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METABOLIC AND CELLULAR BASIS OF 2-BUTOXYETHANOL-INDUCED HEMOLYTIC ANEMIA IN RATS AND ASSESSMENT OF HUMAN RISK *IN VITRO*

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Abstract—Recent work in this laboratory indicated that 2-butoxyethanol (BE) causes acute hemolytic anemia in rats, and activation of BE to butoxyacetic acid (BAA), presumably through the intermediate 2-butoxyacetaldehyde (BAL), is a prerequisite for development of hematotoxicity. In the present studies, the effects of BE and its metabolites, BAL and BAA, on erythrocytes from rats and humans were investigated *in vitro*. Incubation of BE (up to 10 mM) with blood from male F344 rats caused no hemolysis and resulted in no metabolic alteration of BE. Further, addition of alcohol and aldehyde dehydrogenases, with their co-factors, to the incubation mixture failed to alter BE or its effect. At 20 mM, BE caused significant ($P \leq 0.05$) hemolysis of rat erythrocytes accompanied by a significant ($P \leq 0.05$) decrease in hematocrit (HCT). In contrast, incubation of BAL or BAA with rat blood caused time- and concentration-dependent swelling of red blood cells followed by hemolysis; however, BAA was significantly more efficacious than BAL. Addition of aldehyde dehydrogenase and its co-factors significantly ($P \leq 0.05$) potentiated the effect of BAL on rat erythrocytes. Further *in vitro* investigation of the cellular mechanisms involved in the hemolytic effect revealed that incubation of rat blood with BAA or BAL caused a time- and concentration-dependent decrease in blood ATP concentration. As observed with the hemolytic effects, the decrease in blood ATP was significantly ($P \leq 0.05$) greater with BAA than with BAL and was not induced by BE. Further, BAA caused no significant changes in the concentration of reduced glutathione and glucose-6-phosphate dehydrogenase in rat erythrocytes. Assessment of human sensitivity by incubation of human blood with BAA showed minimal swelling or hemolysis of erythrocytes with minimal decline in blood ATP levels at BAA concentrations several-fold higher than required to cause complete hemolysis of rat erythrocytes. In summary, the current studies confirm that the hemolytic effect of BE can be attributed primarily to its metabolite BAA, that hemolysis of rat erythrocytes by BAA or BAL is preceded by swelling and ATP depletion, suggesting that the erythrocyte membrane is the most likely target, and, finally, that human erythrocytes are comparatively insensitive to the hemolytic effects of BAA *in vitro*.

2-Butoxyethanol (BE) is a major industrial chemical which is widely used in the manufacture of a variety of consumer products including cleaning agents and household aerosols. The use of such products provides a high risk of human exposure to BE.

Several reports indicated that BE causes hemolytic anemia in experimental animals [1–5]. In recent studies conducted in this laboratory, we found that gavage administration of a single dose of BE to male F344 rats causes severe acute hemolysis of erythrocytes with subsequent development of hemolytic anemia [1, 6] as evident by a significant ($P \leq 0.05$) decrease in the number of circulating erythrocytes (red blood cells; RBCs), hematocrit (compact cell volume; HCT), and hemoglobin (HGB) concentration. Further, BE causes significant elevation in the concentration of free plasma HGB and secondary hemoglobinuria [1, 6]. In a recent investigation of BE metabolism and disposition, three metabolic pathways of BE metabolism were characterized: (a) metabolism to butoxyacetic acid (BAA) via the alcohol and aldehyde dehydrogenases; (b) conjugation of BE with glucuronic acid; and (c) conjugation of BE with sulfate. All three metabolites were thoroughly characterized and identified [7]. *In vivo* studies strongly suggested that metabolic activation of BE via the alcohol/aldehyde dehydrogenases is a prerequisite for the development of hematotoxicity by BE [1].

