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GLUCOSE METABOLISM AND HEMOGLOBIN REACTIVITY IN HUMAN RED BLOOD CELLS EXPOSED TO THE TRYPTOPHAN METABOLITES 3-HYDROXYANTHRANILATE, QUINOLINATE AND PICOLINATE

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Abstract—Glucose metabolism and hemoglobin reactivity in intact human erythrocytes were assessed in the presence of the tryptophan metabolites, 3-hydroxyanthranilate (3-HAT), quinolinate and picolinate. Of these compounds, only 3-HAT altered red cell oxidative status by inducing, in a dose-dependent manner, formation of methemoglobin and non-functional oxidation products of hemoglobin, and by increasing both net glycolytic flux and flux through the hexose monophosphate shunt. 3-HAT also decreased the normal lactate to pyruvate production ratio with pyruvate accumulating at the expense of lactate. These findings are consistent with the auto-oxidative reactivity of quinolinate, picolinate, and 3-HAT in that only 3-HAT undergoes base-catalyzed auto-oxidation (Dykens *et al.*, *Biochem Pharmacol* **36**: 211–217, 1987). Lactate and pyruvate added to the medium in physiologic concentrations uncoupled oxidative glycolysis from reductive glycolysis, resulting in accumulation of pyruvate in the presence of 3-HAT with little increase in total glycolytic flux. Superoxide dismutase (SOD), which accelerates 3-HAT auto-oxidation *in vitro* (Dykens *et al.*, *Biochem Pharmacol* **36**: 211–217, 1987), exacerbated HAT-mediated oxidative insult by increasing methemoglobin formation, hexose monophosphate shunt flux, and pyruvate accumulation. Persistence of 3-HAT-induced red cell metabolic responses and oxidative damage in the presence of SOD, DETAPAC (diethylenetriaminepentaacetic acid) and formate suggests that an organic-based radical, perhaps the anthranilyl radical produced during 3-HAT auto-oxidation, is the proximate agent exerting oxidative stress. Slow rates of auto-oxidation indicate that 3-HAT may be useful as a probe of antioxidant mechanisms in normal and diseased red blood cells.

The oxidative reactivity of tryptophan metabolites in the kynurenine pathway warrants further examination because tryptophan loading, used as a "natural" soporific, not only induces the enzyme tryptophan oxygenase, the rate-limiting enzyme leading to the kynurenine pathway, but also competitively inhibits tryptophan hydroxylase, the initial enzyme in the pathway leading to serotonin synthesis. Both responses act to increase levels of metabolites from the kynurenine pathway in both blood and brain following tryptophan loading [1–5]. Among the metabolites arising in the kynurenine pathway, 3-hydroxyanthranilate (3-HAT) has been shown to possess auto-oxidative reactivity [6–11], and to be potentially carcinogenic [12–14, but see 15]. Moreover, quinolinate, a dicarboxylic purine also arising in the kynurenine pathway and found in human brain [16], is a potent convulsant [2, 17] that, when introduced directly into the striatum, can induce neuronal degeneration in rats similar to that observed in Huntington's disease [18]. Unlike 3-HAT, however, quinolinate demonstrates no oxidative reactivity *in vitro* [6; present data]. This observation, in light of reports that quinolinate is an agonist at the *N*-methyl-D-aspartate (NMDA) binding site and is physiologically active there [18–20], supports the suggestion [6, 18] that the neurotoxicity of quinolinate is attributable to its structural similarity to other dicarboxylic excitotoxins [18–24], and not to generation of cytotoxic by-products of auto-oxidation.

Earlier cell-free experiments [6] have shown that 3-HAT auto-oxidation is accelerated by both superoxide dismutase (SOD) and catalase, enzymes classically considered antioxidants. 3-HAT auto-oxidation is believed to be a two-step process with formation of superoxide and anthranilyl radicals preceding condensation reactions that eventually yield the pigment cinnabarinate [6–11]. It has been proposed [6, 8, 11] that SOD and catalase potentiate 3-HAT by removing O_2^- and H_2O_2, thereby preventing reverse reactions with initial 3-HAT auto-oxidation products such as the anthranilyl radical and semiquinoneimine. Prevention of reverse reactions probably allows accumulation of other products of 3-HAT auto-oxidation including hydroxyl and/or organic-based radicals in addition to the above-mentioned superoxide, hydrogen peroxide and cinnabarinate [6].

Tomoda *et al.* [11] reported that, when red cells are incubated with 3-HAT, oxyhemoglobin is oxidized to methemoglobin, and other oxidized metabolites, while cinnabarinate accumulates in the medium. However, this work predates publication of data on the pH sensitivity of 3-HAT auto-oxidation [6] and

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does not include assessment of 3-HAT auto-oxidation rates in the absence of hemoglobin. In addition, many studies of red cell metabolic responses to oxidative stress are carried out with cells incubated in buffered phosphate medium despite reports that physiologic (plasma) concentrations of lactate and pyruvate in the incubation medium uncouple the two functions of glycolysis, i.e. ATP and NADH production [25, 26]. Accordingly, we have examined the effects of these tryptophan metabolites on red cell glucose metabolism and hemoglobin reactivity, paying particular attention to the pH sensitivity of 3-HAT auto-oxidation. Likewise, to model *in vivo* conditions more accurately, red cell glycolytic responses to 3-HAT auto-oxidation were also examined in the presence of exogenous lactate and pyruvate.

