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Involvement of oxidoreductive reactions of intracellular haemoglobin in the metabolism of 3-hydroxyanthranilic acid in human erythrocytes

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3-Hydroxyanthranilic acid, a metabolite of tryptophan, was rapidly metabolized by human erythrocytes. The final product was determined to be cinnabarinic acid as detected by spectrophotometry, paper chromatography and t.l.c. The formation of cinnabarinic acid from 3-hydroxyanthranilic acid in the cells was markedly inhibited by CO when intracellular haemoglobin was in a ferrous state, and by cyanide when it was in a ferric state. Ferrous haemoglobin in erythrocytes was oxidized to $(\alpha^{3+}\beta^{2+})_2$, $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{3+})_2$ by 3-hydroxyanthranilic acid, and the oxidation rates were very high, like those of cinnabarinic acid formation, suggesting that the metabolism of 3-hydroxyanthranilic acid is coupled with oxidoreductive reactions of intracellular haemoglobin. This view was further confirmed by the findings that 3-hydroxyanthranilic acid was metabolized by ferrous or ferric haemoglobin and that ferrous and ferric haemoglobins were oxidized and reduced by the compound respectively. The significance of the metabolism of 3-hydroxyanthranilic acid and the oxidoreductive reactions of haemoglobin with this compound may be associated with the pathological conditions with increased 3-hydroxyanthranilic acid levels in the blood of diabetic subjects.

Tryptophan metabolites have been shown to be metabolized by various organs such as liver and brain (Leklem, 1971; Azmitia & McEwen, 1969). 3-Hydroxyanthranilic acid (3-HAT) is especially important as a precursor of NAD, and is firstly metabolized to 2-acroleyl 3-aminofumarate. However, haemoproteins such as catalase and haemoglobin have been shown to metabolize 3-HAT to cinnabarinic acid in the presence of Mn^{2+} (in the several-hundred-micromolar range) (Savage & Prinz, 1977; Ishiguro *et al.*, 1971). The physiological significance of this metabolic process is not still clear.

We have observed that 3-HAT oxidizes intracellular haemoglobin extensively when human erythrocytes are incubated with this compound. This finding suggests that 3-HAT may be metabolized in human erythrocytes, coupled with oxidation of intracellular haemoglobin, though metabolism of tryptophan metabolites in human erythrocytes has not been well studied hitherto. From this viewpoint we investigated 3-HAT metabolism in human erythrocytes and the mechanism of oxidation of haemoglobin. This paper represents the first report on the metabolism of 3-HAT by human erythrocytes.

Abbreviations used: 3-HAT, 3-hydroxyanthranilic acid; P_6-inositol, *myo*-inositol hexakisphosphate; Bis-tris, 2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

Experimental

Metabolism of 3-HAT by haemoglobins and human erythrocytes

ACD (aged citric acid/sodium citrate/dextrose) blood samples, which were obtained from a local blood bank (3 days outdated), were centrifuged at 3000*g* for 10min. The plasma and buffy coats were removed by aspiration, and the erythrocytes thus obtained were washed four times with 7vol. of chilled 0.9% NaCl solution. Then the cells were suspended in a Krebs–Ringer solution to give a haematocrit of 10%. 3-HAT (Wako Junyaku, Tokyo, Japan), which was dissolved in 1M-HCl solution then semi-neutralized with 1M-NaOH solution to make a 38mM solution, was gently