Investigation of the mechanism of BE-induced hemolysis indicated that there are species differences in susceptibility to BE [2]. Further, it was reported that BE causes increased osmotic fragility of erythrocytes which results in increased sensitivity to osmotic lysis [2]; however, the exact mechanism of BE-induced hemolytic anemia remained essentially uncharacterized. Therefore, the current studies were undertaken: (a) to investigate the metabolic basis of BE-induced hematotoxicity *in vitro* by comparing the hematotoxicity of BE with that of its metabolites, butoxyacetaldehyde (BAL) and BAA; (b) to try to reconstitute the proposed metabolizing enzymes (by adding exogenous alcohol/aldehyde dehydrogenases) and to study the effect of these enzymes on hematotoxicity *in vitro*; (c) to determine the sensitivity of human erythrocytes to BAA; and (d) to investigate the mechanisms of the hemolytic effects and to compare these mechanisms in rat and human erythrocytes *in vitro*.

METHODS

Chemicals. 99% Ethylene glycol monobutyl ether (2-butoxyethanol, BE) was purchased from the

Aldrich Chemical Co. (Milwaukee, WI). Alcohol and aldehyde dehydrogenases, NAD, NADP, and cyanamide were all obtained from the Sigma Chemical Co. (St Louis, MO). 2-(1-[^{14}C]Butoxy)ethanol was purchased from New England Nuclear (Boston, MA). The specific activity of BE was 6.32 mCi/mmol with greater than 99% radiochemical purity.

Synthesis of BAL and BAA. BAL and BAA were synthesized as previously reported [1]. Solutions of BE, BAL, and BAA were made by mixing with physiological saline solution (0.9% NaCl) to allow the addition of 10 μl/ml blood. This resulted in a final concentration of 5.0, 10.0, or 20.0 mM BE in whole blood, and 0.5, 1.0, or 2.0 mM BAL and BAA.

In vitro *hematotoxicity in rats.* Blood was collected from anesthetized 9- to 13-week-old F344 male rats (Charles River Breeding Laboratories, Raleigh, NC) by cardiac puncture using EDTA as the anticoagulant. Blood from individual rats was pooled and gently mixed.

Human blood was obtained, using heparin as the anticoagulant, from healthy male and female adult volunteers (18- to 40-years-old). They were questioned as to drug intake and disease status, and those who were taking any medication or had any known disease were excluded from this study.

For a further characterization of the role of alcohol and aldehyde dehydrogenases, alcohol dehydrogenase dissolved in physiological saline was added at 5 units/ml whole blood followed by its co-factor NADP which was added at 4.0 mM. Aldehyde dehydrogenase was suspended in physiological saline and added to the blood at 5 units/ml whole blood followed by its co-factor NAD which was added at 4.0 mM followed by BAL. After addition of the enzyme mixture, BE or BAL was added and the hematotoxicity was evaluated.

To investigate the effect of cyanamide on aldehyde dehydrogenase, cyanamide was dissolved in physiological saline and added to whole blood at 4 mM, or was added to the whole blood/aldehyde dehydrogenase mixture at the same concentration, followed by BAL.

Blood–chemical mixtures were incubated in a shaking water bath at 37°. At the end of the incubation period (0.25 to 4.0 hr), the test tubes containing the chemical-treated blood and their matching controls were removed, and the spun hematocrit was determined. The remaining blood was transferred to a 1-ml microtube and centrifuged using a microfuge (Beckman Instruments). The plasma was carefully transferred to a test tube to be used for the determination of the concentration of free plasma hemoglobin. Free plasma hemoglobin was quantitated colorimetrically using Drabkin's Reagent (Kit No. 525, Sigma Diagnostics, St Louis, MO). The concentration of free HGB in plasma obtained from control untreated blood was generally between 0.1 and 0.2 g/dl. The effect of each concentration was investigated in triplicate and was accompanied by a matching control at each time point investigated.

Determination of blood ATP, reduced glutathione, and glucose-6-phosphate dehydrogenase. Blood was collected from rats (as described above) or humans using acetate-citrate-dextrose (ACD) as anticoagulant and incubated with the test chemical for various periods of time. At the end of the incubation period, the hematocrit was determined and 1 ml of the remaining blood–chemical mixture was immediately added with 1 ml of 12% trichloroacetic acid, placed in an ice water bath for 5 min, and then centrifuged at 3000 rpm for 10 min. The resulting clear supernatant fraction was used for the quantitative determination of blood ATP concentration enzymatically using Sigma Assay Kit No. 366A (Sigma Diagnostics).

