INTERACTION OF BENZYL ALCOHOL WITH HUMAN ERYTHROCYTES

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Abstract—The uptake of benzyl alcohol by human erythrocytes and its binding to cell membranes were examined in vitro in relation to its hemolytic actions. The hemolysis induced by benzyl alcohol was found to be time-, dose- and temperature-dependent. Little or no hemolysis was observed until the concentration of benzyl alcohol increased over a certain level. Radiotracer studies revealed that in contrast to the rapid cellular uptake which was independent of temperature, the binding of benzyl alcohol to membranes increased gradually with time and was dependent mainly on the temperature. The critical hemolytic levels of the alcohol bound to membranes were estimated to be about 500 nmoles/mg protein. The results obtained herein suggest that the process of hemolysis induced by benzyl alcohol depends on the binding of the alcohol to erythrocyte membranes.

Benzyl alcohol has been used extensively as a preservative in the drug preparations of injectable form because of its stability and antibacterial activity. In fact, it has been reported that benzyl alcohol is most frequently added to the injection material in Japan except for agents used to maintain tonicity (1). In the screening experiments, we found that the injections containing benzyl alcohol show an appreciable hemolytic activity (2). Furthermore, in vivo hemolytic actions of benzyl alcohol on rat erythrocytes have been demonstrated in our previous paper (3). In the present work, uptake of benzyl alcohol by human erythrocytes and its binding to the cell membranes were examined in vitro in relation to hemolysis induced by the alcohol.

MATERIALS AND METHODS

Chemicals

\(^{14}\text{C}-\text{benzyl alcohol (carbonyl-}^{14}\text{C, specific activity 14.7 mCi/mmole) was obtained from the Radiochemical Centre, Amersham. Non-labelled benzyl alcohol of reagent grade was purchased from Nakarai Chem. Ltd.}^{14}\text{C-benzyl alcohol was diluted with non-labelled alcohol to achieve the desired concentration. All other high purity chemicals were obtained commercially and used without further purification.}

Preparation of erythrocytes

Fresh human blood was provided by Akita Red Cross Blood Center, stored at 4°C and used within 7 days. The blood was centrifuged to separate the plasma and buffy coat. The packed erythrocytes were washed with phosphate buffered saline (154 mM NaCl, 9.6 mM \(\text{Na}_2\text{HPO}_4\), 1.6 mM \(\text{NaH}_2\text{PO}_4\), pH 7.4) until the supernatant became colorless, and
were then resuspended to make a required hematocrit (Ht) value.

**Determination of hemolysis**

One or 2 ml of the erythrocyte suspension was added to 1 or 2 ml of the buffered saline containing varying concentrations of $^{14}$C- or non-labelled benzyl alcohol and the reaction mixture in a total volume of 3 ml was incubated at 4°C or at 37°C. At the end of the incubation time, the mixture was centrifuged at 10,000 rpm for 5 min in a refrigerated centrifuge and the hemoglobin released was determined by measuring the absorbance of the supernatant at 540 nm in a spectrophotometer. The data were corrected for the spontaneous release of hemoglobin observed in controls without benzyl alcohol added to the medium during an identical incubation period. The total amounts of intracellular hemoglobin were determined by complete lysis of erythrocytes with Triton X-100 at the final concentration of 0.1%.

**Uptake of $^{14}$C-benzyl alcohol by erythrocytes**

After centrifugation of the incubation mixture, aliquots of 0.1 ml supernatant fractions were pipetted into a counting vial and counted in a liquid scintillation spectrometer utilizing 10 ml of the scintillation fluid as described by Bray (4). The differences between the counts in the supernatant and in the total cell suspension were taken as the amount of the alcohol taken up by the cells.

**Binding of $^{14}$C-benzyl alcohol to cell membranes**

The erythrocyte membranes were isolated according to the method of Dodge et al. (5). Shortly after the isolation, the radioactivity in the membrane fractions solubilized in 10 ml of PCS Solubilizer was determined as described above. Protein content was estimated by the method of Lowry et al. (6).

