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STIMULATION OF THE MOUSE HEPATIC MICROSOMAL ANILINE *p*-HYDROXYLASES BY ACETONE AND POLYAMINES

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Abstract—1. The effects of acetone ± spermine on the high (AH-I) and low (AH-II) affinity forms of aniline hydroxylases in the mouse hepatic microsomes were investigated under *in vitro* conditions.

2. The addition of either acetone or spermine alone stimulated both AH-I and AH-II activities at low concentration while some decline in stimulation was noted at higher concentrations.

3. In the presence of both the modifiers the observed monoxygenation rates were greater than those produced by any one enhancer alone for AH-I and more than additive for AH-II.

4. The results suggest that the enhancement of aniline *p*-hydroxylation by the acetone and spermine in the mouse hepatic microsomes involves at least two separate and possibly interdependent mechanisms.

INTRODUCTION

The polyamines, spermine and spermidine are aliphatic nonprotein nitrogeneous bases of ubiquitous occurrence. In mammalian liver, polyamines occur in millimolar concentrations and exhibit dramatic fluctuations under a variety of experimental conditions (Tabor and Tabor, 1976).

Although the physiological role of polyamines is not at present clearly understood, available information suggests their influence on many cellular processes, especially those involving cell membranes (Tabor and Tabor, 1976). Earlier studies indicated that polyamines, in general, enhance cytochrome *P*-450-dependent hepatic microsomal mixed function oxidase system (Chapman, 1976; Kitada *et al.*, 1980; Andersson *et al.*, 1981; Dalet *et al.*, 1983). In this regard, up to 40% increase in aniline *p*-hydroxylase activity of rat liver microsomes was observed in the presence of polyamines by Chapman (1976) and Kitada *et al.* (1980). Besides polyamines, a number of other chemicals have been shown to modify *in vitro* microsomal oxidation of xenobiotics (Cinti, 1978) of which acetone-enhancement of aniline *p*-hydroxylase has been repeatedly documented (Anders, 1968, 1969, 1972; Cinti, 1978; Anders and Gander, 1979).

The presence of (at least) two kinetically distinguishable forms of aniline *p*-hydroxylase, one with high affinity (low Km, AH-I) and the other with low affinity (high Km, AH-II) toward aniline have been described for hamster (McCoy, 1980) rat (Wada *et al.*, 1968; Ebel, 1981; Bidlack and Lowery, 1982; Ortiz *et al.*, 1982) and mouse (Wada *et al.*, 1968) liver microsomes.

Earlier, Anders (1969, 1972) reported that both acetone and 2,2′-bipyridine share the same mechanism while ethylisocyanide brings about enhancement of aniline hydroxylation by another mechanism. Although a possible involvement of multiple forms of aniline *p*-hydroxylases was postulated in different studies (Wada *et al.*, 1968; McCoy, 1980; Ebel, 1981; Bidlack and Lowery, 1982; Ortiz *et al.*, 1982) the question whether AH-I or AH-II or both are affected by the addition of acetone was not examined. Since hepatic endoplasmic reticulum is in constant contact with polyamines it is of interest to examine acetone-enhancement phenomenon in the presence of polyamines. Our data suggest that separate but possibly inter-dependent mechanisms are involved in the stimulatory response of aniline *p*-hydroxylases to polyamines and acetone.

MATERIALS AND METHODS

Animals

Young male mice (CD_1 strain) of approximately 30 g body weight purchased from Charles River Laboratories were used. Animals were kept on a standard laboratory diet and tapwater *ad libitum* for at least 8 days before sacrifice.

Tissue preparation

The animals were stunned by a blow on the head, decapitated, exsanguinated and quickly hepatectomized. Liver microsomes were prepared by differential centrifugation from 20% homogenate in 50 mM Tris buffer, pH 7.4 containing 0.25 M sucrose and 0.1 mM EDTA as described previously (Kulkarni *et al.*, 1975).