MATERIALS AND METHODS

Red cell preparations. Adult human blood from healthy volunteers was drawn daily into tubes containing 3.8% sodium citrate. After centrifugation, plasma and white cells were removed, and the red cells were washed three times in an equal volume of 0.9% NaCl. Methemoglobin-containing red cells were prepared by suspending packed red cells in an equal volume of 0.5% $NaNO_2$, 0.45% NaCl and incubating for 10 min at 25°. To remove excess $NaNO_2$, cells were washed five times in 0.9% NaCl. Red cells containing carbonmonoxyhemoglobin were prepared by sparging a 25% (v/v) red cell suspension in Krebs–Ringer phosphate buffer (120 mM NaCl; 4.7 mM KCl; 1.3 mM $CaCl_2$; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 16.2 mM NaH_2PO_4, pH 7.4) with CO until the visible spectra of red cell lysates reached a maximum at 569 nm. Red cells containing carbonmonoxyhemoglobin were then collected by centrifugation and resuspended in Kreb–Ringer phosphate buffer previously sparged with CO ($P_{O_2} < 5$ torr). Incubations of cells containing carbonmonoxyhemoglobin were carried out under 100% CO.

Incubation conditions and assays. Incubations with the indicated concentrations of tryptophan metabolites were carried out at 37° in stoppered 10-ml Erlenmeyer flasks containing 25% (v/v) red cell suspensions in Krebs–Ringer phosphate as described above containing 5 mM glucose (KRG). 3-Hydroxyanthranilic acid (Sigma Chemical Co., St. Louis, MO) is not very soluble at pH values near neutrality, a factor exacerbated by the pH-dependent nature of 3-HAT auto-oxidation [6]. Accordingly, a 187.5 mM stock solution of 3-HAT was prepared daily in 1.0 mM KCl, 0.1 M HCl, and 10 M HCl was added dropwise until all the 3-HAT dissolved (5–6 drops). The final pH was 2.0, a pH at which 3-HAT auto-oxidation does not occur at appreciable rates [6]. The stock 3-HAT solution was titrated to neutrality with 10 M NaOH immediately before addition to red cell suspensions. Both quinolinate (2,3-pyridinedicarboxylic acid) and picolinate (2-pyridinecarboxylic acid), obtained from the Aldrich Chemical Co. (Milwaukee, WI), were dissolved in 100 mM phosphate buffer and the pH was adjusted to 7.4. Superoxide dismutase (Sigma Chemical Co.) was diluted in 100 mM sodium phosphate buffer (pH 7.4) to a concentration of 10 units/μl. Catalase in phosphate buffer (88,823 units/ml) was obtained from Calbiochem-Behring (La Jolla, CA). DETAPAC (diethylenetriaminepentaacetic acid) and formate were obtained from Sigma.

Oxyhemoglobin (HbO_2), methemoglobin (Hb^{3+}) and intact hemoglobin (defined as the sum, $HbO_2 + Hb^{3+}$) were measured by the method of Harley and Mauer [27] with modifications as previously described [28].

Flux through the hexose monophosphate shunt was monitored by following $^{14}CO_2$ formation from [U-^{14}C]glucose as described by Trotta *et al.* [29]. U-^{14}C-Labeled glucose was used rather than 1-^{14}C-labeled so that all hexose monophosphate shunt (HMS) flux, including recycling of pentoses, could be assessed [25, 26].

Pyruvate and lactate in the medium and red cell compartments were assayed by the method of Beutler [30] with previously described modifications [25, 26].

RESULTS

Red cells incubated in the presence of 3-hydroxyanthranilate accumulated methemoglobin and non-intact hemoglobin in direct proportion to the amount of 3-HAT present in the incubation medium (Table 1). Conversely, at even higher concentrations, neither quinolinate nor picolinate induced formation of methemoglobin or non-intact hemoglobin above control levels (Table 1).

In the presence of 2.5 mM 3-HAT, amounts of oxy-, met- and non-intact hemoglobins were altered significantly by the addition of superoxide dismutase, catalase, DETAPAC and formate in various combinations to the incubation medium (Table 2). Addition of superoxide dismutase, either alone or in conjunction with catalase, increased methemoglobin accumulation 2-fold with no corresponding increase in non-intact hemoglobin. Although catalase appeared to reduce the formation of non-intact hemoglobin by half (Table 2), large variability rendered the effect not significant at $P < 0.05$. DETAPAC and formate, separately or together, prevented all 3-HAT-induced accumulation of methemoglobin and non-intact hemoglobin, except when SOD was also present (Table 2).

The effects of 3-HAT, quinolinate and picolinate on hexose monophosphate shunt activity are presented in Fig. 1. Neither quinolinate nor picolinate altered HMS flux compared to controls. However, HMS activity increased directly with increasing 3-HAT concentrations ($r = 0.90$, $N = 66$, $P < 0.001$), showing an 8- and 17-fold acceleration in the presence of 2.5 and 10.0 mM 3-HAT respectively (Fig. 1). In red cell suspensions depleted of O_2 via sparging with either N_2 or CO, 3-HAT increased shunt activity to approximately one-half the activity seen in the presence of O_2 (Fig. 1). HMS activities in methemoglobin-containing cells exposed to 3-HAT under normoxic conditions were similar to those seen in cells with unaltered hemoglobin red/ox status (Fig. 1).

