THE REGULATION OF RAT LIVER
DT-DIAPHORASE mRNA BY XENOBIOTICS

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BY
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ABSTRACT

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THE REGULATION OF RAT LIVER DT-DIAPHORASE mRNA BY XENOBIOTICS

Advisors: Professor Franklin D. Hamilton and Professor Cecil Pickett
Thesis dated December 1984

In vitro translational analysis and a cDNA probe complementary to DT-diaphorase mRNA have been utilized to quantitate the levels of mRNA specific for DT-diaphorase in control, 3-methylcholanthrene, trans-stilbene oxide and phenobarbital-treated rats and in persistent hepatocyte nodules induced by chemical carcinogens. Quantitation of the in vitro synthesized protein revealed that DT-diaphorase mRNA is elevated eight-fold at eight hours following a single injection of 3-methylcholanthrene, whereas trans-stilbene oxide and phenobarbital elicited only a two- to three-fold elevation of the mRNA. Increases of five- to seven-fold were obtained in nodular mRNA; furthermore, following nodule formation, the mRNA retained the ability to be regulated by 3-methylcholanthrene, as evidence by a 13- to 21-fold increase in mRNA levels. These data correlate well with that obtained via hybridization studies utilizing the cDNA probe.

Restriction endonuclease mapping and Southern blot analysis of the genomic organization of DT-diaphorase, utilizing the cDNA probe, revealed that the DT-diaphorase gene spans ~7200 bp and encodes a 2000 bp mRNA. A minimum of three introns are present within the gene. Analysis of genomic DNA isolated from the nodules suggests that the mechanism underlying the induction of DT-diaphorase following chemical carcinogenesis may involve hypomethylation.
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<tr>
<td>2-AAF</td>
<td>2-acetylaminofluorene</td>
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</tr>
<tr>
<td>BHA</td>
<td>2(3)-terti-butyl-4-hydroxyanisole</td>
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</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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</tr>
<tr>
<td>BP</td>
<td>benzo(a) pyrene</td>
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<tr>
<td>DBM</td>
<td>diazobenzyloxymethyl</td>
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</tr>
<tr>
<td>DCPIP</td>
<td>2,6-dichlorophenolindophenol</td>
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<tr>
<td>DEN</td>
<td>diethylnitrosamine</td>
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<tr>
<td>7,12-DMBA</td>
<td>7,12-dimethylbenzo(a) anthracene</td>
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</tr>
<tr>
<td>DOC</td>
<td>sodium deoxycholate</td>
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<tr>
<td>DPNH</td>
<td>diphosphopyridine nucleotide</td>
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</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
<td></td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<tr>
<td>EtOH</td>
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</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
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<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid</td>
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</tr>
<tr>
<td>KAc</td>
<td>potassium acetate</td>
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<tr>
<td>3MC</td>
<td>3-methylcholanthrene</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
<td></td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NaOAc</td>
<td>sodium acetate</td>
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<tr>
<td>NH$_4$OAc</td>
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<tr>
<td>PB</td>
<td>phenobarbital</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>Pipes</td>
<td>piperazine-N-N'-bis 2-ethanesulfonic acid</td>
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<td>Description</td>
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<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SSC</td>
<td>sodium chloride and trisodium citrate</td>
<td></td>
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<tr>
<td>SSPE</td>
<td>sodium chloride, sodium phosphate and EDTA</td>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<tr>
<td>TPNH</td>
<td>triphosphopyridine nucleotide</td>
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<tr>
<td>TSO</td>
<td>trans-stilbene oxide</td>
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<tr>
<td>Tris</td>
<td>tris (hydroxymethylaminomethane)</td>
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INTRODUCTION

Historical Perspective

The existence of a rat liver cytosolic fraction which possessed the ability to utilize either diphosphopyridine nucleotide (DPNH) or triphosphopyridine nucleotide (TPNH) with equal reactivity in the reduction of 2,6-dichlorophenolindophenol (DCPIP) was reported in 1958.\(^1,2\) (DPNH and TPNH are the previous nomenclature for nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), respectively). The purification and characterization of the enzyme was accomplished several years later and the enzyme was designated DT-diaphorase, due to its reactivity with both DPNH and TPNH.\(^3\) More recently, Huang et al.\(^4\) reported the presence of a methyl red azo-reductase in the cytosol of 3-methylcholanthrene-treated (3MC) rat liver. Its purification and subsequent characterization in 1979 finally established that this enzyme was identical to DT-diaphorase.\(^5,6\) In addition to DT-diaphorase and azo-reductase, the enzyme has also been referred to as: vitamin K reductase, quinone reductase, menadione reductase, NAD(P)H oxidoreductase, NAD(P)H dehydrogenase (quinone), NAD(P)H quinone reductase, NAD(P)H: (quinone acceptor) oxidoreductase, and more recently the Enzyme Commission on Nomenclature of the International Union of Biochemists has designated the enzyme NAD(P)H: quinone oxidoreductase (EC 1.6.99.2). A general mechanism for DT-diaphorase activity is depicted in Fig. 1.

Purification and Characterization

DT-diaphorase was initially purified by Ernster et al.\(^3\) in 1960 from a 105,000 x g rat liver homogenate (in 0.25 M sucrose) by: (1) precipitation
Fig. 1. Proposed mechanism of DT-diaphorase activity.
with nine volumes of acetone, (2) fractionation with neutralized 55-65% saturated ammonium sulfate, and (3) chromatography on DEAE-cellulose.

Huang et al. isolated the enzyme from the livers of rats which had been treated with 3MC. The protein was purified from the 105,000 x g rat liver supernatant by (1) two successive DE-52 cellulose columns, (2) chromatography on silica hydroxyapatite, and (3) chromatography on Sephadex G-100.

The purification of DT-diaphorase has also been achieved via affinity chromatography. Rase et al. purified DT-diaphorase on dicoumarol-linked Sepharose 4B, and Hojebger et al. purified the enzyme on a column of azodicoumarol coupled to divinyl sulfone cross-linked Sepharose 6B followed by further purification on Sephacryl S-200.

The apparent molecular weight of the rat liver enzyme, based on Sephadex G-100 gel filtration, is 50,000 - 55,000 daltons. Electrophoresis on a polyacrylamide gel in the presence of sodium dodecyl sulfate indicates a subunit molecular weight of 27,000 - 32,000 daltons, suggesting that DT-diaphorase is a dimer in the native form.

Purified DT-diaphorase is pale yellow in color, suggesting the presence of flavin. The flavin content of the enzyme was determined by measuring the absorbance at 450 nm and also by fluorometry. Initially, the presence of 1 mole FAD/55,000 M unit was reported, but the existence of 2 moles FAD/mole enzyme was later demonstrated.

Assays

Assays for DT-diaphorase have been generally based on the activity of the enzyme in the reduction of one of several electron acceptors. Some of the available methods are discussed briefly below.
A spectrophotometric assay designed by Ernster\textsuperscript{1} involved the
addition of DT-diaphorase to an electron acceptor, such as DCPIP or
menadione in Tris-HCl or phosphate buffer, pH 7.5 - 8.0, in the presence of
NADH or NADPH and an activator, i.e., bovine serum albumin (BSA) or
polyvinylpyrrolidone (PVP). The reduction of the quinones was assayed
spectrophotometrically in a coupled assay by monitoring the oxidation of
the pyridine nucleotides at 340 nm. The reduction of DCPIP was directly
monitored at 600 nm.

Huang et al.\textsuperscript{4} devised an assay which utilized the methyl red reductase
activity of DT-diaphorase. Briefly, the activity of the enzyme was assayed
via a sensitive fluorometric measure of the coupling of the reduction
product, 4(N,N-dimethylamino) aniline, with fluorescamine. This procedure,
although quite sensitive because it measured the appearance of the product
rather than the disappearance of the substrate, was hampered by the
instability of the product.

