ACTIVATION AND INHIBITION OF MICROSOMAL HYDROXYLATION BY ETHYL ISOCYANIDE

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Liver microsomes catalyze the hydroxylation of various drugs in the presence of NADPH and oxygen. Recent studies have established that a CO-binding hemoprotein called P-450 (Omura and Sato, 1964) is involved in the microsomal hydroxylase system not only as the oxygen activating enzyme (Cooper et al., 1965; Omura et al., 1965) but also as the substrate binding site (Imai and Sato, 1966a; Remmer et al., 1966). It has been reported, on the other hand, that ethyl isocyanide (EtNC) combines with the reduced form of P-450 to give a characteristic spectral change (Omura and Sato, 1964). Spectrophotometric evidence has further been presented that the oxidized form of P-450 is also capable of binding EtNC, with a relatively low affinity (Nishibayashi et al., 1966). The purpose of this communication is to report that EtNC exerts both stimulatory and inhibitory effects at the same time on the microsomal hydroxylase system. The actual consequence caused by the addition of EtNC seems to be determined by the balance of these two effects acting in the opposite directions. It is concluded that competition between EtNC and oxygen for the heme of reduced P-450 is responsible for the inhibitory effect, whereas the stimulatory effect results from the change in reactivity of oxidized P-450 caused by its combination with EtNC.

Liver microsomes were prepared from male rabbits as described by Omura and Sato (1964). Aniline was employed as the substrate for the hydroxylase assay. The standard reaction mixture (final volume, 1.0 ml) contained 0.1 M Tris-acetate buffer (pH 8.0), microsomes (about 1 mg of protein), 20 mM aniline, and an NADPH-

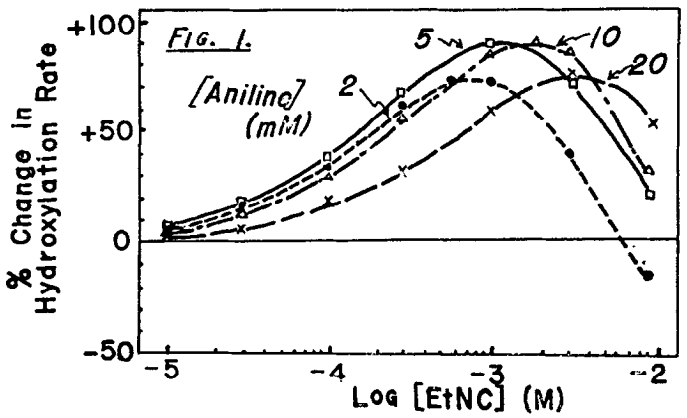

Fig. 1. Effect of EtNC on aniline hydroxylation in air catalyzed by rabbit liver microsomes. The standard assay conditions were used, except that aniline concentration (indicated) was varied and EtNC was added at indicated concentrations.

generating system consisting of 0.5 mM NADP, 2.5 mM $MgCl_2$, 5 mM glucose-6-phosphate, and an excess of glucose-6-phosphate dehydrogenase. The reaction was carried out at 37° for 15 minutes with constant shaking in air, and p-aminophenol formation was determined as described by Imai et al (1966). Thunberg tubes were used for experiments in which the oxygen tension was varied.

Contrary to expectation, it was found that aniline hydroxylation under the standard assay conditions was activated, rather than inhibited, by 10 μM - 1 mM EtNC (Fig. 1). When the EtNC concentration was increased further, gradual decrease in the degree of activation was observed probably due to the appearance of an inhibitory effect. Fig. 1 indicates further that the EtNC concentration giving maximal stimulation was a function of the substrate concentration; the higher the aniline concentration, the more EtNC was needed to attain maximal stimulation. The effect of EtNC was also affected profoundly by the pH of reaction mixture (Fig. 2, Curve A). The hydroxylase activity was activated by 3 mM EtNC at pH values higher than about 7.2, whereas inhibition was observed at lower pH's. In contrast, CO inhibited the reaction to the same extent regardless of the pH employed (pH 6.5 - 9.0). These findings suggested that EtNC, but not

