

Mechanism of *o*-aminophenol metabolism in human erythrocytes

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o-Aminophenol was found to be rapidly metabolized to a brown compound in the presence of purified human oxy- and methemoglobin, coupled with the oxidation and reduction of these hemoglobins by *o*-aminophenol. The final product of *o*-aminophenol was identified as 2-aminophenoxazine-3-one, by using spectrophotometry and HPLC. The metabolism of *o*-aminophenol was also observed in human erythrocytes. The production rates of 2-aminophenoxazine-3-one in the cells were very fast, but these were strongly decreased by bubbling carbon monoxide into the cell suspension when intracellular hemoglobin was in the ferrous state. The production of 2-aminophenoxazine-3-one from *o*-aminophenol in the cells was completely suppressed by cyanide and azide when intracellular hemoglobin was in the ferric state. These results suggest that oxy- and methemoglobin are involved in metabolism of *o*-aminophenol to 2-aminophenoxazine-3-one in human erythrocytes.

o-Aminophenol metabolism 2-Aminophenoxazine-3-one Erythrocyte Hemoglobin (Human)

1. INTRODUCTION

It is well known that *o*-aminophenol causes toxic methemoglobinemia, oxidizing intracellular hemoglobin of human erythrocytes [1]. This compound has been shown to oxidize oxyhemoglobin very rapidly to methemoglobin in vitro [2,3]. However, it has not been determined whether *o*-aminophenol is metabolized by human erythrocytes, coupled with the oxidation of intracellular hemoglobin. We found that *o*-aminophenol is rapidly metabolized to 2-aminophenoxazine-3-one, a brown compound produced by the oxidative dimerization of *o*-aminophenol, in human erythrocytes. This work is a first report on the metabolism of *o*-aminophenol by human erythrocytes.

2. MATERIALS AND METHODS

Human hemoglobin was purified as in [4].

Methemoglobin was obtained by the oxidation of purified hemoglobin with ferricyanide [5]. A solution of 3.0 ml of oxy- or methemoglobin (120 μ M in heme) in 20 mM potassium phosphate buffer (pH 7.0) was mixed with *o*-aminophenol solution (Wako, Tokyo, final concentrations: 1 mM), which was previously neutralized with 0.1 M NaOH. The reaction was performed at 37°C for 60 min. Then 0.5 ml of the reaction mixture was passed through a column (0.5 \times 10 cm) of Sephadex G-25 (fine grade) previously equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The brown solutions which come after hemoglobin and include *o*-aminophenol and its metabolic product were identified using spectrophotometry, and HPLC. Authentic 2-aminophenoxazine-3-one was obtained according to Osman and Bassiouni [6] and used for comparison.

Human erythrocytes were collected from ACD (acid/citrate/dextrose) blood samples, which were obtained from a local blood bank (3 days out-

dated). After washing the cells, the erythrocytes were suspended in a Krebs Ringer solution to give a hematocrit of 18%. *o*-Aminophenol, which was dissolved in 0.9% NaCl solution and then neutralized with 0.1 M NaOH solution to make a 20 mM solution, was gently added to the suspension. The final concentrations of *o*-aminophenol in the suspension were 1 mM. After adjustment of the cell suspension pH to 7.0 at 37°C, the suspension was incubated with *o*-aminophenol at 37°C for 3 h. The experiments were performed in the presence or absence of potassium cyanide (final concentration 1 mM), sodium azide (final concentration 1 mM) and carbon monoxide. Carbon monoxide was bubbled into the cell suspensions. The erythrocytes with methemoglobin were obtained by oxidation of intracellular hemoglobin with nitrite [7]. The samples were taken out at intervals for analysis and were lysed by repeated freeze-thawing. Then the hemolysates were passed through a column of Sephadex G-25 (fine grade) equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The brown fractions which come after hemoglobin and include *o*-aminophenol and its metabolic product, were analyzed by spectrophotometry and HPLC.