The presence in the incubation medium of physiologic concentrations of lactate and pyruvate had no

Table 1. Percentages of oxyhemoglobin, methemoglobin and non-intact hemoglobin isolated from intact RBCs after a 1-hr incubation in the presence of the tryptophan metabolites 3-hydroxyanthranilate (3-HAT), quinolinate, or picolinate at the indicated concentrations

Concentration (mM)	N	Oxyhemoglobin (%)	Methemoglobin (%)	Non-intact hemoglobin (%)
3-HAT				
0.0	30	100.0 ± 2.4[a]	2.3 ± 1.2[a]	0.0 ± 0.0[a]
1.0	5	82.8 ± 4.0[b]	10.8 ± 4.0[b]	6.2 ± 7.2[a]
2.5	11	73.3 ± 14.9[b]	11.6 ± 6.6[b]	12.9 ± 11.7[a]
5.0	5	39.1 ± 8.1[c]	38.2 ± 8.2[c]	22.8 ± 14.8[b]
Quinolinate				
2.5	5	98.5 ± 1.2[a]	1.8 ± 0.9[a]	0.0 ± 0.0[a]
5.0	5	99.0 ± 0.8[a]	2.2 ± 1.1[a]	0.0 ± 0.0[a]
7.5	5	98.8 ± 1.9[a]	2.1 ± 1.3[a]	0.0 ± 0.0[a]
Picolinate				
2.5	5	97.8 ± 1.5[a]	1.7 ± 0.8[a]	0.0 ± 0.0[a]
5.0	5	98.8 ± 1.3[a]	2.1 ± 1.1[a]	0.3 ± 0.2[a]
7.5	5	98.5 ± 0.8[a]	1.9 ± 0.6[a]	0.0 ± 0.0[a]

Values are means (± SD, N indicated). Means significantly different at $P < 0.05$, one-way ANOVA following arcsine transformation, have different superscripts. Temp. = 37°, pH 7.4.

Table 2. Percentages of oxyhemoglobin, methemoglobin and non-intact hemoglobin isolated from intact RBCs following a 1-hr incubation in the presence of auto-oxidizing 3-HAT (2.5 mM) plus excess superoxide dismutase (2000 units/ml, SOD), excess catalase (660 units/ml, CAT), diethylenetriaminepentaacetic acid (DETAPAC, 2.5 mM), and formate (FORM, 2.5 mM)

Addition	N	Oxyhemoglobin (%)	Methemoglobin (%)	Non-intact hemoglobin (%)
Control*	30	100.0 ± 2.4[a]	2.3 ± 1.2[a]	0.0 ± 0.0[a]
3-HAT*	11	73.3 ± 14.9[b]	11.6 ± 6.6[b]	12.9 ± 11.7[a]
3-HAT +				
SOD	8	49.8 ± 17.2[c]	26.4 ± 8.1[c]	12.0 ± 9.1[a]
CAT	11	75.8 ± 15.3[b]	17.6 ± 10.8[bc]	6.7 ± 8.9[a]
SOD + CAT	9	56.3 ± 18.0[v]	27.4 ± 20.0[bc]	13.7 ± 10.9[a]
DETAPAC	4	99.2 ± 1.4[a]	0.5 ± 0.6[a]	0.0 ± 0.0[a]
FORM	4	97.6 ± 2.1[a]	1.3 ± 1.2[a]	0.4 ± 0.6[a]
DETAPAC + FORM	4	98.3 ± 2.2[a]	2.0 ± 1.1[a]	0.0 ± 0.0[a]
SOD + DETAPAC	4	84.6 ± 4.8[ab]	10.9 ± 9.7[b]	7.6 ± 4.2[a]
SOD + FORM	4	71.6 ± 12.4[b]	22.6 ± 18.9[bc]	11.3 ± 9.7[a]
SOD + DETAPAC + FORM	4	69.4 ± 7.5[b]	24.4 ± 11.3[bc]	8.9 ± 8.8[a]

Values are means (± SD, N indicated). Means significantly different at $P < 0.05$, one-way ANOVA following arcsine transformation, have different superscripts. Temp. = 37°, pH 7.4.

* Data from Table 1. Ratios indistinguishable from controls were found when cells were incubated with SOD, catalase, DETAPAC or formate in the absence of 3-HAT.

effect on stimulation of pentose shunt activity by 3-HAT (Table 3). As was the case concerning formation of reduced and non-intact hemoglobin (see Table 1), superoxide dismutase in the medium, either alone or with catalase, increased the 3-HAT-induced acceleration of hexose monophosphate shunt activity (Table 3). However, the presence of catalase alone did not potentiate 3-HAT-induced acceleration of HMS flux (Table 3).

In KRG (Krebs–Ringer phosphate containing 5 mM glucose) lacking pyruvate and lactate, both pyruvate accumulation and total glycolytic flux (= sum of net accumulation of lactate plus pyruvate) increased in direct proportion to 3-HAT concentration (Fig. 2). These increases in pyruvate accumulation and total glycolysis occurred in the absence of significant increases in lactate accumulation (Fig. 2). Physiologically reasonable concentrations of lactate plus pyruvate (2.0 and 0.1 mM respectively) in the incubation medium permitted more substantial accumulation of pyruvate, particularly at 3-HAT concentrations above 1.5 mM, in conjunction with no significant changes in lactate accumulation (Fig. 2). Total glycolytic flux increased in a manner similar to that seen in the absence of exogenous lactate and pyruvate. The effects of exogenous lactate and pyruvate are better revealed by examining the molar ratio between the accumulated lactate and pyruvate. In the absence of lactate and pyruvate, the normal ratio between accumulated lactate and pyruvate, 4.8 (±1.3 SD, N = 16), declined in response to increasing 3-HAT concentrations to 1.33 (±0.5 SD, N = 4) in the presence of 7.5 mM 3-HAT. At no 3-HAT concentration used

