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RED BLOOD CELL OXIDATIVE METABOLISM INDUCED BY HYDROXYPYRUVALDEHYDE

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Abstract—Hydroxypyruvaldehyde is a substrate for the red cell glyoxalase system. It was metabolized by glyoxalase I with reduced glutathione to *S*-glyceroyl glutathione which was subsequently enzymatically hydrolyzed to reduced glutathione and glycerate by glyoxalase II. There was a competing spontaneous reaction of hydroxypyruvaldehyde with oxygen, which produced hydrogen peroxide, inducing oxidative metabolism in hydroxypyruvaldehyde-treated red cells. The incubation of red cells with hydroxypyruvaldehyde produced a stimulation in the flux of glucose oxidized through the hexose monophosphate shunt pathway, a stimulation in lactate production with a decrease in pyruvate production in the Embden–Meyerhoff pathway, an oxidation of reduced pyridine nucleotides and reduced glutathione to their oxidized cogeners, and changes in the oxidative status of hemoglobin. Overall, the majority of hydroxypyruvaldehyde consumption in red cell suspensions appeared to occur via non-oxidative routes, e.g. glyoxalase and/or 2-ketoaldehyde dehydrogenase, and non-enzymic protein binding. Although the observed oxidative metabolism induced by hydroxypyruvaldehyde in red cells was not severe (reduced glutathione levels in hydroxypyruvaldehyde-treated red cells were *ca.* 80% of the control values in untreated cells), the oxidative effects may be important in red cell ageing processes.

Hydroxypyruvaldehyde, also known as triose reductone and hydroxymethylglyoxal, is a simple α-ketoaldehyde which is thought to be predominantly in the hydrated form, $HOCH_2COCH(OH)_2$, in aqueous solution [1]. It is produced non-enzymatically under physiological conditions by the slow reaction of glyceraldehyde and dihydroxyacetone with oxygen [2]:

$$HOCH_2CH(OH)CHO \text{ or } (HOCH_2)_2CO + O_2 \rightarrow HOCH_2COCHO + H_2O_2$$

Recently, interest has been shown in the effects of hydroxypyruvaldehyde on red cell metabolism. The phosphorylated analogue, hydroxypyruvaldehyde-3-phosphate, has been detected in red cells [3] and appears to be a by-product of normal glycolytic metabolism. Red cells treated with high concentrations of DL-glyceraldehyde, an anti-sickling agent [4], have been found to contain relatively large amounts of glyoxalase-reactive material [5], which is presumably hydroxypyruvaldehyde and metabolites formed from the autoxidation of DL-glyceraldehyde [2]. Moreover, in a non-cellular study, hydroxypyruvaldehyde was shown to oxidize oxyhemoglobin [6].

In this study, hydroxypyruvaldehyde was incubated with red cells as a model for studying the effects of slow, chronic production of hydroxypyruvaldehyde-3-phosphate and to judge the extent to which hydroxypyruvaldehyde mediates the deleterious effects of high concentrations of DL-glyceraldehyde on red cell metabolism [5].

The effect of 0.1 to 50 mM hydroxypyruvaldehyde on red cell metabolism was studied. Red cells metabolized relatively large amounts of hydroxypyruvaldehyde, suffering only relatively mild oxidative stress and an increase in methemoglobin reductase activity.

MATERIALS AND METHODS

Red cell preparations

Adult human blood was drawn daily into 3.8% sodium citrate solution. After centrifugation, plasma and white cells were removed and the red cells were washed three times with phosphate-buffered saline (9 parts 0.9% NaCl; 1 part 0.1 M KH_2PO_4/K_2PO_4, pH 7.4). A 25% (v/v) suspension of red cells was prepared in Krebs–Ringer phosphate buffer (120 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 16.5 mM NaH_2PO_4/Na_2HPO_4) from the washed packed cells.

Red cells containing methemoglobin were prepared by incubating a volume of packed red cells with an equal volume of 0.5% $NaNO_2$ in half-concentrated phosphate-buffered saline for 10 min at 25°. After this time, the cells were washed five times in phosphate-buffered saline to remove excess nitrite. A portion of the packed methemoglobin-containing cells thereby produced was analyzed for methemoglobin content (see below) to ensure that complete conversion of the native oxyhemoglobin to methemoglobin had been achieved. A 25% (v/v) suspension of the packed methemoglobin-containing red cells in Krebs–Ringer phosphate buffer was prepared for use in all subsequent incubations.

Red cell suspensions containing carbonmono-

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xyhemoglobin were prepared by blowing carbon monoxide over 25% suspensions of red cells in Krebs–Ringer phosphate buffer until hemolysates of a portion of the cells (see below) gave maximal absorbance at 569 nm. Carbonmonoxyhemoglobin-containing red cells, thereby produced, were washed once in aerated phosphate-buffered saline and resuspended in Krebs–Ringer phosphate buffer.