3. RESULTS

3.1. Metabolism of *o*-aminophenol by oxyhemoglobin and methemoglobin

We studied the reactions of *o*-aminophenol with oxy- and methemoglobin. Fig.1 shows the changes in absorption spectra between 350 and 550 nm of the samples prepared by passing the reaction mixtures of *o*-aminophenol and oxyhemoglobin through a column of Sephadex G-25 (fine grade). The eluates free of oxyhemoglobin showed characteristic spectra with increasing absorbance near 435 nm with time. The absorption spectra of the sample at 120 min were very similar to those of authentic 2-aminophenoxazine-3-one. Nagasawa et al. [8] showed that *o*-aminophenol is converted to 2-aminophenoxazine-3-one by cytochrome *c*, a hemoprotein with protoporphyrin-like hemoglobin and cytochrome oxidase. They showed characteristic absorption spectra of 2-aminophenoxazine-3-one in 1:1 chloroform/ethanol solution, being consistent with our results in the inset to fig.1 (absorption spectra shown by broken lines).

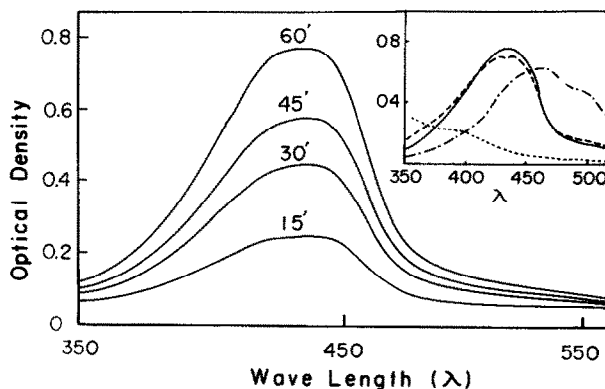


Fig.1. Changes in absorption spectra during the reaction of *o*-aminophenol with oxyhemoglobin. *o*-Aminophenol (final concentration 1 mM) was incubated with oxyhemoglobin (120 μ M in heme) at pH 7.0, 37°C for 60 min. The samples were removed at intervals for analysis and were passed through a column of Sephadex G-25 (fine grade) equilibrated with 50 mM phosphate buffer, pH 7.0. The eluates were subjected to optical measurements. (Inset) Absorption spectra of the sample at 60 min in a variety of solutions: (—) phosphate buffer, (---) 1:1 chloroform/ethanol solution, (.....) dilute HCl solution, (- - -) dilute NaOH solution.

Gerber and Lechevalier [9] obtained the values λ_{\max} in H_2O , 240 and 435 nm; λ_{\max} in diluted HCl solution, 237 and 468 nm; λ_{\max} in diluted NaOH solution, 335 nm. Our results shown in fig.1 (inset) are in good agreement with these values. Therefore, these results suggest that *o*-aminophenol is converted to 2-aminophenoxazine-3-one during incubation of oxyhemoglobin and *o*-aminophenol. Oxyhemoglobin was rapidly oxidized to methemoglobin within 30 min, indicating that oxyhemoglobin is involved in the metabolism of *o*-aminophenol (not shown). To identify the product of *o*-aminophenol with oxyhemoglobin, we studied the HPLC patterns of *o*-aminophenol, *p*-benzoquinone, authentic 2-aminophenoxazine-3-one and the product of *o*-aminophenol with oxyhemoglobin (fig.2). As a result, the patterns of the product of *o*-aminophenol with oxyhemoglobin were in good agreement with those of authentic 2-aminophenoxazine-3-one in position, showing that *o*-aminophenol was converted to 2-aminophenoxazine-3-one by oxyhemoglobin.

We also investigated whether *o*-aminophenol is metabolized by methemoglobin. Consequently,