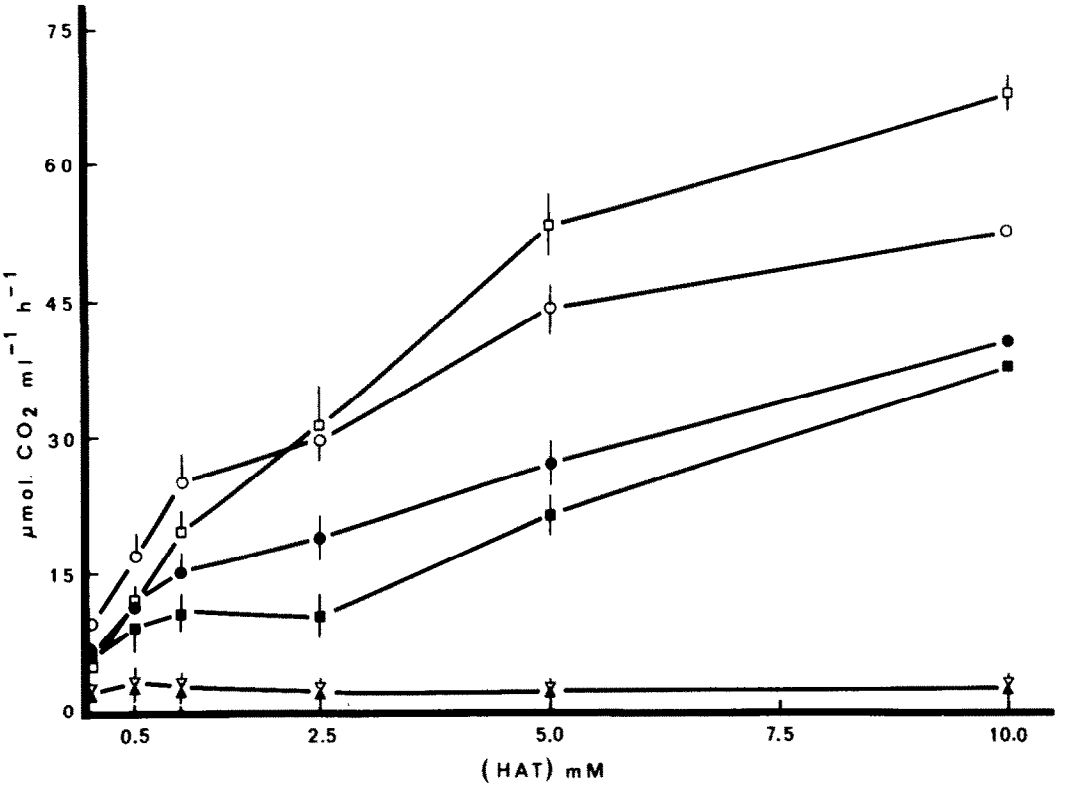

Fig. 1. Hexose monophosphate shunt activity in red blood cells during a 1-hr exposure to the indicated concentrations (mM) of the tryptophan metabolites 3-hydroxyanthranilate (3-HAT) in normoxic cells (□, N = 36) or following either sparging of the flask with N_2 (■, N = 6) or carbon monoxide (●, N = 4), and in the presence of red cells containing methemoglobin (○, N = 6). HMS activity in red blood cells exposed to the indicated concentrations (mM) of quinolinate (▽, N = 6) or picolinate (▲, N = 6). Values are μmol $CO_2 \times 10^{-2}$·ml RBC^{-1}·hr^{-1} (mean ± SD, N indicated). Temp. = 37°, pH 7.4.

Table 3. Hexose monophosphate shunt activity in red blood cells during a 1-hr exposure to 2.5 mM 3-hydroxyanthranilate (3-HAT) plus excess superoxide dismutase (2000 units/ml, SOD), excess catalase (660 units/ml, CAT), or both (SOD/CAT), and in the presence of physiologic concentrations of lactate and pyruvate

Additions	CO_2 [μmol × 10^{-2}·(ml RBC)$^{-1}$·hr^{-1}]	
		2.0 mM Lactate + 0.1 mM Pyruvate
0.0	2.89 ± 1.43 (14)	2.39 ± 0.65[a] (11)
3-HAT	28.31 ± 6.02[b] (21)	23.40 ± 4.74[b] (8)
3-HAT + SOD	63.64 ± 14.81[c] (14)	63.59 ± 7.60[c] (8)
3-HAT + CAT	32.62 ± 8.50[b] (14)	23.83 ± 1.41[b] (8)
3-HAT + SOD + CAT	61.68 ± 15.13[c] (14)	60.06 ± 10.91[c] (7)

Values are means ± SD (N indicated in parentheses). Means not significantly different at $P < 0.001$ (one-way ANOVA, Student–Newman–Keuls) share superscripts. Values indistinguishable from controls were obtained when cells were incubated with SOD, catalase, and both together (data not shown). Temp. = 37°, pH 7.4.

