A POSSIBLE RELATIONSHIP BETWEEN DT DIAPHORASE AND THE ARYL HYDROCARBON HYDROXYLASE SYSTEM

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The enzyme DT diaphorase (E.C.1.6.99.2) is a flavoprotein catalyzing the oxidation of NADH and NADPH by various redox dyes and quinones (1). The high sensitivity of the enzyme to dicoumarol and related anticoagulant drugs has drawn attention to its possible involvement in the vitamin K-dependent biosynthesis of prothrombin and other blood-coagulation factors (1,2). More specifically, the possibility has been considered (3,4) that the enzyme may participate in the metabolism of vitamin K in liver microsomes, with phylloquinone-2,3-epoxide as a possible intermediate (5-7).

In the course of investigating this possibility, the somewhat unexpected finding was made that treatment of rats with 3-methylcholanthrene (MC)[x], an inducer of the microsomal aryl hydrocarbon hydroxylase (AHH) system (8), causes a several-fold increase in liver DT diaphorase activity. The increase occurs both in the microsomes, which contain a small fraction of the enzyme, and in the cytosol where the bulk of DT diaphorase is found. Some preliminary results relating to these findings are presented below, and discussed in terms of a possible functional relationship between DT diaphorase and the AHH system.

Materials and Methods.

Male Sprague-Dawley rats of about 100 g body-weight were used. The animals were kept on standard laboratory diet. Injections with 2 mg MC (dissolved in 0.5 ml corn-oil) and 8 mg sodium phenobarbital (in 1 ml physiological saline) were made intraperitoneally, at intervals as indicated in the table and figure legends. Controls were injected with the appropriate solvents.

Liver microsomes were prepared as described by Ernster *et al.* (9) and washed once with 0.15 M Tris chloride, pH 8. The washed pellet was suspended in 0.25 M sucrose before assay. The supernatant fraction obtained after sedimentation of the microsomes

x) Abbreviations: AHH, aryl hydrocarbon hydroxylase; BP, benzo(a)-pyrene; MC, 3-methylcholanthrene.

was used for the assay of cytosolic enzyme activity. Protein was estimated according to Lowry et al. (10).

DT diaphorase activity was determined at 30°C with NADPH as hydrogen donor and menadione as hydrogen acceptor (2). The menadiol formed was continuously reoxidized by cytochrome c, and the reaction was followed by measuring the formation of reduced cytochrome c spectrophotometrically at 550 nm. The reaction mixture consisted of 50 mM Tris chloride, pH 7.5, 0.5 mM NADPH, 10 µM menadione, 77 µM cytochrome c, 0.5 mM KCN, and 10 mg/ml Tween-20. In some cases, DT diaphorase activity was determined with 2,6-dichlorophenolindophenol as the hydrogen acceptor (2).

The DT diaphorase activity of microsomes, which also contain NADPH-cytochrome c reductase, was assayed in three ways: (a) the decrease in the rate of NADPH oxidation (with menadione + cytochrome c as acceptor) obtained after the addition of 10 µM dicoumarol (cf. ref. 2); (b) after solubilization of NADPH-cytochrome c reductase by treatment of the microsomes with trypsin (11,12), which does not release or inactivate DT diaphorase; (c) by selectively inhibiting NADPH-cytochrome c reductase with $NADP^+$ (13,14). The three assays gave closely comparable results.

Microsomal NADH- and NADPH-cytochrome c reductase activities were determined at 30°C as described by Dallner (15).

The microsomal contents of cytochromes P-450 (or P-448) and b_5 were estimated according to Dallner (15).

Aryl hydrocarbon hydroxylase activity was measured at 37°C with benzo(a)pyrene (BP) as substrate according to Nebert and Gelboin (16).

Results.

