# ISOLATION OF DT-DIAPHORASE [NAD(P)H DEHYDROGENASE (QUINONE)] FROM RAT LIVER CYTOSOL: IDENTIFICATION OF NEW ENZYME SUBSTRATES, CARCINOGENIC ARISTOLOCHIC ACIDS

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Cytosolic fractions isolated from liver and kidney of rats treated with β-naphthoflavone, Sudan I, ellipticine, phenobarbital, ethanol, acetone and natural carcinogenic and nephrotoxic nitroaromatics, aristolochic acids, were tested for the activity of DT-diaphorase [NAD(P)H dehydrogenase (quinone), EC 1.6.99.2]. While the most efficient inducers of DT-diaphorase in liver were Sudan I, ellipticine and aristolochic acids, the highest increase in the DT-diaphorase activity in kidney was produced by aristolochic acids. No increase in the enzyme activity was determined after treatment of rats with acetone. DT-Diaphorase was isolated from liver cytosol of Sudan I-treated rats by the procedure consisting of fractionation with ammonium sulfate, gel permeation chromatography on a Sephadex G-150 column, affinity chromatography on an Affi-Gel Blue (Cibracron Blue Agarose) column and re-chromatography on Sephadex G-150. Rat DT-diaphorase catalyzed NAD(P)H-dependent reduction of menadione (vitamin $K_3$), vitamin $K_1$ and 4-nitrosophenol as substrates. Moreover, we newly identified two carcinogenic nitroaromatic compounds, aristolochic acids, as other substrates of DT-diaphorase. A selective inhibitor of the human DT-diaphorase, dicoumarol, inhibited the catalytic activity of the rat purified enzyme.

**Keywords**: NAD(P)H dehydrogenase (quinone); DT-Diaphorase; Purification procedure; Inducers; Drugs; Carcinogens; Aristolochic acids; Reductive activation; Chinese herbs nephropathy; Carcinogenesis; Enzymes; Oxidases.

Pharmacological efficiencies of many drugs and genotoxic effects of most carcinogens are dependent on their metabolic activation. Although a majority of such xenobiotics is activated by oxidative reactions, participation of reductive metabolism in activation of xenobiotics is unquestionable. The knowledge of enzymes participating in such reductive activations is crucial for many reasons. For example, it is important for elucidation of the fate of protoxicants and procarcinogens, which become toxic after their reductive

activation in organisms. Furthermore, it is essential for the development of an ideal cancer chemotherapeutic prodrug, which is fully inactive until reductively metabolized by a tumor-specific enzyme, or by an enzyme that is only metabolically competent for the prodrug under physiological conditions unique for the tumor. An enzyme system that fulfills one or the other of these criteria might be the cytosolic enzyme, DT-diaphorase [NAD(P)H dehydrogenase (quinone), EC 1.6.99.2]. In general, DT-diaphorase activity is higher in tumors than in the surrounding normal tissues[1]. Schlager and Powis[2] showed that enhanced levels of this enzyme have been found in primary colonic, breast and lung carcinoma as well as human hepatoma, whilst gastric adenocarcinomas had low DT-diaphorase activities compared to adjacent normal tissues[2]. The enzyme is efficient both under aerobic and hypoxic (anaerobic) conditions, the latter being typical features of tumor cells[3,4]. Cytostatic agents have been designed to become activated by this enzyme[5], the prototype compound for "bioreductive activation" being mitomycin C. Conversely, resistance to mitomycin C has been found associated with a decreased activity of DT-diaphorase[6].

DT-diaphorase is a flavoprotein, which catalyzes the two-electron reduction of quinones, quinone epoxides, nitroaromatic compounds, azodyes and certain transition metal ions[7–11]. NADH and NADPH serve as electron donors for such reductions. DT-diaphorase is a homodimer; each subunit contains one molecule of FAD as a prosthetic group. The two identical subunits are in a "head-to-tail" arrangement[11]. Thus, each active site is made of parts of both subunits[11].

Amino acid residues 142–158 of rat liver DT-diaphorase[12] show significant homology to the diphosphate binding regions of other nucleotide-dependent enzymes [NAD(P)H dehydrogenase (quinone) family][11,12].

