al.*, 1977), it is necessary to delineate the changes occurring in the glycyl resonance as a result of (i) the incorporation of $[\text{}^2\text{H}_5]\text{glycine}$ into GSH and (ii) the oxidation of GSH.

Control experiments, run concurrently with the ^1H -n.m.r. experiments, were prepared the same way as described in the legends to the Figures except that there was no added $[\text{}^2\text{H}_5]\text{glycine}$. The results demonstrated that cells with and without $[\text{}^2\text{H}_5]\text{glycine}$ both showed a decrease in the resonance of the GSH glycyl CH_2 with time. However, the greatest decrease occurred with those cells treated with $[\text{}^2\text{H}_5]\text{glycine}$.

An attempt was made to follow directly the incorporation of $[\text{}^2\text{H}_5]\text{glycine}$ into the GSH peptide bond by ^2H n.m.r. In the fully relaxed spectrum (A in

Fig. 3. ^2H n.m.r. inversion-recovery spectrum of erythrocytes

Inversion-recovery spectra (61.4 MHz; 256 transients) of a suspension of human erythrocytes (2.5 ml; haematocrit 0.90), washed free of glucose, in Krebs bicarbonate buffer in $^1\text{H}_2\text{O}$, are shown. Before the analysis, $[\text{}^2\text{H}_5]\text{glycine}$ (final concentration 13.6 mmol/l) was added to the suspension. Spectrum A, $\tau = 1.5$ s; spectrum B, $\tau = 0.3$ s. Assignments are as given in the text.

Fig. 3) the peptide signal appears as a broad shoulder on the low-field side of the free glycine peak (a): the inability to resolve the peaks was also noted by Isab & Rabenstein (1979). However, the difference between the T_1 relaxation times, of GSH glycyl and free glycine, can be exploited to demonstrate the existence of a $[^2\text{H}_3]\text{glycyl}$ resonance. This was achieved by the use of the inversion-recovery pulse sequence ($180^\circ - \tau - 90^\circ$). Spectrum B in Fig. 3 shows that with $\tau = 0.3$ s a spectrum was obtained in which the glycine resonance (c) was reduced and the glycyl resonance (b) at the chemical shift expected for GSH glycyl was relatively enhanced.

^{15}N n.m.r. provides a means for following the incorporation of $[^{15}\text{N}]\text{glycine}$ into peptide bonds. Fig. 4 shows a typical ^{15}N n.m.r. spectrum of

erythrocyte lysate, prepared from cells that had been incubated with $[^{15}\text{N}]\text{glycine}$ (10 mmol/l of suspension) for 3 h in the absence of glucose. The spectrum consists of the resonance of added $[^{15}\text{N}]\text{glycine}$ (30.0 p.p.m.) and that of peptide bond (119.3 p.p.m.) ^{15}N from incorporated $[^{15}\text{N}]\text{glycine}$ plus natural-abundance amide ^{15}N present in haemoglobin. Despite the low natural abundance (0.37%) of ^{15}N , the high concentration of haemoglobin in erythrocytes would give rise to approx. 10 mmol peptide-bound ^{15}N /l. The incorporation of $[^{15}\text{N}]\text{glycine}$ into peptides was followed by measuring the ratio amide peak height/amine peak height. For cells incubated in glucose (10 mmol/l) the ratio remained 0.31 over 3 h. In cells incubated without glucose the ratio increased from 0.28 to 0.53 over 3 h.

Fig. 4. ^{15}N n.m.r. spectrum of erythrocytes

The spectrum (27.36 MHz; 160 000 transients) of human erythrocyte lysate (5 ml; haematocrit of original suspension 0.94) at 4°C is shown. The cells were incubated for 3 h at 37°C with $[^{15}\text{N}]\text{glycine}$ (10 mmol/l). Assignments: a, ^{15}N in peptide bond; b, ^{15}N in free glycine.

Fig. 5. Dependence of exchange rate on intracellular GSH concentration

The Figure shows a plot of ^1H -n.m.r. glycine peak amplitudes versus time of erythrocytes, with no glucose, in Krebs bicarbonate buffer in $^2\text{H}_2\text{O}$ with added *t*-butyl hydroperoxide: O, 0 mmol/l; □, 0.7 mmol/l; △, 1.4 mmol/l. At $t = 0$, $^2\text{H}_3$ glycine (final concentration 10 mmol/l) was added.