Dent et al.,\textsuperscript{9} in a modification of the fluorometric assay of Huang et
al., reported a very sensitive radiometric assay utilizing a \([^{14}\text{C}]\)-labeled
methyl red substrate. The compound was reduced by a rat liver cytosolic
fraction exhibiting DT-diaphorase activity in phosphate buffer in the
presence of NADPH. The labeled reduction product was extracted into
methylene chloride and quantitated via scintillation counting. Enzyme
activity was thus calculated on the basis of the specific activity of the
substrate.
Distribution

1. Subcellular Location and Site of Synthesis

In the rat liver, cytosolic DT-diaphorase is estimated to represent 0.09% of the total cytosolic protein and approximately 95% of the total DT-diaphorase activity. Additionally, a small percentage (2-5%) of diaphorase activity is found in the mitochondria and microsomes. The enzyme is also present in Golgi membranes.

The site of synthesis of microsomal rat liver DT-diaphorase was determined by Edlund et al. by antibody precipitation of peptides released from bound and free ribosomes by puromycin. Both bound and free ribosomes released peptides which were precipitable by DT-diaphorase IgG. Administration of radiolabeled amino acids into the portal vein yielded a high specific labeling of the enzyme in the rough endoplasmic reticulum after five minutes followed by a rapid decrease. The smooth endoplasmic reticulum demonstrated high labeling after twenty minutes. Edlund et al. therefore postulated that microsomal DT-diaphorase is transported sequentially from rough to smooth endoplasmic reticulum. The synthesis of cytosolic DT-diaphorase is presumed to be accomplished on free polysomes, although the site of synthesis has not been directly determined.

Glycosylation of rat liver cytosolic DT-diaphorase was not detected upon labeling in vivo with [3H] glucosamine and [3H] mannose, whereas DT-diaphorase associated radioactivity was observed in enzyme precipitated from rough and smooth microsomes.

2. Tissue and Species Distribution

Rat liver is the most abundant source of DT-diaphorase, but enzyme activity is also detectable in such extrahepatic tissues as lung, kidney,
heart, upper small intestine, thymus, spleen, lymph nodes, mammary glands, and adipose tissue. Additionally, DT-diaphorase is well represented phylogenetically as evidenced by its isolation from pea seeds, Escherichia coli, dog and horse liver, and rabbit kidney.

Electron Acceptors

Equal reactivity is observed between DT-diaphorase and the electron donors NADH and NADPH in the reduction of a wide variety of electron acceptors (Fig. 2). The quinones DCPIP, 2,6-dimethyl-1,4-benzoquinone, 2-methyl-1,4 benzoquinone, p-benzoquinone, menadione, 1,4-napthoquinone, and 1,2-napthoquinone are among the best electron acceptors for DT-diaphorase. In general, quinones which do not possess a side chain in the 3-position are most active; the addition of a side chain elicits a decrease in activity with increasing side chain length. The presence of a methyl group in the 2-position or methoxy group in the 5- and 6-position of benzoquinones does not affect the efficiency as electron acceptors, whereas the substitution of hydroxyl groups in the 2- or 3-positions renders the quinones practically inactive as electron acceptors for DT-diaphorase.

Activators and Inhibitors

The activity of DT-diaphorase can be modulated in vitro by the addition of activators. BSA, PVP, and the nonionic detergents, Tween-20 and Tween-60 increase the $V_{\text{max}}$ of DT-diaphorase 3- to 10-fold. This reversible activation is postulated by Ernster to occur via an increased affinity of the enzyme for NADH and NADPH. Sodium deoxycholate (DOC) on the other hand, does not activate the enzyme; inhibition of the activation of DT-diaphorase by BSA is observed in the presence of DOC.
1,4-Napthoquinone

p-Benzoinone

Methyl Red

Menadione

1,2-Napthoquinone

2,6-Dichlorophenolindophenol

3-Bromomethylmenadione

1,4-Napthoquinone

Fig. 2. Electron acceptors for DT-diaphorase.
Inhibitors of DT-diaphorase can be categorized into three general classes: (1) sulfhydryl reagents (p-chloromercuribenzoate, p-iodosobenzoate), (2) thyronine analogs (thyroxine, desaminothyroxine, 3,3',5-triiodothyronine), and (3) flavin and flavin inhibitors (FAD, FMN, atebrin, chlorpromazine). By far, the most potent inhibitor of DT-diaphorase is dicoumarol (see Fig. 3). The inhibition of DT-diaphorase by dicoumarol is on the order of $K_i = 10^{-8} \text{M}$ and is competitive with respect to NADH and NADPH. Dicoumarol is generally considered to be a selective inhibitor of DT-diaphorase because other diaphorase activities in rat liver are not affected by a 1000-fold excess of the concentration required for complete inhibition of DT-diaphorase.

**Induction by Xenobiotics**

The induction of drug metabolizing enzymes in response to xenobiotic administration is a well-established phenomenon. Several classes of compounds have been identified as inducers of detoxifying enzymes (see Fig. 4). These include: (1) polycyclic aromatic hydrocarbons, (2) barbiturates, and (3) steroid hormones. The induction of DT-diaphorase by xenobiotics is documented below.

Williams-Ashman and Huggins reported increases of up to 7-fold in DT-diaphorase concentration following the administration of a single dose of the polycyclic aromatic hydrocarbons 3MC and 7,12-dimethylbenzo(a)anthracene (7,12-DMBA) in rat mammary gland and adipose tissue. Moreover, when Huggins and Fukunishi administered 3MC to rats prior to injecting 7,12-DMBA, the adrenal cortex was protected from 7,12-DMBA-induced necrosis. The authors proposed that the concomitant increase in DT-diaphorase activity played a role in protection of the adrenal cortex.
**Flavin Adenine Dinucleotide**

**o-Iodosobenzoic Acid**

**Dicoumarol**

*Fig. 3.* Inhibitors of DT-diaphorase.
Fig. 4. Inducers of DT-diaphorase.
from necrosis. This protection could be abolished by ethionine, an inhibitor of protein synthesis. Thus, it was postulated that protection by 3MC of 7,12-DMBA-induced necrosis was elicited by an increase in DT-diaphorase activity due to an increase in protein synthesis.

The protective effect of DT-diaphorase was further supported by Shor et al. Following the injection of rats with 3MC (7.5 x 10^-5 mol/1/kg body weight), a 2.5- to 3.2-fold increase in DT-diaphorase activity was determined spectrophotometrically; the activity of DT-diaphorase was completely abolished by the administration of dicoumarol. The toxicity of 3MC and benzo(a)pyrene (BP) was potentiated (i.e., increased 27% and 20%, respectively) following concomitant administration of the carcinogen and dicoumarol, presumably due to an inhibition of the protective effect of DT-diaphorase.

Lind and Ernster subjected rats to two injections of 3MC at a dosage of 20 mg/kg body weight. A 4- to 5-fold increase in liver DT-diaphorase activity was detected by spectrophotometric assay of the enzyme activity. The same authors later reported that cycloheximide, an inhibitor of protein synthesis, was capable of blocking the increase in DT-diaphorase activity. Therefore induction was proposed to be due to an increased enzyme synthesis.

The fluorometric method was utilized by Huang et al. to demonstrate increases in rat liver azoreductase (DT-diaphorase) activity following 3MC administration (25 mg/kg body weight). 3MC was reported to elevate the activity of DT-diaphorase 7- to 10-fold. More recently, Höjeberg et al. quantitated the levels of DT-diaphorase from 3MC-treated rats by rocket immunoelectrophoresis. The cytosolic concentration of DT-diaphorase was
determined to be 7-fold higher in 3MC-treated as opposed to control animals, supporting the assertion that the drug-induced increase in DT-diaphorase catalytic activity is due to elevation of enzyme protein. Additionally, using Ouchterlony double immunodiffusion, immunological identity between the enzyme isolated from control and induced animals was found.