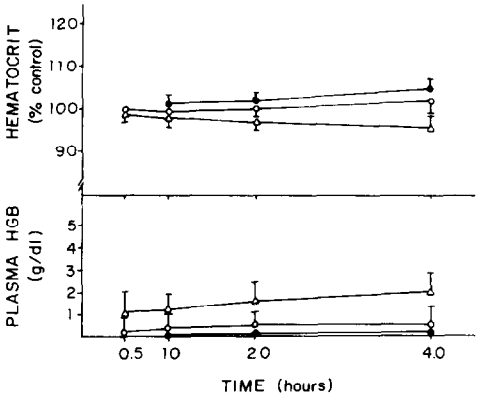

Fig. 1. Effects of concentration and time on hematocrit and free plasma hemoglobin in rat blood incubated with BE *in vitro*. Values are the mean ± SD of ≥ 6 determinations. Key: (●), (○), and (△) represent 5.0, 10.0, and 20.0 mM BE respectively. The concentration of free plasma HGB in the plasma of control rats was 0.1 to 0.2 g/dl.

Reduced glutathione (nonprotein sulfhydryls) was determined in rat erythrocytes using Ellman's reagent, as previously reported [8], and the same concentrations of BAA and BAL described above.

Determination of glucose-6-phosphate dehydrogenase (G6PD) enzymes in rat erythrocytes was performed colorimetrically using Sigma Assay Kit No. 400 (Sigma Diagnostics).

Statistical analysis. Statistical analysis was performed using a pairwise comparison of variance (one-sided *t*-test). Values were considered statistically significant at $P \leq 0.05$.

RESULTS

Effects of BE, BAL, and BAA on rat blood in vitro. Incubation of whole blood from male F344 rats with 5 or 10 mM BE caused a slight (statistically insignificant) increase in HCT and caused no significant hemolysis of erythrocytes as determined by the measurement of the concentration of free HGB in the plasma; however, at 20 mM, BE caused a slight but significant decrease in HCT which was accompanied by significant hemolysis of erythrocytes (Fig. 1). Similar incubations of rat blood with [^{14}C]BE for the same periods of time, followed by HPLC analysis of plasma and blood extracts, showed that no BE metabolites were formed, indicating that the rat blood was unable to metabolize BE *in vitro* (data not shown).

In contrast to BE, incubation of BAL with blood

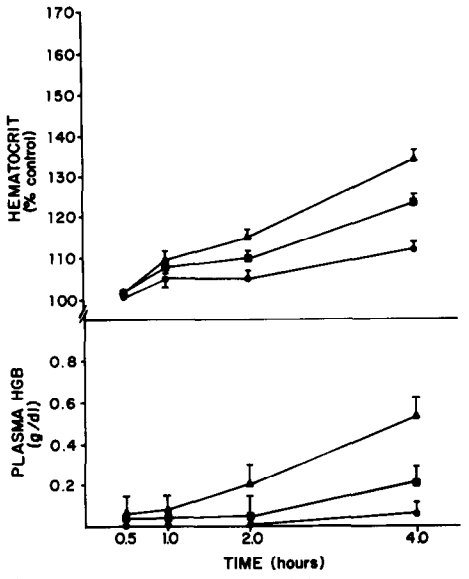

Fig. 2. Effects of concentration and time on hematocrit and the concentration of free plasma hemoglobin in rat blood incubated with butoxyacetaldehyde (BAL) *in vitro*. Values are the means ± SD of ≥ 6 determinations. Key: (●), (■), and (▲) represent 0.5, 1.0, and 2.0 mM BAL respectively.

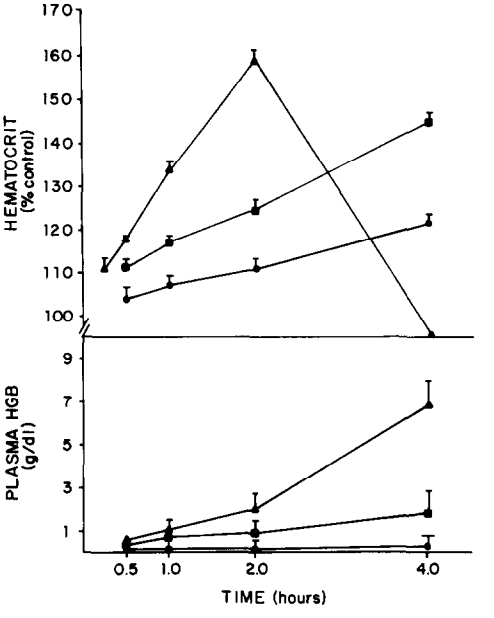

Fig. 3. Effects of concentration and time on hematocrit and the concentration of free plasma hemoglobin in rat blood incubated with butoxyacetic acid (BAA) *in vitro*. Values are the means ± SD of ≥ 6 determinations. Key: (●), (■), and (▲) represent 0.5, 1.0, and 2.0 mM BAA respectively.