Aniline p-*hydroxylase assay*

The standard assay mixtures containined (3.0 ml final volume) 1.0 mM $NADP^+$, 2.5 mM glucose-6-phosphate; 1.5 units of glucose-6-phosphate dehydrogenase, 0.1 M Tris buffer, pH 8.0; 2–3 mg microsomal protein and indicated final concentrations of aniline, acetone and polyamine. Complete assay mixtures minus aniline were preincubated for 5 min at 37°C. Reactions were initiated by the addition of substrate. Following incubation for 20 min at 37°C, the amount of *p*-aminophenol produced was estimated as reported earlier (Kulkarni and Hodgson, 1982). Incubation mixtures without NADPH generating system served as controls. Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin, fraction V, as standard. The results are reported as relative activity (Mean ± SEM). Relative Activity =

$$\frac{\text{Specific Activity in the Presence of Acetone} \pm \text{Polyamine}}{\text{Basal Activity}}$$

where basal activity represents the specific activity (nmol of *p*-aminophenol produced/hr/mg microsomal protein) observed in the absence of both acetone and polyamine.

RESULTS AND DISCUSSION

Wada *et al.* (1968) observed a biphasic response curve when reciprocal of the velocity was plotted against the reciprocal of aniline concentration and concluded the existance of two or more forms of aniline *p*-hydroxylase activity in the mouse liver microsomes. Recently, McCoy (1980) also reported similar observations and proposed the presence of two aniline *p*-hydroxylases in the hamster liver microsomes. He recommended the use of 0.1 and 20 mM aniline in assay media to estimate the high affinity form (AH-I) and total aniline *p*-hydroxylase (TAH) activity, respectively. The calculated difference between the two activities was presumed to represent the titer of the low affinity form (AH-II). Consistent with this view, Ortiz *et al.* (1982) employed 0.1 and 16 mM aniline to evaluate AH-I, TAH and AH-II activities in the rat liver microsomes. Other induction and inhibition studies (McCoy, 1980; Ebel, 1981; Bidlack and Lowery, 1982) also support the concept of multiplicity of aniline *p*-hydroxylase in the mammalian hepatic microsomes.

Previous reports have shown the relative titer of AH-II in hepatic microsomes to be about 40% in rats (Ortiz *et al.*, 1982) and 40–64% in hamster (McCoy, 1980). In our study we used 0.1 and 15 mM aniline and found that of the total mouse hepatic microsomal *p*-hydroxylase activity (29.1 ± 2.0 nmol of *p*-aminophenol formed/hr/mg microsomal protein), the mean relative contribution of AH-II was about 43% and it varied between 23 and 51% in different microsomal preparations ($n = 15$ each). In view of the considerable variation in AH-II activity, no attempt was made to establish K_m and V_{max} values for the isozymes of aniline hydroxylase present in the mouse hepatic microsomes. It should also be pointed out that the concept of multiplicity of aniline *p*-hydroxylase is not yet firmly established by, for example, reconstitution studies utilizing different forms of purified cytochrome *P*-450 species specific for aniline *p*-hydroxylation to evaluate relative affinity and oxidation rates or by appropriate antibody experiments. Therefore, the usage of the terms AH-I and AH-II in this paper although is in accordance with current convention, possibility exists that these two forms actually represent one or more species of cytochrome *P*-450.

Consistant with previous reports (Anders, 1968, 1969, 1972; Anders and Gander, 1979; Vianio and Hanninen, 1972; Powis *et al.*, 1977; Bidlack and Lowery, 1982) acetone alone was found to stimulate TAH (Fig. 1). Based on observations that acetone increases both K_m for aniline and V_{max} in rat liver microsomes Anders (1968) suggested a possibility of unique susceptibility of AH-I to acetone inhibition leading to its suppression concomitant with an increased expression of AH-II activity. However, our data (Figs 2 and 3) on the mouse hepatic microsomes indicate that both the isozymes are stimulated by acetone. The magnitude of AH-I and AH-II stimulation however, was not equal and was related to acetone concentration used. A maximum increase of

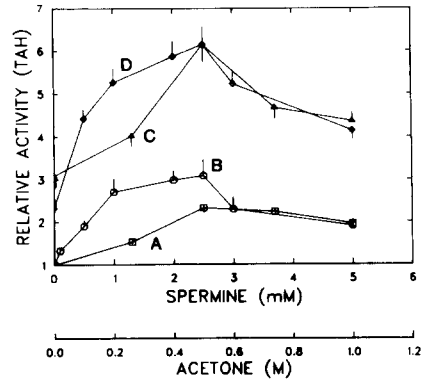

Fig. 1. Effects of acetone and spermine on the total aniline *p*-hydroxylase activity (TAH) of the mouse hepatic microsomes. The incubation mixtures contained microsomes, 15 mM aniline, NADPH generating system and either 0–0.9 M acetone (line A); 0–5.0 mM spermine (line B); 2.5 mM spermine and 0–0.9 M spermine (line C) or 0.45 M acetone and 0–5.0 mM spermine (line D). Each point represents mean of relative specific activity (= 1 in control) ± SE ($N = 4$–8 separate experiments). The basal (TAH) activity in the absence of both acetone and spermine was 29.1 ± 2.0 nmol of *p*-aminophenol produced/hr/mg microsomal protein ($N = 15$). See Materials and Methods for further details.