The antioxidant food additive 2(3)-tert-butyl-4-hydroxy anisole (BHA) has been demonstrated to increase the activity of several detoxifying enzymes, including the glutathione S-transferases, epoxide hydrolase, and UDP glucuronyltransferase. Benson et al.\textsuperscript{12} treated mice with a chronic dosage of BHA (7.5 g/kg in the diet for 14 days). Tissue specific induction of DT-diaphorase activity by BHA, assayed spectrophotometrically, was reported to occur. The highest activities were detected in the gastrointestinal and urinary tracts. DT-diaphorase activity was increased 10-fold in liver, 5.7-fold in small intestine, 4.2-fold in kidney, 3.6-fold in lung, and 2-fold in forestomach and glandular stomach. The induction of DT-diaphorase activity was postulated to be responsible, in part, for the protective properties of BHA against tumor production because of the enhanced ability of the induced enzyme to inactivate electrophiles. Further studies by these authors\textsuperscript{21} extended the list of antioxidant inducers to include ethoxyquin and disulfiram.

Support for the assertion that DT-diaphorase has a role in protection against the production of electrophiles has come from the laboratory of Wefers et al.\textsuperscript{22} They quantitated the level of $O_2^-$ production by measuring menadione-induced low-level chemiluminescence in preparations of supernatant from control and BHA-treated mice. Low level chemiluminescence
is produced by the photoemission of excited species such as singlet
molecular oxygen generated by the redox cycling of the semiquinone.
Reactions containing menadione and supernatant from control animals
exhibited an increase of low-level chemiluminescence, whereas virtually no
chemiluminescence was detected with supernatants obtained from BHA-treated
mice. The level of chemiluminescence was increased by the inhibition of
DT-diaphorase activity by dicoumarol in the BHA supernatants. Thus, the
increase of DT-diaphorase activity by BHA yields a decrease in chemi-
luminescence indicating that the semiquinone radical is not formed.

A class of inducers which appears to be separate from that of
polycyclic aromatic hydrocarbons includes trans-stilbene oxide (TSO). In
contrast to the immediate response of 3MC and other aromatics, Lind
et al.\textsuperscript{22} utilized rocket immunoelectrophoresis and spectrophotometry to
detect a 4- to 5-fold increase in the rat liver enzyme by TSO after a delay
of 24 hrs. The level of enzyme remained elevated for five days, after
which time a slow decline was observed. It is not known whether the
difference in induction is due to the rate of distribution of the inducers
or to actual differences in the mechanism of induction.

Several xenobiotics, in addition to those mentioned previously, have
been shown to induce DT-diaphorase activity. Benzo(a) pyrene, 2-acetyl-
aminofluorene, and 2,3,7,8-tetrachlorodibenzo-p-dioxin have each been
reported to increase the activity of cytosolic rat liver
DT-diaphorase.\textsuperscript{24-26} The induction by phenobarbital (PB) has been reported
to be minimal (1.5- to 2-fold).\textsuperscript{4,20}
Proposed Role of DT-Diaphorase in Detoxication

DT-diaphorase catalyzes the two-electron reduction of quinones to hydroquinones, utilizing either NADH or NADPH as electron donors as shown in Fig. 5. An alternate pathway involving NADPH cytochrome P-450 reductase [NADPH: ferri- cytochrome P-450 reductase, EC 1.6.2.4], a microsomal enzyme, results in the metabolism of quinones to labile semiquinone radical intermediates via a one-electron reduction. Semiquinone radicals readily donate one electron to molecular oxygen to generate superoxide radicals (O$_2^-$) and, ultimately, singlet molecular oxygen ($^{1\Delta}O_2$). The redox cycling between quinone/semiquinone and semiquinone/hydroquinone is thought to bring about oxidative stress. Additionally, semiquinones have been demonstrated to interact with DNA by abstraction of hydrogen leading to strand scission, cross-linkage, and, subsequently, cytotoxicity.

Recent evidence suggests that DT-diaphorase acts in competition with NADPH-cytochrome P-450 reductase to protect against menadione cytotoxicity. Thor et al. compared the metabolism of menadione in hepatocytes and subcellular fractions from 3MC and PB-induced rat liver. (NADPH cytochrome P-450 reductase is inducible by PB, but 3MC has no effect on the enzyme.) Menadione elicited a 50% increase in O$_2$ uptake in hepatocytes isolated from PB-treated rats. However, no change in O$_2$ uptake in hepatocytes from 3MC-treated rats was detectable. The inclusion of dicoumarol caused a further increase in O$_2$ uptake in both hepatocyte preparations, with the greatest increase occurring in the 3MC-treated hepatocyte. The reduction of extracellular acetylated cytochrome c mediated via menadione in PB-treated rat liver hepatocytes and subcellular fractions was inhibitable
Fig. 5. Predominant pathways for quinone metabolism in rat liver cytosol.
by the addition of superoxide dismutase. This investigation confirms that DT-diaphorase reduces quinones to hydroquinones via a two electron pathway, and thus does not generate semiquinone radical intermediates. NADPH cytochrome P-450 reductase catalyzes the one-electron reduction of menadione to form menasemiquinone, as evidenced by the increased uptake of O_2 due to redox cycling in the PB-induced liver. Therefore, the cytotoxic effects mediated via the one-electron reduction by NADPH cytochrome P-450 reductase may be protected against by DT-diaphorase.

Studies completed simultaneously in the laboratory of Lind et al.\textsuperscript{31} corroborate the assertion that DT-diaphorase may exert a protective function.

The mutagenicity of various quinones was assayed in the Salmonella typhimurium tester strain TA104.\textsuperscript{32} Several quinones, including menadione, phenanthrenequinone, benzo(a) pyrene 6,12-quinone, and danthron were added to the cultures in the presence of S9 liver homogenate (9,000 x g fraction) from polychlorobiphenyl-induced rats and in the presence and absence of dicoumarol. Mutagenicity was assessed on the basis of the number of His\textsuperscript{+} revertants obtained. Support for the postulated protective nature of DT-diaphorase comes from the following evidence:

1. Menadione was twice as mutagenic when dicoumarol was present, but the other quinones assayed were not affected by the presence of dicoumarol,
2. NADPH cytochrome P-450 reductase has the predominant role in quinone mutagenicity due to enhanced mutagenicity in the presence of NADPH rather than NADH,
3. Oxygen radical plays a major role in mutagenicity as assessed by the ability of superoxide dismutase and catalase to completely inhibit mutagenicity, and
4. A dual role of cytochrome P-450 in the
metabolism of some quinones was identified. The complexity of quinone metabolism was demonstrated by the differential activation or detoxification of structurally diverse quinones. Thus, four pathways for quinone metabolism were proposed to account for the roles of cytochrome P-450, NADPH cytochrome P-450 reductase, and DT-diaphorase in bioactivation and detoxification.

Morrison et al. monitored the extent of DNA damage following the metabolic activation of menadione in rat hepatocytes isolated from 3MC- and PB-induced animals. The increased sensitivity of hepatocytes from PB-treated rats to menadione was reflected by an increased percentage of single-stranded DNA and a decreased percentage of viable cells. Incubation of hepatic microsomes from PB-induced rats with [3H] menadione revealed a greatly increased binding of menadione to DNA. This binding is presumed to be facilitated via the generation of a semiquinone radical intermediate. Furthermore, binding of menadione metabolites to DNA could be reduced by the addition of rat liver cytosol to the system, again demonstrating DT-diaphorase's role in detoxification and protection from cytotoxicity.