from male F344 rats at one-tenth the concentrations used for BE (0.5, 1.0, or 2.0 mM) resulted in both time- and concentration-dependent swelling of erythrocytes (as evidenced by increased HCT). Hemolysis was statistically significant at 4 hr after incubation with 2 mM BAL only (as evidenced by increased concentrations of free plasma HGB) (Fig. 2). Similarly, incubation of BAA with rat blood at 0.5 or 1.0 mM resulted in a time- and concentration-dependent increase in HCT followed by hemolysis (Fig. 3). However, incubation of 2 mM BAA with blood resulted in a faster time-dependent increase in HCT, reaching maximum at 2 hr, followed by a nearly complete hemolysis by 4 hr (HCT < 10%). This hemolysis was evident by the drastic increase in the concentration of free HGB in plasma and the decrease in HCT (Fig. 3). Results presented in Figs. 1–3 clearly indicate that BAA was the most efficacious hemolytic species followed by BAL. Further, these data indicate that hemolysis of erythrocytes by BAL or BAA was preceded by swelling.

Effects of adding exogenous alcohol and aldehyde dehydrogenases. To investigate further the role of metabolism in BE toxicity, exogenous alcohol and aldehyde dehydrogenases and their cofactors, NADP and NAD, respectively, were added to rat blood followed by 5.0, 10.0, or 20.0 mM BE. Addition of exogenous enzymes had no effect on BE activity on blood (i.e. BE had no hemolytic activity) (data not shown). In contrast, addition of aldehyde dehydrogenase and its cofactor, NAD, to rat blood followed by BAL resulted in significant potentiation of BAL-induced hemolytic activity (Fig. 4). Addition of cyanamide, an aldehyde dehydrogenase inhibitor, significantly decreased the activity of BAL in the presence or absence of exogenous aldehyde dehydrogenase (Fig. 4).

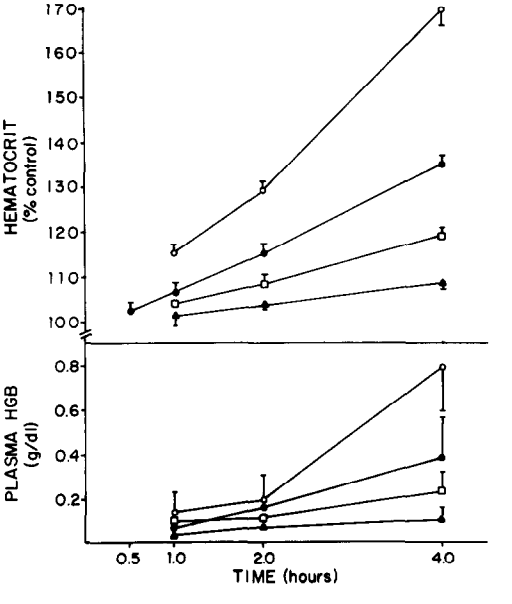

Fig. 4. Effects of addition and/or inhibition of aldehyde dehydrogenase on hematocrit and the concentration of free plasma hemoglobin in rat blood incubated with BAL *in vitro*. Values are the means ± SD of ≥ 6 determinations. Key: (●) 2 mM BAL; (▲) cyanamide + 2 mM BAL; (○) aldehyde dehydrogenase and NAD + 2 mM BAL; and (□) aldehyde dehydrogenase and NAD + 2 mM BAL + cyanamide.