Enzymes and reagents

Lactate dehydrogenase (EC 1.1.1.27) from beef heart, glyoxalase I (EC 4.4.1.5)-grade IV from yeast, glyoxalase II (EC 3.1.2.6) from beef liver, and glutathione reductase (EC 1.6.4.2)-type III were supplied by the Sigma Chemical Co., St. Louis, MO. Glutamate dehydrogenase (EC 1.4.1.3) from beef liver and alcohol dehydrogenase (EC 1.1.1.2) from yeast were supplied by Boehringer Mannheim (Indianapolis, IN). Reduced and oxidized pyridine nucleotides [$NAD(P)^+$ and NAD(P)H], reduced and oxidized glutathione [GSH and GSSG], sodium α-ketoglutarate, glucose-6-phosphate, and sodium lactate and pyruvate were purchased from Sigma. Hydroxypyruvaldehyde was prepared from dihydroxyacetone by the method of Reeves and Ajl [7]. The nitrone spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from the Aldrich Chemical Co., Milwaukee, WI and was purified further by published methods [8].

Hydroxypyruvaldehyde concentration in supernatant fraction

A 25% suspension of red cells in Krebs–Ringer phosphate buffer was incubated with 10 mM hydroxypyruvaldehyde at 37° for 1 hr. During this time, aliquots were withdrawn after 0, 30 and 60 min of reaction time and immediately centrifuged for 5 min at 2000 *g*. The supernatant fraction was then analyzed for hydroxypyruvaldehyde content using glyoxalases I and II in a sequential assay system, following the *S*-glutathione adduct of hydroxypyruvaldehyde at 240 nm. Cuvette assay mixtures contained: 100 mM sodium phosphate, (pH 6.6), 2 mM GSH, and 1 mM diethylenetriaminepentaacetic acid (DETAPAC) plus (test cuvette only) 1% of the cell supernatant fraction. The absorbance at 240 nm was recorded before (baseline) and after the addition of 12.5 units/ml glyoxalase I (to test and reference cuvettes) until a maximum absorbance was attained. A further addition of 2.5 units/ml of glyoxalase II was then made. The fall in absorbance at 240 nm was followed until a minimum was attained. The concentration of hydroxypyruvaldehyde (no α-ketoaldehyde was detected in untreated red cells) was calculated using the fall in A_{240} after addition of glyoxalase II and $E_{240} = 3.95\ mM^{-1}\ cm^{-1}$ [9], with the appropriate dilution factors.

Hydroxypyruvaldehyde consumption was measured in Krebs–Ringer phosphate buffer with and without 25% suspension of red cells. Glyoxalase-reactive material in red cells with no added hydroxypyruvaldehyde was also measured.

Measurement of the flux of glucose oxidized through the hexose monophosphate shunt

The flux of glucose oxidized through the hexose monophosphate shunt (HMS) was measured by collecting $^{14}CO_2$, released from D-[U-^{14}C]glucose, as previously described [10]. One milliliter of 25% red cell suspensions with 5 mM glucose containing 0.33 μCi of D-[U-^{14}C]glucose (purchased from New England Nuclear, Boston, MA), in the presence and absence of hydroxypyruvaldehyde, was incubated for 1 hr at 37° in 10-ml stoppered Erlenmeyer flasks containing a well of 0.2 ml of 2 M KOH. The concentration of NaCl in Krebs–Ringer phosphate buffer was adjusted accordingly to preserve the isotonicity of the cell.

Determination of lactate and pyruvate concentrations

The concentrations of lactate and pyruvate in 25% red cell suspensions with 5 mM glucose, in the presence and absence of 10 mM hydroxypyruvaldehyde, were determined by published methods [11]. Lactate and pyruvate concentrations in red cell suspensions at $t = 0$ and after 1 hr of incubation at 37° were determined.

Determination of the levels of oxidized and reduced pyridine nucleotides

Cellular levels of NAD^+, $NADP^+$, NADH and NADPH, in control and in hydroxypyruvaldehyde-treated red cell suspensions, were determined by a modification of the ethanolic extraction procedure of Sander *et al.* [12]. The enzymatic assays for pyridine nucleotides were essentially those of Segal *et al.* [13], as previously modified by the authors [5].

The reaction between autoxidizing hydroxypyruvaldehyde and pyridine nucleotides in Krebs–Ringer phosphate buffer, pH 7.4 and 37°, was followed by a similar enzymatic analysis procedure. Reaction mixtures (5 ml) containing 20 μM pyridine nucleotide (NAD^+, $NADP^+$, NADH and NADPH) and 10 mM hydroxypyruvaldehyde were incubated for 1 hr. The yield of oxidized, reduced and enzymatically inactive pyridine nucleotide produced was determined as described in Ref. 5.

Determination of the levels of oxidized and reduced glutathione

The concentrations of oxidized and reduced glutathione, GSSG and GSH, respectively, in red cell suspensions with and without incubation with hydroxypyruvaldehyde (10 mM) were determined by previously published methods [10]. GSH was determined in acidified red cell extracts by reaction with Ellmans' reagent, $E_{412} = 13.6\ mM^{-1}\ cm^{-1}$ [14].

Oxygen consumption

The rate of oxygen consumption by autoxidizing hydroxypyruvaldehyde in Krebs–Ringer phosphate buffer, pH 7.4 and 37°, was followed on a Clark-type oxygen electrode (YSI model 53, Yellow Springs Instrument Co. Ltd., Yellow Springs, OH).