added to the human erythrocyte suspension. The final concentration of 3-HAT in the suspension was 3mM. After adjustment of the suspension pH to 7.0 at 37°C, the suspension was incubated for 2h with 3-HAT at 37°C. The samples were taken out at intervals for analysis, and were lysed by repeating freezing and thawing. Then the haemolysates were passed through a column of Sephadex G-25 (fine grade) equilibrated with 10mM-potassium phosphate buffer, pH7.0. The fractions containing haemoglobins were subjected to isoelectric-focusing electrophoresis (LKB PAG plates, pH3.5–9.5). After electrophoresis, the gels were fixed and scanned at 630nm as described previously (Tomoda *et al.*, 1978). The percentage of each component, including oxyhaemoglobin, half-oxidized haemoglobins and methaemoglobin, was estimated after cutting and weighing the chart paper. The orange-coloured fractions which come after haemoglobins, and include 3-HAT and its metabolites, were analysed by spectrophotometry, t.l.c. and paper chromatography. Authentic cinnabarinic acid was prepared by the method of Butenandt *et al.* (1957). T.l.c. of the sample was performed in ascending systems (butanol/acetic acid/water, 4:1:1, by vol.) using silica gel.

Identification of 3-HAT metabolites with oxyhaemoglobin or methaemoglobin

Stripped and purified haemoglobin solutions (ferrous and ferric forms) were obtained by the method previously described (Tomoda *et al.*, 1977, 1978). A solution of 2.2ml of ferrous or ferric haemoglobin (100μM in haem) in 0.05M-Bistris buffer with 0.1M-NaCl was mixed with 3-HAT (final concn. 2.3mM). The assay was performed at 25°C for 90min. Then the reaction mixture was passed through a column (0.5cm×10cm) of Sephadex G-25 (fine grade) previously equilibrated with 10mM-phosphate buffer, pH7.0. The orange-coloured solutions including 3-HAT and metabolic product of 3-HAT were identified spectrophotometrically (Ishiguro *et al.*, 1971). The samples were also subjected to t.l.c. and paper chromatography.

Results and discussion

Oxidation of erythrocyte haemoglobin with 3-HAT

When human erythrocytes were incubated with 3-HAT at 37°C, the intracellular haemoglobin was rapidly oxidized to half-oxidized haemoglobins [$(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$] and methaemoglobin

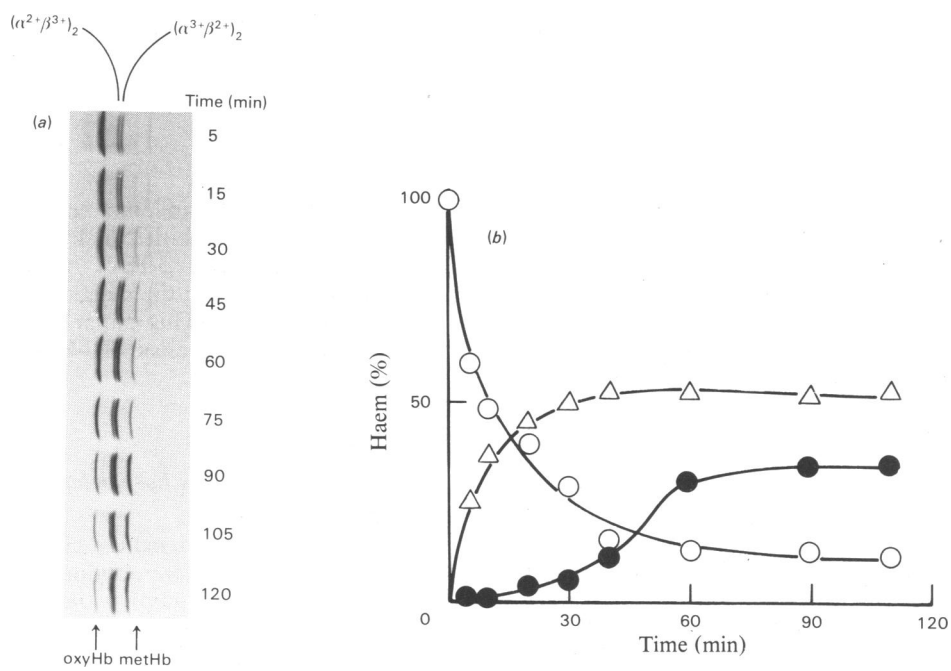


Fig. 1. *Analysis of isoelectric-focusing patterns of haemoglobins which were obtained during incubation of erythrocytes with 3-HAT*

