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NADH SYNERGISM OF MICROSOMAL ANILINE METABOLISM IN THE PRESENCE OF ENHANCING AGENTS

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Abstract—NADH, which normally has no effect upon the NADPH-dependent metabolism of aniline by the microsomal fraction, exerted a synergistic effect in the presence of various enhancing agents, including acetone. Acetone enhanced the rate of aniline metabolism but inhibited the NADPH-dependent reduction of cytochrome P-450, showing that this is not the rate-limiting step in the metabolism of aniline. It is suggested, however, that acetone might facilitate the transfer of the second electron to cytochrome P-450 and that this may normally be the rate limiting step in the metabolism of aniline. The increase in metabolism did not, of itself, appear to be responsible for the synergistic effect exhibited by NADH in the presence of enhancing agents.

Various compounds have been found to enhance the metabolism of aniline, a type II substrate, by the hepatic microsomal fraction *in vitro*, following their addition to the reaction mixture [1, 2]. The metabolism of type I substrates is however, unaffected or even inhibited by these enhancing agents.

The metabolism of type I substrates by the microsomal fraction in the presence of an excess of NADPH is further stimulated by the addition of NADH [3, 4]. The metabolism of type II substrates, on the other hand, does not exhibit this rate-increasing synergism between NADH and NADPH [5]. It has been suggested that the synergistic effect of NADH may be explained by NADH supplying the second electron to the oxygenated cytochrome P-450-substrate complex preferentially to, and more rapidly than, NADPH [6, 7], or that it may represent an electron-sparing effect by NADH for NADPH [8, 9].

The present work suggests that there may be more than just a casual relationship between these two apparently unrelated phenomena, the enhancement of aniline metabolism and NADH synergism. In the presence of enhancing agents NADH exerts a synergistic effect on the metabolism of a type II substrate, and this may not simply be a consequence of the increased rate of metabolism. It is suggested that enhancing agents might be producing their effect on the metabolism of type II substrates by facilitating the transfer of the second electron to the cytochrome P-450 complex.

MATERIALS AND METHODS

Preparation of the microsomal fraction. The microsomal fraction from the livers of male Wistar albino rats, weighing between 200 and 250 g, was prepared by the method of Ernster *et al.* [10] in 0.25 M sucrose containing 0.05 M Tris–HCl buffer, pH 7.4, and adjusted to give a protein concentration of around 8 mg/ml. Protein was determined by the method of Lowry *et al.* [11] with crystalline bovine serum albumin as a standard.

Assay. The metabolism of foreign compounds by the freshly prepared hepatic microsomal fraction was measured over 30 min at 37° with a reaction mixture containing 4 mg of microsomal protein, Tris–HCl buffer, pH 7.4 (112 μmole), $MgCl_2$(10 μmole), nicotinamide (50 μmole), KCN (0.8 μmole), glucose-6-phosphate (25 μmole), glucose-6-phosphate: $NADP^+$ oxidoreductase (EC 1.1.1.49) (2 units), $NADP^+$ (1 μmole), NADH (2 μmole) as appropriate, and either ethylmorphine HCl (10 μmole) or aniline HCl (5 μmole), all in a final incubation vol of 2 ml. Formaldehyde formed from ethylmorphine was trapped with semicarbazide HCl (10 μmole) and measured by the method of Nash [12]. The formation of *p*-aminophenol from aniline was measured by the method of Schenkman *et al.* [13]. The enhancing agent paraoxon interferes with the colour reaction for *p*-aminophenol and in these studies *p*-aminophenol was first extracted with organic solvent as described by Mazel [14]. In some studies the disappearance of aniline from the reaction mixture was measured directly by the method of Bratton and Marshall [15]. The oxidation of NADH and NADPH by the microsomal fraction was measured at 340 nm by the method of Netter and Illing [16] in the presence of 0.2 mM EDTA to inhibit the microsomal nucleotide pyrophosphatase [17]. NADPH-cytochrome P-450 reductase was measured by a modification of the method of Gigon *et al.* [18] as described previously [19]. The enhancing agents employed were acetone [20], 2,2′-bipyridyl [21], metyrapone [22], paraoxon [23], and 1,10-phenanthroline [21], all at concentrations previously found to produce the maximum increase in aniline metabolism [24].