Free radical production from hydroxypyruvaldehyde

Free radical production from hydroxypyruvaldehyde in phosphate-buffered solutions at 37° was investigated by the electron spin resonance (ESR) technique of spin trapping [15]. Reaction mixtures containing 10 mM hydroxypyruvaldehyde, 100 mM sodium phosphate, pH 7.4, and 100 mM DMPO

(spin trapping agent) were incubated for 10 min at 37°. After this time, the ESR spectrum of the reaction mixture was recorded. All ESR spectra were recorded on a Varian E109, dual cavity, X-band ESR spectrometer with typical instrument settings: field set 3385 G, field scan 100 G, modulation frequency 100 kHz, modulation amplitude 0.5 G, microwave frequency 9.514 GHz, microwave power 20 mW, time constant 1.0 sec, and scan time 4 min with variable receiver gain. Spectra presented are representative of four experiments.

Analysis of red cells hemoglobin

The analysis of hemoglobin in 25% red cell suspensions, with and without hydroxypyruvaldehyde and incubated at 37° for 1 hr in Krebs–Ringer phosphate buffer (pH 7.4), was performed as previously described [5]. Visible absorption spectra of all lysates were recorded on a Cary 14 spectrophotometer.

Non-intact hemoglobin (hemoglobin metabolites other than oxyhemoglobin and methemoglobin) was calculated from the difference between the total hemoglobin at $t = 0$ and the sum of the oxyhemoglobin and methemoglobin after incubation for 1 hr.

RESULTS

Consumption of hydroxypyruvaldehyde by red cells

The concentration of glyoxalase-reactive material found in the supernatant fraction of 25% red cell suspensions, treated with 10 mM hydroxypyruvaldehyde, is shown in Fig. 1, curve *b*. No glyoxalase-reactive material was found in the control red cell suspensions incubated without hydroxypyruvaldehyde (curve *d*). However, 10 mM hydroxypyruvaldehyde incubated in Krebs–Ringer phosphate buffer at 37° exhibited a loss of *ca.* 2 mM in concentration over a 1-hr period (curve *a*). This may be explained as the consumption of hydroxypyruvaldehyde by autoxidation [2, 16]. The red cells in the 25% red cell incubation, therefore, accounted for a further (*ca.* 7 mM) drop in hydroxypyruvaldehyde concentration over that due to spontaneous autoxidation.

Hydroxypyruvaldehyde was added to red cells in the form of a concentrated aqueous solution. Dilution factors were calculated assuming free transport of hydroxypyruvaldehyde into the red cell. The assays with glyoxalase at $t = 0$ gave an initial assay of 10 mM. This suggests that hydroxypyruvaldehyde was readily transported across the red cell membrane (if this were not the case, that $t = 0$ assay should have been *ca.* 13.3 mM hydroxypyruvaldehyde).

Addition of 5 mM glucose to the 25% red cell suspensions with hydroxypyruvaldehyde gave no detectable changes from the incubation without added glucose in the glyoxalase-reactive material assays (curve *c*).

Oxidation of glucose via the hexose monophosphate shunt

The effect of hydroxypyruvaldehyde on the flux of glucose oxidized via the hexose monophosphate shunt (HMS), in red cells, is shown in Fig. 2. Even at very low concentrations of hydroxypyruvaldehyde (0.1 mM), the HMS was stimulated in oxyhemoglobin- and carbonmonoxyhemoglobin-containing red cell suspensions, and was relatively unchanged in methemoglobin-containing red cell suspensions. Increasing the hydroxypyruvaldehyde concentration gave an increased stimulation of the HMS. The maximum stimulation of the HMS in oxyhemoglobin-containing red cell suspensions was observed for incubations with 10 mM hydroxypyruvaldehyde, $\Delta HMS \approx +0.4$ μmole glucose/ml packed red cells/hr. For all hydroxypyruvaldehyde concentrations (0.1 to 50 mM), the stimulation in the HMS for the three different cell types studied was in the order:

oxyhemoglobin-containing cells

> carbonmonoxyhemoglobin-containing cells

> methemoglobin-containing cells

The mean values for the HMS in untreated cells were

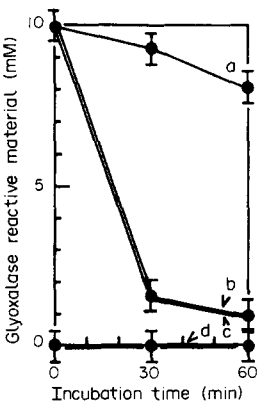

Fig. 1. Consumption of hydroxypyruvaldehyde by red cell suspensions. Reaction mixtures contained: (a) 10 mM hydroxypyruvaldehyde, (b) 10 mM hydroxypyruvaldehyde + 25% packed red cells, (c) 10 mM hydroxypyruvaldehyde + 25% packed red cells + 5 mM D-glucose, and (d) 25% packed red cells only; all reaction mixtures were in Krebs–Ringer phosphate buffer, pH 7.4 and 37°. Data given are means ± S.D. of four independent determinations.