The role of DT-diaphorase in the rabbit kidney was recently addressed by Mohandas et al. Quantitation of renal DT-diaphorase activity revealed a differential distribution of DT-diaphorase within the kidney. The highest activity of DT-diaphorase was detected in the renal inner medulla, whereas very little activity was found in the renal cortex. This distribution corresponds to that of prostaglandin H synthase, an enzyme which has been demonstrated to metabolize BP to quinones. Thus, prostaglandin H synthase and DT-diaphorase coexist in such proportions as to provide maximal protection against the formation of semiquinone radicals.
and superoxide radicals in the renal inner medulla. This may explain, in part, why kidney toxins such as phenol and p-aminophenol exert their effects primarily in the renal cortex.

The possibility that DT-diaphorase participates in the bioactivation of the anti-tumor quinone 3-bromomethylmenadione was reported by Talcott et al.\textsuperscript{34} The mutagenicity of the quinone was assayed in \textit{Salmonella typhimurium} TA97 in the presence of an S9 supernatant and in the absence or presence of dicoumarol. Metabolism of the quinone resulted in an increase in mutagenicity in the absence of dicoumarol, suggesting that DT-diaphorase was responsible for generating the reactive species. This seemingly anomalous behavior for DT-diaphorase can be rationalized by noting the presence of the reactive leaving group (bromine) in the 3-position (Fig. 2).

**Induction of DT-Diaphorase in Persistent Hepatocyte Nodules**

The administration of a single dose of the initiator diethylnitrosamine (DEN) to rats plus the promoter 2-acetylaminofluorene (2-AAF) followed by a partial hepatectomy induces the formation of hyperplastic liver nodules.\textsuperscript{35} The hepatocyte nodules are thought to represent a preneoplastic state and as such are widely used as model systems for chemical carcinogenesis. In addition to the morphological changes accompanying nodule formation, profound biochemical alterations are detectable in the nodules. The enzyme activities of various phase II drug metabolizing enzymes, including epoxide hydrolase and the glutathione transferases, are elevated in the nodules, whereas cytochrome P-450 and NADPH cytochrome P-450 reductase activities are decreased.\textsuperscript{36}
of DT-diaphorase has been reported to be increased 12.1-fold following induction of nodules by DEN, 2-AAF, and a partial hepatectomy.\textsuperscript{36,37}

**Rationale**

DT-diaphorase has been proposed to play a predominant role in protection from semiquinone radical-mediated cytotoxicity. It is, therefore, vital to obtain an understanding of the processes involved in the regulation of this enzyme.

The inducibility of DT-diaphorase by numerous xenobiotics, including 3-methylcholanthrene, benzo(a)pyrene, and trans-stilbene oxide has been suggested to be due to an increased synthesis of the enzyme. Whether this elevation is regulated at the transcriptional or translational level has not yet been elucidated. It has been previously demonstrated that xenobiotic induction of NADPH cytochrome P-450 reductase,\textsuperscript{38} epoxide hydrolase,\textsuperscript{39} and the glutathione transferases\textsuperscript{40,41} occurs via an increase in the functional mRNA specific for the enzymes. Therefore, in order to determine the site of regulation of DT-diaphorase, I propose to utilize in vitro translational analysis and a cDNA probe complementary to DT-diaphorase mRNA to quantitate the level of the mRNA in 3-methylcholanthrene, trans-stilbene oxide, and phenobarbital-induced rat liver.

Increased enzymatic activity of DT-diaphorase,\textsuperscript{36,37} as well as epoxide hydrolase and the glutathione transferases,\textsuperscript{36} has been reported to exist in persistent hyperplastic liver nodules induced by chemical carcinogens. In contrast to the transient elevation of the enzymes present following xenobiotic induction, the increase in the enzyme levels in the nodules persists over a substantial time period. Thus, it appears that a more permanent alteration of gene expression has occurred. Several mechanisms
have been proposed to account for alterations of gene expression, including gene amplification, gene rearrangement, altered regulatory regions and modified methylation patterns. In order to determine the mechanism underlying the induction of nodular DT-diaphorase mRNA, genomic DNA will be isolated from the nodules and subjected to restriction endonuclease analysis. In addition, the elevation of DT-diaphorase mRNA in the nodules, as well as in nodular tissue following induction by 3-methylcholanthrene, will be quantitated by in vitro translational analysis and hybridization assay utilizing the cDNA probe. These studies should provide insight into the mechanism of xenobiotic induction and chemical carcinogenesis.
METHODS

Purification of DT-Diaphorase and Preparation and Purification of Antibodies

DT-diaphorase was purified from the livers of male Long-Evans rats which had been administered 3MC intraperitoneally for 4 days at a dosage of 25 mg/kg body weight by the method of Huang et al. Antibodies against the purified rat liver DT-diaphorase were raised in New Zealand rabbits by intradermal injections of 200–300 µg of the protein mixed with Freund's complete adjuvant, followed by weekly subcutaneous booster injections of 70–80 µg of protein mixed with Freund's incomplete adjuvant. The IgG was purified by chromatography in DEAE Affi-Gel Blue equilibrated in 0.02 M Tris-HCl, pH 8.0/0.028 M NaCl.

Isolation and Purification of Poly (A)+ RNA

Total RNA was isolated by the method of Chirgwin. Male Long-Evans rats were injected intraperitoneally with either 40 mg/kg body weight of 3MC, 300 mg/kg body weight of TSO or 80 mg/kg body weight PB. The rats were sacrificed at either 8, 16 or 24 hr post-injection and the livers were obtained. The livers were washed in ice cold saline and any adhering connective tissue was removed. The livers were minced and homogenized in 4 M guanidine thiocyanate/0.5% sodium lauroyl sarcosine/0.025 M sodium citrate/0.1 M β-mercaptoethanol. Homogenates were centrifuged at 8,000 rpm for 10 min at 10°C in a Sorvall SS-34 rotor. The supernatants were decanted and the nucleic acids were precipitated at −20°C overnight by the addition of 0.025 volume of 1 M acetic acid and 0.75 volume of 100% ethanol (EtOH). The nucleic acid was pelleted as above and then dissolved in 7.5 M
guanidine hydrochloride/0.025 M sodium citrate/5 mM dithiothreitol (DTT). The ribonucleic acid was reprecipitated at -20°C overnight by the addition of 0.025 volume of 1 M acetic acid and 0.5 volume of 100% EtOH. The RNA was pelleted and reprecipitated as above. The final RNA pellet was rinsed with 100% EtOH, dissolved in sterile water and precipitated at -20°C overnight with 0.1 volume of 2 M potassium acetate (KAc) pH 5.0 and 2 volumes of 100% EtOH. The RNA was collected by centrifugation at 10,000 rpm, dissolved in 5 ml sterile water and frozen at -20°C.

Oligo (dT)-Cellulose Chromatography

Poly (A⁺)-RNA was obtained by affinity chromatography on oligo (dT) cellulose. The RNA was heated at 68°C for 5 min and cooled to room temperature on ice. After the addition of an equal volume of 20 mM Tris-HCl, pH 7.5/1% sodium dodecyl sulfate (SDS)/2 mM EDTA/1 M LiCl the RNA was applied to an oligo (dT)-cellulose affinity column which was equilibrated in 10 mM Tris-HCl, pH 7.5/0.5% SDS/1 mM EDTA/0.5 M LiCl. The column was rinsed with the same buffer. Poly (A⁺)-RNA was eluted with 10 mM Tris-HCl, pH 7.5. Fractions which contained the RNA peak, as determined by the absorbance at 260 nm, were pooled and the RNA precipitated at -20°C overnight with 0.1 volume of 2 M KAc, pH 5.0 and 2 volumes of 100% EtOH. The RNA was recovered by centrifugation at 12,000 rpm and the pellet was dissolved in sterile H₂O.