All experimental samples were studied in quadruplicate. Results are presented as mean ± standard error.

**RESULTS**

**Benzyl alcohol-induced hemolysis**

The time course of benzyl alcohol-induced hemolysis of human erythrocytes (Ht 16.6%) is shown in Fig. 1. At a benzyl alcohol concentration of 100 mM, the hemolysis induced by the alcohol was relatively steady and a progressive release of hemoglobin was observed at 37°C with time. On the contrary, little or no hemolysis occurred at 4°C for 60 min at the same alcohol concentration.

The effect of benzyl alcohol concentrations on the hemolysis is shown in Fig. 2. It can be seen that the extent of hemolysis was markedly influenced by the alcohol concentrations. The temperature-dependent hemolysis by benzyl alcohol was reconfirmed herein. The concentration required for 50% hemolysis at 37°C after 60 min was found to be about 100 mM as shown in Fig. 1. When the benzyl alcohol concentration was kept constant, the extent of hemolysis was proportionally increased with decreasing concentration of erythrocytes (Fig. 3).
**Uptake of benzyl alcohol by erythrocytes**

Radiotracer studies revealed that about 25% of benzyl alcohol added to erythrocyte suspensions of Ht 16.6% was transferred to the cells within 60 min over the wide range of

**Fig. 1.** Time course of benzyl alcohol-induced hemolysis of human erythrocytes. Erythrocyte suspensions of Ht 16.6% were incubated with 100 mM benzyl alcohol at 37°C (—●—) or 4°C (—○—).

**Fig. 2.** Hemolysis of human erythrocytes induced by benzyl alcohol. Erythrocyte suspensions of Ht 16.6% were incubated with varying concentrations of benzyl alcohol for 60 min at 37°C (—●—) or 4°C (—○—).

**Fig. 3.** Effect of erythrocyte concentrations on benzyl alcohol-induced hemolysis. Erythrocyte suspensions of varying Ht values were incubated with 100 mM benzyl alcohol for 60 min at 37°C.

**Fig. 4.** Uptake of varying concentrations of benzyl alcohol by human erythrocytes. Erythrocyte suspensions of Ht 16.6% were incubated with 10⁻²−10⁻⁵ M benzyl alcohol for 60 min at 37°C.
non-hemolytic concentrations of $10^{-6}$–$10^{-2}$ M (Fig. 4). This result suggested that benzyl alcohol was transported into the cells principally according to a simple diffusion.

**Binding of benzyl alcohol to cell membranes**

The data concerning the binding of benzyl alcohol to cell membranes of varying concentrations of erythrocytes are shown in Fig. 5. It is well known that the yield of membrane components may be variable if the conditions of preparing erythrocyte membranes are different (7, 8). The linear relationship between original erythrocyte concentrations and the recovery of membrane proteins strongly suggested the relative accuracy and reproducibility of our experimental procedures.

The bindings of a fixed concentration (10 mM) of benzyl alcohol to cell membranes are presented in terms of nmoles per mg protein as indicated on the left ordinate in Fig. 5. The amounts of benzyl alcohol bound to membranes increased with the decreasing concentrations of erythrocytes.

**Time course of uptake by the erythrocytes and binding to membranes of benzyl alcohol**

As demonstrated in Fig. 6, about 20% of benzyl alcohol added was transferred to erythrocytes of Ht 13.3% within 15 min. This level did not change during further incubation. A similar pattern of benzyl alcohol uptake was observed at 4°C with the exception of an initial slower rate than that at 37°C. In contrast, the binding of benzyl alcohol to cell membranes increased gradually with time. The alcohol binding to membranes at 4°C was approx. 40% lower than that at 37°C. These findings corresponded well with the characteristics of time- and temperature-dependent hemolysis elicited by benzyl alcohol.

