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ACTIVATION OF A CATALASE PEROXIDATIVE PATHWAY FOR THE OXIDATION OF ALCOHOLS IN MAMMALIAN ERYTHROCYTES*

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Abstract—Alcohol oxidation has been induced in hemolyzed and intact mammalian erythrocytes by activating a catalase peroxidative pathway. Although erythrocytes contain high concentrations of catalase they ordinarily do not oxidize alcohols, presumably because of low hydrogen peroxide levels. Activation of the hexose monophosphate shunt with methylene blue and glucose induced the oxidation of methanol and ethanol in intact red cells. Inosine and hexoses which are readily converted to glucose-6-phosphate replaced glucose, but they were less effective. Galactose, a sugar poorly metabolized by erythrocytes, did not replace glucose. 2-Deoxyglucose competitively inhibited the effect of glucose but did not alter the response due to inosine. Furthermore, the optimal rate of alcohol oxidation achieved with glucose and methylene blue was augmented by the addition of inosine. Requirements for alcohol oxidation by hemolysates include methylene blue and NADPH. Inhibition of catalase activity by 3-amino-1,2,4-triazole or reduction of hydrogen peroxide levels with reduced glutathione inhibited alcohol oxidation.

THE peroxidative oxidation of alcohols *in vitro* was first described by Keilin and Hartree,[1, 2] and recently has been shown to be a major pathway for the oxidation of methanol in the rat.[3, 4] Although alcohol metabolism takes place primarily in the liver, one might expect that methanol and other alcohols could be oxidized by erythrocytes as well, since these cells contain high concentrations of catalase. That this does not normally occur has long been known. Earlier studies[5–8] showed that the inactivation of crystalline catalase by 3-amino-1,2,4-triazole (AT) was dependent upon a steady source of hydrogen peroxide in low concentration, such as that generated by reactions involving flavoproteins (xanthine oxidase, aldehyde oxidase, etc.). Experiments with AT *in vivo* presented a seeming enigma; when AT was administered to rats i.p., hepatic catalase was profoundly inhibited, but the catalase of intact erythrocytes was unaffected, even though purified catalase preparations from both sources were susceptible to inhibition by AT in the presence of a hydrogen peroxide-generating system.[5, 6, 9]

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To explain both the failure of erythrocytes to oxidize alcohols and the apparent insusceptibility of catalase in erythrocytes to inhibition by AT, it was postulated that the concentration of hydrogen peroxide in erythrocytes is normally very low.[5] A method of testing this hypothesis was suggested by the classic observations that addition of methylene blue to erythrocytes causes a marked stimulation of oxygen uptake and of carbon dioxide production, an increase in glucose consumption, and decrease in lactate formation (Harrop and Barron[10] and Barron and Harrop[11]) Mature, non-nucleated mammalian erythrocytes do not possess a cytochrome system or an intact citric acid cycle and do not readily transfer electrons to atmospheric oxygen. The auto-oxidizable dye, methylene blue, can serve as an electron carrier in erythrocytes, thus facilitating the transfer of electrons to oxygen. The generation of hydrogen peroxide often occurs in such electron-transfer reactions. The striking alteration in erythrocytic metabolism induced by methylene blue has recently been shown to be due to the stimulation of the hexose monophosphate oxidative pathway[12, 13] (pentose shunt) that occurs when methylene blue mediates the reoxidation of NADPH. Previous reports from this laboratory showed that, when mammalian erythrocytes are incubated with methylene blue and glucose, these cells become able to oxidize alcohols, and the erythrocytic catalase becomes susceptible to inhibition by AT.[14–16] The current investigations, which are extensions of these studies, strengthen the view that the induction of alcohol metabolism in erythrocytes is implemented through activation of the pentose shunt.

EXPERIMENTAL

Chemicals. NAD, NADP, NADPH, reduced glutathione (GSH), glucose-6-phosphate, fructose, mannose, galactose, 2-deoxyglucose, and inosine were purchased from California Biochemical Corp. and were of the highest purity obtainable from the company. All other agents were of analytical grade. The 3-amino-1,2,4-triazole (AT) was generously supplied by the American Cyanamide Co. and was purified as described previously.[3] Phosphate buffer solutions were prepared from monobasic potassium phosphate and dibasic sodium phosphate.