(*a*) Time course of isoelectric-focusing patterns of haemoglobins. (*b*) Fractional changes in oxyhaemoglobin (oxyHb), half-oxidized haemoglobins and methaemoglobin (metHb) during incubation of erythrocytes with 3-HAT. The isoelectric-focusing patterns were analysed by gel-scanning at 630nm, and the haem contents (%) of each of the components [oxyhaemoglobin, $(\alpha^{2+}\beta^{3+})_2+(\alpha^{3+}\beta^{2+})_2$ and methaemoglobin] were estimated. The results were plotted against time. ○, Oxyhaemoglobin; △, $(\alpha^{2+}\beta^{3+})_2+(\alpha^{3+}\beta^{2+})_2$; ●, methaemoglobin.

$[(\alpha^{3+}\beta^{3+})_2]$. Fig. 1(*a*) shows the isoelectric-focusing patterns of lysates of erythrocytes incubated with 3-HAT. The four major bands, including oxyhaemoglobin, $(\alpha^{2+}\ \beta^{3+})_2$, $(\alpha^{3+}\ \beta^{2+})_2$ and methaemoglobin, were observed on the gel. From the gel-scanning patterns of the result, it is possible to estimate the fractional changes in each component (Fig. 1*b*). Consequently, oxyhaemoglobin was promptly converted into half-oxidized haemoglobins. The latter were also converted into methaemoglobin consecutively. However, the amounts of half-oxidized haemoglobins and methaemoglobin were kept constant after their rapid formation from oxyhaemoglobin. This result suggests that met-form haemoglobins are also involved in the metabolism of 3-HAT as well as oxyhaemoglobin, and are reduced by 3-HAT. We also observed that purified oxyhaemoglobin is oxidized by 3-HAT, and that purified methaemoglobin is reduced by the same compound (A. Tomoda & E. Shirasawa, unpublished work). These findings suggest that 3-HAT may be metabolized coupled with the oxidation and reduction of intracellular haemoglobins in erythrocytes. We therefore determined the products of 3-HAT during oxidation and reduction of oxy- and met-haemoglobin.

Determination of the metabolic product of 3-HAT

Fig. 2 shows the absorption spectra between 440nm and 500nm of the samples prepared by passing the reaction mixtures of 3-HAT and oxy- or met-haemoglobin through a column of Sephadex G-25 (fine grade). The eluates free of oxy- and met-haemoglobin showed spectra characteristic of authentic cinnabarinic acid, with a peak at 455nm (Ishiguro *et al.*, 1971). Our results are very consistent with those of Ishiguro *et al.* (1971) in showing that a small amount of haemoglobin catalyses 3-HAT conversion into cinnabarinic acid in the presence of 0.1mM-Mn^{2+}. The amounts of cinnabarinic acid produced by reduction of methaemoglobin with 3-HAT were 2-fold higher than those produced by oxidation of ferrous haemoglobin with 3-HAT. This result suggests that methaemoglobin has higher capacity to metabolize 3-HAT. We determined the product of 3-HAT during the oxidation and reduction of oxy- and methaemoglobin with 3-HAT by t.l.c. and paper chromatography. The product of 3-HAT was found to be cinnabarinic acid, since its chromatographic mobility was in good accordance with that of authentic cinnabarinic acid (results not shown).

Metabolism of 3-HAT in human red cells

Since we found that haemoglobins are able to catabolize 3-HAT, we decided to discover whether 3-HAT metabolism occurs in human red cells (Fig. 3*a*). The absorption spectra characteristic of