The major cytosolic form of the enzyme in humans is specifically inhibited by anticoagulants such as dicoumarol and is encoded by a gene located on chromosome 16q2.2. The enzyme is inducible by a variety of agents[13]. Rat DT-diaphorase is coinduced with aromatic hydrocarbon hydroxylase and glutathione-S-transferases by pretreatment with inducers causing the activation of the *Ah*-locus, such as 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo[1,4]dioxin[14]. 1,1′-Azonaphthalenes, Sudan I, Sudan III, flavonoids, Aroclor-1254, coumarins, and the antioxidants butylated hydroquinone and butylated hydroxyanisole are additional compounds efficiently inducing the enzyme[14]. The precise mechanism of induction of DT-diaphorase has not been clearly established[13,14]; however, the existence of multiple genes in rat and human liver suggests the presence of multiple forms of the enzyme[14,15]. One gene appeared to be closely linked to a gene

regulating aromatic hydrocarbon monooxygenase induction (see above), another is unlinked and located on another chromosome. Indeed, antiestrogens tamoxifen and hydroxytamoxifen stimulate expression of DT-diaphorase by activation of a receptor specific for estrogens (the ER receptor), which is different from the *Ah*-locus[16].

The obligatory two-electron reduction of quinones catalyzed by DT-diaphorase circumvents the semiquinone stage and thereby prevents redox cycling and alkylation by these highly reactive compounds[17]. This is well documented for many quinones. However, some hydroquinones are also autoxidizable or can act as alkylating agents. Such compounds are activated by DT-diaphorase to their ultimately toxic form[18]. Likewise, reductive activation of many other compounds such as anticancer drugs (*e.g.* prodrug mitomycin C) or toxic compounds (azo dyes and nitroso- or nitroaromatics) was discovered as a function of DT-diaphorase[3,19–23].

In order to extend our knowledge on the ability of DT-diaphorase to metabolize prodrugs or toxic and carcinogenic xenobiotics functionally dependent on reductive activation, the purified enzyme in sufficient amounts and purity is needed for *in vitro* studies dealing with such problems. Although the enzyme was purified in a number of laboratories[12,22–34], most of the purification procedures either did not lead to isolation of the homogeneous enzyme[12,22,31,34] or were rather complicated[31,34]. Thus, development of simple and effective isolation procedures for purification of the enzyme is highly desirable.

This paper reports a novel, simple purification procedure for DT-diaphorase from liver cytosol of rats induced with an azo dye, Sudan I. Furthermore, the substrate specificity of the enzyme and the potential of several inducers of enzymes metabolizing xenobiotics to induce DT-diaphorase in rats are shown.

## EXPERIMENTAL

*Abbreviations used*: AAI, Aristolochic acid I, 8-methoxy-6-nitrophenanthro[3,4-*d*][1,3]dioxole-5-carboxylic acid; AAII, aristolochic acid II, 6-nitrophenanthro[3,4-*d*][1,3]dioxole-5-carboxylic acid; $AA_M$, a natural mixture of AAI and AAII; β-NF, β-naphthoflavone; CHN, Chinese herbs nephropathy; CYP, cytochrome P450; PB, phenobarbital; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

### Chemicals

Chemicals were obtained from the following sources: NADH, NADPH, ellipticine, β-naphthoflavone (β-NF; 3-phenyl-1*H*-benzo[*f*]chroman-1-one), vitamin $K_1$, cytochrome c, sodium dodecylsulfate (SDS) and dicoumarol from Sigma Chemical Co. (St. Louis, MO),

bicinchoninic acid from Pierce, (Rockford, IL), Sephadex G-150 from Pharmacia (Uppsala, Sweden), menadione from Merck (Darmstadt, Germany), Affi-Gel Blue (Cibacron Blue Agarose, Porcine Blue HB, C.T. 61211 Agarose) from Bio-Rad (Richmond, CA), Sudan I from the BDH (U.K.) and 4-nitrosophenol from Aldrich Chemical Company (Milwauke, WI). The natural mixture of aristolochic acids ($AA_M$) consisting of 65% aristolochic acid I (AAI) and 34% aristolochic acid II (AAII) was a gift from Madaus, Cologne, Germany. AAI and AAII were isolated from the mixture by preparative HPLC (ref.[35]); their purity was 99.7% as estimated by HPLC. All chemicals were of analytical purity or better.