Enzymes. Catalase (Worthington Biochemical Corp.) was a twice-crystallized preparation from beef liver containing 4,000 Kat. f. units/ml.[17] Yeast glucose-6-phosphate dehydrogenase (California Biochemical Corp.) contained 70 units/mg.[18]

Erythrocytic preparations. Blood was obtained in heparinized syringes by abdominal aortic puncture from rats under ether anesthesia. The red cells were washed three times with cold 0·9% NaCl and then were brought to a hematocrit of 10% in 0·1 M phosphate buffer, pH 7·4. Removal of the buffy coat had no effect on the methanol oxidation rates. Hemolysates were made by mixing washed, packed red cells with 5 volumes of ice-cold water and dialyzing against 0·1 M phosphate buffer (500 ml, pH 7·4) for 3 hr; a magnetic stirrer was used. The buffer solution was replaced every 30 min.

Measurement of alcohol oxidation. Methanol oxidation was studied by a modification of a method, previously reported, which uses liver homogenates.[5] Reaction mixtures containing whole or hemolyzed rat erythrocytes equivalent to 1 ml of whole blood, semicarbazide (25 μmoles), and other substances as indicated, were preincubated for 15 min in a Dubnoff metabolic shaker at 37° in unstoppered 25-ml Erlenmeyer flasks, after which time 100 μmoles of methanol was added. The final volume

was 10 ml in 0·1 M phosphate buffer, pH 7·4. Two-ml aliquots removed at zero time and at measured time intervals after the addition of methanol were added immediately to 3 ml of 30% (w/v) trichloroacetic acid solution. Four ml of distillate was collected from the trichloroacetic acid solution, and the formaldehyde content was determined by the chromotropic acid method of MacFadyen.[19] That formaldehyde formation is a valid measure of methanol oxidation was shown by recovery experiments in which no appreciable loss of formaldehyde occurred when determined amounts of formaldehyde were incubated in erythrocyte systems known to promote maximal oxidation of methanol.

RESULTS

Studies with intact erythrocytes

Activation of methanol oxidation. Results from typical experiments shown in Table 1 reveal several basic features of the activation of methanol oxidation in mammalian erythrocytes.

TABLE 1. ACTIVATION OF METHANOL OXIDATION IN MAMMALIAN ERYTHROCYTES

Additions	Methanol oxidation* (μmoles HCHO formed/ml whole blood/hr)
1. None	
2. Methylene blue	0 ± 0
3. Glucose	0 ± 0
4. Methylene blue + glucose	1·70 ± 0·31
5. Ascorbic acid	1·45 ± 0·25
6. Glucose + ascorbic acid	0·99 ± 0·26
7. Methylene blue + glucose + ascorbic acid	4·26 ± 0·48
8. Methylene blue + ascorbic acid	4·78 ± 0·28
9. Boiled erythrocytes + methylene blue + glucose + crystalline catalase (30 Kat. f. units)	0·12 ± 0·02
10. Boiled erythrocytes + methylene blue + ascorbic acid + crystalline catalase (30 Kat. f. units)	3·4 ± 0

Reaction mixtures contained rat erythrocytes equivalent to 1 ml of whole blood, semicarbazide (2·5 mM), and methanol (10 mM); final volume 10 ml, pH 7·4, 0·1 M phosphate buffer. The concentrations of other reactants were: methylene blue (3·5 mM), glucose (55·0 mM), and ascorbic acid (5 mM).

* Each value represents the mean of 4 determinations ± S.D.

Addition of both methylene blue and glucose (no. 4 in Table 1) induced the oxidation of methanol to formaldehyde at a significant rate, whereas methylene blue or glucose alone was without effect (no. 2 and 3). Many other substrates with oxidation-reduction potentials reasonably close to that of methylene blue were also tested. A compound of considerable interest is ascorbic acid, which promoted methanol oxidation in the absence of methylene blue (no. 5). When ascorbic acid was added with methylene blue (no. 8), a marked potentiation was observed, the rate being greater than the sum of the rates in no. 4 and 5. When glucose was added, the effect of ascorbic acid was diminished (compare 5 and 6, and 7 and 8).