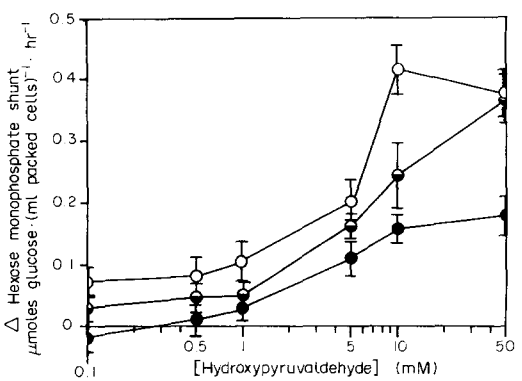

Fig. 2. Effect of hydroxypyruvaldehyde on the flux of glucose oxidized by the hexose monophosphate shunt in red cells. The hexose monophosphate shunt was measured at pH 7.4 and 37° in Krebs–Ringer phosphate buffer. Data given are the means ± S.D. of four independent determinations. Key: (○) oxyhemoglobin-containing cells, (◒) carbonmonoxyhemoglobin-containing cells, and (●) methemoglobin-containing red cells.

Table 1. Effect of hydroxypyruvaldehyde on lactate and pyruvate production by red cells.*

Additions	Lactate production (μmole/ml packed red cells/hr)	Pyruvate production (μmole/ml packed red cells/hr)
None (control)	3.27 ± 0.23	0.035 ± 0.031
+10 mM Hydroxypyruvaldehyde	9.04 ± 1.10	−0.002 ± 0.008†

* Values are means ± S.D.

† Indicates that the pyruvate concentration was reduced below the initial $t = 0$ value (0.030 ± 0.004 μmole/ml red cells).

0.17 ± 0.10, 0.18 ± 0.10, and 0.22 ± 0.20 μmoles glucose/ml cells/hr for oxyhemoglobin-, carbonmonoxyhemoglobin-, and methemoglobin-containing cells respectively.

Production of lactate and pyruvate by red cell glycolysis

Incubation of washed red cells with 5 mM D-glucose in the extracellular medium for 1 hr at 37° increased the concentration of lactate and decreased the concentration of pyruvate in red cells (Table 1). Addition of 10 mM hydroxypyruvaldehyde to red cells in a similar incubation gave a further increase in red cell lactate and a further decrease in red cell pyruvate concentrations (Table 1).

Oxidative status of hemoglobin

The oxidation of oxyhemoglobin to methemoglobin and hemichromes by hydroxypyruvaldehyde has been reported previously by the authors [6]. Incubation of a 25% suspension of oxyhemoglobin-containing red cells with 0.1 to 50 mM hydroxypyruvaldehyde for 1 hr at 37° converted oxyhemoglobin to non-intact hemoglobin (although the maximum conversion observed as only *ca.* 20% of the total hemoglobin). Very little methemoglobin formation occurred (Fig. 3, open circles) (cf. glyceraldehyde-treated red cells [5]). Red cell hemoglobin was protected from oxidation by hydroxypyruvaldehyde upon the addition of 5 mM glucose (Fig. 3, closed circles). The treatment of methemoglobin-containing red cell suspensions with hydroxypyruvaldehyde stimulated the reduction of methemoglobin to oxyhemoglobin (Fig. 4A). Similar incubations with 5 mM glucose added to the external medium further stimulated the rate of reduction of methemoglobin to oxyhemoglobin. Carbonmonoxyhemoglobin-containing red cells showed little change in the oxidative status of hemoglobin when treated with hydroxypyruvaldehyde (Fig. 4B).

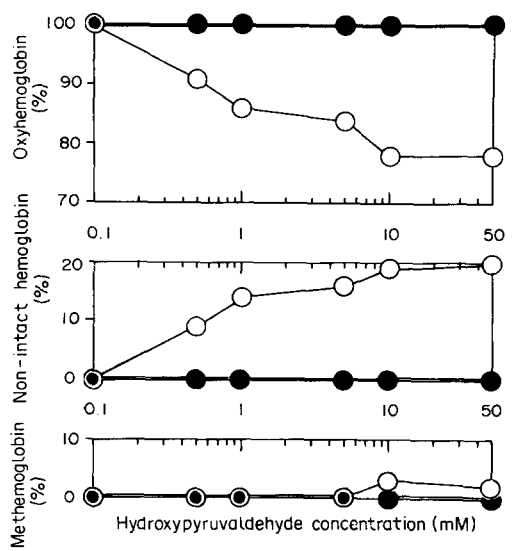

Fig. 3. Effect of hydroxypyruvaldehyde on the oxidative status of hemoglobin in oxyhemoglobin-containing red cells. Inubations contained the given hydroxypyruvaldehyde concentration and 25% (V/V) oxyhemoglobin-containing red cells in Krebs–Ringer phosphate buffer, pH 7.4 and 37°. The cell suspensions were incubated for 1 hr and then were analyzed for hemoglobin derivatives. Data are the means ± S.D. of four independent determinations. Key: (○) red cells + hydroxypyruvaldehyde, and (●) red cells + hydroxypyruvaldehyde + 5 mM glucose.