Treatment of rats with two injections of MC (5 and 3 days before sacrifice) resulted in a 4-5-fold increase in liver DT diaphorase activity, expressed on the protein basis, of both the cytosolic and microsomal fractions (Table I). The increase in DT diaphorase activity was also observed with 2,6-dichlorophenolindophenol (rather than menadione + cytochrome c) as hydrogen acceptor (not shown). As expected (8), this treatment caused a substantial increase of the AHH activity (measured here with BP as substrate; cf. legend of Table I). The content of cytochrome P-448 of the liver microsomes was likewise increased, whereas the NADPH-cytochrome c reductase activity showed a slight decrease (Table II). Treatment of rats with 3 daily injections of phenobarbital also gave rise to an increase in the liver cytosolic and microsomal DT diaphorase activities, but this was only 1.5-2-fold over the controls and was accompanied by an increase in both cytochrome P-450 content and NADPH-cytochrome c reductase activity (cf. Table II); similar results were obtained when the phenobarbital treatment was extended to 6 days.

Table I

Liver DT diaphorase activities of rats treated with MC.

MC was injected 5 and 3 days before sacrifice. The average AHH activities of the control and MC-treated microsomes, respectively, were 0.15 and 1.11 nanomoles hydroxy-BP formed/min/mg protein.

DT diaphorase activity (nanomoles NADPH oxidized/min/mg protein)			
Cytosol		Microsomes	
Control	MC-treated	Control	MC-treated
1980	12590	24.8	103.8
2070	10810	23.2	109.4
2610	8030	29.5	108.1
1850	10340	25.4	118.8
1810	10880	23.6	102.3
M = 2060	M = 10530	M = 25.3	M = 108.5

Table II

Liver DT diaphorase and NADPH-cytochrome c reductase activities and cytochrome P-450 (or P-448) contents in rats treated with MC or phenobarbital (PB).

MC treatment was as in Table I. PB was injected daily during 3 days. Each value represents the average of the individual values obtained with a group of three animals.

	DT diaphorase[a]		NADPH-cyt.c red[a]	Cyt.P-450 (or P-448)[b]
	Cytosol	Microsomes		
Control	2090	26.2	196	0.75
MC	9750	109.7	143	1.78
PB	3050	57.4	335	2.24

[a] nanomoles/min/mg protein [b] nanomoles/mg protein

Fig. 1 shows the time course of the increase in microsomal and cytosolic DT diaphorase activities following a single injection of MC. The microsomal DT diaphorase activity showed a relatively early increase after which it tended to reach an asymptote after 12 hrs. In contrast, the cytosolic DT diaphorase activity displayed

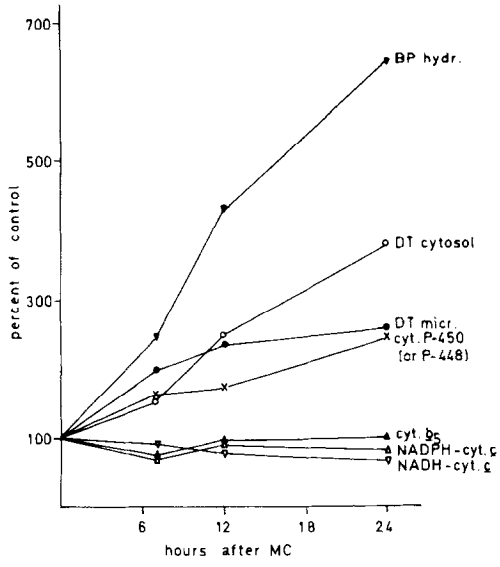

Fig. 1. Time course of changes in enzyme levels following a single injection of MC.

Each value represents the average of the individual values obtained with a group of 3 rats. Control activities (nanomoles/min/mg protein) or, in the cases of cytochromes b_5 and P-450 (or P-448), amounts (nanomoles/mg protein) were: DT diaphorase, cytosol: 1670; DT diaphorase, microsomes: 37.5; NADH-cytochrome c reductase: 569; NADPH-cytochrome c reductase: 167; BP hydroxylation: 0.12; cytochrome P-450 (or P-448): 0.45; cytochrome b_5: 0.57.

a progressive increase which showed no tendency of levelling off even after 24 hrs. Subject to more detailed investigation, these kinetics would be compatible with a precursor-product relationship between the microsomal and cytosolic fractions of DT diaphorase. The content of cytochrome P-450 (P-448) and the overall AHH activity also increased progressively, whereas the NADH- and NADPH cytochrome c reductase activities and the content of cytochrome b_5 revealed, as expected, no increase.