### Animal Experiments

Gavage with aristolochic acid I and a natural mixture of both acids ($AA_M$) dissolved in 0.15 mM NaCl (10 mg/kg body weight) was administered to six male Wistar rats (100–150 g) once a day for four consecutive days. Six control animals received an equal volume of 0.15 mM NaCl. Rats were placed in cages in temperature- and humidity-controlled rooms. Ten male Wistar rats were injected i.p. with β-NF or Sudan I in maize oil (60 mg β-NF/kg body weight or 20 mg Sudan I/kg body weight) once a day for three consecutive days or with ellipticine in maize oil–dimethyl sulfoxide (1 : 1, v/v) (40 mg ellipticine/kg body weight) once a day for two consecutive days. Pretreatment of rats with phenobarbital (PB) and ethanol or acetone was carried out by procedures described by Hodek *et al.*[36] and Yang *et al.*[37], respectively. Control rats obtained the solvents only. Standardized diet and water were provided *ad libitum*. Animals were killed 24 h after the last treatment by cervical dislocation[38]. Liver and kidney of animals were excised immediately after sacrifice, quickly frozen in liquid nitrogen, and stored at –80 °C until the isolation of cytosolic and microsomal fractions.

### Preparation of Cytosolic Fractions

Liver and renal fractions (cytosol and microsomes) were prepared by differential centrifugation as described previously[39]. The 105 000 *g* supernatant was taken as cytosol and used for studies presented in the paper. All tissue fractions were stored at –80 °C.

### DT-Diaphorase Activity and Assays

DT-diaphorase activity was measured essentially as described by Ernster[40]. The standard assay system contained 25 mM Tris-HCl (pH 7.4), 0.2% Tween 20, 0.07% bovine serum albumin, 400 μM NADH (or NADPH) and 100 μM menadione (2-methyl-1,4-naphthoquinone) dissolved in methanol. The enzyme activity was determined by following the oxidation of NADH (NADPH) spectrophotometrically at 340 nm on a Hewlett–Packard 8453 diode array spectrophotometer. One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH (molar absorption coefficient = 6.27 $mmol^{-1}$ l $cm^{-1}$) per minute. When vitamin $K_1$ or 4-nitrosophenol was used as an electron acceptor, these compounds were added instead of menadione. The activity of DT-diaphorase with respect to aristolochic acid I and II was measured essentially as described for other substrates by Hajos and Winston[22]. The assay system contained 25 mM Tris-HCl (pH 7.4), 0.2% Tween 20, 0.07% bovine serum albumin, 400 μM NADH, 200 μM cytochrome c and 5–100 μM aristolochic acid I or II dissolved in distilled water. The enzyme activity with respect to these substances was determined by following the reduction of cytochrome c spectrophoto-

metrically as an increase in absorbance at 550 nm on the spectrophotometer mentioned above. An absorption coefficient of cytochrome c (21 $mmol^{-1}$ l $cm^{-1}$) was used for quantitation[22]. Protein concentration was assessed using the bicinchoninic acid protein assay with serum albumin as a standard[41].

Isolation of DT-Diaphorase

Throughout this investigation, liver cytosol from Sudan I-treated rats has been used. Cytosolic DT-diaphorase was purified as follows. Proteins of cytosol (20 ml, 24 mg $ml^{-1}$) were fractionated with ammonium sulfate and the fraction of 30–90% saturation containing most of the DT-diaphorase activity was dialyzed against 2 000 ml of a buffer containing 150 mM KCl and 50 mM Tris-HCl (pH 7.4). The dialyzed enzyme preparation was chromatographed on a Sephadex G-150 column and DT-diaphorase eluted with the same buffer. Pooled fractions containing the DT-diaphorase activity were applied onto a column of Affi-Gel Blue and non-DT-diaphorase proteins were eluted with the same buffer and subsequently with a gradient of NaCl (0–3.5 mol $l^{-1}$) in the buffer. DT-Diaphorase was eluted from Affi-Gel Blue with 20 mM Tris-HCl buffer (pH 10.0) containing 1 mM NADH. The eluate was concentrated by ultrafiltration in a diaflo cell (Amicon, Lexington, MA) equipped with a PM-30 filter. In order to remove residual protein impurities, the concentrated sample was applied onto a Sephadex G-150 column and re-chromatographed. Elution was performed as described above. The eluate was concentrated by ultrafiltration (see above) and stored at –80 °C.

Electrophoresis

Discontinuous SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli[42] and gels were stained with Comassie Blue.