did pyruvate account for more than 45% of the total glycolytic flux when lactate and pyruvate were absent (Fig. 2). However, in the presence of exogenous lactate and pyruvate, the molar ratio between accumulated lactate and pyruvate declined even further, to 0.44 (±0.09 SD, N = 4) at 7.5 mM 3-HAT, with pyruvate accounting for fully 81% of the total glycolytic flux and lactate for less than 20% (Fig. 2). Thus, although total glycolytic flux increased similarly in the absence and presence of exogenous

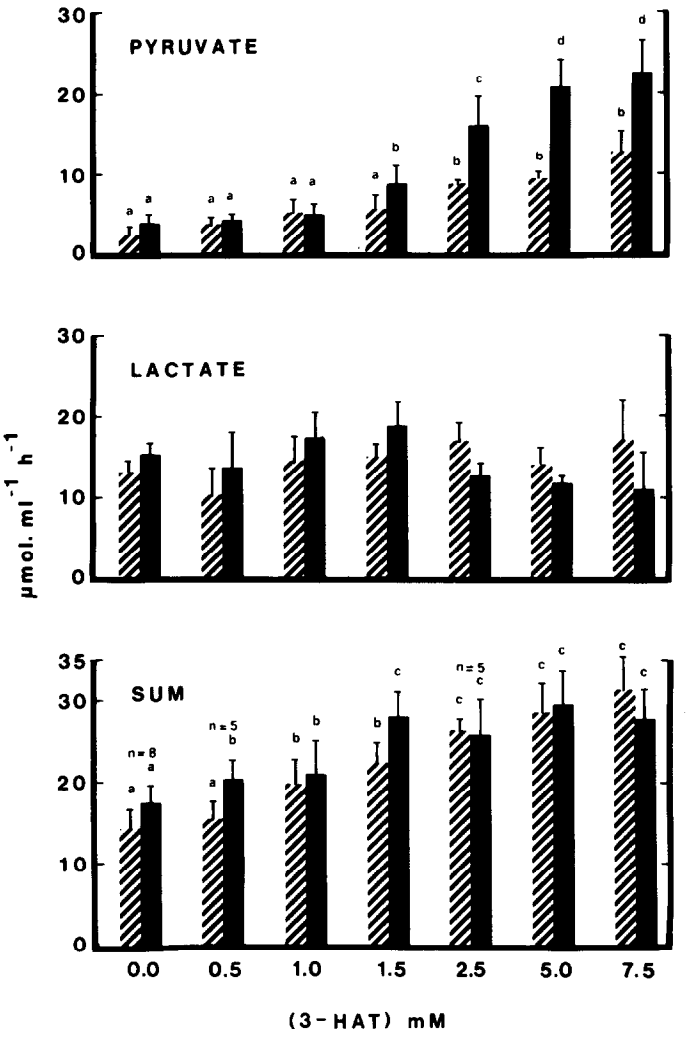

Fig. 2. Net pyruvate and lactate production in intact red blood cells during a 1-hr incubation in KRG containing the indicated concentrations of 3-hydroxyanthranilate (3-HAT) either in the absence (striped bars), or in the presence (solid bars), of physiologic concentrations of pyruvate and lactate (2.0 and 0.1 mM respectively). Values are μmol pyruvate or lactate · ml $RBC^{-1} \cdot hr^{-1}$ (mean ± SE, N = 4 except where indicated). Temp. = 37°, pH 7.4. Two-way ANOVA for pooled lactate data reveals no significant effects of 3-HAT concentration or exogenous lactate and pyruvate. However, both pyruvate accumulation and total glycolytic flux increased directly with 3-HAT concentration (pyruvate: $y = 0.11x + 0.33$, $r = 0.73$, $N = 34$, $P < 0.001$; total flux: $y = 0.18x + 1.68$, $r = 0.84$, $N = 34$, $P < 0.001$). Means not significantly different at $P < 0.05$ (two-way ANOVA, Student–Newman–Keuls) share superscripts.

lactate and pyruvate, when lactate and pyruvate were present, increases in total flux were due to preferential accumulation of pyruvate over lactate.

Total glycolytic flux and the ratio between accumulated lactate and pyruvate in cells incubated with quinolinate or picolinate at 5.0 and 7.5 mM were indistinguishable from controls (data not shown). This was true regardless of whether lactate and pyruvate were present in the medium.

The presence of SOD increased production of pyruvate in cells incubated with 3-HAT without a corresponding increase in lactate accumulation (Table 4). However, this increase in pyruvate, coupled with slightly higher lactate production, resulted in a significant increase in total glycolytic flux. Exogenous catalase did not elevate pyruvate or lactate production above levels seen with 3-HAT alone. Addition of both SOD and catalase did not increase pyruvate accumulation or total glycolytic flux above levels seen with only SOD present (Table 4).