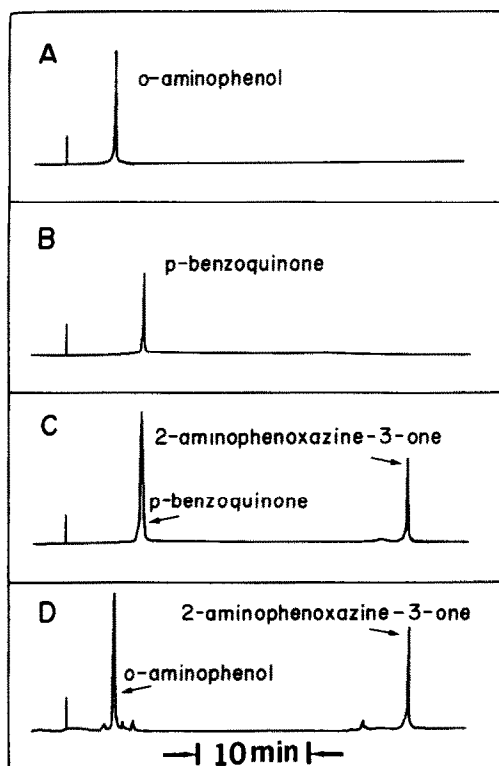


Fig.2. HPLC patterns of *o*-aminophenol, *p*-benzoquinone, authentic 2-aminophenoxazine-3-one and the product of *o*-aminophenol with oxyhemoglobin. The samples were applied to an HPLC apparatus (Toyo Soda, Japan) with a column (type ODS 120 T). (A) Authentic *o*-aminophenol, (B) authentic *p*-benzoquinone, (C) products of the reactions of *o*-aminophenol with *p*-benzoquinone, (D) products of the reactions of *o*-aminophenol with purified hemoglobin for 30 min.

2-aminophenoxazine-3-one was produced when *o*-aminophenol was incubated with methemoglobin, because characteristic spectra with peaks at 240 and 435 nm for 2-aminophenoxazine-3-one were observed in the samples including *o*-aminophenol and methemoglobin. Fig.3 shows the comparison of the 2-aminophenoxazine-3-one formation as detected by the changes in absorbance at 435 nm when *o*-aminophenol was incubated with oxy- and methemoglobin. The rates of 2-aminophenoxazine-3-one formation with oxyhemoglobin were faster than those with methemoglobin. We also observed that methemoglobin was reduced by *o*-

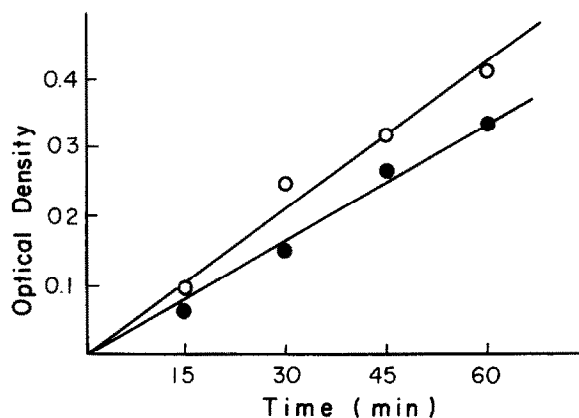


Fig.3. Changes in absorbance at 435 nm during incubation of *o*-aminophenol with oxyhemoglobin or methemoglobin. Changes in absorbance at 435 nm during incubation of *o*-aminophenol with oxyhemoglobin (○) or methemoglobin (●) were plotted vs time.

aminophenol, indicating that the reduction of methemoglobin is involved in metabolism of *o*-aminophenol (not shown).

3.2. Metabolism of *o*-aminophenol in human erythrocytes

Since we observed that *o*-aminophenol is metabolized by oxy- or methemoglobin, we investigated whether *o*-aminophenol is converted to 2-aminophenoxazine-3-one in human erythrocytes, where there is abundant hemoglobin. The absorption spectra characteristic of 2-aminophenoxazine-3-one increased with time between 350 and 550 nm (typical peak at 435 nm), when erythrocytes were incubated with *o*-aminophenol for 150 min at 37°C, indicating that 2-aminophenoxazine-3-one is progressively produced in human erythrocytes coupled with the oxidation of intracellular hemoglobin (we observed that intracellular oxyhemoglobin was oxidized to methemoglobin by *o*-aminophenol within 60 min; not shown here). Thus, the rates of 2-aminophenoxazine-3-one formation were estimated by the changes in absorbance at 435 nm.