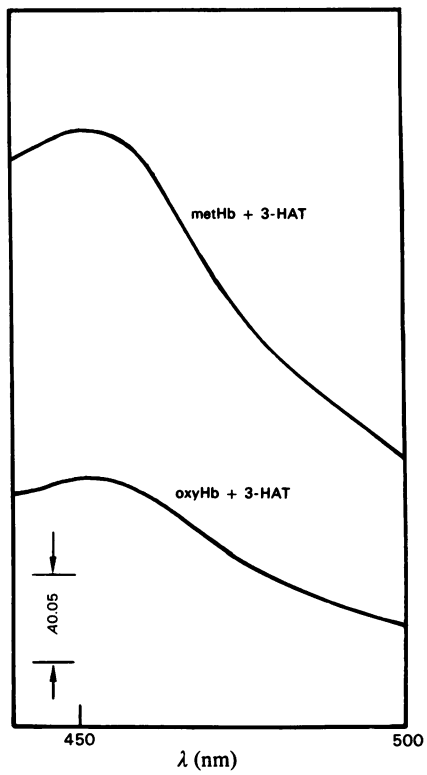


Fig. 2. *Absorption spectra of the samples obtained by the reaction of oxyhaemoglobin or methaemoglobin with 3-HAT*
After oxy- or met-haemoglobin was allowed to react with 3-HAT at 25°C for 90min, the reaction mixture was passed through a column (0.8cm × 10cm) of Sephadex G-25 (fine grade). The orange-coloured portion of the effluent was collected and measured spectrophotometrically between 440 and 500nm.

cinnabarinic acid increased with time between 440 and 500nm (typical peak at 455nm) when erythrocytes were incubated with 3-HAT for 2h at 37°C. This fact shows that cinnabarinic acid is progressively produced by human erythrocytes coupled with the oxidation of intracellular haemoglobin, as shown in Figs. 1(*a*) and 2.

The time course of cinnabarinic acid production during 2h incubation of erythrocytes with 3-HAT is shown in Fig. 3(*b*). The rates of cinnabarinic acid production by the cells were very high and almost comparable with rates of glucose metabolism by human erythrocytes at pH7.0 and 37°C (about 200nmol/ml of erythrocytes).

Fig. 4 shows the position of the 3-HAT metabolite on t.l.c. In this case we studied the metabolism of 3-HAT in human erythrocytes with oxyhaemoglobin or methaemoglobin. The t.l.c. was performed after putting the samples with authentic 3-HAT, cinnabarinic acid, anthranilic

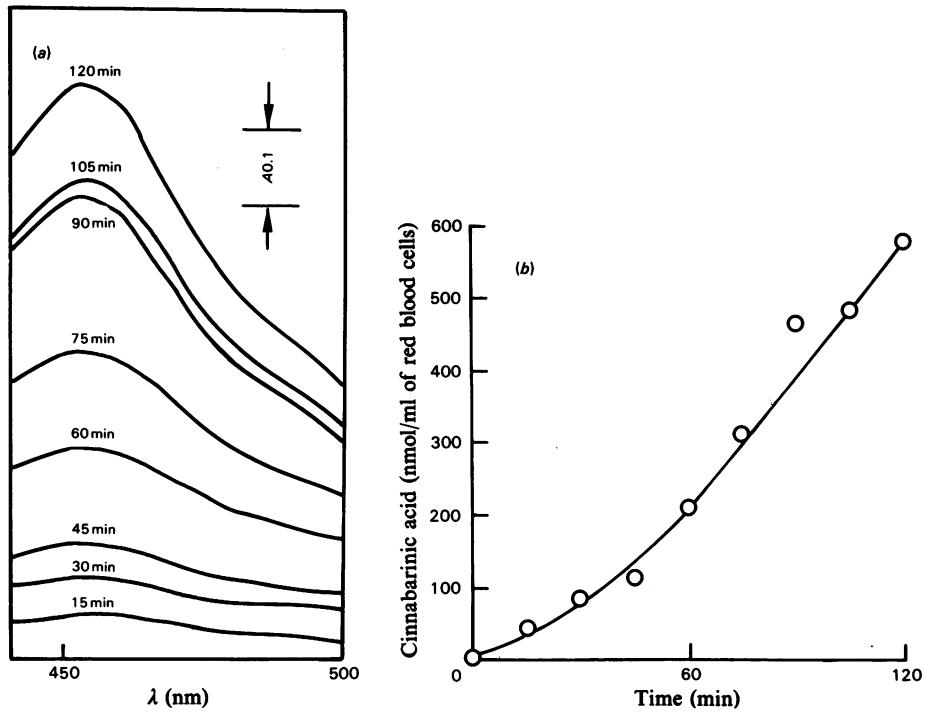
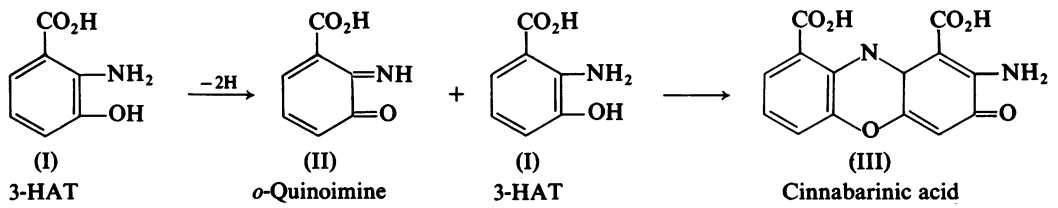