Effect of hydroxypyruvaldehyde on the levels of oxidized and reduced pyridine nucleotides glutathione in red cells

The concentrations of reduced and oxidized pyridine nucleotides in red cells incubated with and

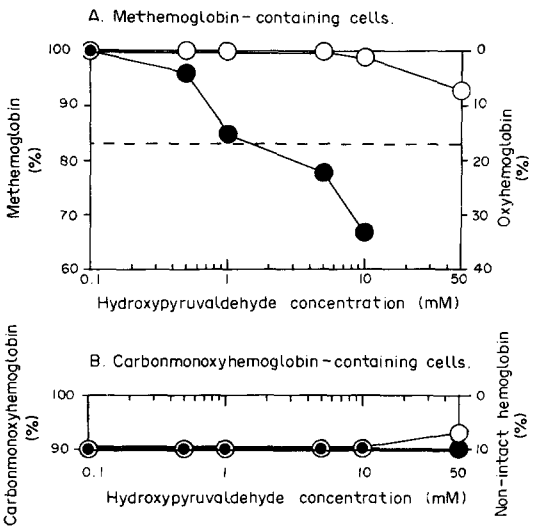

Fig. 4. Effect of hydroxypyruvaldehyde on the oxidative status of hemoglobin in (A) methemoglobin and (B) carbonmonoxyhemoglobin-containing cells. Incubation conditions and data analysis were as for Fig. 3. Key: (○) red cells + hydroxypyruvaldehyde, and (●) red cells + hydroxypyruvaldehyde + 5 mM glucose; --- methemoglobin reductase activity.

Table 2. Hydroxypyruvaldehyde-induced changes in red cells concentrations of reduced and oxidized pyridine nucleotides*

Additions	NAD^+	NADH	$NADP^+$	NADPH
	(nmoles/ml packed cells)			
None (control)	30.0 ± 1.8	2.6 ± 0.4	7.5 ± 0.9	5.0 ± 0.6
+10 mM Hydroxypyruvaldehyde	24.9 ± 1.5	2.3 ± 0.5	12.0 ± 1.3	0.1 ± 0.4

* Reduced and oxidized pyridine nucleotides were determined as described in Materials and Methods. Data presented are the means ± S.D. of four determinations.

without hydroxypyruvaldehyde are given in Table 2. Hydroxypyruvaldehyde-treated cells showed a marked oxidation of NADPH to $NADP^+$ but only a very small decrease in the concentration of NADH. It appears that the NADPH was more susceptible than NADH to oxidation by hydroxypyruvaldehyde in red cells. This was not reflected in the non-cellular reaction of hydroxypyruvaldehyde with NAD(P)H. Both NADH and NADPH were readily oxidized to NAD^+ and $NADP^+$, respectively (some enzymatically inactive nucleotide was also formed, Table 3), by incubation with hydroxypyruvaldehyde at 37°. Hydroxypyruvaldehyde gave a slow reaction with NAD^+ and $NADP^+$, converting both oxidized nucleotides to unidentified enzymatically inactive derivatives (Table 3).

Red cells treated with 10 mM hydroxypyruvaldehyde showed a sharp fall in the cellular concentration of reduced glutathione early in the incubation ($\Delta GSH = -0.3$ mM), followed by a lower fall in GSH concentration over the next hour incubation period ($\Delta GSH = -0.36$ mM) (Fig. 5, curve *b*). The control cells showed very little loss in reduced glutathione over a 1-hr incubation period ($\Delta GSH = -0.05$ mM). With the loss of reduced glutathione in hydroxypyruvaldehyde-treated red cells, there was a concomitant increase in cellular levels of GSSG (the concentration of GSSG increased from *ca.* 60 μM in control cells to *ca.* 170 μM in hydroxypyruvaldehyde-treated cells). The loss of GSH seems to be, for the most part, accounted for by the increase in GSSG in hydroxypyruvaldehyde-treated red cells, $2GSH \xrightarrow{\text{oxidant}} GSSG$.

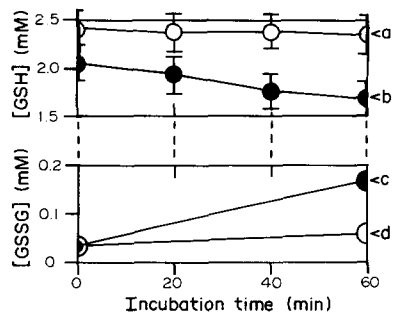

Fig. 5. Effect of hydroxypyruvaldehyde on cellular concentrations of reduced and oxidized glutathione. Reaction mixtures: (1) 25% suspension of red cells in Krebs–Ringer phosphate buffer with 5 mM glucose, curve *a* GSH, curve *d* GSSG. (2) 25% red cell suspension, 5 mM glucose with 10 mM hydroxypyruvaldehyde in Krebs–Ringer phosphate buffer, pH 7.4 and 37°, *b* GSH and *c* GSSG. Data given are the means ± S.D. of four independent experiments.