## RESULTS

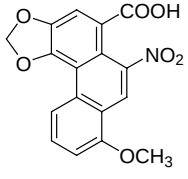
In order to obtain cytosolic fractions possessing high levels of DT-diaphorase for the enzyme isolation, we examined potentials of some of DT-diaphorase inducers (Sudan I, β-NF) and compounds with unknown effects (ellipticine, PB, ethanol, acetone, aristolochic acids) to increase the activities of the enzyme in cytosols isolated from liver and kidney of rats pretreated with such compounds. While some of these compounds are known inducers of rat cytochrome P450 (CYP) isoenzymes[43], the effects of most of them on DT-diaphorase have not been established as yet. Cytosolic fractions of rats pretreated with β-NF, Sudan I, ellipticine (inducers of CYP1A1/2), PB (inducer of CYP2B1/2), ethanol and acetone (inducers of CYP2E1) and with two natural nitroaromatics with unknown induction potentials, aristolochic acids I (Fig. 1) and a natural mixture of aristolochic acids I and II ($AA_M$, Fig. 1), were tested for the activity of DT-diaphorase. The DT-diaphorase activity was an order of magnitude higher in cytosol of liver than in that of kidney (Table I). In comparison with the activity of the en-

zyme in the liver cytosol of control (untreated) rats, the most efficient enzyme inducer was Sudan I, followed by ellipticine, aristolochic acids, β-NF and PB. No increase in the DT-diaphorase activity was determined for acetone (Table I). Interestingly, natural nephrotoxins and carcinogens, aristolochic acids, and an anticancer agent, ellipticine, exhibited a great potential to increase the DT-diaphorase activity, comparable with a strong DT-diaphorase inducer, Sudan I (Table I). Moreover, we found that both carcinogenic aristolochic acids were the most efficient compounds increas-

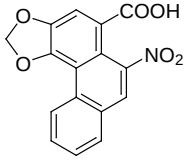
TABLE I
DT-Diaphorase activity in rat hepatic and renal cytosol. (Enzyme activities with menadione as a substrate and protein contents were assayed as described in Experimental)

| Inducer | Hepatic cytosol | | | Renal cytosol | | |
|---|---|---|---|---|---|---|
| | protein mg $ml^{-1}$ | specific activity units $ml^{-1}$ | fold | protein mg $ml^{-1}$ | specific activity units $ml^{-1}$ | fold |
| None | 19.0 | 0.79 ± 0.05[a] | – | 14.4 | 0.081 ± 0.01[a] | – |
| Sudan I | 24.0 | 3.55 ± 0.25 | 4.49 | 29.9 | 0.138 ± 0.021 | 1.58 |
| Ellipticine | 26.4 | 3.51 ± 0.24 | 4.44 | 23.5 | 0.164 ± 0.025 | 2.02 |
| AAI | 18.9 | 2.69 ± 0.20 | 3.41 | 15.0 | 0.380 ± 0.036 | 4.90 |
| $AA_M$ | 19.0 | 2.42 ± 0.20 | 3.06 | 21.0 | 0.328 ± 0.039 | 4.09 |
| β-NF | 20.0 | 2.20 ± 0.22 | 2.78 | 20.0 | 0.135 ± 0.019 | 1.67 |
| PB | 20.2 | 2.12 ± 0.24 | 2.68 | –[b] | –[b] | –[b] |
| Ethanol | 15.1 | 1.37 ± 0.28 | 1.73 | –[b] | –[b] | –[b] |
| Acetone | 23.1 | 0.67 ± 0.07 | 0.85 | –[b] | –[b] | –[b] |

[a] Averages and standard deviations of five parallel measurements. [b] Not determined.



AAI



AAII

FIG. 1
Structures of aristolochic acids I and II

ing the DT-diaphorase activity in cytosol of the renal tissue, the target tissue of their toxic and carcinogenic effects[35] (Table I).

DT-Diaphorase can be isolated in a high purity from rat liver cytosol by purification procedures where the key isolation step is the biospecific adsorption of the enzyme on immobilized competitive inhibitors of the enzyme with respect to NAD(P)H (dicoumarol[34] or a triazine dye, Cibacron Blue (ref.[33]). Nevertheless, the enzyme purified by such one-step procedures was still contaminated with proteins, different from DT-diaphorase[31,34].