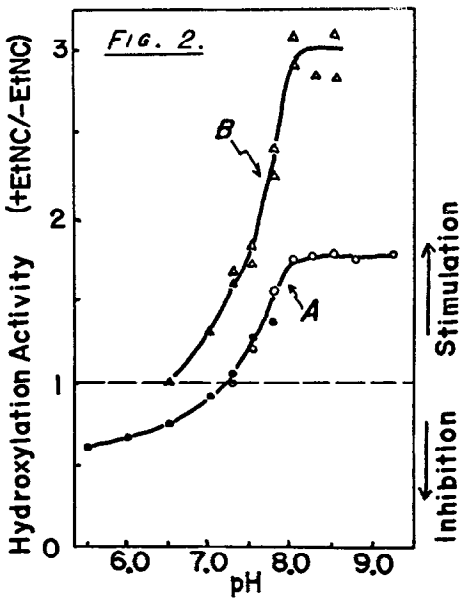

Fig. 2. pH dependence of EtNC effect on aniline hydroxylation. Curve A: Results obtained in air and in the presence of 3 mM EtNC. Standard assay conditions were used except that pH was varied. Curve B: Effect of pH on ideal hydroxylase activity at infinite oxygen tension. Calculated from data such as shown in Fig. 4 (Curve A).
o,△; 0.1 M Tris-acetate.
●,▲; 0.1 M K-phosphate.

CO, exerts an inhibitory effect as well as a stimulatory effect on the hydroxylase system and the two effects are differently influenced by reaction conditions such as pH and the concentrations of substrate and EtNC.

EtNC has been shown to combine with reduced P-450 in competition with CO (Omura and Sato, 1964) and the latter is believed to compete with oxygen for the heme of P-450. It may therefore be assumed that EtNC competes with oxygen for the ferrous iron of the heme of reduced P-450 and this competition is responsible for the inhibitory effect. In accordance with this assumption, it was in fact found that the effect of EtNC on aniline hydroxylation was influenced by the partial pressure of oxygen in the gas phase (Fig. 3). Although the hydroxylase activity in the absence of EtNC was virtually unaffected by changing the oxygen concentration from 5 to 100 %, the activity in the presence of EtNC was increased progressively as the oxygen tension was increased. Moreover, the increase in the activity was more pronounced in the presence of higher concentrations of EtNC. It may therefore be concluded that there is actually competition between EtNC

and oxygen. Fig. 3 also indicates that the apparent K_m of the hydroxylase system for oxygen was dependent on the concentration of EtNC. For instance, the apparent K_m in the presence of 9 mM EtNC was determined to be 288 μM. Such a high value is undoubtedly caused by the competition between EtNC and oxygen for reduced P-450. By assuming such competition and employing the reported value of 6 μM for the dissociation constant of the EtNC compound of reduced P-450 (Imai and Sato, 1966b), the K_m for oxygen in the absence of EtNC was calculated to be 0.19 μM. This value is quite reasonable for the hydroxylase system and supports the validity of the assumed competition.

If the inhibitory effect of EtNC is due to the competition between this reagent and oxygen as concluded above, this effect could be eliminated by running the experiment at infinite oxygen tension; under such an extreme condition EtNC would exert only its stimulatory effect on the hydroxylase activity. The hydroxylase activity at infinite oxygen tension could be readily determined from the Lineweaver-Burk plots of the dependence of activity on oxygen tension (data such as shown in Fig. 3). This "ideal" activity at infinite oxygen tension thus determined would be of great value for the elucidation of the mechanism of stimulation by EtNC. As shown in Fig. 4 (Curves A and B), this ideal activity was a function of both EtNC and aniline concentrations. From the dependence on the EtNC concentration, it was possible to estimate the "K_m" for EtNC in the stimulatory effect ("K_m for stimulation") at a given aniline concentration. Thus, a value of about 1 mM was obtained for the K_m for stimulation at 20 mM aniline. Although the maximal extent of stimulation by EtNC was not very much affected by the aniline concentration, the K_m for stimulation was strongly dependent on it as illustrated in Fig. 5 (Curve A). It therefore seems that the stimulatory effect of EtNC is interfered with by aniline in a sort of "competitive" manner.

Since the oxidized form of P-450 has been reported to combine with EtNC

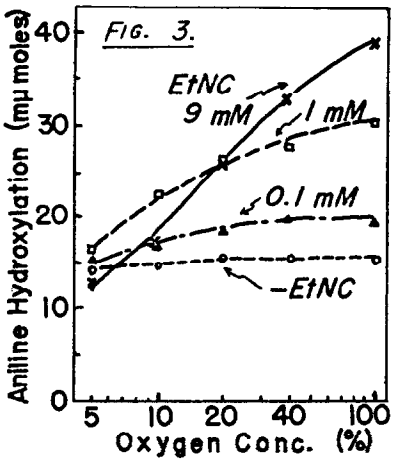

Fig. 3. Effect of oxygen concentration on aniline hydroxylation in the presence and absence of EtNC. Standard assay conditions were used, except that oxygen concentration in the gas phase was varied and EtNC was added as indicated. The gas phase consisted of $O_2 - N_2$ mixture at 1 atm.