Table 3. Reaction of hydroxypyruvaldehyde with pyridine nucleotides in Krebs–Ringer phosphate buffer under physiological conditions*

Initial nucleotide	Nucleotide analysis	Control	+10 mM hydroxypyruvaldehyde
NADH	NADH (μM)	1.0 ± 0.3	9.3 ± 0.5
	NAD^+ (μM)	0.7 ± 0.5	6.0 ± 0.5
	Non-intact (μM)	0.3 ± 0.8	3.3 ± 1.0
NADPH	NADPH (μM)	15.2 ± 0.6	18.7 ± 1.3
	$NADP^+$ (μM)	8.8 ± 0.6	8.5 ± 0.9
	Non-intact (μM)	6.4 ± 1.2	10.2 ± 2.2
NAD^+	NAD^+ (μM)	0.3 ± 0.2	1.4 ± 0.2
	Non-intact (μM)	0.3 ± 0.3	1.4 ± 0.3
$NADP^+$	$NADP^+$ (μM)	0.0 ± 0.1	1.1 ± 0.6
	Non-intact (μM)	0.0 ± 0.4	1.1 ± 0.9

* All incubations initially contained 20 μM pyridine nucleotide with and without 10 mM hydroxypyruvaldehyde in Krebs–Ringer phosphate buffer, pH 7.4 and 37°. Samples were incubated for 1 hr and analyzed enzymatically (see Materials and Methods) for pyridine nucleotides. Values are means ±S.D. Data presented were calculated as follows:

$$NAD(P)H = NAD(P)H_{t=0} - NAD(P)H_{t=60};\ NAD(P)^+_{t=0} - NAD(P)^+_{t=60};$$

$$\text{Non-intact} = NAD(P)H - NAD(P)^+.$$

Table 4. Oxygen consumption by hydroxypyruvaldehyde in phosphate-buffered systems at pH 7.4 and 37°

System	$\frac{-d[O_2]_0}{dt}$ (μM min^{-1})
50 mM Hydroxypyruvaldehyde in 100 mM sodium phosphate, pH 7.4 and 37°	28.7 ± 3.1*
10 mM Hydroxypyruvaldehyde in 100 mM sodium phosphate, pH 7.4 and 37°	5.9 ± 0.7
50 mM Hydroxypyruvaldehyde in Krebs–Ringer phosphate buffer, pH 7.4 and 37°†	9.6 ± 1.1
10 mM Hydroxypyruvaldehyde in Krebs–Ringer phosphate buffer, pH 7.4 and 37°†	2.0 ± 0.2

* Values are means ± S.D.
† Krebs–Ringer phosphate buffer contained 16.5 mM $HPO_4^{2-}/H_2PO_4^-$.

Autoxidation of hydroxypyruvaldehyde: Oxygen consumption and free radical production

Table 4 shows the initial rates of oxygen consumption by phosphate-buffered solutions of hydroxypyruvaldehyde at 37°. The rate of oxygen consumption by hydroxypyruvaldehyde was dependent on both the concentrations of hydroxypyruvaldehyde and phosphate buffer, as previously observed by the authors for other simple monosaccharides [2]. Over a 1-hr incubation period, 10 mM hydroxypyruvaldehyde in Krebs–Ringer phosphate buffer consumed sufficient oxygen to decrease the initial oxygen concentration by 120 μ M, at 37°.

Figure 6 shows the free radical production from hydroxypyruvaldehyde when incubated in phosphate-buffered systems at 37° with the spin trap DMPO. The incubation of 10 mM hydroxypyruvaldehyde with 100 mM DMPO in 100 mM sodium phosphate, pH 7.4 and 37°, for 10 min gave the ESR spectrum reported in Fig. 6. The ESR spectrum can be assigned to a composite spectrum of two components—referred to as components DMPO-R_1 and DMPO-R_2 and given as discrete computer-simulated spectra in Fig. 6, *b* and *c*, respectively. The composite of the computer-simulated components is given in Fig. 6*d* and is in good agreement with the experimental spectrum 6*a*. Component DMPO-R_1 was characterized by the ESR spectral parameters $g = 2.0056$, $a_N = 14.9$ G, and $a_H = 19.6$ G; these are similar to the parameters previously reported for carbonyl spin adducts of DMPO [17]. Components DMPO-R_2 was characterized by the ESR spectral parameters $g = 2.0055$, $a_N = 15.9$ G, and $a_H = 23.0$ G; these parameters are similar to those for carbon-centred free radical-derived spin adducts of DMPO [17], particularly those previously reported from spin-trapping studies on the autoxidation of simple monosaccharides [2].

Free radical production from hydroxypyruvaldehyde in Krebs–Ringer phosphate buffer at pH 7.4 and 37° was also investigated. Figure 7*a* and *b* show the production of DMPO spin adduct ESR spectra from the incubation of hydroxypyruvaldehyde in Krebs–Ringer phosphate buffer, with and without a 25% suspension of red cells respectively. The spin adduct production was inhibited by the red cells. Red cells may suppress free radical formation (and spin adduct production) by rapidly metabolizing the free radical precursor (hydroxypyruvaldehyde) and, possibly, also by quenching any residual free radical formation; it is known [18] that the spin trap DMPO readily equilibrates across the red cell membrane, in the cytosol and extracellular medium.