We combined the affinity chromatography on an Affi-Gel Blue (Cibracron Blue agarose) column with gel permeation chromatography on a column of Sephadex G-150 (Table II). The isolation procedure utilized for purification of rat liver cytosolic DT-diaphorase induced by Sudan I consisted of several steps. A protein fraction enriched with DT-diaphorase by precipitation with ammonium sulfate was chromatographed on a Sephadex G-150 column and the eluted DT-diaphorase (Fig. 2) was applied onto an Affi-Gel Blue column. DT-Diaphorase was eluted from the column using a buffer containing the enzyme cofactor, NADH (Fig. 3). Re-chromatography of the enzyme preparation on Sephadex G-150 was the final step of the isolation procedure (Fig. 4, Table II). The purified enzyme showed a characteristic absorption spectrum of a flavoprotein[44]. The preparation was pure using the criterion of homogeneity on SDS–PAGE (Fig. 5) and its mono-

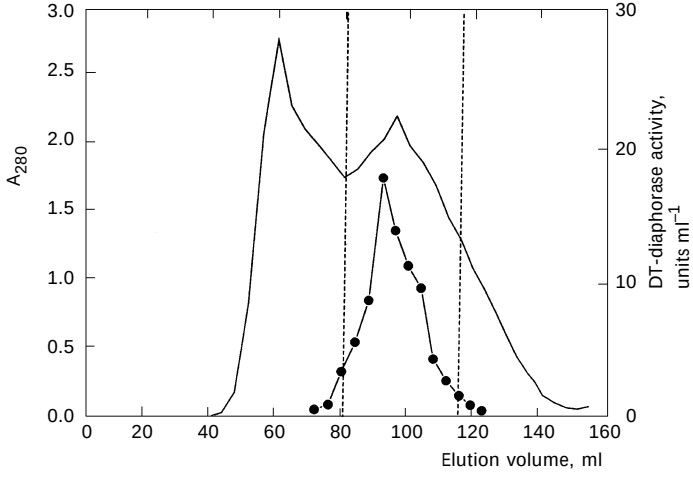



FIG. 2
Gel permeation chromatography of rat hepatic DT-diaphorase on Sephadex G-150. Absorbance at 280 nm (———), DT-diaphorase activity (●)

TABLE II

Purification of DT-diaphorase from cytosol of rats pretreated with Sudan I. (Enzyme activities with menadione as a substrate and protein contents were assayed as described in Experimental)

| Purification step | Total protein mg | Total activity units | Specific activity units $mg^{-1}$ | Purification fold | Yield % |
|---|---|---|---|---|---|
| Cytosol | 480.00 | 1 704.00 | 3.55 | – | 100.00 |
| 35–90% $(NH_4)_2SO_4$ | –[a] | 1 224.36 | –[a] | – | 71.85 |
| Sephadex G-150 | 129.20 | 578.39 | 4.48 | 1.26 | 33.94 |
| Affi-Gel Blue | 5.68 | 526.97 | 91.89 | 25.89 | 30.63 |
| Sephadex G-150 | 1.14 | 220.07 | 193.04 | 54.38 | 12.91 |
| Final preparation stored at –80 °C for 12 months | 1.04 | 216.60 | 190.10 | 53.55 | 12.71 |

[a] Not measured.

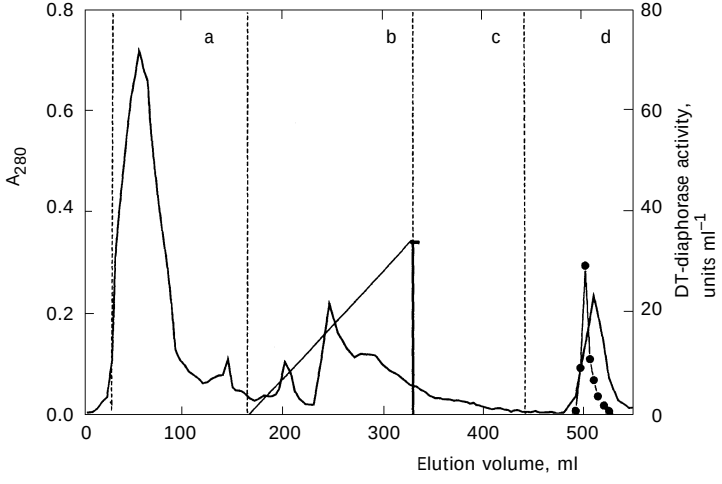



FIG. 3

Affinity chromatography of rat hepatic DT-diaphorase on Affi-Gel Blue (Cibacron Blue agarose). Absorbance at 280 nm (———), DT-diaphorase activity (●). a 150 mM KCl/50 mM Tris-HCl, pH 7.4 (buffer A); b gradient 0–3.5 M NaCl in buffer A; c buffer A; d 1 mM NaDH in 20 mM Tris-HCl (pH 10.0)

meric relative molecular weight was estimated at ≈32 000, essentially consistent with previous reports on DT-diaphorase induced by 3-methylcholanthrene or Aroclor-1254 (refs[11,20,33]). The enzyme is stable when stored at –80 °C. Essentially no decrease in the enzyme activity towards