Although the ^2H - and ^{15}N -n.m.r. experiments demonstrate that glycine is incorporated into peptide bonds, they do not prove that the reaction is entirely due to incorporation of glycine into GSH. Although the $^2\text{H}_3$ glycyl resonance observed in the ^2H n.m.r. spectrum is at the chemical shift expected for GSH glycyl, the chemical-shift range of ^2H is small; therefore it could be possible that glycine is incorporated into a number of peptides.

To test whether glycine is incorporated only into GSH by the exchange reaction, the dependence of the exchange rate on GSH concentration was studied. Various amounts of GSH, in erythrocytes washed free of glucose, were oxidized by *t*-butyl hydroperoxide. This compound, at the low concentrations used, selectively oxidizes GSH to GSSG by serving as a substrate for GSH peroxidase (EC 1.11.1.9) (Srivastava *et al.*, 1974). Intra-erythrocyte GSH is normally maintained in its reduced state by GSH reductase (EC 1.6.4.2) in the presence of NADPH, which is one product of glucose 6-phosphate dehydrogenase. Therefore in erythrocytes depleted of glucose this system cannot operate.

Fig. 5 shows that as the concentration of GSH is decreased the rate at which protonated glycine is released, by exchange with $^2\text{H}_3$ glycine, is also decreased. This indicates that the reaction involves glycine-GSH exchange.

The dependence of the rate of incorporation of $^2\text{H}_3$ glycine into GSH on the concentration of $^2\text{H}_3$ glycine is shown in Fig. 6. The data set was

Fig. 6. Dependence of the GSH-glycine exchange rate on $^2\text{H}_3$ glycine concentration

The Figure shows a plot of the rate of change of protonated glycine concentration versus $^2\text{H}_3$ glycine concentration in erythrocyte suspensions incubated at 37°C . The smooth curve was calculated by non-linear regression analysis of the data on the Michaelis-Menten equation.

analysed by non-linear regression (Osborne, 1976) on the Michaelis-Menten equation and yielded an apparent K_m of 2.44 ± 0.31 mmol/l and $V_{\max}$ of 5.29 ± 0.19 $\mu\text{mol}/\text{min}$ per l. The n.m.r. peak intensities were converted into concentrations by reference to the glycine calibration curve and corrected for haematocrit.

The relevance, in the present experimental context, of high-energy phosphate compounds in GSH synthesis was assessed in 6 h incubations in the presence and in the absence of glucose. The ATP concentration was initially stable in both cell suspensions, but after 1 h the concentration in the glucose-deficient cells declined at the rate of 3.48 ± 0.12 $\mu\text{mol}/\text{l}$ of packed cells per min. The 2,3-bisphosphoglycerate concentration fell in both suspensions of erythrocytes but at a greater rate (10.5 ± 0.7 $\mu\text{mol}/\text{l}$ of packed cells per min) than in glucose-supplied cells (4.8 ± 0.6 $\mu\text{mol}/\text{l}$ of packed cells per min).

Discussion

Close inspection of the ^1H n.m.r. spectra presented by Isab & Rabenstein (1979) indicated that exchange of $^2\text{H}_3$ glycine with erythrocyte GSH occurred after all the glucose, initially supplied to the human erythrocytes, had been utilized. Results reported in the present paper confirmed that more

rapid exchange occurs in erythrocytes deficient in glucose (Fig. 2). The decrease in intensity of the GSH glycol CH_2 in cells with $[^2\text{H}_3]\text{glycine}$ did not correlate with the concomitant increase in the CH_2 resonance of free glycine (Fig. 1), raising the question whether the reaction is entirely exchange between glycine and the GSH glycine residue.

The $[^{15}\text{N}]\text{glycine}$ experiments indicate that glycine is incorporated into peptides. The ^2H -n.m.r. results show that the glycol resonance from incorporated $[^2\text{H}_3]\text{glycine}$ is at the chemical shift expected for GSH glycol. The demonstrated dependence of the rate of glycine exchange on the intracellular concentration of GSH (Fig. 5) is evidence that GSH is the principal substrate in the exchange process, rather than some other protein or peptide (including GSSG and GSH conjugates). In addition, preliminary investigations have shown that the CH_2 of $[^2\text{H}_3]\text{glycine}$ does not become protonated when the labelled compound is incubated with haemoglobin.