RESULTS

Effects of acetone and cyanide upon the synergistic effect of NADH. Figure 1 shows that NADH failed to exert a synergistic effect upon the metabolism of aniline, a type II substrate, thus confirming previous reports [5, 25]. The addition of acetone to the reac-

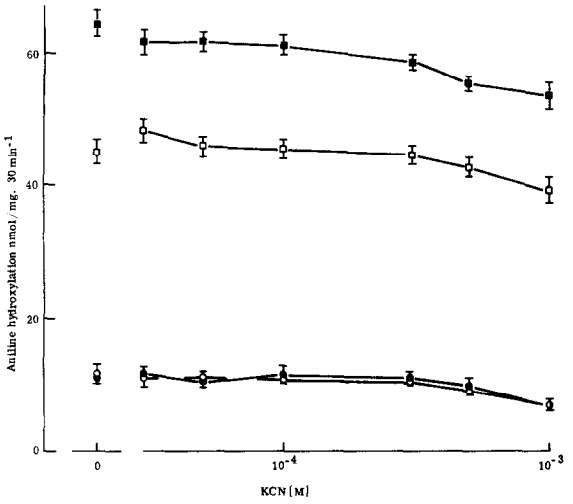

Fig. 1. Effect of acetone and cyanide upon the synergistic effect of NADH on the metabolism of aniline. Metabolism was determined as described in the text at various cyanide concentrations ○, with a NADPH-generating system; ●, with a NADPH-generating system and 1 mM NADH; □, with a NADPH-generating system in the presence of 0.75 M acetone; ■, with a NADPH-generating system and 1 mM NADH in the presence of 0.75 M acetone. Each point is the mean of four observations and bars represent S.E.M.

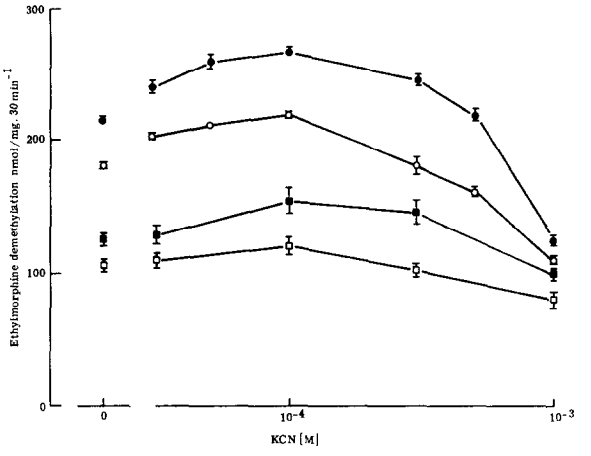

Fig. 2. Effect of acetone and cyanide upon the synergistic effect of NADH on the metabolism of ethylmorphine. Metabolism was determined as described in the text at various cyanide concentrations ○, with a NADPH-generating system; ●, with a NADPH-generating system and 1 mM NADH; □, with a NADPH-generating system in the presence of 0.75 M acetone; ■, with a NADPH-generating system and 1 mM NADH in the presence of 0.75 acetone. Each point is the mean of four observations and bars represent S.E.M.

tion mixture resulted in the enhancement of aniline metabolism, as expected [1, 2], and also in the appearance of a synergistic effect by NADH. Similar effects were noted when aniline metabolism was measured over a much shorter period, 10 min, when the rate of metabolism was linear. For convenience and ease of measurement, however, a 30-min incubation period was routinely employed. Acetone also produced an inhibition in the metabolism of ethylmorphine, a type I substrate, and produced no further increase in the synergistic effect of NADH upon the metabolism of ethylmorphine (Fig. 2).

Cyanide had no effect upon the metabolism of aniline, apart from a small inhibition at higher concentrations, nor upon the enhancement of aniline metabolism by acetone, nor upon the synergistic effect of NADH in the presence of acetone (Fig. 1). This contrasts with the small potentiation of the synergistic effect of NADH upon the metabolism of ethylmorphine by cyanide, an effect attributed to an inhibition of the microsomal stearate desaturase system [5] and an inhibition of ethylmorphine metabolism in the absence of acetone above 0.3 mM KCN (Fig. 2). The effects of cyanide were, however, somewhat variable and in this series of experiments cyanide had less of an inhibitory effect upon ethylmorphine metabolism than in another series of experiments shown in Table 1.