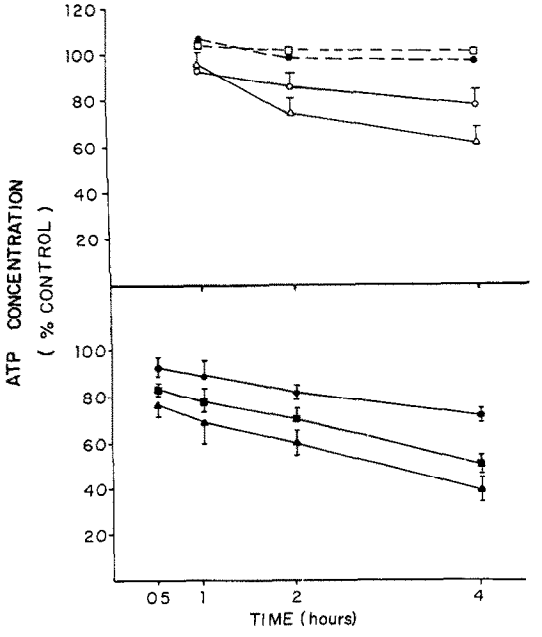

Fig. 5. Effects of BE, BAL (top) and BAA (bottom) on the concentration of ATP in rat blood *in vitro*. Values are the means ± SD of ≥ 6 determinations. Top panel: (○) and (△) represent 1 and 2 mM BAL, respectively, while (□) and (●) represent 5 and 10 mM BE respectively. Bottom panel: (●), (■), and (▲) represent 0.5, 1.0, and 2.0 mM BAA respectively. Control ATP concentration was 48.0 ± 2.3 μmol/dl (mean ± SD).

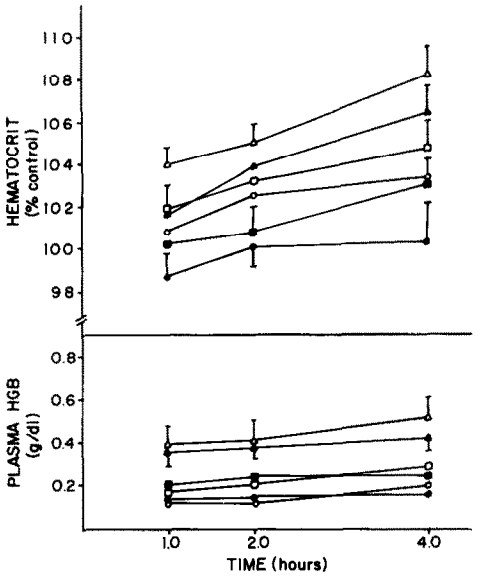

Fig. 6. Effects of concentration, time and sex on hematocrit and the concentration of free plasma hemoglobin in human blood incubated with BAA *in vitro*. Values are the means ± SD of ≥ 3 individuals. Key (○, ●), (□, ■), and (△, ▲) represent 2, 4, and 8 mM BAA respectively. Closed symbols represent males and open symbols represent females.

Effects of BE, BAL, and BAA on blood ATP concentration in vitro. Investigation of the mechanisms of erythrocyte hemolysis revealed that there was a time- and concentration-dependent decrease in the concentration of blood ATP caused by both BAL and BAA (Fig. 5). Further, there appeared to be a correlation between the decline in blood ATP and the hemolytic activity of BAL and BAA. As observed in the erythrocyte hemolysis studies, BAA was significantly more efficacious than BAL, while BE had no significant effect on blood ATP levels even at ten times higher concentration (Fig. 5).

Effects of BE, BAL, and BAA on the concentration of GSH and G6PD in rat erythrocytes in vitro. None of the three chemicals (at the concentrations employed above which caused swelling and hemolysis of rat erythrocytes) resulted in any significant changes in the concentration of GSH or G6PD in rat erythrocytes (data not shown).

Effects of BAA on human blood in vitro. To assess the susceptibility of human erythrocytes to the hemolytic effect of BAA, blood from young healthy male and female volunteers was incubated with the BAA concentrations that were used in the rat study (0.5, 1.0, or 2.0 mM) plus two higher concentrations (4.0 and 8.0 mM). BAA caused no significant changes in HCT or hemolysis of erythrocytes at BAA concentrations below 4 mM. However, there was a slight but significant increase in HCT followed by minimal but significant hemolysis of erythrocytes at the highest BAA concentration employed (Fig. 6). At all concentrations investigated, female erythrocytes were slightly more sensitive than male erythrocytes.