The relative intensities of peaks in spin-echo spectra depend on the T_1 and T_2 values of the different spins. Furthermore, with the rapid pulse repetition rates used in the present work partial saturation of spins, especially those with longer T_1 values, occurs. It was therefore important to construct concentration calibration curves for GSH and glycine under the particular pulse/experimental conditions employed. A calibration curve of the kind obtained for glycine cannot be obtained for GSH because the tripeptide is not transported across the erythrocyte membrane. However, various concentrations of GSH were added to a lysate and a linear plot of relative peak height versus concentration was obtained (coefficient of variation of slope ± 0.07). The ratio of peak height of GSH glycol to ergothioneine was not significantly different (1.06) between whole cells and lysates, validating the use of the calibration line for determining GSH concentrations in whole cells.

The ratio of the slopes of the GSH and glycine calibration lines was 1.24 ± 0.09 , and in a series of five exchange experiments the ratio of the rates of change of the corresponding relative peak amplitude, -1.4 ± 0.3 , was in good agreement.

Therefore, within experimental error, the decrease in protonated glycol concentration was equal to the increase in protonated glycine. This result, taken in conjunction with the results of the ^{15}N -, ^2H - and *t*-butyl hydroperoxide ^1H -n.m.r. experiments, strongly supports the notion that the reaction is entirely glycine–GSH exchange.

The determination of the K_m and $V_{\max}$ values reported in the present paper for the exchange process illustrates the utility of n.m.r. for characterizing the kinetics *in vivo* of enzymic reactions. Snoke & Bloch (1955) reported that the exchange

reaction catalysed by GSH synthetase *in vitro* required Mg^{2+} , ADP and phosphate (or arsenate) and involves partial reversal of the overall GSH synthesis reaction, to form enzyme-bound γ -glutamylcysteinyl phosphate and free glycine. In our experiments $[^2\text{H}_3]\text{glycine}$ is believed to react with the enzyme-bound intermediate to form $[^2\text{H}_3]\text{GSH}$. The K_m of glycine, in the synthesis of GSH *in vitro* in the presence of ATP (35 mmol/l), γ -glutamylcysteine (0.6 mmol/l) and purified human glutathione synthetase, was reported to be 0.36 mmol/l (Majerus *et al.*, 1971). The higher K_m found for the exchange reaction in the present study *in vitro* (2.44 ± 0.31 mmol/l of intact cells) with ATP (0.6–1.2 mmol/l), γ -glutamylcysteine (2 $\mu\text{mol/l}$) (Fahey *et al.*, 1981) and GSH (2–3 mmol/l) is consistent with the finding that the exchange reaction proceeds at only 10% of the rate of GSH synthesis *in vitro* (Snoke & Bloch, 1955).

The exchange reaction predominates in the erythrocyte *in vivo* because the concentration of γ -glutamylcysteine is so low (2 $\mu\text{mol/l}$) (Fahey *et al.*, 1981) that the maximal rate of GSH synthesis cannot be expressed (Griffith, 1981).

The values of K_m and $V_{\max}$ estimated in the present work for the exchange reaction are valid only if the transport of glycine into the erythrocyte is not rate-limiting. Young & Ellory (1977) have shown that glycine transport into the erythrocyte occurs via at least two pathways: (i) a low-capacity saturable system (K_m 0.3 mmol/l; $V_{\max}$ 1.2 $\mu\text{mol/min per l}$ at 37°C) and (ii) a high-capacity non-saturable system with an apparent diffusion constant for glycine of 0.004 min^{-1} . Solution of the differential equations describing the two-compartment exchange system indicates that glycine equilibration occurs sufficiently rapidly to ensure that $[\text{glycine}]_{\text{outside}} \approx [\text{glycine}]_{\text{inside}}$.

The more rapid exchange in erythrocytes deficient in glucose indicates that the factors controlling the activity of GSH synthetase and associated reactions must be modulated in some way by glucose.

The concentration of high-energy phosphate compounds was investigated as being a possible factor controlling GSH synthetase activity. The time-dependent changes in the concentration of ATP in erythrocytes with and without glucose indicated that the erythrocyte is able to maintain near-normal concentrations of ATP for 2–3 h after being washed free of glucose. The more rapid decline in concentration of 2,3-bisphosphoglycerate in erythrocytes deficient in glucose, compared with those with glucose, is consistent with 2,3-bisphosphoglycerate supplying a source of free energy via glycolysis, for use in the phosphorylation of ADP via pyruvate kinase. These results therefore give no indication that the activity of GSH synthetase is modulated by

the concentration of ATP in glucose-free erythrocytes.

Other possible modulators of the exchange reaction, apart from glucose itself, are NADPH or one or more of the intermediates of glycolysis, and such factors require further investigation.

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