Effect of various enhancing agents upon the synergistic effect of NADH. Table 1 shows the effect of various enhancing agents upon the synergistic effect of NADH upon the metabolism of aniline and ethylmorphine. KCN was included in the reaction mixture at a concentration, 0.4 mM, known to produce almost a 90 per cent inhibition of the microsomal stearate desaturase pathway [25] although it had no observable effect upon aniline metabolism. In the presence of all the enhancing agents tested, excepted for metyrapone, NADH exerted a synergistic effect upon the metabolism of aniline, of a similar magnitude to its effect upon the metabolism of ethylmorphine. This suggests therefore that enhancing agents produce their effect by stimulating the transfer of electrons to

Table 1. Effect of enhancing agents upon the NADH-mediated synergism of the hepatic microsomal mixed function oxidase

	Aniline hydroxylation		Ethylmorphine demethylation	
	NADPH (nmole/mg 30 min^{-1})	NADPH + NADH (nmole/mg 30 min^{-1})	NADPH (nmole/mg 30 min^{-1})	NADPH + NADH (nmole/mg 30 min^{-1})
Control	11.1 ± 1.0	12.5 ± 1.0	131.3 ± 4.5	179.3 ± 9.9†
Acetone 0.75 M	44.1 ± 1.2	58.5 ± 0.9†	104.5 ± 3.0	146.0 ± 9.9†
2,2'-bipyridyl 2.5 mM	28.9 ± 1.7	35.0 ± 0.7*	97.8 ± 4.5	123.0 ± 3.5†
Metyrapone 1 mM	13.8 ± 0.3	14.5 ± 0.9	72.8 ± 9.9	93.8 ± 5.3
Paraoxon 5 mM	31.0 ± 3.7	52.8 ± 2.4†	85.3 ± 5.3	114.8 ± 4.5†
1,10-phenathroline 4 mM	21.9 ± 0.3	26.0 ± 0.3†	79.5 ± 3.0	62.8 ± 8.6

Metabolism was determined as described in the text, in the presence of 0.4 mM KCN, with a NADPH-generating system. NADH was added at a concentration of 1 mM. Values are the mean ± S.E.M. of four observations. *$P < 0.05$, †$P < 0.01$ compared to the value in the absence of NADH.

Table 2. Effect of enhancing agents upon the oxidation of NADPH and NADH by the hepatic microsomal fraction

	NADH metabolism (nmole/mg min^{-1})	NADPH metabolism (nmole/mg min^{-1})
Control	7.7 ± 1.4	18.4 ± 0.8
Acetone 0.75 M	15.7 ± 1.7*	25.6 ± 1.4†
2,2'-bipyridyl 2.5 mM	11.6 ± 0.4*	25.8 ± 1.1†
Paraoxon 5 mM	16.5 ± 0.5†	29.7 ± 0.5†

The metabolism of NADH or NADPH, at an initial concentration of 33 μM, was measured by the decreased absorption at 340 nm over 90 s, at 25°. The reaction mixture contained Tris–HCl buffer pH 7.4, 0.1 M, nicotinamide 0.4 mM, EDTA disodium 0.2 mM, potassium cyanide 0.4 mM, $MgCl_2$ 3 mM, and a microsomal protein concentration of 0.5 mg/ml. Values are the mean ±S.E.M. of four determinations. *$P < 0.05$, †$P < 0.01$ compared to the appropriate control.

cytochrome P-450 rather than through an action on the stearate desaturase pathway, as has been suggested by Soliman *et al.* [26]. A similar effect of acetone and NADH was observed when the disappearance of aniline from the reaction mixture was measured directly (results not shown). The synergistic effect of NADH upon the metabolism of ethylmorphine was relatively unaffected by the presence of acetone, 2,2'-bipyridyl and paraoxon but was inhibited by metyrapone and 1,10-phenanthroline.

Effect of enhancing agents upon the oxidation of NADH and NADPH by the hepatic microsomal fraction. The enhancing agents tested all produced an increase in the oxidation of both NADH and NADPH by the microsomal fraction in the absence of any substrate for the mixed function oxidase (Table 2) although of course the concentration of nucleotides used was much lower than those employed in the studies on ethylmorphine and aniline metabolism. Similar changes were found in the presence of either 5 mM aminopyrine or 5 mM aniline, both of which themselves produced a small increase in the metabolism of NADH and NADPH (these results are not shown).

Effect of acetone upon NADPH-cytochrome P-450 reductase. Type II substrates such as aniline have been found to inhibit the NADPH dependent reduction of microsomal cytochrome P-450 [18] which, it has been suggested, may represent the rate-limiting step in the microsomal metabolism of foreign compounds [27]. Vainio and Hanninen [8] have reported that acetone partly reverses the inhibition of NADPH–cytochrome P-450 reductase produced by aniline and they have suggested that this might explain, at least in part, the enhancing effect of acetone. We have been unable to confirm this finding and we have found that acetone inhibits NADPH–cytochrome P-450 reductase, in the absence and in the presence of a type I substrate such as aminopyrine (Fig. 3). The inhibitory effects of aniline and acetone do not however, appear to be additive.