about 160% in AH-I activity (Fig. 2, line A) and of about 100% in AH-II activity (Fig. 3, line A) over control values was observed in the presence of 0.45 M and 0.68 M acetone respectively. Apparently, these observations do not represent a species-specific difference in response to acetone since with rat liver microsomes also, Bidlack and Lowery (1982) observed acetone to produce a general increase in hydroxylase activity at a lower aniline concentration and a specific increase in activity with >0.75 mM

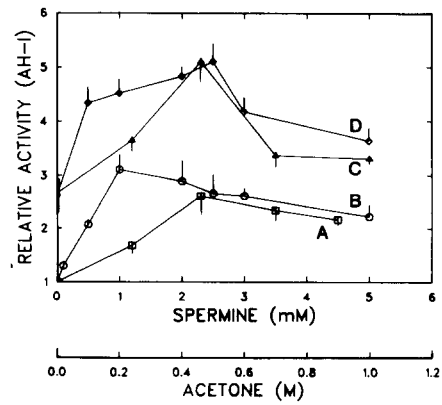

Fig. 2. Effects of acetone and spermine on the AH-I activity of the mouse hepatic microsomes. The assay media contained microsomes, 0.1 mM aniline, NADPH generating system and either 0–0.9 M acetone (line A); 0–5.0 mM spermine (line B); 2.5 mM spermine and 0–0.9 M acetone (line C) or 0.45 M acetone and 0–5.0 mM spermine (line D). each point represents mean of relative specific activity (= 1.0 control) ± SE ($N = 4$–8 separate experiments). The basal AH-I activity in the absence of both acetone and spermine was 16.5 ± 1.3 nmol of *p*-aminophenol produced/hr/mg microsomal protein $N = 15$). See Materials and Methods for futher details.

aniline. Similarly, Anders and Gander (1979) reported a marked dependence of acetone enhancement on aniline concentration and the stimulation, in general, was greater in NADPH/oxygen as well as cumene hydroperoxide supported reactions when lower aniline concentrations were used.

Although in all the previous investigations (Anders, 1968, 1969, 1972; Anders and Gander, 1979; Vianio and Hanninen, 1972; Powis *et al.*, 1977; Bidlack and Lowery, 1982), only the stimulatory effect of acetone had been the focal point of proposed mechanisms, our data (Figs 1–3) indicate that at higher acetone concentrations, the activity–response curves for TAH, AH-I as well as AH-II do not plateau but rather exhibit a gradual decline in stimulation. This was also noted by Anders (1968) with rat liver microsomes and by Kitada *et al.* (1983) in the reconstituted systems. Our data, considered together with the known inhibitory effects of acetone on several other monooxygenase reactions (Cinti, 1978; Kawalek and Andrews, 1980) suggest that at any given concentration acetone exerts both stimulatory as well as inhibitory effects on aniline *p*-hydroxylation. The observed activity, therefore, represents the net balance between these two opposing effects. In the mouse liver microsomes, the equilibrium appears to be in favor of maximum stimulation when an acetone concentration of about 0.45 M in the case of AH-I and 0.68 M in the case of AH-II was used.

It is currently believed that acetone enhancement of aniline *p*-hydroxylation probably involves either facilitated introduction of second electron (Powis *et al.*, 1977), a site for formation or insertion of activated oxygen into the substrate (Anders and Gander, 1979) and/or multiple forms of aniline *p*-hydroxylase