BAA also resulted in a slight but significant decrease in ATP concentration in blood from a young male human volunteer at 8.0 mM BAA; however, this decrease was not time dependent, being the same at 1, 2, and 4 hr (data not shown).

DISCUSSION

Investigation of the metabolic basis of BE-induced acute hemolytic anemia in experimental animals has revealed that BE activation to BAA via the alcohol and aldehyde dehydrogenases is required for the development of hemolytic anemia, with BAA being more efficacious than BAL [1, 2]. To minimize the effect of *in vivo* factors like metabolism, distribution, and elimination, studies were undertaken to compare the effects of BE, BAL, and BAA on blood from male F344 rats *in vitro*. Incubation of blood from male F344 rats with 5 or 10 mM BE caused a slight increase in HCT which resulted in no significant hemolysis of erythrocytes. At 20 mM BE, a significant decrease in HCT was observed which was accompanied by significant hemolysis. BE activity was characterized by: (a) no significant swelling of erythrocytes at all BE concentrations investigated; (b) significant hemolysis at the highest concentration of BE only; and (c) BE-induced hemolysis that was neither time nor concentration dependent. These characteristics suggest that BE-induced hemolysis is most likely to be non-specific in nature. Similar incubations of rat blood with BE, with and without exogenous alcohol and aldehyde dehydrogenases, followed by HPLC analysis caused neither modi-

fication of BE activity nor metabolic alteration of BE. This clearly indicated that blood is not capable of metabolizing BE *in vitro* and that BE is essentially devoid of the hemolytic activity that was observed *in vivo*. This suggested that the *in vivo* hemolytic activity of BE observed earlier [1, 6] can be attributed to one or more of the BE metabolites. As a result, we investigated the effects of BAL and BAA on blood from male rats *in vitro*. Incubation of 0.5, 1.0, or 2.0 mM BAL or BAA (concentrations selected in light of our *in vivo* pharmacokinetic studies [9]) with rat blood resulted in both concentration- and time-dependent increases in HCT, indicating swelling of erythrocytes. Maximum swelling of erythrocytes was observed 4 hr after addition of BAL; however, minimal hemolysis of erythrocytes was observed at that time point compared to that caused by BAA. In contrast, significantly higher levels of erythrocyte swelling were observed after the addition of BAA at all three concentrations investigated. Further, maximum swelling was observed at 2 hr after 2 mM BAA followed by a near complete hemolysis of erythrocytes as evident by the dramatic decrease in HCT and the corresponding increase in the concentration of free plasma HGB. This implies that the mechanism of erythrocyte hemolysis by BAA is different from that caused by BE.

In summary, *in vitro* comparison of the activities of BE, BAL and BAA clearly indicated that: (a) BAL and/or BAA are responsible for the hemolysis caused by BE *in vivo*; (b) BAA is more efficacious than BAL, whereas BE is essentially inactive; (c) the hemolytic metabolite is formed *(in vivo)* by BE metabolism at an extrahematic site(s), with the liver being the most likely candidate; and (d) hemolysis of erythrocytes is preceded by swelling, which strongly suggests that the erythrocyte membrane is the most likely target of the hemolytic species. The erythrocyte membrane was suspected of being the target from earlier findings of increased osmotic fragility [2] and increased mean copuscular volume (MCV) [5] of erythrocytes in laboratory animals. In earlier *in vivo* studies [1, 6], we observed a decrease in HCT, HGB concentration, and number of circulating erythrocytes accompanied by significant increases in the spleen wt/body wt ratio. In light of the present findings, we have reassessed the effect of BE on HCT *in vivo* and found that gavage administration of BE to male rats caused an early increase in HCT followed by a subsequent decrease (data not shown). However, the increase in HCT *in vivo* was less pronounced than that observed in the current *in vitro* investigations. This apparent difference can be explained by the fact that, although swelling of erythrocytes occurs in both the *in vivo* and the *in vitro* systems, most swollen erythrocytes are removed immediately from the systemic circulation by the spleen as evidenced by the drastic increase in the spleen wt/body wt ratio [1].