Experiments with boiled erythrocytes to which crystalline catalase was added permitted further insight into the mechanisms of activation of methanol oxidation by methylene blue and ascorbic acid. Methylene blue and glucose (no. 9) were

ineffective, whereas the addition of methylene blue and ascorbic acid (no. 10) gave a rate of methanol oxidation which approached that seen with normal erythrocytes (no. 8). This indicated that, although the induction of methanol oxidation by methylene blue plus glucose apparently is dependent upon other erythrocytic enzymes as well as catalase, the induction by ascorbic acid plus methylene blue might result from a nonenzymic event. This suggestion was supported further by the demonstration that a combination of methylene blue (0·035 mM), ascorbic acid (5 mM), NAD (0·26 mM), and crystalline catalase (100 Kat. f. units) catalyzed methanol oxidation at a rate of about 3·2 μmoles/hr in the absence of erythrocytes. Thus it appears that a direct chemical reaction of methylene blue, ascorbate, and NAD can result in the generation of hydrogen peroxide and a peroxidative oxidation by catalase.

Several other dyes structurally similar to methylene blue can activate the oxidation of methanol in intact mammalian erythrocytes (Table 2). The addition of azure A

TABLE 2. DYES THAT ACTIVATE METHANOL OXIDATION IN MAMMALIAN ERYTHROCYTES

Dye added	Methanol oxidation	
	Glucose	Glucose + ascorbate
	(μmoles/ml whole blood/hr)	
None	0	0·64*
Methylene blue (0·03 mM)†	1·70	3·50
Azure A (1 mM)	1·50	1·90
Thionine (1 mM)	1·55	1·60
Neutral red (3m M)	0	2·60

Unless otherwise noted, the final concentrations of each reactant was the same as in Table 1. Each reaction mixture contained semicarbazide (2·5 mM), methanol (10 mM), and erythrocytes equivalent to 1 ml of whole blood in a final volume of 10 ml, pH 7·4, 0·1 M phosphate buffer.

* Values represent the average of 3 experiments.

† Figures in parentheses indicate final concentration.

or thionine produced a significant rate of methanol oxidation in the presence of glucose, but only in concentrations many times greater than that of methylene blue. Neutral red did not activate the oxidation of methanol in the absence of ascorbic acid. Many other compounds, such as gallocyanine, acriflavin, hydrazine, primaquine, and pentaquine, stimulated methanol oxidation in mammalian erythrocytes only when ascorbic acid was present.

Role of carbohydrates. In Table 3 it is shown that fructose and mannose can replace glucose with lesser effectiveness as a co-requirement for the activation of alcohol oxidation in erythrocytes. All three sugars may be converted metabolically to glucose-6-phosphate and then be oxidized via the pentose shunt. Galactose, a hexose which is poorly metabolized by many tissues, including erythrocytes, has little or no capacity to replace glucose in the stimulation of methanol oxidation.

The experiments with 2-deoxyglucose are of special interest (Fig. 1). It can be seen that 2-deoxyglucose inhibits competitively the effect of glucose in its role as an activator of methanol oxidation by intact erythrocytes. The Michaelis constant (K_m) for glucose and inhibitor constant (K_i) for 2-deoxyglucose in this whole-cell system were 2·7 mM and 3·9 mM respectively. While it is not possible from this experiment

to locate the site of competition between glucose and 2-deoxyglucose, several possibilities can be mentioned. Hexokinase readily mediates the phosphorylation of both glucose and 2-deoxyglucose.[20] Kirkman showed that 2-deoxyglucose-6-phosphate binds relatively well with glucose-6-phosphate dehydrogenase ($K_m = 6{\cdot}9 \times 10^{-4}$ M) although its maximal velocity was only about 9% of that seen when glucose-6-phos-

TABLE 3. SUGARS ACTIVATING METHANOL OXIDATION IN INTACT MAMMALIAN ERYTHROCYTES

Sugar		Methanol oxidation (μmoles/ml whole blood/hr)
Glucose	(2·7 mM)*	1·70†
Fructose	(5·5 mM)	0·90
Mannose	(5·5 mM)	0·74
Galactose	(5·5 mM)	0·14

Each reaction mixture contained methylene blue (0·03 mM), semicarbazide (2·5 mM), methanol (10 mM), and rat erythrocytes equivalent to 1 ml of whole blood in a final volume of 10 ml, pH 7·4, 0·1 M phosphate buffer.

* Figures in parentheses indicate final concentrations.

† Each value represents the average of 3 experiments.