Isolation of Genomic DNA

High molecular weight genomic DNA was prepared by the method of Blin and Stafford. Rat liver was isolated from control animals and finely pulverized in liquid N₂. The tissue was transferred into a lysis buffer
containing 50% phenol/50% aqueous buffer (20 mM Tris-HCl, pH 7.8/0.5% SDS/0.5 mM EDTA/1.0 M NaCl). The mixture was extracted twice and the aqueous phases pooled and extracted three times with two volumes of diethyl ether. The mixture was then treated with RNase H (50 μg/ml) at 37°C for 16 hr. The DNA solution was adjusted to 50 mM EDTA/0.5% SDS, 50 μg/ml proteinase K was added, and the solution was incubated at 37°C for 6 hr. The DNA was extracted twice with phenol lysis buffer as above and dialyzed against 20 mM Tris-HCl, pH 7.8/0.5 mM EDTA/10 mM NaCl until the absorbance at 270 nm of the dialysate was less than 0.050.

Isolation and Purification of Polysome mRNA

Purified DT-diaphorase mRNA was obtained via polysome immunoabsorption using protein A-sepharose affinity chromatography. Male Sprague-Dawley rats were administered a single injection of 3MC at a dosage of 40 mg/kg body weight and sacrificed at 16 hr post-injection. Livers were obtained and homogenized in 6.7 volumes of 50 mM Tris, pH 7.5/25 mM NaCl/5 mM MgCl₂/0.25 M sucrose/0.2 mg/ml heparin/1 μg/ml cycloheximide. The homogenate was centrifuged at 10,000 rpm for 10 min in a Sorvall SS34 rotor. The supernatant was collected and diluted with 0.1 volume of 10% sodium deoxycholate (DOC)/10% Triton X-100. Aliquots (30 ml) of supernatant were layered over a discontinuous sucrose gradient (3 ml 2.5 M sucrose and 3 ml 2.0 M sucrose). The gradients were centrifuged at 20,000 rpm for 17 hr in a Beckman SW28 rotor. Following centrifugation, the supernatants and the 2.0 M sucrose layer were aspirated. The 2.5 M sucrose layer containing the polysomes was diluted 1:1 with 25 mM Tris-HCl, pH 7.5/150 mM NaCl/5 mM MgCl₂/0.1% Nonidet P-40 (NP-40)/1 μg/ml cycloheximide/0.2 mg/ml heparin. The solution was dialyzed against 21 of the same buffer at
4°C for 20 hr. The polysomes were quick frozen in dry ice/ethanol and stored at -70°C. Polysomes were thawed, centrifuged at 12,000 rpm for 10 min at 4°C and reacted with DT-diaphorase IgG at a ratio of 160 A₂₆₀ U/mg IgG. Polysomes which were synthesizing DT-diaphorase were immobilized on a protein A-sepharose column which was equilibrated in polysome buffer. The column was unpacked and the resin was washed with 2 volumes of polysome buffer. The column was repacked and washed overnight with polysome buffer. RNA was eluted from the column in the presence of 25 mM Tris-HCl/20 mM EDTA, pH 7.5/0.2 mg/ml heparin and poly (A)^+—RNA was isolated by oligo (dT)—cellulose affinity chromatography as described previously.

**In Vitro Translations**

Poly (A)^+—RNA was utilized to program a rabbit reticulocyte lysate in vitro translation system.³⁹ The reaction (270 μl) consisted of 25 mM Hepes/40 mM KCl/10 mM creatine phosphate/50 μM of all amino acids (minus methionine)/87 mM KAc, pH 7.2 and the rabbit reticulocyte lysate, and [³⁵S]—methionine was used to radiolabel the in vitro synthesized protein. Reactions were incubated at 30°C for 90 min. The cell-free translation products were quantitated via scintillation counting of the trichloroacetic acid (TCA) precipitable protein in aliquots extracted from the reaction at 0 and 90 min.

**Immunoprecipitation of DT-Diaphorase**

In vitro synthesized DT-diaphorase was recovered from the translation mixture by indirect immunoprecipitation.³⁹ In vitro translation mixtures were diluted 1:4 with 1% Triton X-100/1% DOC and centrifuged at 40,000 rpm for 60 min at 4°C to pellet ribosomes. The supernatant was removed to
another tube and 15 μl rabbit anti-rat DT-diaphorase IgG and 100 μl protein A-sepharose slurry (in 150 mM NaCl/5 mM EDTA/50 mM Tris-HCl, pH 7.4/0.05% NP-40) were added. The tubes were rotated end-to-end at 4°C overnight. The protein A-sepharose beads were spun in a microfuge for 5 min and washed five times with phosphate buffered saline (PBS) containing 0.1% SDS and 0.5% NP-40 to remove non-specifically bound proteins. The in vitro synthesized DT-diaphorase was eluted from the beads by boiling in 190 mM NaCl/50 mM Tris-HCl, pH 7.4/6 mM EDTA/1% Triton X-100/1% DOC for 5 min. The beads were respun for 5 min and the supernatant removed to another tube. The supernatants were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. The gel was fluorographed in Enlightening (New England Nuclear) and subjected to autoradiography overnight at -70°C. Additionally, the relative amount of DT-diaphorase synthesized was quantitated via scintillation counting of the gel slices containing the radiolabeled protein. The gel slices were dissolved at 60°C with shaking in 200 μl 60% perchloric acid and 400 μl 30% H₂O₂. The samples were counted in 10 ml Aquasol II. Background radioactivity was determined by excising a gel slice above and below the DT-diaphorase band.

Peptide Mapping of DT-Diaphorase

In order to determine the identity of the cell-free translation product, purified DT-diaphorase and in vitro synthesized DT-diaphorase were subjected to limited proteolysis utilizing Staphylococcus aureus V8 protease. Purified DT-diaphorase and in vitro synthesized DT-diaphorase were electrophoresed on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue R-250 for 60 min, destained in 45% methanol/1.5 M acetic acid and rinsed in cold water. Bands containing DT-diaphorase were
excised and soaked in 0.125 M Tris-HCl, pH 6.8/0.1% SDS/1 mM EDTA for 30 min.

The excised bands were placed into the wells of a 15% SDS-polyacrylamide gel, overlaid with 10% glycerol and 4 μg S. aureus V8 protease and electrophoresed. The gel was stained, destained and fluorographed as described previously.

Isolation of Nodular RNA and DNA

Persistent hepatocyte nodules were induced using the Solt-Farber model. Male Sprague-Dawley rats were administered diethylnitrosamine (200 mg/kg body weight) intraperitoneally, followed one to two weeks later by three daily doses of 2-acetylaminofluorene (20 mg/kg body weight) intragastrically. A partial hepatectomy was performed on the fourth day to stimulate hepatic regeneration. Occasionally, carbon tetrachloride was administered after partial hepatectomy in order to increase cellular proliferation and increase the size of the nodules. One group of rats was administered 3-methylcholanthrene (80 mg/kg body weight) for five days in addition to the above regimen. The animals were sacrificed and RNA and DNA were isolated from the nodules as described in the above sections.

Construction of cDNA Clones

RNA which was isolated by polysome immunoabsorption was used as a template for first strand cDNA synthesis by the method illustrated in Fig. 6. The reaction consisted of 50 mM Tris-HCl, pH 8.0/10 mM MgCl₂/10 mM DTT/1.25 mM dATP/0.5 mM dCTP/1.25 mM dGTP/1.25 mM dTTP/100 μCi ³²P-dCTP (800 Ci/m mole)/150 ng poly (A)⁺ RNA and 1 μg oligo dT₁₂₋₁₈ (to serve as primer) in a total volume of 100 μl. Reverse transcriptase (10 U) was
Construction of cDNA clones.
added and the reaction was incubated at 43°C for 30 min. The reaction was stopped by the addition of 20 mM EDTA and then was phenol extracted with an equal volume of buffer-saturated phenol. The phenol phase was back extracted with an equal volume of 10 mM Tris-HCl, pH 7.5/5 mM EDTA and both aqueous phases were pooled. The single-stranded cDNA in the aqueous phase was precipitated with 0.1 volume 2 M NH₄OAc and 2 volumes of 100% EtOH.