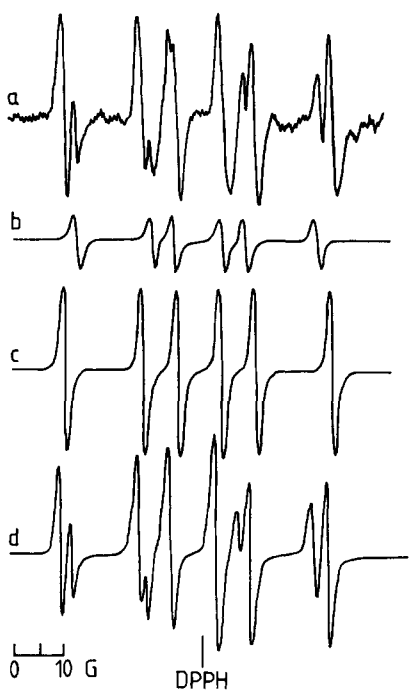

Fig. 6. Spin trapped intermediates from the autoxidation of hydroxypyruvaldehyde in sodium phosphate buffer at pH 7.4 and 37°. (*a*) Experimental spectrum. Reaction mixture contained: 10 mM hydroxypyruvaldehyde, 100 mM DMPO in 100 mM sodium phosphate buffer, pH 7.4. The reaction mixture was incubated for 10 min at 37° and the ESR spectrum recorded. Receiver gain = 2×10^4. (*b*) Computer simulation of component DMPO-R_1 with parameters set at $g = 2.0056$, $a_N = 14.9$ G and $a_H = 19.6$ G. (*c*) Computer simulation of component DMPO-R_2 with parameters set at DMPO-R_2: $g = 2.0055$, $a_N = 15.9$ G, and $a_H = 23.0$ G. (*d*) Addition spectrum of simulated components DMPO-R_1 + DMPO-R_2 (cf. experimental spectrum, *a*).

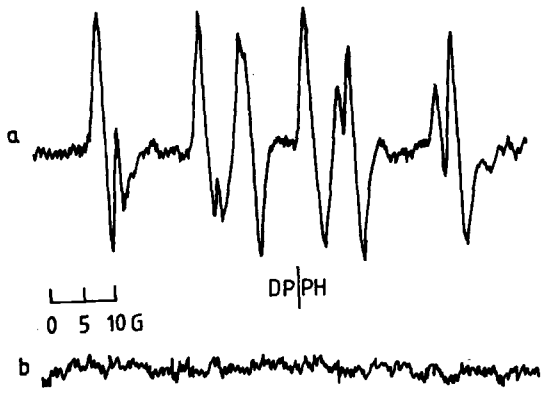

Fig. 7. Spin-trapped intermediates from the autoxidation of hydroxypyruvaldehyde in Krebs–Ringer phosphate buffer at pH 7.4 and 37°. Reaction mixtures: 10 mM hydroxypyruvaldehyde, 100 mM DMPO in Krebs–Ringer phosphate buffer with (*a*) control and (*b*) 25% red cells. Receiver gain = 4×10^4. Reaction mixtures were incubated for 30 min at 37° and then the ESR spectrum was immediately recorded.

DISCUSSION

Production and consumption of hydroxypyruvaldehyde in biological systems

The production of hydroxypyruvaldehyde has been reported for the following reactions:

(i) the spontaneous autoxidation of glyceraldehyde and dihydroxyacetone under physiological conditions [2],
(ii) the paracatalytic reactions of transaldolase [19], and
(iii) the metabolism of lactyl coenzyme A by a cell-free extract of *Escherichia coli* [20].

Hydroxypyruvaldehyde may be consumed by the following processes:

(i) a slow spontaneous autoxidation reaction [2] which is thought to proceed via hydroxypyruvate to glycollate [16], and
(ii) enzymatic metabolism—by the glyoxalase system [21] to glycerate,

$$HOCH_2COCHO \xrightarrow[GSH]{\text{Glyoxalase I}} HOCH_2CH(OH)CO{-}SG$$

$$\xrightarrow[+H_2O]{\text{Glyoxalase II}} HOCH_2CH(OH)CO_2H + GSH$$

by 2-oxoaldehyde dehydrogenase [22] and aldehyde dehydrogenase [23] to hydroxypyruvate,

$$NAD(P)^+ + HOCH_2COCHO \rightarrow NAD(P)H + HOCH_2COCO_2H$$

and by aldehyde reductase [24] to dihydroxyacetone. Only the glyoxalase enzymatic metabolism of hydroxypyruvaldehyde has been demonstrated for a red cell [3].

Effect of hydroxypyruvaldehyde on red cell metabolism

Stimulation of the hexose monophosphate shunt. In principle, the hexose monophosphate may be stimulated by enzymatic and non-enzymatic oxidation of NADPH and GSH, and by the enzymatic detoxication of peroxides with glutathione peroxidase [25]. NADPH was oxidized by non-enzymatic reaction with autoxidizing hydroxypyruvaldehyde (Table 3) and by enzymatic oxidation of NADPH with glutathione reductase; some enzymatic oxidation of NADPH by aldehyde reductase cannot be discounted. GSH may be oxidized to GSSG by the enzymatic reaction of glutathione peroxidase; the GSSG is then reduced back to GSH with glutathione reductase. Glutathione detoxifies low levels of hydrogen peroxide in the red cell [26]; this may be particularly pertinent to the relatively slow production of hydrogen peroxide by the reactions of hydroxypyruvaldehyde with oxygen [2] and oxyhemoglobin [6].