7,8-Benzoflavone, a known inhibitor of the microsomal AHH system(17), was found to inhibit DT diaphorase, both in the cytosolic and microsomal fractions (Fig. 2). Furthermore, whereas the inhibition of the AHH activity has been shown to occur only in induced animals (18), DT diaphorase was inhibited both in the control and the MC-treated rats. In all cases, the inhibition was competitive

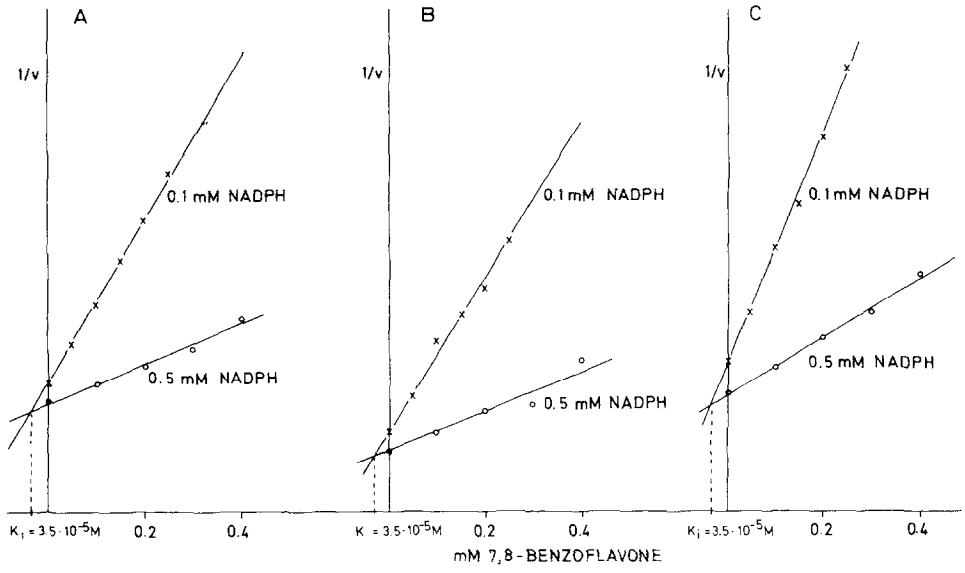

Fig. 2. Inhibition of DT diaphorase by 7,8-benzoflavone. (A) Control rats, cytosol; (B) MC-treated rats, cytosol; (C) MC-treated rats, microsomes.

MC was injected 1 and 2 days before sacrifice. Microsomal DT diaphorase was assayed by procedure (c) in Materials and Methods. 1/v is expressed in arbitrary units.

with respect to NADPH. The K_i value was 3.5×10^{-5} M in the case of the control cytosol and the MC-treated cytosol and microsomes (Fig. 2); preliminary estimates indicate that the same K_i value holds for the low DT diaphorase activity of the control microsomes. The inhibition by 7,8-benzoflavone of DT diaphorase is comparable in potency to that reported for the liver-microsomal AHH system of MC--treated rats (cf. ref. 18).

The effects of known inhibitors of DT diaphorase on the AHH system were also tested. Dicoumarol, a very potent competitive inhibitor of DT diaphorase with respect to NAD(P)H (K_i of the order of 10^{-8} M; cf. ref. 2), gave a variable, relatively weak inhibition (30-60 % at 10 μM dicoumarol) of BP hydroxylation in liver microsomes from control rats, and virtually no inhibition in those from MC-treated rats. These data thus showed no direct correlation between the effects of dicoumarol on the microsomal AHH system and DT diaphorase. An explanation of the effect of dicoumarol on BP hydroxylation in control microsomes may be that

dicoumarol, which is a substrate of the microsomal hydroxylating system (19), competes with the cytochrome P-450-linked hydroxylation of BP in control rats, but not with the cytochrome P-448--linked hydroxylation of BP in MC-induced rats. Preliminary results based on measurements of the effects of dicoumarol and BP on the NADPH-cytochrome P-450 (or P-448) reductase activities of microsomes from control and MC-treated rats are in line with this explanation. An inhibition of microsomal cytochrome P-450-linked activities by dicoumarol has recently been reported by Christensen and Wissing (20,21). Two other classes of anticoagulants inhibiting DT diaphorase, 2-phenyl-1,3-indandione (4) and 2,3,5,6-tetrachloro-4-pyridinol (22), were likewise ineffective as inhibitors of microsomal BP hydroxylation.