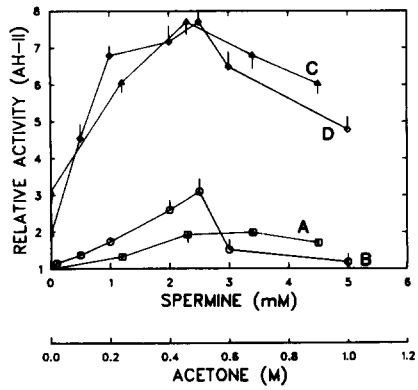

Fig. 3. Effects of acetone and spermine on the AH-II activity of the mouse hepatic microsomes. The AH-II activity was calculated by substracting AH-I activity from TAH activity observed in the assay media containing microsomes, 0.1 or 15 mM aniline, NADPH generating system and either 0–0.9 M acetone (line A); 0–5.0 mM spermine (line B); 2.5 mM spermine and 0–0.9 M acetone (line C) or 0.45 M acetone and 0–5.0 mM spermine. Each value represents mean of relative AH-II activity (= 1.0 in control ± SEM). The calculated basal AH-II activity in the absence of both acetone and spermine was 12.5 ± 2.8 nmol of *p*-aminophenol produced/hr/mg microsomal protein ($N = 15$). The data on TAH and AH-I activity are given in Figs 1 and 2. See text for further details.

(Bidlack and Lowery, 1982). For a variety of reasons, it is believed that the type-I and type-II binding of aniline to cytochrome *P*-450, and lipid peroxidation do not play a significant role (Cinti, 1978; Anders and Gander, 1979; Bidlack and Lowery, 1982). Conflicting results have been reported in the literature as regards the role of cytochrome *P*-450 reduction rate in the acetone stimulated aniline hydroxylation. Thus Vainio and Hanninen (1972) reported that acetone partly reverses the inhibition of NADPH-cytochrome *P*-450 reductase produced by aniline and suggested that this might partly explain the enhancing effects of acetone. The results of reconstitution studies (Kitada *et al.*, 1983) with phenobarbital inducible form of cytochrome *P*-450 are in favor of this view. In contrast to this, Powis *et al.* (1977) reported that acetone inhibits NADPH-cytochrome *P*-450 reductase and the inhibitory effects were not additive in the presence of aniline. A possibility that this reductase may not be the locus of enhancement is apparent from the reported stimulation of hepatic microsomal aniline hydroxylation by acetone in the cumene hydroperoxide-mediated reaction (Anders and Gander, 1979). However, it should be pointed out that Kitada *et al.* (1983) failed to observe such a stimulation in the cumene hydroperoxide-supplemented reconstituted systems containing either cytochrome *P*-450 or *P*-448.

Similar to acetone, spermine alone also caused significant enhancement of aniline *p*-hydroxylase activity of the mouse hepatic microsomes (Figs 1–3). The spermine concentration as low as 100 μM produced a detectable increase (15–30%) in activity. In contrast to acetone which caused relatively more stimulation of AH-I activity, the stimulatory effects of spermine were nearly equal on both the forms (Figs 2 and 3). A maximum of about a 3-fold increase was observed when optimal concentration of spermine was present in the incubation medium. Earlier, Chapman (1976) and Kitada *et al.* (1983) reported, respectively, only about a 40 and 8% increase in aniline *p*-hydroxylation by rat liver preparations in the presence of 1–5 mM spermine. A higher degree of stimulation noted in our study with the mouse hepatic microsomes, therefore, may be due to differences in the methodology or represents a true species-specific difference.

The effects of both acetone and spermine on aniline *p*-hydroxylation are also shown in Figs 1–3. In each case, the observed value for TAH activity (Fig. 1) was found to be either equal to or greater than the expected value (based on activity noted in the presence of either acetone or spermine). The two forms of the enzyme, however, were not equally affected and as compared to AH-I the magnitude of AH-II stimulation was much greater (Figs 2 and 3 lines C and D). Except for the reaction media containing 0.45 M acetone, the results, in general show a simple additive or less than additive effect on AH-I (Fig. 2) and greater than additive effects on AH-II (Fig. 3). The decrease in the magnitude of stimulation of TAH, AH-I and AH-II when >2.5 mM spermine was used in the presence or absence of acetone (Figs 1–3; lines B and D) may be related to aggregating effects of spermine on microsomal vesicles (Tadolini, 1980). Such an alteration may decrease the total number of

accessible binding sites which in turn will render availability of NADPH and possibly aniline rate limiting in overall monooxygenation process.