The DNA was pelleted in a microfuge for 15 min, resuspended in 10 mM Tris-HCl, pH 7.5/1 mM EDTA and reprecipitated and pelleted as above. The pellet was rinsed with cold 100% EtOH and dried under vacuum. Second strand synthesis of cDNA was mediated by digestion of the mRNA strand by RNase H and replacement with cDNA by E. coli DNA polymerase I and E. coli DNA ligase. The reaction mixture, containing 20 mM Tris-HCl, pH 7.5/5 mM MgCl₂/10 mM (NH₄)₂SO₄/100 mM KCl/0.15 mM β-NAD/ 50 μg/ml BSA/1 mM dATP/1 mM dCTP/1 mM dGTP/1 mM dTTP, plus the single-stranded DNA in sterile water was incubated at 12°C for 60 min and at 22°C for 60 min in the presence of 0.85 U E. coli RNaseH, 23 U E. coli DNA polymerase I and 1 U E. coli DNA ligase. The reaction was stopped by the addition of 20 mM EDTA. Phenol extractions and precipitations were as described above. The double-stranded cDNA was tailed by the addition of homopolymer tracts of deoxycytidine. The cDNA was dissolved in sterile water and added to a mixture containing 0.2 M potassium cacodylate, pH 6.9/1 mM CoCl₂/1 mM dCTP/50 μCi α³²P-dCTP (800 Ci/mmole). The reaction was initiated at 37°C with 40 U terminal deoxynucleotidyl transferase for 2 min and then quick frozen in dry ice/ethanol to inactivate the enzyme. The tail length was determined by specific activity measurements by scintillation counting of ³²P bound to Whatman DE-81. Briefly, the DE-81 filters were washed in 0.5 M sodium
phosphate. The filters were counted in Aquasol II. The specific activity (dpm/mole) was calculated based on 50 μCi $^{32}$P-dCTP and 1.25 mM dCTP. Total dpm, obtained by multiplying the specific activity times moles ds cDNA, was divided by the total volume to obtain the incorporation of $^{32}$P required for the addition of one deoxycytidine residue per μl per 2 ends cDNA. This value was divided by 2 (to obtain dpm/endeDNA) and then multiplied by 20 to determine dpm required for the addition of 20 deoxycytidine residues. The reaction mixture was thawed and then phenol extracted and precipitated and the pellet was resuspended in sterile water as above.

The chimeric plasmid was prepared by annealing an equimolar quantity of Pst I-cut dG-tailed pBr322 to 160 ng dC-tailed double-stranded cDNA in 0.15 M NaCl/10 mM Tris-HCl, pH 7.6/1 mM EDTA by first incubating the reaction at 70°C for 10 min, then at 48°C for 2 hr and at room temperature for 2 hr.

Annealed chimeric plasmid was utilized to transform E. coli RR1 made competent by calcium chloride/heat shock treatment. All procedures were performed under NIH guidelines in P1 containment. Transformants were identified by plating onto L-agar containing 15 μg/ml tetracycline.

**Colony Hybridization**

Colonies which contained inserts which were complementary to DT-diaphorase mRNA were identified by in situ hybridization. Transformants were picked from the original transformation plates onto sterile gridded nitrocellulose which had been layered onto L-agar plus tetracycline. The plates were incubated at 37°C for 3 hr. Plates and nitrocellulose were keyed with India ink and the filters were carefully
peeled from the agar. The nitrocellulose filters were placed for 15 min at room temperature onto Whatman 3 MM, which had been wetted with 0.5 M NaOH/1.5 M NaCl. The filters were transferred to dry Whatman 3 MM for 5 min and then Whatman 3 MM, which had been wetted with 1 M Tris, pH 7.4, for 5 min. The filters were again transferred to dry Whatman 3 MM and then Whatman 3 MM, wetted with 0.5 M Tris-HCl, pH 7.4/1.5 M NaCl, for 5 min. After blotting onto Whatman 3 MM, the filters were washed three times with 50 ml chloroform and air dried. The nitrocellulose was baked for 2 hr at 80°C in vacuo.

The polysome immunoabsorbed mRNA was utilized to prepare a $^{32}$P-radio-labeled probe to screen the colonies. The mRNA ($\sim 15$ ng) was pre-incubated with 10 µg of the oligo (dT)$_{12-18}$ primer at room temperature for 10 min. To the primed poly(A$^+$)-RNA was added, 50 mM Tris-HCl, pH 8.0, 0.125 mM each of dATP, dCTP, dGTP and dTTP, 10 mM β-mercaptoethanol, 10 mM MgCl$_2$ and 90 µCi each of $\alpha^{32}$P-dATP, $\alpha^{32}$P-dCTP, $\alpha^{32}$P-dGTP and $\alpha^{32}$P-dTTP. The reaction was initiated by the addition of 20 U of reverse transcriptase and was incubated at 42°C for 30 min. Progress of the reaction was monitored by the amount of TCA-precipitable nucleic acid at 0 and 30 min. The reaction was stopped by the addition of 8 µl of 0.1 M EDTA, pH 8.0, and extracted with buffer-saturated phenol. The $^{32}$P-labeled cDNA:mRNA hybrid was subjected to gel filtration on an AcA-54 column which was equilibrated in 10 mM Tris-HCl, pH 8.0/2 mM EDTA/0.1 M NaCl. Fractions were collected (500 µl) and those containing the peak of radioactivity were pooled. The cDNA:mRNA hybrid was precipitated at -20°C overnight by the addition of 0.1 volume of 2 M NaOAc, pH 5.5 and 2 volumes of 100% EtOH. The cDNA:mRNA hybrid was pelleted at 15,000 rpm for 30 min in a Sorvall SS-34 rotor at
4°C. The supernatant was decanted and the pellet was dried under vacuum. The dried pellet was resuspended in 180 μl sterile water and the mRNA was hydrolyzed by incubating the cDNA:mRNA hybrid at 37°C for 1 hr in the presence of 20 μl 3 N NaOH. The 32P cDNA was precipitated at -20°C overnight by the addition of 4 μl glacial acetic acid, 18.4 μl 2 M NaOAc, pH 5.5 and 404 μl 100% EtOH. The cDNA was pelleted in a microfuge at 4°C for 30 min. The supernatant was decanted and the pellet was dried under vacuum. The dried pellet was resuspended in 6X SSC (1X SSC = 0.75 M NaCl and 75 mM sodium citrate, pH 7.0).

The nitrocellulose filters were pre-hybridized at 68°C for 15 hr in 6X SSC/0.2% PVP/0.2% Ficoll/0.2% BSA. The pre-hybridization solution was removed and replaced by 6X SSC/0.2% PVP/0.2% Ficoll/0.2% BSA/0.5% SDS/1 mM EDTA and 2 x 10^6 cpm pre-boiled 32P c-DNA probe. The filters were incubated at 68°C overnight and then washed at 68°C in 2X SSC/0.5% SDS for 1 hr and at room temperature in 1X SSC for 1 hr. The filters were air dried and exposed for autoradiography.