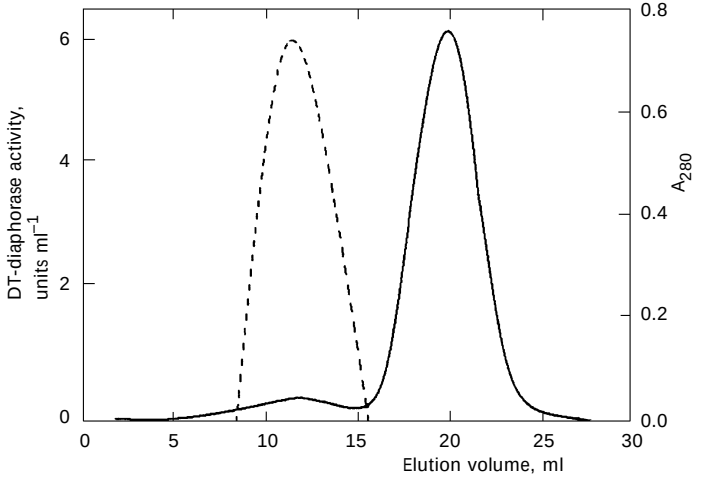



FIG. 4
Re-chromatography of rat hepatic DT-diaphorase on Sephadex G-150. DT-Diaphorase activity (- - - - -), absorbance at 280 nm (———)

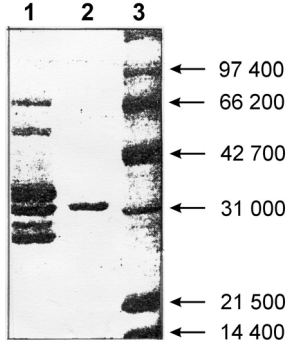



FIG. 5
SDS–PAGE of purified rat liver DT-diaphorase. Hepatic cytosol of rats induced with Sudan I (lane 1), purified rat DT-diaphorase (1 μg) (lane 2) and molecular weight standards (lane 3)

menadione was observed after several months of its storage under these conditions (12 months).

Purified DT-diaphorase-catalyzed reduction of menadione was sensitive to an inhibitor of the human enzyme, dicoumarol. In the presence of this inhibitor in 20 μM concentration, the reduction of the substrate decreased to 9% of the control (without inhibitor). In addition to menadione, the purified enzyme catalyzed the NAD(P)$H_2$-dependent reduction of additional substrates; another quinone, vitamin $K_1$ and a nitrosocompound, 4-nitrosophenol. Moreover, we were interested in establishing whether two carcinogenic nitroaromatics, aristolochic acids (Fig. 1), whose genotoxicity is dependent on reductive activation[35,45,46], are substrates for DT-diaphorase. We found that both the carcinogens are reduced by the purified enzyme. Of the tested compounds, the highest activity of DT-diaphorase was observed with menadione as an electron acceptor (Table III).

## DISCUSSION

The present results show a simple isolation procedure of rat liver cytosolic DT-diaphorase induced with a strong enzyme inducer, Sudan I. The procedure consisting of fractionation with ammonium sulfate, gel permeation chromatography on a Sephadex G-150 column, affinity chromatography on an Affi-Gel Blue (Cibracron Blue Agarose) column and re-chromato-