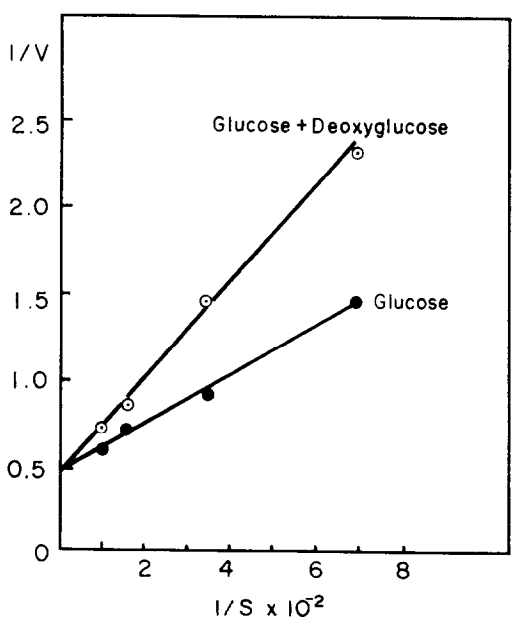

FIG. 1. Effect of 2-deoxyglucose on the oxidation of methanol in mammalian erythrocytes: ●—●, glucose; ⊙—⊙, glucose + 2-deoxyglucose. Varying concentrations of glucose were employed with 2-deoxyglucose (3 mM), methylene blue (0·3 mM) and methanol (10 mM) with the equivalent of 1 ml of rat erythrocytes in a final volume of 10 ml, pH 7·4, 0·1 M phosphate buffer. V = μmoles formaldehyde formed/ml of whole blood per hr. S = moles per liter. Each point represents the average value of at least 3 determinations. The K_m for glucose was 2·7 (±S.E. 0·43) mM and the K_i for 2-deoxyglucose was 3·9 (±S.E. 0·77) mM. The values were obtained by means of a computer program suggested by Cleland.[28]

phate was the substrate.[21] However, since 2-deoxyglucose does not inhibit the action of inosine (see below), it appears unlikely that the inhibition of the glucose effect occurs through the inhibition of glucose-6-phosphate dehydrogenase. More attractive possibilities are that 2-deoxyglucose acts as a competitive substrate for hexokinase or for a component of the complex sugar transport system known to occur in mammalian erythrocytes.[22]

Effect of inosine. Inosine can serve as a source of energy for erythrocytes. Ribose 5-phosphate may be metabolized by the pentose shunt after it is formed from inosine by the following reactions:

1. Inosine + orthophosphate $\xrightleftharpoons{\text{purine nucleoside phosphorylase}}$ Hypoxanthine + ribose-1-phosphate

2. Ribose-1-phosphate $\xrightleftharpoons{\text{phosphoribomutase}}$ ribose-5-phosphate

Inosine is an excellent energy-yielding substrate for erythrocytic metabolism because it makes available a phosphorylated sugar without the consumption of a high-energy phosphate bond of ATP. Figure 2 shows that inosine can replace glucose in the activation of methanol oxidation, but less effectively. That a separate

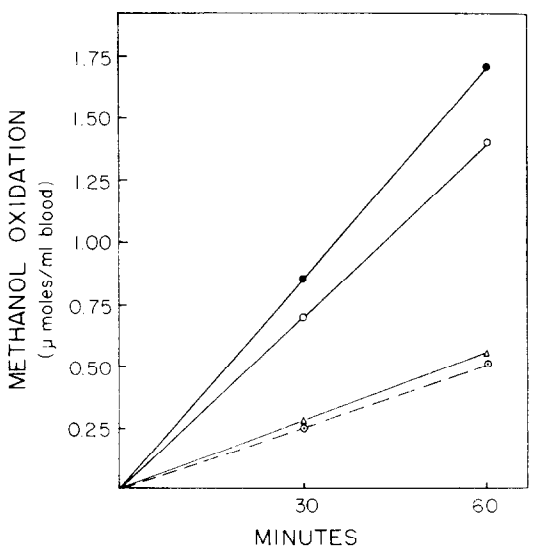

FIG. 2. Activation of methanol oxidation in mammalian erythrocytes by methylene blue and inosine: ●—●, methylene blue + glucose + inosine; ○—○, methylene blue + glucose; △—△ methylene blue + inosine; ○--○ methylene blue + inosine + 2-deoxyglucose. Each system contained methanol (10 mM), methylene blue (0·03 mM), and washed rat erythrocytes equivalent to 1 ml of whole blood in a final volume of 10 ml, pH 7·4, 0·1 M phosphate buffer. The final concentration of other additions was: glucose, 10 mM; 2-deoxyglucose, 15 mM, and inosine, 5 mM. Each point represents the average of at least 2 determinations. Reaction mixtures were incubated for 2 hr prior to the addition of methanol.