Fig. 3. *Time course of cinnabarinic acid formation during incubation of erythrocytes with 3-HAT*
(*a*) Absorption spectra of the samples obtained by the reaction of erythrocytes with 3-HAT. The erythrocytes were incubated with 3-HAT at 37°C for 2h. The samples were taken out at intervals for analysis. The haemolysates were passed through a column of Sephadex G-25 (fine grade). The orange-coloured portions of the effluent were collected and measured spectrophotometrically between 440 and 500nm. (*b*) Production of cinnabarinic acid during incubation of erythrocytes with 3-HAT. The amounts of cinnabarinic acid were calculated from A_{455} shown in (*a*) ($\varepsilon^{mM}_{455} = 23$; Ishiguro *et al.*, 1971) and were plotted against time.

acid, 3-hydroxykynurenine and kynurenine for comparison. As a result, it was found that the position of 3-HAT metabolite was in good agreement with that of authentic cinnabarinic acid, showing that 3-HAT is metabolized to cinnabarinic acid in human erythrocytes.

In order to elucidate the mechanism of 3-HAT metabolism in human erythrocytes, we studied the formation of cinnabarinic acid in the cells under various conditions. When oxyhaemoglobin was converted into carboxyhaemoglobin by CO in the cells, the formation rates of cinnabarinic acid from 3-HAT were extensively suppressed (Fig. 5). This result suggests that oxyhaemoglobin acts as a catalyst of 3-HAT conversion into cinnabarinic acid. However, KCN and NaN_3 had little effect on the conversion rates. Though Savage & Prinz (1977) showed that catalase of baboon liver has a capacity to metabolize 3-HAT to cinnabarinic acid, it is not likely that catalase is involved in the reaction in human red cells, since cyanide, a strong inhibitor of catalase, did not affect the formation rates of cinnabarinic acid. On the other hand, the formation rates of cinnabarinic acid were markedly accelerated when red-cell haemoglobin was converted into methaemoglobin. This result is consistent with that shown in Fig. 2. In this case, KCN suppressed the formation rates of cinnabarinic acid in erythrocytes, showing that cyanide–methaemoglobin complex is inactive for the metabolism of 3-HAT. These results demonstrate that 3-HAT is mainly metabolized by oxyhaemoglobin and methaemoglobin in human erythrocytes, and not by the catalase. The process of 3-HAT metabolism to cinnabarinic acid may be visualized as proposed by Subba Rao & Vaidyanathan (1966) (Scheme 1).

Though the metabolism of tryptophan and its metabolites such as 3-HAT, 3-hydroxykynurenine etc. has been extensively studied in various organs, there is little information for human erythrocytes. Furthermore, cinnabarinic acid seems not to be taken into account when the metabolism of tryptophan metabolites in various organs is discussed. In the present study we showed that erythrocytes have a high capacity to metabolize 3-



Scheme 1. *Process of cinnabarinic acid formation from 3-HAT*
The Scheme was derived from that of Subba Rao & Vaidyanathan (1966) on the metabolism of 3-HAT to cinnabarinic acid by catalase.