TABLE III

The values of the Michaelis constant ($K_M$) and maximal velocity ($V_{max}$) for reduction of various electron acceptors with purified DT-diaphorase. Experimental conditions: 25 mM Tris-HCl (pH 7.4), 0.2% Tween 20, 0.07% bovine serum albumin, 400 μM NADH, 1–100 μM menadione, 20–300 μM vitamin $K_1$, 5–100 μM 4-nitrosophenol, AAI or AAII. The reduction of DT-diaphorase substrates was assayed as described in Experimental. The values in the table are averages and standard deviations of three parallel measurements

| Electron acceptor | $K_M$ μmol $l^{-1}$ | $V_{max}$ μmol $min^{-1}$ $mg^{-1}$ |
|---|---|---|
| Menadione | 2.5 ± 0.1 | 193.3 ± 8.7 |
| Vitamin $K_1$ | 222 ± 21.8 | 2.0 ± 0.2 |
| 4-Nitrosophenol | 20.0 ± 2.1 | 26.3 ± 2.5 |
| Aristolochic acid I | 27.7 ± 2.3 | 20.4 ± 2.0 |
| Aristolochic acid II | 45.4 ± 4.2 | 11.8 ± 1.6 |

graphy on Sephadex G-150 led to the electrophoretically homogeneous enzyme. The most efficient purification step was affinity chromatography on Affi-Gel Blue (see Table II). The enzyme purified using such a procedure is enzymatically active with respect to three known enzyme substrates (menadione, vitamin $K_1$, 4-nitrosophenol). Therefore, it can be utilized for evaluation of DT-diaphorase efficiency in activating physiologically important compounds (drugs, toxicants, carcinogens).

We tested this purified enzyme preparation for its ability to reduce two naturally occurring nitrophenanthrene derivatives, carcinogenic and nephrotoxic aristolochic acids. Both these acids were recently found to be involved in an endemic renal fibrosis, designated Chinese herbs nephropathy (CHN) and urothelial cancer in young Belgian women who had followed a slimming regimen including Chinese herbs[45–48] containing aristolochic acids (refs[45–48]). The carcinogens are known to be *in vitro* and *in vivo* activated by reductive reactions to form DNA adducts. We found that both carcinogens are efficiently reduced by DT-diaphorase. Hence, our results suggest that DT-diaphorase-mediated reduction of both carcinogens might be important for DNA adduct formation with aristolochic acids. Indeed, preliminary results indicate that DT-diaphorase of rat cytosol is able to bioactivate aristolochic acids to form the same DNA adducts found in rodents treated with aristolochic acids and humans exposed to these acids[49]. Nevertheless, a detailed study to evaluate the efficiency of DT-diaphorase in mediating the formation of DNA adducts with aristolochic acids is still missing and is planned to be carried out in future. The DT-diaphorase preparation isolated in the present work will be used for such a detailed study.

Another important result of our present study is the finding that the activity of DT-diaphorase in cytosol is increased by compounds, whose efficiencies in inducing the enzyme were not known. We found that both the tested carcinogenic aristolochic acids increase dramatically the activity of DT-diaphorase in liver and kidney of rats treated with these compounds. This fact has great importance in the light of their physiological activities. Not all those undergoing slimming using Chinese herbs containing aristolochic acids are affected by CHN (ref.[48]). Thus, the treatment of women with aristolochic acids exhibited individual differences in response to the carcinogens. Differences in levels of the enzymes responsible for the carcinogen activation or detoxication (mainly in the target organ, kidney) could be the reason for individual susceptibility. Levels of expression and activities of DT-diaphorase in humans differ considerably among individuals being influenced by several factors (smoking, drugs, environmental chemicals and genetic polymorphisms)[6,50]. Because in rats the DT-dia-

phorase is increased by aristolochic acids (the present paper), their possible effects on the enzyme activity also in humans might be considered. Collectively, all these data suggest that variations of this enzyme might play a role in the risk of the carcinogenicity of aristolochic acids in slimming. To test this hypothesis, CHN patients and other participants in the slimming regimen will be screened for genetic polymorphisms of genes of DT-diaphorase in the next phase of our work.

## CONCLUSIONS

The results presented in this paper demonstrate the capability of naturally occurring aristolochic acids I and II, plant products associated with the development of a renal fibrosis (Chinese herbs nephropathy) and urothelial cancer in organisms including human, to induce DT-diaphorase in rat liver and kidney. Moreover, both these acids are substrates of the purified DT-diaphorase, being reduced by the enzyme. The reduction of the acids generates reactive species forming DNA adducts[35,45,46], which are responsible for initiation of carcinogenic processes. Collectively, these results are potentially important for understanding the mechanism and etiology of the diseases generated by these carcinogenic compounds (Chinese herbs nephropathy, urothelial cancer). Therefore, a detailed study to evaluate the role of DT-diaphorase in the clinical occurrence of these AA-dependent diseases in humans, is the aim of our future work.

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