DISCUSSION

Red cells incubated in the presence of 3-HAT accumulated methemoglobin and non-intact hemoglobin in direct proportion to 3-HAT concentration. In a dose-dependent manner, exposure to 3-HAT also increased flux through hexose monophosphate shunt, pyruvate formation, and net glycolytic flux as reflected by the accumulation of pyruvate plus lactate. In contrast, even at concentrations of up to

Table 4. Net pyruvate and lactate production in intact red blood cells during a 1-hr incubation in the presence of auto-oxidizing 3-HAT (2.5 mM) plus excess superoxide dismutase (2000 units/ml, SOD), or excess catalase (660 units/ml, CAT)

Addition	N	Pyruvate*	Lactate*	Sum of lactate + pyruvate*
Control	9	0.30 ± 0.05^{a}	1.37 ± 0.73^{a}	2.04 ± 0.53^{a}
3-HAT	6	0.56 ± 0.15^{b}	1.55 ± 0.54^{a}	2.70 ± 1.26^{b}
3-HAT + SOD	6	0.75 ± 0.18^{c}	1.79 ± 0.54^{a}	2.51 ± 0.63^{c}
3-HAT + CAT	6	0.63 ± 0.13^{bc}	1.68 ± 0.54^{a}	2.40 ± 0.61^{c}
3-HAT + SOD + CAT	6	0.74 ± 0.09^{c}	1.63 ± 0.49^{a}	2.37 ± 0.51^{c}

Values are means ± SD (N indicated). Temp. = 37°, pH 7.4. Means not significantly different at $P < 0.05$ (one-way ANOVA, Student–Newman–Keuls) share superscripts.
* Expressed in $\mu mol \cdot (ml\ RBC)^{-1} \cdot hr^{-1}$.

7.5 mM, neither quinolinate nor picolinate had any effect on hemoglobin status, HMS activity or glycolytic flux. These findings are in accord with the previously-reported oxidative reactivity of these compounds: 3-HAT undergoes base-catalyzed auto-oxidation [6–11], whereas neither quinolinate nor picolinate exhibits oxidative reactivity under identical conditions [6]. Although 3-HAT auto-oxidation eventually yields the pigment cinnabarinate via condensation reactions, during auto-oxidation a variety of reactive intermediates including H_2O_2, superoxide, anthranilyl and semiquinoneimine radicals are generated [6–11]. These reactive species are capable of oxidizing hemoglobin in solution to non-functional forms and can bleach the final product, cinnabarinate [6, 11].

In cell-free experiments, 3-HAT auto-oxidation was not accelerated by catalase or by either hemoglobin or methemoglobin in solution, although interconversion between the two forms of hemoglobin occurs [6]. Conversely, superoxide dismutase accelerated 3-HAT auto-oxidation 400%, probably by preventing back reactions between superoxide and anthranilyl radicals (or quinoneimine) formed during the initial events of auto-oxidation [6]. Acceleration of 3-HAT auto-oxidation by SOD was also shown in the present experiments using intact red cells in that, at all 3-HAT concentrations examined, addition of SOD to the incubation medium exacerbated methemoglobin formation (Table 2), increased hexose monophosphate shunt activity (Table 3), and stimulated net glycolytic flux with preferential accumulation of pyruvate (Table 4). The worsening of hemoglobin oxidative damage and increased mobilization of red cell metabolic oxidative defenses observed in the presence of SOD argues that the major agent exerting oxidative stress is not superoxide anion, but rather either H_2O_2 or an organic-based radical such as semiquinoneimine or anthranilyl.

Catalase prevents bleaching of cinnabarinate by H_2O_2 in cell-free experiments, but does not accelerate 3-HAT auto-oxidation [6]. Likewise, catalase had no effect on the formation of met- and non-intact hemoglobin in intact cells, or on the stimulation of hexose monophosphate shunt and glycolysis induced by 3-HAT. The failure of catalase to diminish red cell metabolic responses to auto-oxidizing 3-HAT, coupled with the observed worsening of hemoglobin oxidative damage in the presence of SOD, provides additional circumstantial evidence that an organic-based radical, not H_2O_2 or superoxide anion, is the agent inflicting oxidative stress.

Except when SOD was also present, both DETAPAC, a chelator which removes iron reactivity, and formate, an effective hydroxyl radical scavenger, prevented the accumulation of methemoglobin and non-intact hemoglobin normally induced in red cell suspensions by 3-HAT auto-oxidation (Table 2). Interconversion between oxyhemoglobin and methemoglobin observed in cell-free experiments suggests the presence of one-electron donors and acceptors such as 3-HAT, quinoneimine, superoxide and anthranilyl radicals, as well as oxidized or reduced transition metal cations [6, 11]. Formation of non-functional hemoglobin derivatives in solution [6] and in intact cells implies irreversible oxidative attack by intermediates such as H_2O_2 and hydroxyl radicals. Complete protection against hemoglobin oxidation in intact cells during 3-HAT exposure by both DETAPAC and formate (Table 2) implicate transition metals and hydroxyl radicals in 3-HAT-mediated oxidative damage. However, the failure of catalase to prevent hemoglobin oxidative damage in intact cells suggests that direct attack by H_2O_2 is not the primary mechanism of hemoglobin oxidation, and also that hydroxyl radical from metal-catalyzed Fenton reactions which require H_2O_2 is not the major oxidatively damaging compound. Indeed, hemoglobin oxidation continued unabated even when SOD, formate and DETAPAC were added together, further supporting the suggestion that an organic, not oxygen-based, radical is the agent responsible for oxidative damage.

