INHIBITORY ACTION OF CYANIDE ON ANILINE HYDROXYLASE SYSTEM

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1. Introduction

Cyanide is known to bind with hemeproteins, such as catalase and peroxidase [1,2], and inhibits the activity of mitochondrial cytochrome oxidase [3]. However, the microsomal electron transport system associated with drug hydroxylation has been reported to be insensitive to cyanide [4].

Cytochrome *P*-450, the terminal oxidase of microsomal hydroxylase, has been reported to bind with certain compounds. Many xenobiotics, known to be substrates for the hepatic microsomal hydroxylase, have been shown to interact with oxidized cytochrome *P*-450, which is detected spectrophotometrically as a 'type I' spectral change. The second group of compounds is composed of basic amines, of which only aniline is a known substrate for microsomal hydroxylase, and these form the 'type II' spectral change [5–7]. Cyanide was classified as a type II compound like aniline, although the spectral dissociation constant of cyanide was very different from that of aniline [5]. Thus, cyanide is supposed to interfere with the microsomal aniline hydroxylase system.

The present paper provides evidence that the activity of hepatic aniline hydroxylase was decreased markedly by cyanide. The inhibitory effect of cyanide was observed clearly at low concentrations of aniline and the inhibitory pattern was non-competitive. On the other hand, a mixed type inhibitory pattern was observed when higher concentrations of aniline were added to the reaction mixture. Aniline-induced difference spectra showed abnormal type II spectra in the presence of cyanide as the modifier, which suggested the release of cyanide from the cyanide-cytochrome *P*-450 complex with the addition of aniline.

2. Materials and methods

Normal male rats of the Wistar strain, weighing 250–300 g, were used for the experiments and liver microsomes were prepared as reported previously [8]. Microsomal protein concentration was determined by the biuret reaction [9] using bovine serum albumin as standard.

The activity of aniline hydroxylase was determined by measuring the formation of *p*-aminophenol from aniline. The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.4), 150 mM KCl, 10 mM $MgCl_2$, 5 mM glucose 6-phosphate, 0.5 mM NADP, 1.5 units glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 2 mg protein of microsomes and substrate, aniline, in a final volume of 1 ml. The reaction was carried out at 37°C aerobically with moderate shaking and stopped by adding 0.5 ml of 20% trichloroacetic acid. The *p*-aminophenol formed was measured according to the method of Brodie and Axelrod [10] as modified by Imai et al. [11]. Difference spectra were recorded with a Hitachi Two-Wavelength Double Beam Spectrophotometer, Model 356, in a split-beam mode.

Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADPH and NADP were purchased from C. F. Boehringer and Sohne GmbH. Aniline was distilled under reduced pressure and used for the substrate. Whenever solutions of potassium cyanide were required, they were made up and carefully adjusted to pH 7.5 with hydrochloric acid just prior to use. Other chemicals of the purest grade available were obtained commercially and not further purified.

3. Results

The time course of aniline hydroxylation by liver microsomes was linear for 10 min under the conditions used, and the amount of *p*-aminophenol formed was directly proportional to the amount of microsomal protein from 1–4 mg per ml of incubation mixture. The amount of *p*-aminophenol formed was decreased markedly by the addition of 10 mM cyanide when low concentrations of aniline were used for the experiment, and the inhibition was observed similarly in the reaction system containing either NADPH or NADPH-generating system as a source of reducing equivalent. No inhibitory action of cyanide, on the other hand, was observed in the reaction system containing higher concentrations (5-10 mM) of aniline. Fig.1 indicates the relationship between the hydroxylase activity and the inhibitory effect of cyanide at varying concentrations of aniline. A Lineweaver-Burk plot of the aniline hydroxylation showed two distinct phases, suggesting two sites of hydroxylation with different affinities (fig.1B). Cyanide interfered differently with these two reaction phases: i.e., non-competitive and mixed-type inhibitions of hydroxylases by cyanide were observed in the reaction system containing low and high concentrations of aniline, respectively. Using a fixed concentration of aniline, the effect of varying concentrations of cyanide on the aniline hydroxylase activity was observed as shown in fig.2. Dixon's plot showed two distinct patterns of cyanide inhibition. Inhibitory patterns of aniline hydroxylases observed at lower and higher concentrations of cyanide were non-competitive and competitive types, respectively. The inhibitor constants of cyanide for non-competitive and competitive inhibitions were determined graphically to be 7 and 31 mM, respectively.

The effect of cyanide on the binding of aniline with cytochrome *P*-450 was observed spectrophotometrically A typical type II binding spectrum was observed by adding either cyanide or aniline to the microsomal suspension (fig.3A and B). In the presence of aniline as a modifier [12], the extent of cyanide-induced difference spectrum decreased clearly, although the wavelengths of peak and trough were not different (fig.3C). We can conclude that some of the binding sites of cytochrome *P*-450 are saturated by aniline and only a small amount of cyanide can bind with the remaining binding sites.