The time course of 2-aminophenoxazine-3-one production during 150 min incubation of erythrocytes with *o*-aminophenol under different conditions is shown in fig.4. When intracellular hemoglobin was in the oxy form in erythrocytes, the

rates of 2-aminophenoxazine-3-one formation were very fast (about $900 \mu\text{M}/\text{ml}$ cells per h; this value was estimated taking account of the $\epsilon_{\text{mM}} = 1.1$ for 2-aminophenoxazine-3-one [9]), being comparable to the rates of glucose consumption in human erythrocytes at physiological pH. However, when intracellular hemoglobin was converted to the carboxy form, the reaction was greatly suppressed. This fact supports the views that oxyhemoglobin is responsible for the formation of 2-aminophenoxazine-3-one from *o*-aminophenol, and carboxyhemoglobin is not. Sodium azide, an inhibitor of catalase, did not influence 2-aminophenoxazine-3-one. On the other hand, when intracellular hemoglobin was converted to methemoglobin and incubated with *o*-aminophenol, 2-aminophenoxazine-3-one was produced, though the production rates were somewhat slower compared with the case of the control experiment with oxyhemoglobin, being consistent with the results in fig.3. However, the production of 2-aminophenoxazine-3-one was completely suppressed when sodium azide or potassium cyanide was previously added to the erythrocyte suspension containing methemoglobin. These results mean that methemoglobin is also involved in the metabolism of *o*-aminophenol to 2-aminophenoxazine-3-one in human erythrocytes, but that the cyanide or azide methemoglobin complex is inert to the metabolism of the compound.

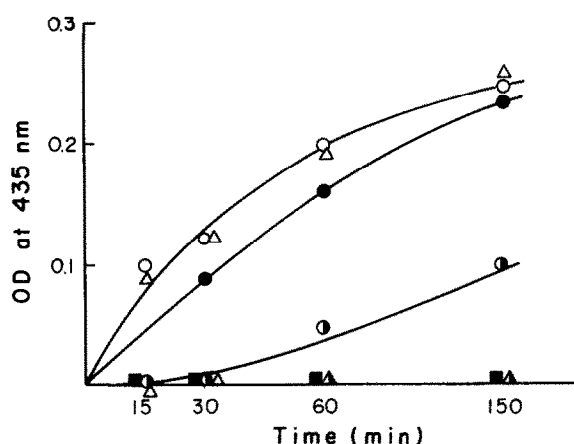
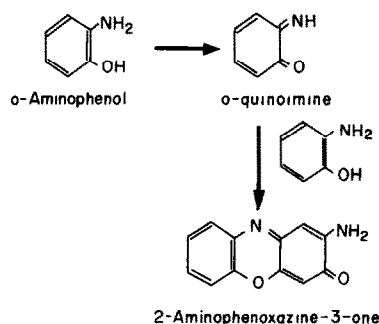


Fig.4. Metabolism of *o*-aminophenol in human erythrocytes under different conditions. Human erythrocytes were incubated with *o*-aminophenol at pH 7.0 and 37°C for 150 min under various conditions.

4. DISCUSSION

Our results show that *o*-aminophenol is metabolized to 2-aminophenoxazine-3-one by purified human oxy- and methemoglobin (figs 1–3). The same results were observed in human erythrocytes (fig.4). Since human erythrocytes contain a large quantity of hemoglobin inside, it is possible that this hemoprotein plays an important role in the metabolism of *o*-aminophenol to 2-aminophenoxazine-3-one in the cells. This view is supported by the results that the metabolism of *o*-aminophenol was much suppressed in the presence of CO gas when intracellular hemoglobin was in the ferrous state, and completely suppressed when intracellular hemoglobin was in the ferric state. We previously reported that 3-hydroxyanthranilic acid, which is an important tryptophan metabolite and has an analogous structure to *o*-aminophenol, is oxidatively dimerized to cinnabaric acid by human hemoglobin and erythrocytes [10]. Our results are also very analogous to the reaction of *o*-aminophenol with cytochrome *c* and cytochrome-*c* oxidase [8], and with isophenoxazine synthase from *Pycnoporus coccineus* [11]. From these reports, the metabolism of *o*-aminophenol to 2-aminophenoxazine-3-one by human hemoglobin may be visualized as follows.

The physiological significance of *o*-aminophenol metabolism by human erythrocytes remains



Scheme 1. Metabolism of *o*-aminophenol to 2-aminophenoxazine-3-one by human hemoglobin.

unclear, but it is possible that a quantity of 2-aminophenoxazine-3-one might be produced in a patient of toxic methemoglobinemia with *o*-aminophenol.

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