When aniline concentration was varied (0.1–15.0 mM) in the reaction media containing 2.5 mM spermine and 0.45 M acetone, a maximum stimulation of about 6-fold was observed with 2.5 mM aniline (Fig. 4, line A). The magnitude of stimulation did not increase further with higher aniline concentration. This suggests a general increased affinity of isozymes for aniline. Under these conditions, 2.5 mM aniline apparently saturates both the isozymes, and the observed rate, therefore, represents a total aniline *p*-hydroxylase activity. The results also suggest that in the presence of both acetone and spermine, high aniline concentration (5.0–15.0 mM) is not inhibitory. In the absence of enhancing agents, aniline has been shown to cause substrate inhibition at high concentrations (Ebel, 1981). The data given in Fig. 4, indicate that at 2.5 mM aniline, maximum stimulation is observed when 0.45 M acetone (line C) or 2.5 mM spermine (line B) alone was used. However, in the presence of both the enhancing agents greater than additive effects were observed. The stimulation was dependent upon amount of acetone present into the incubation media and peaked at 0.45 M (line D).

Similar experiments were conducted in the presence of spermidine and putrescine (the data not shown). A maximum stimulation of about 5 fold in the aniline *p*-hydroxylases was observed in the presence of 0.68 M acetone, 3.0 mM spermidine and 2.5 mM aniline. A similarity between the effects of spermidine and spermine includes that both caused

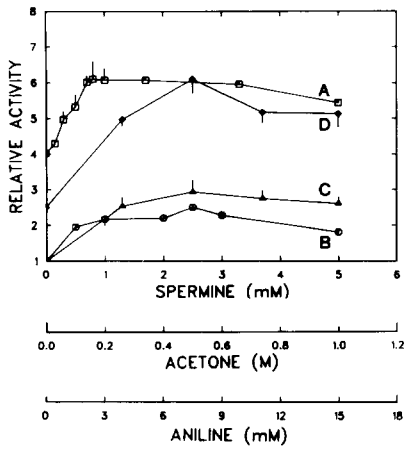

Fig. 4. Effects of acetone and spermine on the aniline *p*-hydroxylase activity of the mouse hepatic microsomes. The incubation media contained microsomes, NADPH generating system and either 2.5 mM spermine, 0.45 M acetone and 0.1–15 mM aniline (line A); 2.5 mM aniline and 0–5 mM spermine (line B); 2.5 mM aniline and 0–0.9 M acetone (line-C); or 2.5 mM aniline, 2.5 mM spermine and 0–0.9 M acetone (line D). Each point represents mean of relative specific activity (= 1.0 in control) ± SE. The basal activities in the absence of both acetone and spermine using 2.5 (lines B, C and D) and 0.1 (line A) mM aniline were 36.6 ± 4.1 and 21.2 ± 2.7 nmol of *p*-aminophenol produced/hr/mg microsomal protein ($N = 4$–8 separate experiments). See text for further details.

relatively more stimulation of AH-II activity. Only marginal increase in the aniline *p*-hydroxylases were observed in the presence of low concentrations of putrescine while high levels were found to be inhibitory.

Kitada *et al.* (1980) observed no significant increase in ethylmorphine N-demethylation by either spermine, spermidine or putrescine in a reconstituted monooxygenase system consisting of purified cytochrome *P*-450, NADPH-cytochrome *P*-450 reductase, both isolated from phenobarbital pretreated rats and either extracted lipids or dilauroyl-L-3-phosphatidylcholine. However, stimulation was observed when hepatic microsomes were used. In contrast to these results, Andersson *et al.* (1981) working with a reconstituted system containing cytochrome *P*-450 LM_2 or LM_4, NADPH-cytochrome *P*-450 reductase, both isolated from either untreated or phenobarbital pretreated rabbits and dilauroylglyceryl-3-phosphorylcholine observed 3- to 4-fold stimulation of benzphetamine and 7-ethoxycoumarin dealkylation by spermine. Similar data were also reported for the monooxygenation of benzphetamine, 7-ethoxycoumarin, benzo(a)pyrene and testosterone by liver microsomes from human and untreated or phenobarbital pretreated rabbits (Dalet *et al.*, 1983). These results suggest some specificity in the stimulation of monooxygenation reactions by polyamines. In mammalian hepatic microsomes phosphatidyl serine is localized primarily on the cytosolic side (Nilsson and Dallner, 1975) giving this surface a higher negative charge density than the canalicular surface. Neutralization of these negative charges by organic polycations such as polyamines is expected to bring about stabilizing effects on membranes. Thus, removal of physical constraints from microsomal membranes and/or an allosteric effect of polyamines on the cytochrome *P*-450 molecule may be involved. Apparently, the specificity and the magnitude of this effect depend not only upon the species of cytochrome *P*-450 in question but also upon the substrate and other factors such as its surrounding phospholipid environment.