An abnormal aniline-induced difference spectrum was obtained in the presence of cyanide as a spectral modifier (fig.3D). The wavelengths of peak and trough of aniline-induced difference spectrum shifted to short wavelengths, 424 and 382 nm, respectively, and another trough appeared at 447 nm. This may suggest that aniline is not only capable of binding with the remaining binding sites of cytochrome *P*-450 but also can release cyanide from the cyanide–cytochrome *P*-450 complex, because of higher affinity of aniline

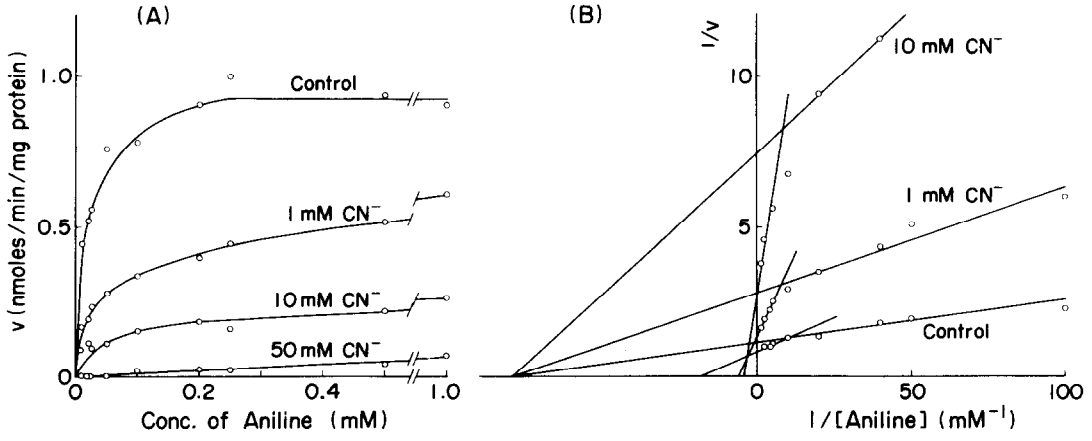

Fig.1. Inhibition of aniline hydroxylase activity by cyanide. The activity of microsomal aniline hydroxylase with or without cyanide was estimated in the presence of varying concentrations of aniline (A), and an inverse plot (B) indicating the effect of cyanide on the velocity of aniline hydroxylation was obtained from the data shown in A.

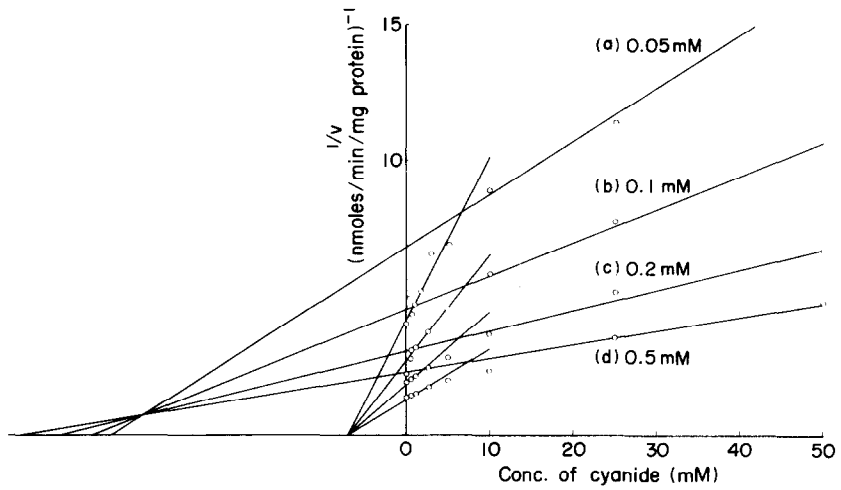

Fig.2. Dixon plot showing effect of cyanide on velocity of aniline hydroxylation. Formation of *p*-aminophenol was determined in the presence of 0.05, 0.1, 0.2 or 0.5 mM aniline under varying concentrations of cyanide.