Experiments on HMS activity in hypoxic cells (either N_2 or CO sparged) exposed to 3-HAT also support findings from cell-free experiments [6] with several interesting exceptions. In the absence of cells, 3-HAT auto-oxidation can be followed by monitoring either O_2 consumption or cinnabarinate formation [6]. When cell-free suspensions are depleted of oxygen by N_2-sparging, no cinnabarinate is formed. However, in red cell suspensions depleted of oxygen by sparging with N_2 or CO, HMS flux was accelerated to fully half the rates obtained under normoxic conditions, indicating that 3-HAT auto-oxidation persists during anoxia (Fig. 1). It should be noted in this context that the red cell provides

electron sinks other than oxygen capable of supporting 3-HAT auto-oxidation [29]. Acceleration of HMS due to 3-HAT was also not affected by the hemoglobin oxidation state: rates of HMS flux in cells containing either oxyhemoglobin or methemoglobin increased with 3-HAT concentration in a similar manner. This is consistent with results from cell-free experiments in which both oxyhemoglobin and methemoglobin are shown to interact with products of 3-HAT auto-oxidation, but not to alter rates of overall auto-oxidation [6].

The role of red cell glycolysis as a source of antioxidant reducing equivalents has been subject to misinterpretation because glycolytic responses to oxidative stress are often expressed in terms of glucose utilization, which serves as an index of red cell ATP and/or 2,3-diphosphoglycerate (2,3-DPG) production, but does not necessarily reflect utilization of reducing potential [25, 26]. Likewise, assessment of glycolytic antioxidant activity by monitoring lactate accumulation is also misleading because lactate production represents storage of reducing equivalents and implies no net utilization of reducing potential by the red cell. Conversely, pyruvate accumulation directly reflects glycolytic antioxidant potential because each mole of pyruvate accumulated during oxidative stress indicates utilization of 2 electron equivalents. In the absence of exogenous lactate and pyruvate, increased glycolytic production of reducing equivalents during oxidative stress is unavoidably coupled with increased ATP or 2,3-DPG turnover. When present at close to equilibrium concentrations for lactate dehydrogenase (LDH), exogenous lactate and pyruvate allow utilization of reducing equivalents formed in glycolysis, i.e. preferential accumulation of pyruvate, without concomitant increases in glycolytic flux or ATP production. In this way, exogenous lactate and pyruvate uncouple the two functions of glycolysis, i.e. ATP and NADH production [25, 26].

Red cells exposed to 2.5 mM 3-HAT showed a 4-fold increase in pyruvate accumulation which, coupled with a slight but insignificant increase in lactate, yielded a significant increase in total end-product accumulation (Fig. 2). This preferential accumulation of pyruvate at the expense of lactate indicates use of reducing potential generated by glycolysis. However, when lactate and pyruvate were available in the medium in physiologic concentrations, pyruvate accumulated to an even greater extent without concomitant increases in lactate accumulation: total glycolytic flux increased similarly in the presence and absence of exogenous lactate and pyruvate, but the proportion of this increase due to pyruvate was doubled when exogenous lactate and pyruvate were available (Fig. 2). Thus, exogenous lactate and pyruvate uncouple red cell oxidative glycolysis from reductive glycolysis, allowing production of reducing potential without associated ATP turnover [25, 26]. It should be emphasized that exogenous lactate and pyruvate did not serve as a net electron source or sink because the amounts of both increased in the red cell suspensions during the 1-hr incubation, i.e. the data presented are *net* production.

Finally, red cell responses to 3-HAT auto-oxidation (pyruvate accumulation, increased HMS flux, and hemoglobin oxidation and/or decomposition) all increased in a roughly linear manner with increasing 3-HAT concentration. This correlation suggests that the mechanism(s) whereby 3-HAT induces oxidative stress is consistent over the range of concentrations used in the present study. This consistency, coupled with slower rates of auto-oxidation compared to other compounds used to study red cell oxidative metabolism [29, 31], suggest that 3-HAT may be useful as a probe of antioxidant mechanisms not only in red blood cells, but in other cells as well.

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REFERENCES

1. Yuwiler A, Brammer GL, Morley JE, Raleigh MJ, Flannery JW and Geller E, Short-term and repetitive administration of oral tryptophan in normal men. *Arch Gen Psychiatry* **38**: 619–626, 1981.
2. Lapin IP, Kynurenines and seizures. *Epilepsia* **22**: 257–265, 1981.
3. Sourkes TL, Toxicology of serotonin precursors. *Adv Biol Psychiatry* **10**: 160, 1983.
4. Green AR and Aronson JK, The pharmacokinetics of oral l-tryptophan; Effects of dose and of concomitant pyridoxine, allopurinol or nicotinamide administration. *Adv Biol Psychiatry* **10**: 67, 1983.
5. Gal EM and Sherman AD, L-Kynurenine; Its synthesis and possible regulatory function in brain. *Neurochem Res* **5**: 223–239, 1980.
6. Dykens JA, Sullivan SG and Stern A, Oxidative reactivity of the tryptophan metabolites 3-hydroxyanthranilate, cinnabarinate, quinolinate and picolinate. *Biochem Pharmacol* **36**: 211–217, 1987.
7. Ogawa H, Nagamura Y and Ishiguro I, Cinnabarinate formation in Malpighian tubules of the silkworm, *Bombyx mori*: Reaction mechanism of cinnabarinate formation in the presence of catalase and manganese ions. *Hoppe-Seyler's Z Physiol Chem* **364**: 1507–1518, 1983.
8. Ehrensvard G, Liljekvist J and Heath RG, Oxidation of 3-hydroxy-anthranilic acid by human serum. *Acta Chem Scand* **14**: 2081–2088, 1960.
9. Subba Rao PV and Vaidyanathan CS, Enzymic conversion of 3-hydroxyanthranilic acid into cinnabarinic acid. Partial purification and properties of rat-liver cinnabarinate synthase. *Biochem J* **99**: 317–322, 1966.
10. Ogawa H, Nagamura Y and Ishiguro I, Cinnabarinic acid formation in Malpighian tubules of the silkworm, *Bombyx mori*: Participation of catalase in cinnabarinic acid formation in the presence of manganese ion. *Hoppe-Seyler's Z Physiol Chem* **364**: 1059–1066, 1983.
11. Tomoda A, Shirasawa E, Nagao S, Minami M and Yoneyama Y, Involvement of oxidoreductive reactions of intracellular hemoglobin in the metabolism of 3-hydroxyanthranilic acid in human erythrocytes. *Biochem J* **222**: 755–760, 1984.
12. Allen MJ, Boyland E, Dukes CE, Horning ES and Watson JG, Cancer of the urinary bladder induced in mice with metabolites of aromatic amines and tryptophan. *Br J Cancer* **11**: 212–228, 1957.
13. Bryan GT, Brown RR and Price JM, Mouse bladder carcinogenicity of certain tryptophan metabolites and other aromatic nitrogen compounds suspended in cholesterol. *Cancer Res* **24**: 596–602, 1964.
14. Ehrhart H, Ried W, Benoit W and Dröfler H, Ünter-