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**Fig. 5.** Binding of benzyl alcohol to erythrocyte membranes. Erythrocyte suspensions of varying Ht values were incubated with 10 mM benzyl alcohol for 60 min at 37°C.
Hemolytic effects and binding to cell membranes of benzyl alcohol

When the human erythrocyte suspensions (Ht 16.6%) were exposed to less than 80 mM of benzyl alcohol, little or no distinct hemolysis was noted within 60 min at 37°C. The hemolytic effects increased sharply with the alcohol concentrations above 80 mM as shown in Fig. 7. On the other hand, the binding of benzyl alcohol to membranes increased in proportion to the increasing alcohol concentrations in the suspending media. The alcohol
threshold concentration in the membranes was estimated to be $5 \times 10^{-7}$ moles/mg protein. Assuming that 1 ml of packed erythrocytes is equivalent to $10^{10}$ cells and contains 5 mg of membrane protein (9), the critical hemolytic level can be calculated to be $1.5 \times 10^{8}$ molecules ($2.5 \times 10^{-16}$ moles)/cell membrane.

**DISCUSSION**

In the present experiments, it was clearly shown that benzyl alcohol produced a hemolysis of human erythrocytes, the extent of which was dependent on time, dose and temperature (Figs. 1 and 2). The findings that the concentrations of erythrocytes had a profound effect on hemolysis indicated that benzyl alcohol was consumed by the cells (Fig. 3). In fact, the uptake level of benzyl alcohol by the red cells was somewhat higher than the theoretical diffusion values calculated on the assumption that the cell is 100% water (Figs. 4 and 6). However, benzyl alcohol appeared to be transported into the cell by a passive diffusion, as suggested by independency of the alcohol concentrations (Fig. 4) and temperature (Fig. 6). It thus seemed likely that accumulation above the diffusion values was due to the binding of the alcohol by the cellular constituents.

The rate of uptake of benzyl alcohol by the erythrocytes was found to be relatively rapid and the uptake level reached a plateau within 15 min (Fig. 6). These findings were essentially consistent with those of Lang and Rye (10) who reported that the uptake of benzyl alcohol by the cells of *Escherichia coli* was independent of contact time in the range of 10-90 min. The cell/medium concentration ratio was calculated to be about 1.6 over the wide range of concentrations when 25% of the benzyl alcohol added was transferred to the erythrocytes (Ht 16.6%) (Fig. 4). This value was roughly equal to that seen in the cells of *Escherichia coli* (10).

Contrary to the rapid cellular uptake, the binding of benzyl alcohol to membranes increased progressively with time and depended mainly on the temperature (Fig. 6), in correspondence to the time- and temperature-dependent hemolysis.

With regard to the intracellular distribution, it is estimated that benzyl alcohol bound to the membranes is 120, 240 and 360 nmoles/mg protein at 20, 40 and 60 mM, respectively, assuming that the weight of membrane is equivalent to 2% of the red cell (11) and the drug is uniformly distributed between the membrane and non-membraneous fractions. All these values are in good agreement with the actual ones shown in Fig. 7. At the higher concentrations, however, discrepancies were noted between the theoretical value and experimental one: 480 vs. 520 nmoles/mg protein at 80 mM and 660 vs. 650 nmoles/mg protein at 100 mM. This is consistent with the report of Colley et al. (12) who found an increase in partition of benzyl alcohol into the isolated erythrocyte membranes at higher concentrations than 80 mM. They stated that the disruption by above 80 mM benzyl alcohol of hydrophobic interactions between lipids and proteins, which seal the membrane protein in a defined conformation into the normal structure, leads to a breakdown in the structural integrity. In addition, their finding that the binding of the alcohol is dependent on temperature also coincides with the present results with intact membranes.
Using a centrifugation method, Colley et al. (12) reported that the partition coefficient of benzyl alcohol between the isolated membranes and buffer is a little over 3 at 25°C at 5-80 mM and the binding is reversible. Since the cell membranes were obtained by washing the erythrocytes 3 or more times with 10 mOsm phosphate buffer according to the method of Dodge et al. (5), loss of the drug bound to the membranes may have occurred in the present experiment. In view of the fact that benzyl alcohol is metabolized principally in the liver by alcohol dehydrogenase (13) which is absent in the erythrocytes (9), unchanged benzyl alcohol per se must exert the membrane damaging action.

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