metabolic pathway may be used by inosine and glucose is indicated by the finding that the effects of glucose and inosine are additive and that, although 2-deoxyglucose is a potent competitive inhibitor of glucose, no inhibition by 2-deoxyglucose occurred when inosine was the substrate (Fig. 2). It should be noted that, to achieve maximal activation of methanol oxidation in erythrocytes by the addition of inosine and methylene blue, a 2-hr preincubation period prior to addition to the methanol was required. A possible explanation is offered by the finding that a marked increase in the concentration of glucose-6-phosphate occurs when erythrocytes are incubated with inosine.[23]

Studies with hemolysates

The preceding studies with intact erythrocytes indicated that the activation of methanol oxidation by the addition of a metabolizable hexose or nucleoside and an auto-oxidizable dye such as methylene blue was mediated through the enzymes of the pentose shunt. In order to explore this question further, experiments were performed with dialyzed hemolysates.

TABLE 4. ACTIVATION OF METHANOL OXIDATION IN MAMMALIAN HEMOLYSATES

Additions	Methanol oxidation (μmoles/ml whole blood/hr)
1. Erythrocytes + methylene blue + glucose	1·70*
2. Dialyzed hemolysate + methylene blue + glucose	0
3. Dialyzed hemolysate + methylene blue + glucose-6-PO_4	0·10
4. Dialyzed hemolysate + methylene blue + NADP	0·30
5. Dialyzed hemolysate + methylene blue + glucose-6-PO_4 + NAD	0·30
6. Dialyzed hemolysate + methylene blue + glucose-6-PO_4 + NADP	1·50
7. Dialyzed hemolysate + methylene blue + 6-phosphogluconate + NADP	1·80
8. Dialyzed hemolysate + methylene blue + NADPH	2·60
9. 6 + GSH	0·30
10. Boiled hemolysate + methylene blue + glucose-6-PO_4 + crystalline catalase (30 Kat. f. units) + NADP + glucose-6-PO_4 dehydrogenase (0·5 mg)	0·15

Each reaction mixture contained rat erythrocytes or dialyzed hemolysate equivalent to 1 ml of whole blood with semicarbazide (2·5 mM) and methanol (10 mM) in a final volume of 10 ml, pH 7·4, 0·1 M phosphate buffer. Concentrations of other reactants were: 0·03 mM, methylene blue; 16 mM, glucose; 16 mM, glucose-6-phosphate; 0·26 mM, NADP; 0·26 mM, NAD; 16 mM, 6-phosphogluconate; 1·75 mM, NADPH; and 0·32 mM, GSH.

* Each value represents the average of 3 experiments.

In Table 4 it may be seen that no methanol oxidation occurred when dialyzed hemolysates were supplemented with glucose and methylene blue (no. 2). However, an oxidation rate comparable to that obtained with intact cells was achieved (no. 6) when hemolysates were supplemented with glucose-6-phosphate (G-6P), NADP, and methylene blue. When either G-6P or NADP was omitted (no. 3, 4) or when NAD

replaced NADP (no. 5) only slight methanol oxidation occurred. However, 6-phosphogluconate (6-PG) completely replaced G-6P, which is not surprising, since 6-PG dehydrogenase catalyzed the reduction of NADP (no. 7). As expected, NADPH (1·75 mM) was fully effective in replacing the NADPH-generating system (no. 8).

Mills[24–26] and Cohen and Hochstein[27] have demonstrated the importance of glutathione peroxidase in the elimination of hydrogen peroxide in erythrocytes. This enzyme catalyzes the following reaction:

$$2GSH + H_2O_2 \xrightarrow[\text{peroxidase}]{\text{glutathione}} GSSG + H_2O$$

When reduced glutathione was added to dialyzed hemolysates with methylene blue, G-6P, and NADP, the rate of methanol oxidation was markedly diminished (Table 4, compare 6 and 9). Boiled hemolysates with an added source of catalase and G-6P dehydrogenase in the presence of methylene blue, G-6P, and NADP did not oxidize methanol (no. 10).