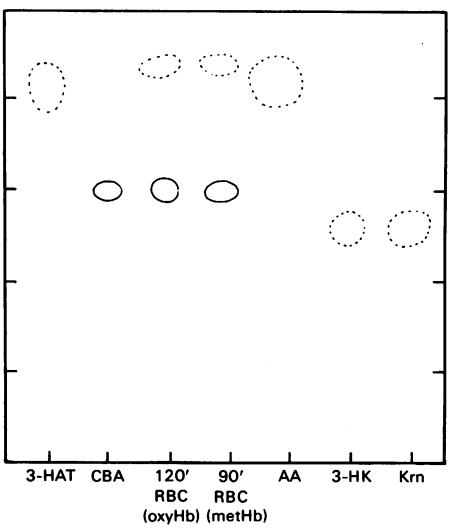


Fig. 4. *T.l.c. of the samples obtained after incubation of erythrocytes [with oxy- (oxyHb) or met-haemoglobin (metHb)] with 3-HAT*
After incubation of erythrocytes (which contain ferrous or ferric haemoglobin inside) with 3-HAT, the samples were removed after 90 (90′ RBC) and 120min (120′ RBC). Then they were lysed, and passed through a column of Sephadex G-25 (fine grade). The orange-coloured portion of the effluent was put on silica gel, and t.l.c. was performed with authentic 3-HAT, cinnabarinic acid (CBA), 3-hydroxykynurenine (3-HK), anthranilic acid (AA) and kynurenine (Krn) for comparison. The solvent was butanol/acetic acid/water (4:1:1, by vol.).

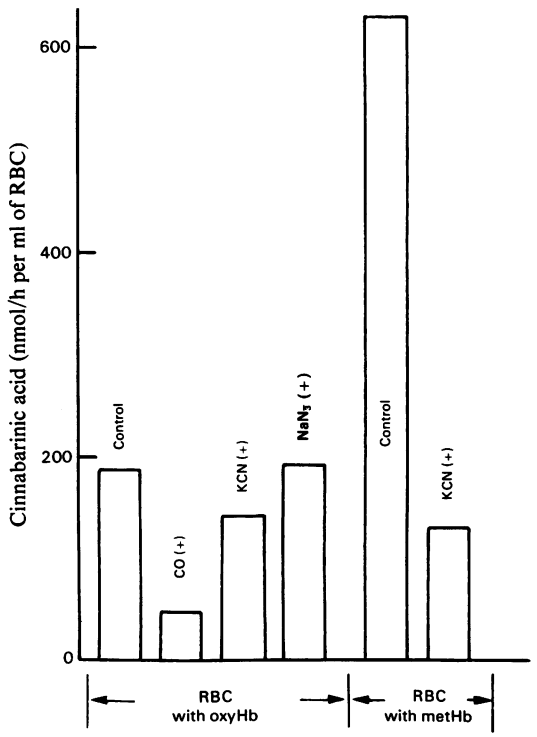


Fig. 5. *Rates of formation of cinnabarinic acid by human erythrocytes under various conditions*
The erythrocytes [RBC; with oxy- (oxyHb) or met-haemoglobin (metHb)] were incubated at 37°C for 2h with 3-HAT, after addition of KCN, NaN_3 and bubbling with CO. Then the rates of formation of cinnabarinic acid were determined by monitoring the changes in A_{455} as described in the legend to Fig. 3.

HAT to cinnabarinic acid. This fact suggests that erythrocytes contribute to the metabolism of 3-HAT in human blood. The accumulation of cinnabarinic acid may be especially possible in the red cells of diabetic patients, because it has been shown that tryptophan metabolites increase in their blood (Khattab *et al.*, 1972).

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