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Oxidative activation of 2-aminofluorene by human red blood cell cytosol

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Abstract—Purified red blood cell cytosol is able to activate 2-aminofluorene (2AF) to *N*-hydroxy-2-aminofluorene. Apparent kinetic parameters are determined with and without methylene blue. The latter, which maintains haemoglobin in the reduced form and stimulates NADPH production, increases the affinity of the enzyme for the 2AF. This activity is inhibited by carbon monoxide while cyanide is without effect. The involvement of a peroxidative reaction or a one-electron oxidative mechanism involving free radicals may be excluded.

Primary arylamines were among the first chemicals to be identified as carcinogens in human and experimental animals [1, 2]. Most studies on the mechanism of activation of carcinogenic arylamines have been concerned with the cytochrome P450-dependent mixed function oxidase, which is mainly located in the liver [3]. However, experimental data suggested that flavin-containing monooxygenase [4] and prostaglandin H synthase are also involved, a fact which would explain the activation of arylamines in extrahepatic tissues [5].

Many arylamines produce typical toxic effects in blood, e.g. oxidation of haemoglobin and haemolysis [6]. These effects were thought to result from the production of hydroxylated metabolites escaping the liver and taken up by erythrocytes. Once within the erythrocyte, these metabolites are rapidly oxidized to the nitroso derivative by oxyhaemoglobin with concurrent formation of methaemoglobin [7].

Recently, we demonstrated that human red blood cell cytosol can activate 2-aminofluorene (2AF*) to a mutagenic intermediate, and we detected *N*-hydroxy-2-aminofluorene (*N*-hydroxy-2AF) in the incubation mixture [8].

In this study, we report that cytosol from human red blood cells catalyses the N-hydroxylation of 2AF and the dependence on 2AF concentration displays typical Michaelis–Menten kinetics. Whether this reaction plays an important role for the *in vivo* drug metabolism remains to be determined.

Material and Methods

Chemicals. 2AF and methylene blue were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) *N*-Hydroxy-2AF was a gift of Dr Ch. Razzouk (School of Pharmacy, UCL, Brussels, Belgium). All other reagents of analytical grade were purchased from Merck (Darmstadt, Germany).

Preparation of red blood cell cytosol. Human blood was obtained from a healthy young male donor. Red blood cells were purified and cytosol was obtained as described previously [9]. In these conditions, 85% of haemoglobin was in the oxy form.

Assay of N-hydroxylation activity. N-Hydroxylation activity was assayed by gas liquid chromatography–electron capture detection (GLC–ECD) as described previously [10]. An isotonic phosphate buffer (400 μL) containing 37 mM K_2HPO_4 mM, 3 mM KCl, 69 mM NaCl, 10 mM glucose) at pH 7.3 was preincubated for 5 min in a shaking water bath at 38°. When specified, methylene blue was added (19 μM) as an artificial electron carrier.

After addition of red blood cell cytosol (0.7 mg protein/assay), the reaction was initiated by the addition of 2AF dissolved in methanol. The reaction was stopped by the addition of concentrated HCl (250 μL). Extraction and derivatization of *N*-hydroxy-2AF for GLC–ECD were as described by Razzouk *et al.* [10].

The protein content was determined by the method of Lowry *et al.* [11] and oxyhaemoglobin was measured by the method of Drabkin [12].

The influence of iron ligands (CO and CN) were tested in order to understand what form of haemoglobin is involved in the N-hydroxylation of 2AF.

Data analysis. Apparent kinetic constants were calculated by means of a non-linear regression program [13].

Results and Discussion

The gas chromatographic method described by Razzouk *et al.* [10] was initially designed to assay the liver microsomal N-hydroxylation activity. As we were interested in measuring N-hydroxylation activity in a cytosolic fraction of red blood cells, we redefined the optimal conditions. *N*-Hydroxy-2AF formation was optimal at pH 7.3 (Fig. 1). The time course of this metabolite formation was linear up to 2 min for a protein concentration of 1.5 mg/assay and 0.01 mM concentration of 2AF (Fig. 2a). The relationship between *N*-hydroxy-2AF formed and protein concentration was found to be linear up to 1 mg of protein (Fig. 2b).

Red blood cell cytosolic 2AF *N*-hydroxylase activity values fitted Michaelis–Menten kinetics in the presence and in the absence of methylene blue (apparent V_{max} and K_m are given in Fig. 3).