Large Scale Plasmid Isolation

Intensely hybridizing colonies were sized by electrophoresis of a small quantity of plasmid DNA from the colonies. The DNA was fractionated on a 1% agarose gel in 40 mM Tris-OH/20 mM acetic acid/2 mM EDTA in the presence of 0.5 μg/ml ethidium bromide. Colonies whose inserts appeared to be full length were grown on a large scale and the plasmid DNA was isolated. L-broth containing 15 μg/ml tetracycline (100 ml) was inoculated with a single colony and incubated at 37°C overnight. The overnight culture was diluted 1:100 into M9 (M9 is 18.7 mM NH4Cl, 423 mM Na2HPO4, 21.7 mM KH₂PO₄, 85 mM NaCl, 2% casamino acids, 0.4 mM MgSO₄, 0.1
mM CaCl$_2$, 0.2% glucose, 10 g/ml B1) containing 1 mg/ml uridine. The culture was incubated at 37°C until the optical density at 550 nm was 1.0. Chloramphenicol (250 μg/ml) was added to the culture and incubation was continued at 37°C overnight. The cells were pelleted at 5,000 rpm for 5 min, resuspended in 1/20 the original volume of 10 mM Tris-HCl, pH 8.0/1 mM EDTA and respun as above. The cells were resuspended in 25% sucrose/50 mM Tris-HCl, pH 8.0 and 2 mg/ml lysozyme were added. The cellular suspension was placed on ice for 10 min with occasional agitation, 0.25 M EDTA, pH 8.0 was added and the suspension was again placed on ice with agitation for 5 min. Brij solution was added and the suspension was placed on ice with occasional mixing. The cellular debris was pelleted at 35,000 rpm in a Beckman 50.2 Ti rotor for 45 min at 4°C. Ethidium bromide (500 μg/ml) was added to the lysate and the refractive index was adjusted to 1.394 with cesium chloride (0.8 g/ml). The lysate was cleared by centrifugation at 12,000 rpm for 15 min and then the DNA was isolated by centrifugation at 42,000 rpm in a Beckman 50.2 Ti rotor for 48 hr at 20°C. The band containing the plasmid DNA was removed and the refractive index was adjusted to 1.390 with cesium chloride. The DNA was centrifuged at 42,000 rpm in a Beckman 80 Ti rotor for 48 hr at 20°C. The plasmid DNA was collected, extracted four times with cesium chloride-saturated isopropanol and dialyzed overnight against 10 mM Tris-HCl/1 mM EDTA, pH 7.8. The plasmid DNA was extracted with an equal volume of 50:24:1 phenol-chloroform-isoamyl alcohol. The aqueous phase was extracted three times with an equal volume of buffer-saturated ether, and the DNA was precipitated at -20°C overnight by the addition of 0.1 volume 3 M NaOAc, pH 7.0 and 2 volumes 100% EtOH. The DNA was recovered by pelleting at 12,000
rpm for 30 min at 4°C. The DNA pellet was dried under vacuum and resuspended in 10 mM Tris-HCl, pH 7.5/1 mM EDTA.

Hybrid Select Translation

The specificity of one of the inserts was ascertained by hybrid select translation.\textsuperscript{55} The plasmid, designated pDTD55, was linearized with Bam HI, brought up in 0.2 N NaOH and adjusted to 3X SSC. The DNA was boiled for 60 sec, quick frozen in dry ice/ethanol and then thawed and applied to a 13 mm nitrocellulose filter. The filter was air dried, rinsed in 6X SSC and baked at 80°C for 2 hr in vacuo. The filter was hybridized at 37°C for 12 hr in 50% formamide/1 mM Pipes, pH 6.4/0.4 M NaCl, and in the presence of 60 μg of mRNA isolated from 3MC-treated rats. The filters were washed ten times in 1 ml 1X SSC/0.5% SDS at 60°C and rinsed in 2 mM EDTA. Filter-bound mRNA was eluted for 60 sec at 100°C in 300 μl 1 mM EDTA, pH 7.9 containing 10 μg calf liver tRNA. The mRNA was quick frozen in dry ice/ethanol, and then thawed and precipitated with 0.1 volume 2 M KAc, pH 5.5 and 2 volumes 100% EtOH. The RNA was pelleted at 4°C for 15 min, the supernatant was decanted and the pellet was dried under vacuum. The pellet was resuspended in 10 μl sterile water. The mRNA was utilized to program a rabbit reticulocyte lysate cell free translation system. The in vitro synthesized protein was immunoprecipitated, subjected to electrophoresis on an SDS-polyacrylamide gel and fluorographed as described above.

Restriction Endonuclease Mapping

A partial restriction map was constructed by limited digestion of a \textsuperscript{32}P 5'-end labeled fragment.\textsuperscript{56} The plasmid, pDTD55, was digested with Sca I and Eco RI. The digest was fractionated on a 1% agarose gel and the band
of interest was excised from the gel. The agarose was dissolved in saturated sodium iodide solution by heating at 37°C. Processed glass beads were added to the solutions and the tubes containing the solutions were rotated end-to-end at 4°C overnight. The beads were spun 1 min in a microfuge, washed with cold sodium iodide solution, respun and washed twice with cold 50% ethanol containing 0.1 M NaCl/10 mM Tris-HCl, pH 7.5/1 mM EDTA. The DNA was eluted from the glass beads twice at 37°C for 1 hr in 10 mM Tris-HCl, pH 7.5/1 mM EDTA. The DNA was precipitated from the pooled elutions at -20°C overnight by the addition of 0.1 volume 3 M NaOAc, pH 7.0 and 2 volumes of 100% EtOH. The DNA was pelleted, the supernatant was decanted, and the pellet was dried under vacuum. The DNA was resuspended in 10 mM Tris-HCl, pH 7.5/1 mM EDTA, adjusted to 0.5 M Tris-HCl, pH 8.5, and 2 volumes of isopropanol were added. The DNA was placed at -70°C for 5 min and pelleted for 15 min. The supernatant was decanted and the pellet was dried under vacuum. The DNA was resuspended in 10 mM Tris-HCl, pH 7.5/1 mM EDTA, reprecipitated, and pelleted as above.

The DNA was labeled at the 5'-end by replacement of the 5'-phosphate with $^32$P-dATP. Briefly, the DNA was dephosphorylated at 65°C by bacterial alkaline phosphatase for 1 hr. The DNA was rephosphorylated at 37°C by polynucleotide kinase for 30 min in the presence of 60 mM Tris-HCl, pH 7.8, 15 mM β-mercaptoethanol, 10 mM MgCl$_2$, 0.33 μM ATP and $^32$P-dATP. The $^32$P-labeled DNA fragment was digested secondarily with Cla I to remove the label at the Eco RI end. The $^32$P-labeled Sca I-Cla I fragment was subjected to limited digestion with Ava II, Hinf I, Pst I and Dde I over a 60 min time course. The digests were subjected to electrophoresis on a 1% agarose gel. The gel was dried and autoradiographed.
Northern Blot

Poly(A⁺)-RNA (5 µg) isolated from rats which had received 3MC was fractioned on a 1.5% agarose gel containing 10 mM methylmercury hydroxide. The RNA was transferred to diazobenzyloxymethyl paper (DBM) overnight in the presence of 0.2 M NaOAc and the paper was prehybridized at 42°C for 24 hr in 50% formamide/1% glycine/1% SDS/0.02% BSA/0.02% Ficoll/0.02% PVP/50 mM sodium phosphate, pH 6.5/0.75 M NaCl/75 mM sodium citrate/1 mg/ml denatured salmon sperm DNA. Hybridization was at 42°C for 16 hr in the same buffer as above minus glycine and in the presence of 2 x 10⁶ cpm ³²P-pmCTD55. The ³²P-labeled probe was prepared by nick translation of the plasmid by the method of Rigby et al. The blot was washed twice at room temperature for 15 min in 0.36 M NaCl/20 mM Na₂HPO₄/2 mM EDTA/0.1% SDS and twice at 50°C for 30 min in 18 mM NaCl/1 mM Na₂HPO₄/0.1 mM EDTA/0.1% SDS. The paper was air dried and autoradiographed.