TABLE 5. THE EFFECT OF AMINOTRIAZOLE (AT) ON METHANOL OXIDIZATION IN MAMMALIAN ERYTHROCYTES

Additions	Methanol oxidation (μmoles/ml whole blood/hr)
1. Erythrocytes + methylene blue + glucose	1·70*
2. Dialyzed hemolysate + methylene blue + glucose	0
3. Dialyzed hemolysate + glucose-6-PO_4 + NADP + methylene blue	1·50
4. AT-treated erythrocytes† + glucose + methylene blue	0·12
5. Dialyzed hemolysate of 4 + NADP + glucose-6-PO_4 + methylene blue	0·03
6. Dialyzed hemolysate of 4 + NADP + methylene blue + glucose-6-PO_4 + crystalline catalase (30 Kat. f. units)	1·40

Each reaction mixture contained methylene blue (0·03 mM), semicarbazide (2·5 mM), and methanol (10 mM) equivalent to 1 ml of whole blood in a final volume of 10 ml, pH 7·4, 0·1 M phosphate buffer. Concentrations of other reactants were: 16 mM, glucose; 16 mM, glucose-6-phosphate; and 0·26 mM, NADP.

* Each value represents the average of 3 experiments.

† Erythrocytes equivalent to 2 ml of whole blood and washed in the usual fashion were incubated with ascorbic acid (40 mM), glucose (55 mM), AT (40 mM), and methylene blue (0·03 mM) in 0·1 M phosphate buffer, pH 7·4 at 37° for 60 min in a Dubnoff metabolic shaker. After this time no erythrocytic catalase activity could be demonstrated. These cells were then washed 3 times in iced saline, reconstituted in 0·1 M phosphate buffer or hemolyzed with iced water. Reaction mixtures outlined in nos. 4, 5, and 6 employed AT-treated erythrocytes or hemolysates. Erythrocytes treated in a similar manner but without AT yielded reaction rates similar to those seen in no. 1.

Role of catalase. The importance of catalase in the erythrocytic metabolism of alcohol is shown in Table 5. Washed rat erythrocytes were treated with the catalase inhibitor AT, glucose, ascorbic acid, and methylene blue. After an incubation period sufficient to inactivate the catalase, the cells were washed free of unbound AT. When incubated with methylene blue and glucose, these cells were incapable of

oxidizing methanol (no. 4). When these cells were hemolyzed, dialyzed, and resupplemented with glucose-6-phosphate, methylene blue, and NADP, alcohol-oxidizing capacity was absent (no. 5). The addition of crystalline catalase to this mixture restored methanol oxidation (no. 6).

DISCUSSION

These experiments show that erythrocytes have the potential to oxidize alcohols at significant rates via a catalase–peroxide reaction. It has become increasingly apparent that the hydrogen peroxide concentration in normal mammalian erythrocytes is exceedingly low. The finding that alcohol oxidation may be induced in erythrocytes demonstrates that hydrogen peroxide may be produced by enzyme systems present in the erythrocyte. An electron acceptor, such as methylene blue, and NADPH are required.

Recently, Mills[24–26] and Cohen and Hochstein[27] have established that the erythrocytic GSH peroxidase reaction is the primary means by which hydrogen peroxide is decomposed in the erythrocyte. It is possible that methylene blue lowers the level of GSH in mammalian red cells to a concentration where GSH peroxidase can no longer function effectively, with the result that hydrogen peroxide generated in other reactions accumulates. Another possibility is that methylene blue stimulates hydrogen peroxide-generating reactions which normally function at minimal rates. Perhaps both factors are involved.

Most of the experiments reported here using rat blood have been repeated with human erythrocytes, and no basic differences were observed. Several experiments were performed with ethanol as the substrate. The rate of oxidation of ethanol measured by acetaldehyde formation proceeded at about the same rate as that reported for methanol. While the clinical significance of these studies has not been evaluated, the findings show that erythrocytes have the potential to participate in the metabolism of alcohols. It should be pointed out, however, that erythrocytes cannot oxidize formaldehyde or acetaldehyde at significant rates. Since these substances are considerably more toxic than their related alcohols it might be harmful if the oxidation of alcohols took place readily.

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