Relationship between the rate of metabolism and NADH synergism. There appears to be no direct relationship between the rate of ethylmorphine metabolism and the magnitude of the synergistic effect of

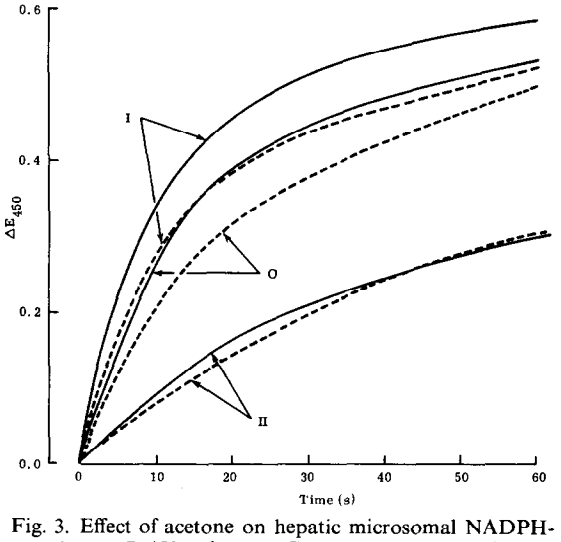

Fig. 3. Effect of acetone on hepatic microsomal NADPH-cytochrome P-450 reductase. Cytochrome P-450 reductase was determined as described in the text, 0 in the absence of any added substrate, I, in the presence of a type I substrate (3 mM aminopyrine) and II, in the presence of type II substrate (12 mM aniline). The broken line shows the effect of 0.75 M acetone. Each line represents the mean of four determinations.

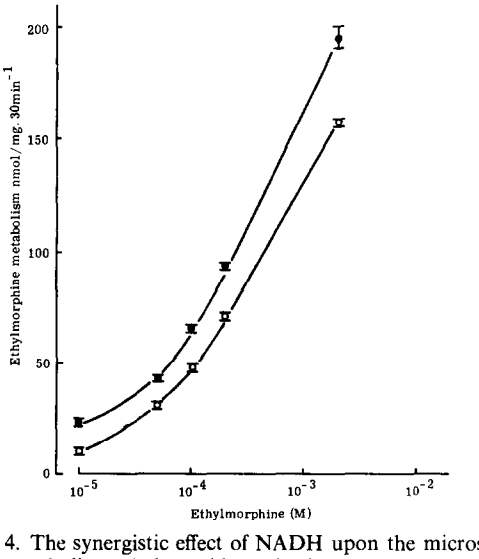

Fig. 4. The synergistic effect of NADH upon the microsomal metabolism ethylmorphine. Metabolism was measured as described in the text over a period of 30 min with either O, a NADPH-generating system or ●, a NADPH-generating system and NADH added at an initial concentration of 1 mM. Each point is the mean of six observations and bars represent S.E.M.

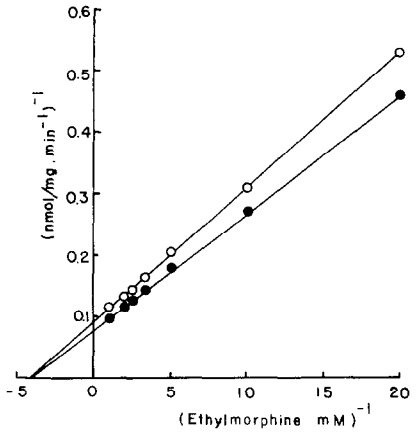

Fig. 5. The synergistic effect of NADH upon the intial rate of ethylmorphine metabolism. Metabolism was measured over a period of 5 min with either ○, a NADPH-generating system or ●, a NADPH-generating system and NADH added at an initial concentration of 1 mM.

NADH. When the concentration of ethylmorphine was lowered and became rate-limiting NADH still exerted a synergistic effect, even at the lowest rates of metabolism (Fig. 4). These lower rates are comparable with the control rates of aniline metabolism where NADH exerted no synergistic effect. Fig. 5 is included to show the effect of NADH upon the initial rates of ethylmorphine metabolism, measured over the first 5 min when the rate of metabolism is linear. NADH had no effect upon the affinity of ethylmorphine for the enzyme system but increased the maximal rate of metabolism. The synergistic effect of NADH was apparent even at the lowest rates of metabolism. NADH exerted no synergistic effect upon the initial rate of aniline metabolism measured in a similar manner (these results are not shown).