The rate of hydrogen peroxide production from the reaction of hydroxypyruvaldehyde with oxygen is influenced by the presence of hemoglobin derivatives. Oxyheme groups in oxyhemoglobin are readily reduced to hydrogen peroxide by hydroxypyruvaldehyde [2] and, so, oxyhemoglobin stimulates hydrogen peroxide production from autoxidizing hydroxypyruvaldehyde. Carbonmonoxyheme groups in carbonmonoxyhemoglobin do not react with hydroxypyruvaldehyde and, so, hydrogen peroxide production is slower than with oxyhemoglobin. Methemoglobin was reduced by hydroxypyruvaldehyde (Fig. 4), diverted reducing equivalents from oxygen (in the autoxidation reaction), and thereby suppressed the rate of hydrogen peroxide production to less than that observed for both oxyhemoglobin and carbonmonoxyhemoglobin with hydroxypyruvaldehyde (P. J. Thornalley and A. Stern, unpublished observation).

Overall, the rate of hydrogen peroxide production from hydroxypyruvaldehyde in aerobic hemoglobin solutions is expected to be in the order:

oxyhemoglobin > carbonmonoxyhemoglobin > methemoglobin

The exact same order was observed for the stimulation of the hexose monophosphate shunt in hydroxypyruvaldehyde-treated red cells (see Results).

Non-enzymatic oxidation of NADPH by hydroxypyruvaldehyde and the detoxication of hydrogen peroxide (produced by the reaction of hydroxypyruvaldehyde with oxygen and hemoglobin derivatives) appear to be major factors involved in the stimulation of the hexose monophosphate shunt in hydroxypyruvaldehyde-treated red cells.

Stimulation of lactate production by the Embden–Meyerhoff pathway. Hydroxypyruvaldehyde stimulated the production of lactate in red cell glycolysis (Table 1). The reason for this is not clear. A plausible explanation for this effect is the generation of NADH (and NADPH) by the enzymatic action of 2-oxoaldehyde dehydrogenase. This putative production of NADH is consistent with the apparent resistance of NADH to oxidation by hydroxypyruvaldehyde, where no similar resistance was observed in a simple non-cellular incubation (see Tables 2 and 3). NADPH may also be produced but the oxidative effects would be expected to redress this effect

through the stimulation of NADPH oxidation in the hexose monophosphate shunt.

Autoxidation of hydroxypyruvaldehyde: Oxygen reduction, free radical prodution, and oxidation of oxyhemoglobin. Hydroxypyruvaldehyde autoxidizes in Krebs–Ringer phosphate buffer at 37°, producing carbon-centred free radical intermediates. A similar free radical-mediated process has been described for the autoxidation of other simple monosaccharides [2, 6]. The free radicals produced from hydroxypyruvaldehyde autoxidation are expected to be damaging to the red cell. Carbon-centred free radicals have been implicated in the oxidation of reduced pyridine nucleotides [27] and the alkylation of heme groups [28]. Hydroxypyruvaldehyde is cytotoxic. It binds irreversibly to protein [1] and (although not in the red cell) nucleic acids [29].

Major sites of interaction of hydroxypyruvaldehyde in red cells. The incubation of a 25% suspension of red cells in Krebs–Ringer phosphate buffer at 37° for 1 hr, with 10 mM hydroxypyruvaldehyde, produced a fall in hydroxypyruvaldehyde concentration of 9 mM. The reaction of hydroxypyruvaldehyde with oxygen consumed 2 mM hydroxypyruvaldehyde and the reaction with oxyhemoglobin consumed *ca.* 1 mM hydroxypyruvaldehyde (assuming a 1:1 stoichiometry for the reaction of hydroxypyruvaldehyde with oxyheme groups) in this incubation. The majority (*ca.* 70%) of the hydroxypyruvaldehyde consumption appeared to occur via other than oxidative routes, e.g. the red cell glyoxalase system and 2-ketoaldehyde dehydrogenase.

Oxidative metabolism stimulated in red cells by hydroxypyruvaldehyde was relatively mild (cf. the phenylhydrazine reaction [30]). Moreover, in methemoglobin-containing cells, methemoglobin reductase activity was apparently enhanced (Fig. 4). This may indicate that through either non-enzymatic reduction reactions, as for the reaction of ascorbic acid with methemoglobin [31], or enzymatic production of NADH [22, 23], hydroxypyruvaldehyde can stimulate lactate production and methemoglobin reductase activity. Hence, hydroxypyruvaldehyde appears to have both oxidative and reductive metabolic effects in the red cell.

Hydroxypyruvaldehyde and hydroxypyruvaldehyde-3-phosphate production in red cells. Cogoli-Greuter and Christen [3] detected low levels of hydroxypyruvaldehyde-3-phosphate in normal red cell metabolism. The mechanism of formation is not known although glyceraldehyde-3-phosphate and dihydroxyacetone phosphate were found to undergo a spontaneous, slow autoxidation reaction, similar to their non-phosphorylated analogues [2]. Hydroxypyruvaldehyde-3-phosphate is thought not to be a substrate for red cell glyoxalase [3]; other routes for detoxication of this α-ketoaldehyde are not known in the red cell at present.

Glyoxalase-reactive material accumulates in glyceraldehyde-treated red cells—the autoxidation of glyceraldehyde in this system to hydroxypyruvaldehyde has been demonstrated [5]. It is clear that the use of high plasma concentrations of glyceraldehyde in the prevention of sickling will effectively administer a chronic low dose of hydroxypyruvaldehyde to the red cells.

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