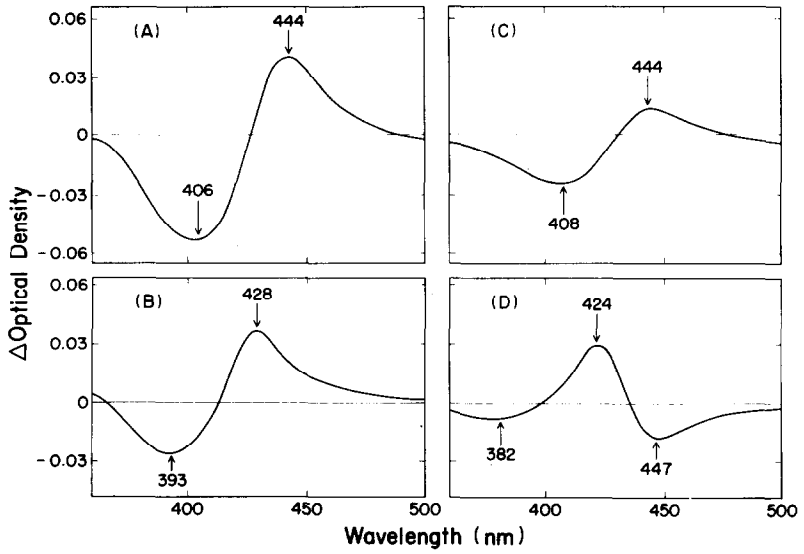

Fig.3. Substrate-induced difference spectra of microsomes. Hepatic microsomes were diluted to a concentration of 2 mg protein per ml (1.48 nmol cytochrome *P*-450/ml) in 50 mM Tris–HCl buffer (pH 7.4) containing 150 mM KCl and 10 mM $MgCl_2$ and the samples were divided between two cuvettes. Cyanide, 6.25 mM, (A) of 8 mM aniline (B) was added to the sample cuvette and the resultant spectrum recorded; (C) Aniline was added to the microsomal suspension at a concentration of 8 mM and the mixture was divided between two cuvettes. Cyanide (6.25 mM) was added to the sample cuvette and the difference spectrum was observed; (D) Cyanide (6.25 mM) was added first to the microsomal suspension and the samples were divided equally into sample and reference cuvettes. The difference spectrum was recorded after addition of 8 mM aniline to the sample cuvette.

for cytochrome *P*-450 compared to that of cyanide. Thus, the abnormal aniline-induced difference spectrum shown in fig.3D is supposed to be the complex form of aniline-induced and negative cyanide-induced difference spectra.

4. Discussion

The method employed to detect and determine *p*-aminophenol was not affected by cyanide and no activity change of membrane-bound NADPH-cytochrome *c* reductase was observed by adding cyanide. Thus, cyanide was supposed to interfere directly with the binding sites of cytochrome *P*-450. Cyanide is well known to be a potent inhibitor of many hemeproteins and is considered to bind directly with the sixth ligand of heme-iron [13]. Type II compounds of the microsomal hydroxylase system including aniline have been reported to bind directly with the same ligand of cytochrome *P*-450 [12]. If cyanide and aniline bind with the sixth ligand of cytochrome *P*-450, the overall inhibitory pattern of cyanide on aniline hydroxylation should be competitive [14]. As shown in fig.1 and 2, inhibition of hydroxylation by cyanide was observed to be non-competitive in the presence of low concentrations of aniline, suggesting a different binding site of aniline from that of cyanide. We can thus conclude that the specific binding site of aniline is not the sixth ligand of heme-iron, assuming that this is the specific binding site of cyanide. Upon addition of higher concentrations of aniline, the compound is supposed to bind not only with its specific binding site but with heme-iron directly, showing two distinct phases of hydroxylation and different inhibitory patterns by cyanide.

The microsomal hydroxylase system is generally considered to be insensitive to cyanide. Spectrophotometrical study revealed that cyanide was released from the complex of cyanide and cytochrome *P*-450 by the addition of aniline (fig.3D). Release of cyanide from the complex may, in turn, cause an insensitivity of hydroxylase to cyanide. Detailed studies on the binding of cyanide and aniline with cytochrome *P*-450 will be reported elsewhere.

References

[1] Chance, B. (1952) J. Biol. Chem. 194, 483–496.
[2] Keilin, D. and Hartree, E. F. (1955) Biochem. J. 61, 153–171.
[3] Racker, E. (1965) Mechanisms in Bioenergetics, p.14, Academic Press, New York.
[4] Oshino, N., Imai, Y. and Sato, R. (1966) Biochim. Biophys. Acta 128, 13–28.
[5] Schenkman, J. B., Remmer, H. and Estabrook, R. W. (1967) Mol. Pharmacol. 3, 113–123.
[6] Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H. Gillette, J., Narasimhulu, S., Cooper, D. Y. and Rosenthal, O. (1966) Mol. Pharmacol. 2, 187–190.
[7] Imai, Y. and Sato, R. (1966) Biochem. Biophys. Res. Commun. 22, 620–626.
[8] Remmer, H., Greim, H., Schenkman, J. B. and Estabrook, R. W. (1967) in: Methods in Enzymol. (Estabrook, R. W. and Pullman, M. E., eds.), Vol. X, pp. 703–708, Academic Press, New York.
[9] Gornall, A. G., BarDawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–766.
[10] Brodie, B. B. and Axelrod, J. (1948) J. Pharmacol. Exptl. Therap. 94, 22–28.
[11] Imai, Y., Ito, A. and Sato, R. (1966) J. Biochem. 60, 417–428.
[12] Schenkman, J. B. (1970) Biochemistry 9, 2081–2091.
[13] Orii, Y. and Okunuki, K. (1964) J. Biochem. 55, 37–48.
[14] Ullrich, V. and Schnabel, K. H. (1973) Drug Metab. Disposition 1, 176–182.