DISCUSSION

NADPH–cytochrome P-450 reductase has been suggested to be the rate-limiting step in the microsomal metabolism of foreign compounds [27] and it is thought that the binding of type I substrates to cytochrome P-450 may change the oxidation–reduction potential of the complex to a level where it can more readily accept electrons at the first and also the second electron transfer sites [5]. NADPH–cytochrome P-450 reductase is, however, unlikely to be the rate-limiting step in the metabolism of aniline since the results obtained in the present study show that acetone, which enhances the metabolism of aniline, fails to stimulate NADPH–cytochrome P-450 reductase. Correia and Mannering [5] have pointed out that there is evidence from work on cytochrome $P\text{-}450_{cam}$ [29] that the overall rate of hydroxylation reactions may be controlled by the rate of introduction of the second rather than the first electron. Aniline normally appears to inhibit the transfer of the first electron, as shown by a decreased rate of reduction of cytochrome P-450 by NADPH, and it might be having a similar inhibitory effect upon the transfer of the second electron. Enhancing agents possibly act to overcome this inhibition.

It has been suggested that NADH can supply the second electron to the oxygenated cytochrome P-450 substrate complex preferentially to, and more rapidly than NADPH [6, 7], and the appearance of a synergistic effect of NADH upon the metabolism of aniline in the presence of enhancing agents argues for a facilitation of the transfer of the second electron being the mechanism of action of enhancing agents. There is however a caveat to such a suggestion for it is implicit in the original suggestion that NADH may supply the second electron at a faster rate than NADPH [5, 6, 7] that the synergistic effect of NADH is dependent upon the rate of turnover of cytochrome P-450. With a type II substrate, such as aniline it was suggested that the rate of metabolism would not be sufficient to increase the demand for the second electron above that which could be supplied by NADPH [5]. An increase in the rate of metabolism of aniline leading to an increased turnover of cytochrome P-450 would therefore be expected to lead to an increased synergistic effect of NADH. Results obtained in the present study would suggest however, that there is no simple relationship between the rate of metabolism and the extent of NADH synergism, at least for the metabolism of ethylmorphine and the same may be true for aniline. The simplest explanation for the effect of enhancing agents in promoting NADH synergism and aniline metabolism is that they act at a point common to both processes, that is by facilitating transfer of the second electron.

The synergistic effect of NADH upon the metabolism of type I substrates has also been explained as an electron sparing effect by NADH for NADPH when the substrate is a partial uncoupler of the microsomal mixed function oxidase [8]. On the basis of this theory it might be expected that substrates such as aniline, which exhibit no synergistic effect by NADH, are tightly coupled and that enhancing agents can promote an uncoupling leading to the appearance of a synergistic effect. It would also be expected that uncoupling by enhancing agents would then lead to a decrease in the rate of aniline metabolism rather than an increase, as is observed. It is only possible to reconcile the findings of both enhancement and synergism with this theory if it is assumed that they are two completely independent phenomena.

There remains the problem of the relationship of the type I binding site on the cytochrome P-450 to the phenomenon of enhancement. It is known that enhancing agents require an intact type I binding site to produce their effect [24, 2] and Vainio and Hanninen [28] have suggested that there might be a correlation between the enhancement of aniline metabolism by acetone and their observation of an increased type I spectral change produced by a mixture of aniline and acetone. We have, however, been unable to repeat this observation. Netter and Illing [16] have suggested that the second electron is preferentially donated by NADH only to a type I substrate-cytochrome P-450-oxygen complex, which can be detected by its absorption at 440 nm [28]. Type II substrates, such as aniline, do not form such intermediary complexes. There is however, no evidence for the formation of an intermediary complex by aniline in the presence of acetone, or by acetone itself (Powis, un-

published observations). Thus, although a type I site is required for the enhancing action of acetone it does not appear that aniline is transformed into a type I substrate.

In conclusion therefore it has been shown that enhancing agents lead to an increase in the rate of aniline metabolism and the appearance of a synergistic effect by NADH, but indirect evidence suggests that the increase in the rate of metabolism may not be directly related to the appearance of NADH synergism. Rather, it is suggested, enhancing agents act to facilitate the transfer of the second electron to the cytochrome P-450 complex, by a mechanism as yet unknown but probably involving an interaction with the type I binding site, and that this accounts for the observed increase in the rate of aniline metabolism and the synergistic effect of NADH.

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