suchungen über experimentelle Leukämien (VII). Adenocarcinome, sarkome und leukämien bei mäusen nach injektionen von 3-hydroxy-kynurenin. *Z Krebsforsch* **71**: 1, 1968.
15. Fujii K and Watanabe M, Comparative study of tumorigenicity in mice administered transplacentally or neonatally with metabolites of tryptophan and its related compounds. *J Cancer Res Clin Oncol* **96**: 163, 1980.
16. Wolfensberger M, Amslaer U, Cuenod M, Foster GA, Whetsell WO and Schwarcz R, Identification of quinolinic acid in rat and human brain tissue. *Neurosci Lett* **41**: 247–252, 1983.
17. Lapin IP, Prakhie IB and Kiseleva IP, Excitatory effects of kynurenine and its metabolites, amino acids, and convulsants administered into brain ventricles: Differences between rats and mice. *J Neural Transm* **54**: 229–238, 1982.
18. Schwarcz R, Whetsell W and Mangano M, Quinolinic acid: An endogenous metabolite that produces axon-sparing lesions in rat brain. *Science* **219**: 316–318, 1983.
19. Coyle JT, Bird SJ, Evans RH, Gulley RL, Nadler JV, Nicklas WJ and Olney JW, Excitatory amino acid neurotoxins: Selectivity, specificity, and mechanisms of action. *Neurosci Res Prog Bull* **19**: 330–427, 1981.
20. Kohler C, Neuronal degeneration after intracerebral injections of excitotoxins. A histological analysis of kainic acid, ibotenic acid and quinolinic acid lesions in the rat brain. In: *Excitotoxins* (Eds. Fuxe K, Roberts P and Schwarcz R), pp. 99–111. Plenum Press, New York, 1984.
21. Olney JW, Neurotoxicity of excitatory amino acids. In: *Kainic Acid as a Tool in Neurobiology* (Eds. McGeer EG, Olney JW and McGeer PL), pp. 95–121. Raven Press, New York, 1978.
22. Olney JW, Excitotoxins: An overview. In: *Excitotoxins* (Eds. Fuxe K, Roberts P and Schwarcz R), pp. 82–96. Plenum Press, New York, 1984.
23. Schwarcz R, Whetsell WO and Foster GA, The neurodegenerative properties of intracerebral quinolinic acid and its structural analog *cis*-2,3-piperidine dicarboxylic acid. In: *Excitotoxins* (Eds. Fuxe K, Roberts P and Schwarcz R), pp. 122–137. Plenum Press, New York, 1984.
24. Watkins JC, Excitatory amino acids. In: *Kainic Acid as a Tool in Neurobiology* (Eds. McGeer EG, Olney JW and McGeer PL), pp. 37–69. Raven Press, New York, 1978.
25. Sullivan SG and Stern A, Effects of physiologic concentrations of lactate, pyruvate and ascorbate on glucose metabolism in unstressed and oxidatively stressed human red blood cells. *Biochem Pharmacol* **32**: 2891–2902, 1983.
26. Sullivan SG and Stern A, Glucose metabolism of oxidatively stressed human red blood cells incubated in plasma or medium containing physiologic concentrations of lactate, pyruvate and ascorbate. *Biochem Pharmacol* **33**: 1417–1421, 1984.
27. Harley JD and Mauer AM, Studies on the formation of Heinz bodies. I. Methemoglobin production and oxyhemoglobin destruction. *Blood* **16**: 1722–1735, 1960.
28. Sullivan SG, McMahon S and Stern A, Restoration of red cell catalase activity by glucose metabolism after exposure to a vitamin K analog. *Biochem Pharmacol* **28**: 3403–3407, 1979.
29. Trotta RJ, Sullivan SG and Stern A, Lipid peroxidation and hemoglobin degradation in red cells exposed to *t*-butyl hydroperoxide. Effects of the hexose monophosphate shunt as mediated by glutathione and ascorbate. *Biochem J* **204**: 405–415, 1982.
30. Beutler E, *Red Cell Metabolism, A Manual of Biochemical Methods*. Grune & Stratton, New York, 1975.
31. Stern A, Red cell oxidative damage. In: *Oxidative Stress* (Ed. Sies H), pp. 331–349. Academic Press, New York, 1985.