The actual underlying molecular events leading to increased enzyme activity by polyamines are unclear at present and do not probably include effects on substrate binding to cytochrome *P*-450 (Kitada *et al.*, 1980; Andersson *et al.*, 1981; Dalet *et al.*, 1983; Osimitz and Kulkarni, 1984). Recently Kitada *et al.* (1983) have proposed that the inhibitory effects of polyamines on lipid peroxidation might be responsible for their stimulatory effects on monooxygenation reactions. The role of this factor in the observed stimulation of aniline *p*-hydroxylation is questionable since we (Kulkarni and Hodgson, 1981) have earlier shown that in the mouse hepatic microsomes aniline itself inhibits lipid peroxidation. Our laboratory has investigated the effects of polyamines on the different steps of cytochrome *P*-450-mediated reaction in the mouse hepatic microsomes (Osimitz and Kulkarni, 1984). For example, spermidine significantly increased the rate of NADPH oxidation and NADPH-dependent cytochrome *P*-450 reduction while redox behaviour of cytochrome b_5 and H_2O_2 generation rates were unaffected suggesting that efficient coupling of NADPH utilization may be

one of the mechanisms of polyamine-caused stimulation of aniline hydroxylation. In view of the complexity noted in the interpretation of data from preliminary experiments, further attempts to elucidate the effects of acetone–polyamine combination on the individual steps of monooxygenation reaction cycle were discontinued.

The observed additive effects clearly suggest that two separate mechanisms are involved in the enhancement of aniline *p*-hydroxylation by acetone and polyamines. The greater than additive effects indicate inter-dependency of the polyamine stimulation on acetone effects. Anders and Gander (1979) suggested that acetone removes a rate limiting factor by exerting its effect at or near the formation or insertion of the activation oxygen. The reported lack of stimulation by polyamines of either H_2O_2 (Kitada *et al.*, 1980) or cumene hydroperoxide (Dalet *et al.*, 1983) supported monooxygenation reactions and the fact that some of the experiments described in this paper were performed in the presence of acetone concentrations that produced maximum stimulation (in the absence of polyamines), suggest that the polyamine-caused further stimulation of aniline *p*-hydroxylation involves additional mechanism(s) other than the sites for oxygen activation or insertion. Alternately, it must be presumed that these steps remain rate limiting in NADPH supported systems even in the presence of acetone.

Dalet *et al.* (1983) noted increased rates of 7-ethoxycoumarin O-deethylation by rabbit liver microsomes due to relatively smaller spermine addition to reaction media containing NADH as well as NADPH. Based on the observations that the NADH–NADPH synergism can only be observed in the presence of acetone Powis *et al.* (1977) postulated that acetone facilitates the transfer of second electron to oxyferrous complex and this may be the rate limiting step in aniline *p*-hydroxylation. These authors also suggested that enhancing agents relieve the aniline caused inhibition of transfer of second electron derived from NADPH. Earlier Oshino and Sato (1971) reported enhancement of microsomal cytochrome b_5 reoxidation by aniline as well as *p*-aminophenol, the oxidation product of aniline. An increase in aniline hydroxylation rate is expected if the enhancing agents produce "sparing effects" on electrons derived from NADPH by blocking uncoupling caused by aniline and *p*-aminophenol. Although the reported stimulation of NADH oxidation by the enhancing agents (Powis *et al.*, 1977) is in favor of this hypothesis, Stevens *et al.* (1972) did not observe inhibition of aniline *p*-hydroxylation by the exogenously added excess *p*-aminophenol. In any case the role of cytochrome b_5 becomes obscure since polyamines do not alter reduction and reoxidation rate of cytochrome b_5 in the mouse hepatic microsomes (Osimitz and Kulkarni, 1984) but can stimulate monooxygenation reaction in the reconstituted system in the absence of this hemoprotein (Andersson *et al.*, 1981; Dalet *et al.*, 1983). However, a faster introduction of second electron via NADPH-cytochrome *P*-450 reductase is likely since we (Osimitz and Kulkarni, 1984) have observed that polyamines significantly stimulate this activity in the mouse hepatic microsomes. Thus, in conclusion it is believed that the stimulation of aniline *p*-hydroxylation in the presence of both acetone and polyamines involves more than one of these mechanisms which operate in a concerted fashion. The results also clearly indicate that mammalian hepatic microsomes possess much greater capacity to hydroxylate aniline than previously realized by routine assays in the presence of either NADPH alone or NADPH plus acetone.

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