To characterize further the activity of the aldehyde intermediate (BAL) and the role of its metabolism in the observed hemolytic activity, we used cyanamide, an aldehyde dehydrogenase inhibitor, to inhibit BAL metabolism to BAA. Addition of cyanamide significantly decreased the activity of BAL. Furthermore, addition of exogenous aldehyde dehydrogenase and its co-factors to rat blood, followed by BAL, significantly potentiated the activity of BAL. This suggested that erythrocyte swelling and hemolysis induced by BAL *in vitro* are probably caused by its metabolism in rat blood to BAA.

In the course of investigating the cellular basis of BE-induced hemolytic anemia, several biochemical parameters were quantitated in rat erythrocytes. BAA had no effect on reduced glutathione or glucose-6-phosphate dehydrogenase levels in rat blood. Further, we measured the concentration of ATP in blood incubated with BE, BAL, and BAA. There was a time- and concentration-dependent decrease in the concentration of ATP following incubation of blood with BAL and BAA; however, BAA was significantly more efficacious than BAL, while BE had minimal or no effect. There was an apparent correlation between swelling of erythrocytes and the decrease in blood ATP, but it is not clear at this stage whether ATP depletion is the primary effect or secondary to erythrocyte swelling. Addition of exogenous ATP to blood plus BAA incubation mixtures failed to prevent erythrocyte swelling (data not shown); however, the intracellular accessibility of the added ATP is questionable and did not support either argument.

Because the ultimate objective of the current studies was to extrapolate from animals to humans, we investigated the effects of the proximate hemolytic metabolite of BE, namely BAA, on blood from healthy human volunteers. BAA caused minimal swelling or hemolysis of human erythrocytes at concentrations several-fold higher than required to cause complete hemolysis of rat erythrocytes. Furthermore, BAA caused no significant changes in human blood ATP levels. However, the highest BAA concentration (8 mM) decreased blood ATP by about 20% at all time points investigated. This decline was comparatively small compared to that observed in rat erythrocytes and was most likely non-specific. This observation is in agreement with previous reports which indicated that there are species differences in the sensitivity of erythrocytes to the hemolytic effects of BAA, and that humans are comparatively less sensitive than animals [10]. A recent report described a case of severe human poisoning with BE after a woman drank 250–300 ml of a window cleaner containing 12% BE. She suffered, among other symptoms, hemoglobinuria which suggests that exposure of humans to BE causes hemolysis [11]. The present results did not resolve the question as to why human erythrocytes are relatively insensitive to the hemolytic effect of BAA *in vitro*. It has been speculated that toxicokinetic parameters such as metabolism, distribution, and clearance may play a role in the species differences in susceptibility to the hemolytic effects of BE *in vivo* [1, 3, 12]. However, the observations made here with BAA *in vitro* unequivocally demonstrate that human erythrocytes were less sensitive to BAA-induced hemolysis which argues against the involvement of toxicokinetic factors in the species differences in sensitivity to BE. Comparison of the biochemical and anatomical characteristics of erythrocytes from a sensitive (rat) and an insensitive (human) species may provide a good model to inves-

tigate the species differences in sensitivity to BAA. The relative low sensitivity of human erythrocytes to the hemolytic effects of BAA *in vitro* should minimize our concern of hemolytic anemia in healthy young humans exposed to BE. However, humans predisposed to hemolytic anemia by certain disorders such as hereditary spherocytosis, glucose-6-phosphate dehydrogenase deficiency, and sickle cell anemia may have high sensitivity and could be at a higher risk than healthy humans. Another group of the human population that could be at a higher risk are the elderly, especially since BE-induced hemolytic anemia is age dependent in animals [6], and several biochemical defense mechanisms are reported to be compromised with age in both humans [13–15] and animals [16–18].

In conclusion, the current *in vitro* findings support earlier *in vivo* findings which indicated that metabolic activation of BE to BAA is required for the pathogenesis of hemolytic anemia and that BAA is the proximate hemolytic metabolite. Furthermore, we report here for the first time that BE-induced hemolysis was preceded by swelling of erythrocytes and that there was an apparent association between the swelling and the subsequent hemolysis of erythrocytes and ATP depletion. However, it is not clear at this time which one of the two effects of BAA is the primary effect. This mechanism of hemolysis may provide a model in investigating the mechanism of hemolytic anemia induced by other drugs and toxins. Finally, humans of both sexes are comparatively insensitive to the erythrocyte swelling and hemolysis as well as to ATP depletion by BAA.

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