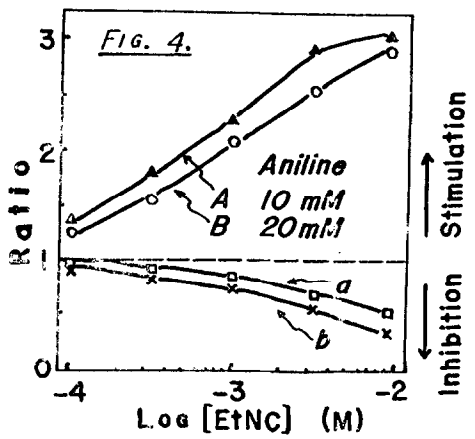

Fig. 4. Concentration dependence of stimulatory and inhibitory effects of EtNC on aniline hydroxylation. Curves A and B: Effect of EtNC concentration on the ratio [ideal activity (+EtNC)] / [ideal activity(-EtNC)]. Curves a and b: Effect of EtNC on the ratio [activity(measured)] / [activity ($O_2 \rightarrow \infty$)]. O_2 concentration: a, 20 %, b, 10 %.

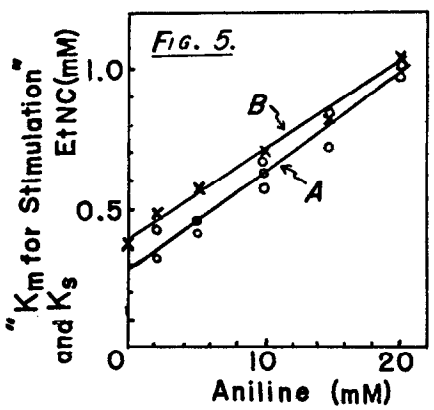

Fig. 5. Effects of aniline concentration on "K_m for stimulation"(Curve A) and apparent dissociation constant (K_s) of EtNC compound of oxidized P-450 (Curve B). K_s was determined spectrophotometrically.

(Nishibayashi et al., 1966) as well as with aniline (Imai and Sato, 1966a; Remmer et al., 1966), it seemed likely that the stimulation is caused by the interaction of EtNC with oxidized P-450 and this interaction is "competitively" prevented by aniline. It was in fact found that the spectrophotometrically determined dissociation constant (K_s) of the EtNC compound of oxidized P-450 was affected by aniline as shown in Fig. 5 (Curve B). The close similarity of this

dependence to that of K_m for stimulation on aniline (Fig. 5, Curve A) supports the aforementioned view. Available circumstantial evidence suggests that the rate-limiting step of the overall hydroxylation process is the reduction of oxidized P-450. It seems therefore likely that the combination of EtNC with oxidized P-450 results in an increase in the rate of reduction of the hemoprotein probably due to either certain structural alterations or changes in oxidation-reduction potential. This was actually supported by the following spectrophotometric observations; while microsomal P-450 remained essentially in the oxidized form in the presence of NADPH under aerobic conditions, the addition of EtNC to this system caused a considerable increase in the reduction level of the hemeprotein.

As mentioned above, pH has a profound effect on the stimulation of the hydroxylase activity by EtNC. This effect could be visualized more clearly by plotting the ideal activity at infinite oxygen tension against pH as shown in Fig. 2 (Curve B). It will be seen that EtNC shows practically no stimulatory effect at pH 6.5, whereas the stimulatory effect becomes maximal at pH 8.0. It may be understood that the pH curve obtained in air (Fig. 2, Curve A) corresponds to balance between the ideal activity and the inhibition. These results suggest that pH exerts a profound effect on the interaction of EtNC with oxidized P-450, but nothing is as yet known of the mechanism of this phenomenon. It should be pointed out here that the interaction of EtNC with reduced P-450 is also affected by pH in a characteristic way (Imai and Sato, 1966b). Although this observation has led to the suggestion that reduced P-450 exists in two forms which are in a pH-dependent equilibrium (Imai and Sato, 1966b), much is still to be explored concerning the effects of pH on P-450.

The use of ideal activity at infinite oxygen tension also permitted the estimation of the inhibition degree by EtNC at a given oxygen tension. This could be determined from the ratio [activity at a given oxygen tension] / [ideal activi-

ty at infinite oxygen tension]. As shown in Fig. 4 (Curves a and b), this ratio decreased with increasing EtNC concentration according to the simple inhibition mechanism. The results shown in Fig. 1 represent the sum of the stimulation (as shown in Fig. 4, Curves A and B) and the inhibition (as shown in Fig. 4, Curve a).

Preliminary experiments showed that similar activation-inhibition effects of EtNC were also observed in the oxidative demethylation of aminopyrine by rabbit liver microsomes. It was further found that in aniline hydroxylation by rat and guinea pig liver microsomes the inhibitory effect of EtNC was considerably more pronounced than that observed in rabbit liver microsomes.

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