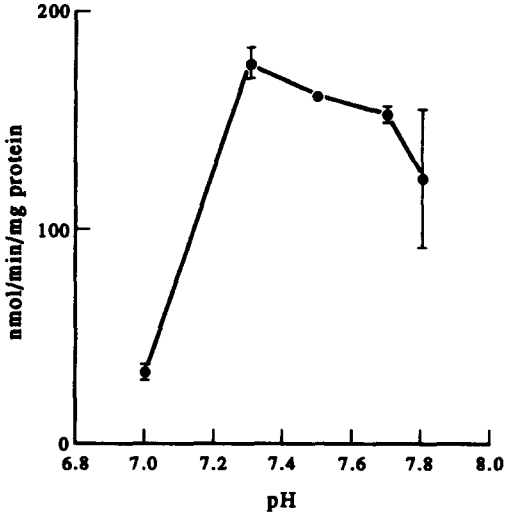

Fig. 1. Effect of pH on 2AF N-hydroxylation.

* Abbreviations: 2AF, 2-aminofluorene; *N*-hydroxy-2AF, *N*-hydroxy-2-aminofluorene; GLC-ECD, gas liquid chromatography–electron capture detection.

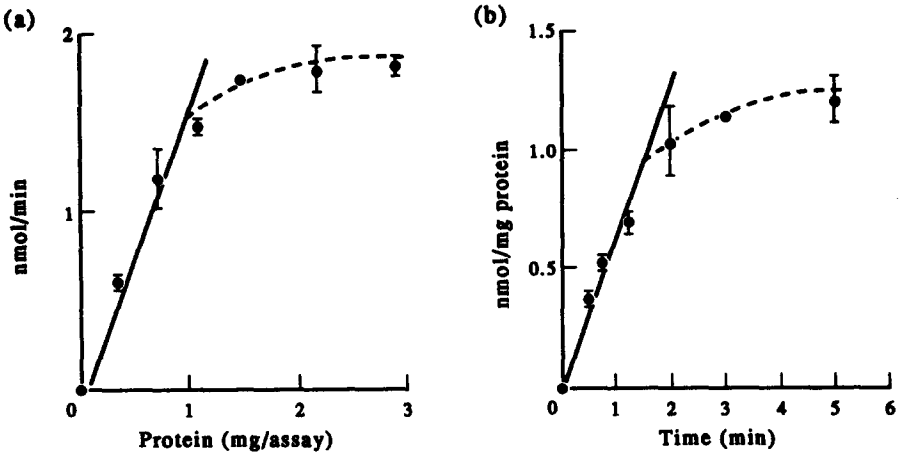

Fig. 2. N-Hydroxylation of 2AF as a function of protein content (a) and of time (b).

Although mature red cells do not contain an effective cytochrome P450 system, various reports suggest that erythrocytes might be a site of xenobiotic metabolism where haemoglobin is the key molecule involved in the reaction (see Ref. 14 for review). Two electron transport systems contribute to maintaining haemoglobin in the reduced form: the primary pathway involves NADH and the secondary NADPH-dependent pathway can be recruited by the administration of the exogeneous electron carrier methylene blue [15]. Increasing the levels of NADPH by methylene blue through activation of the pentose phosphate shunt leads to a higher affinity for 2AF with a slight reduction in *N*-hydroxy-2AF formation.

These results suggest that NADH or NADPH are effective electron donors, with NADPH giving a higher enzyme affinity for 2AF.

While 2AF *N*-hydroxylase activity was inhibited by CO, CN was without effect (Table 1) suggesting that active form of the catalyst is probably reduced haemoglobin.

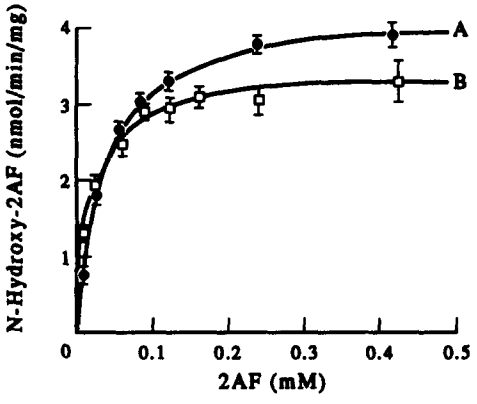

Fig. 3. Activity versus 2AF concentration plots for cytosolic 2AF N-hydroxylation in the absence (A) or in the presence (B) of methylene blue. Each point is the mean of three experiments ± SE. The K_m and V_{max} values determined via non-linear regression analysis [13] were 0.05 ± 0.005 mM and 4.71 ± 0.159 nmol/min/mg protein (A) and 0.019 ± 0.002 mM and 3.43 ± 0.099 nmol/min/mg protein (B).

Table 1. Dependence of *N*-hydroxy-2AF formation on the components of the incubation medium

System	% Activity
Complete	100
Minus methylene blue	137
+CO	34
+KCN (1 mM)	90

One hundred per cent activity = 3.43 ± 0.099 nmol/min/mg protein.

CO was bubbled in the incubation medium for 30 sec just before addition of 2AF (0.42 mM).

The involvement of a peroxidative reaction or a one-electron oxidative mechanism involving free radicals may be excluded since these reactions never lead to the production of an *N*-hydroxy-2AF intermediate [16, 17].

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