Discussion.

The results reported here reveal an increase in liver DT diaphorase activity following treatment of rats with compounds which act as inducers of the microsomal hydroxylating enzyme systems. The increase in the activity of this flavoprotein is moderate after treatment with phenobarbital, a known inducer of NADPH-cytochrome c reductase (23), and even escaped attention in earlier studies (24). In the case of MC as the inducer, however, DT diaphorase shows a marked increase in activity, in contrast to NADPH-cytochrome c reductase, which undergoes even a slight decrease. This pattern of response suggests a functional relationship between DT diaphorase and the AHH system, the latter being known to increase moderately after phenobarbital treatment and markedly after the administration of MC (25).

The existence of such a relationship is further supported by the finding that 7,8-benzoflavone, an inhibitor of the AHH system (17,18), is an equally potent inhibitor of DT diaphorase. The same compound did not inhibit NADPH-cytochrome c reductase. The fact that the inhibition of DT diaphorase is not restricted to MC-induced animals, as is the inhibition of BP hydroxylation (18), may be explained by assuming that DT diaphorase is involved in the MC-induced, cytochrome P-448-linked hydroxylation of BP, but not in the basal BP hydroxylating activity of the control microsomes, proceeding via cytochrome P-450; or, in other words, that DT diaphorase might act as the cytochrome P-448 reductase component of

the AHH system, in a way analogous to the generally assumed role of NADPH-cytochrome c reductase in cytochrome P-450-linked hydroxylations. Alternatively, DT diaphorase may be involved in the induction of the AHH system caused by MC, perhaps by providing a component necessary for its biosynthesis.

Against a direct role of DT diaphorase as cytochrome P-448 reductase speaks the virtual lack of inhibition of BP hydroxylation in microsomes from MC-induced rats by dicoumarol and other known inhibitors of DT diaphorase as described above. Furthermore, if DT diaphorase were involved in this reaction, NADH should efficiently replace NADPH as the hydrogen donor for BP hydroxylation in microsomes from MC-treated rats. In testing this possibility it was found that the reaction rate with NADH was only some 10 % of that with NADPH, in accordance with earlier reports (8). It should be pointed out, however, that most of the information regarding the properties of DT diaphorase originates from studies with the soluble enzyme and does not necessarily apply to its membrane--bound counterpart. Certain phospholipids have recently been found to act as potent modifiers of the enzyme (26), which thus may possess greatly altered properties when associated with microsomes.

From experiments with isolated cytochromes P-450 and P-448 Lu et al. (27) have recently concluded that a common "reductase" catalyzes the reduction of the two species of cytochromes. However, this reductase was only 5-fold enriched from microsomes, and thus may well contain both NADPH-cytochrome c reductase and DT diaphorase. Relevant to this possibility are preliminary experiments in this laboratory indicating that BP hydroxylation is insensitive to concentrations of $NADP^+$ that strongly inhibit the hydroxylation of aminopyrine. $NADP^+$ is a potent inhibitor of NADPH-cytochrome c reductase (13,14), the enzyme involved in cytochrome P-450 reduction, but not of DT diaphorase. Further investigations of a possible role of DT diaphorase in the reconstructed system of Lu et al. (27) are in progress.

In conclusion, the present results suggest a functional relationship between DT diaphorase and the AHH system. Elucidation of the nature of this relationship may shed light not only on the underlying reaction mechanisms but also on the sofar unknown physiological functions of these enzymes, and their possible role in the metabolism of vitamin K.

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