RNA Dot Blots

Poly (A⁺)-RNA (5 µg) was dissolved in 0.2 M NaOAc, pH 5.5 and pipetted onto DBM paper. The paper was rinsed twice in 0.2 M NaOAc and air dried. Prehybridization, hybridization, and wash conditions were as described in Northern Blot section.

Genomic Blots

Genomic DNA (15 µg) was digested to completion with one of several restriction endonucleases and the digests were subjected to electrophoresis on a 1% agarose gel. The DNA was transferred to nitrocellulose paper overnight in the presence of 20X SSC and the paper was baked at 80°C in vacuo for 2 hr. Prehybridization was at 42°C for 24 hr in 50%
formamide/5X SSPE/5X Denhardt's solution/100 μg/ml denatured salmon sperm DNA. (1X SSPE = 0.18 M NaCl, 1 mM EDTA, 10 mM NaPO₄, pH 7.4 and 1X Denhardt's = 0.02% BSA, 0.02% Ficoll, 0.02% PVP). Hybridization was at 42°C for 16 hr in the same buffer in the presence of 5% dextran sulfate and 20 x 10⁶ cpm ³²P-nick translated pDTD55. The paper was washed twice at room temperature for 15 min in 2X SSC/0.1% SDS and twice and 68°C for 30 min in 1X SSC/0.1% SDS. The nitrocellulose was then air dried and autoradiographed.

Slot Blot

Genomic DNA (1-12 g) was dissolved in 10 mM Tris–HCl, pH 7.5/1 mM EDTA, adjusted to 0.3 M NaOH and incubated at 60-70°C for 60 min. The DNA was cooled to room temperature and neutralized with an equal volume of 2 M NH₄OAc, pH 7.0. Nitrocellulose paper which had been wet with 1 M NH₄OAc was placed onto a Slot Blot apparatus (Schliecher and Schuell) and the DNA was pipetted into the wells under vacuum. The paper was air dried and baked at 80°C for 2 hr. Prehybridization, hybridization and washes of the paper were as described in the Genomic Blot section.
RESULTS AND DISCUSSION

Mobility of DT-Diaphorase on SDS-Polyacrylamide and Immunoreactivity with DT-Diaphorase IgG

Purified rat liver DT-diaphorase was subjected to electrophoresis on a 10% SDS-polyacrylamide gel in order to estimate the molecular weight and judge the purity of the preparation (Fig. 7). The subunits migrated as a single polypeptide at a molecular weight of 32,000 daltons (Fig. 8A). Antiserum obtained from rabbits was reacted with the purified protein and produced a single precipitin band (Fig. 8B). This antiserum was purified and utilized in all subsequent immunoprecipitations.

Peptide Mapping of DT-Diaphorase

Total poly (A⁺)-RNA was utilized to direct the synthesis of a rabbit reticulocyte lysate in vitro translation system in the presence of [35S]-methionine. DT-diaphorase was recovered by indirect immunoprecipitation utilizing protein A-sepharose and DT-diaphorase IgG and the immunoprecipitated protein was electrophoresed on 10% SDS-polyacrylamide. The identity of the immunoprecipitated product was confirmed by peptide mapping of the in vitro synthesized protein and purified DT-diaphorase. As can be seen in Fig. 9, each [35S]-methionine containing peptide of the in vitro translated protein corresponds to a Coomassie Blue stained fragment of the purified protein.

Purification of Polysome Immunoprecipitated DT-Diaphorase mRNA

In order to obtain highly purified mRNA for cloning, polysome immunoadsorption techniques were utilized. Polysomes were isolated from the
Molecular weight determination for DT-diaphorase. The relative mobility, $R_m = (R_i/R_f)$, was calculated based on the migration of the protein standards ($R_i$) in reference to the dye front ($R_f$). Relative mobility is plotted versus the log of the molecular weight. Molecular weight markers: bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500). Closed circle indicates migration of purified DT-diaphorase subunits.
Fig. 8A. SDS/polyacrylamide gel electrophoresis of purified rat liver DT-diaphorase. Lane 1, molecular weight markers: phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500). Lane 2, 5 μg of purified DT-diaphorase stained with Coomassie Blue R250.

Fig. 8B. Ouchterlony double immunodiffusion analysis of purified DT-diaphorase. The center well contained antiserum against purified DT-diaphorase. The outer wells contained various dilutions of the purified protein. Well 1, 0.91 mg/ml; well 2, 0.45 mg/ml; well 3, 0.091 mg/ml; well 4, 0.009 mg/ml; well 5, 0.9 g/ml. The precipitin bands were visualized by staining with Coomassie Blue G250.
Peptide maps of purified DT-diaphorase and in vitro synthesized DT-diaphorase. Purified DT-diaphorase and in vitro synthesized and immunoprecipitated DT-diaphorase were subjected to limited proteolysis using S. aureus V-8 protease (4 µg). Lane 1, purified DT-diaphorase (10 µg); lane 2, in vitro synthesized DT-diaphorase. The proteolytic fragments generated from the purified protein were detected by Coomassie Blue staining whereas fragments generated from the in vitro synthesized DT-diaphorase were visualized by fluorography. All of the peptides were resolved on a 15% w/v SDS-polyacrylamide gel. Closed circles represent peptides which are common to both digestions.
livers of rats which had received a single injection of 3MC sixteen hours prior to sacrifice. The polysomes were reacted with DT-diaphorase IgG, immobilized on a protein A-sepharose column, and eluted from the column in the presence of EDTA. Poly (A⁺)-RNA was selected on oligo (dT) cellulose and the purity of the RNA preparation was assessed by in vitro translation and immunoprecipitation. As can be seen in the autoradiograph of the gel (Fig. 10), a single major band was obtained denoting a high degree of purity of this mRNA preparation. It was not possible to determine the quantity of poly (A⁺)-RNA obtained, but based on the yield from the cDNA cloning discussed later, it is estimated to be 100-200 ng. This highly purified mRNA was utilized in subsequent cloning techniques.

Construction, Identification and Characterization of the cDNA Clone

A novel technique which utilizes ribonuclease H and DNA polymerase I was used to synthesize double-stranded cDNA. This procedure obviates the need for S1 nuclease digestion and assures the synthesis of cDNA clones which contain sequences complementary to the 5'-ends of the corresponding mRNAs. The addition of 20 oligo (dC) residues to the cDNA was monitored by scintillation counting as described in Methods. The cDNA was annealed to an equimolar quantity of Pst I-cut dG-tailed pBr322 and utilized to transform E. coli RR1. Since the inserts were cloned into the Pst I site, the site of ampicillin resistance, transformants were selected which were tetracycline resistant and ampicillin sensitive. Of the greater than 1300 transformants obtained, 75% of the colonies possessed an insert. Screening of 500 of these colonies by in situ hybridization to a ³²P-cDNA probe prepared by reverse transcription of polysome immunoprecipitated poly (A⁺)-RNA resulted in the identification of eighty-six colonies which
Fig. 10. Autoradiograph of SDS/polyacrylamide gel of in vitro synthesized DT-diaphorase immunoprecipitated from translation system programmed with rat liver poly (A')-RNA isolated by polysome immunoabsorption. Lane 1, total endogenous translation products; lane 2, total translation products from system programmed with purified mRNA; lane 3, DT-diaphorase immunoprecipitated from total translation products. Molecular weight standards are identical to those presented in Fig. 8A. Arrow indicates migration of purified DT-diaphorase subunits.
possessed sequences complementary to DT-diaphorase mRNA (Fig. 11). The colonies were sized on 1% agarose gels as described above and a range of cDNA inserts from 400 to 2000 bp was obtained, with 60% of these in excess of 1000 bp. Based on a molecular weight of 32,000 daltons, an mRNA of approximately 1000 bp would be required to encode the protein. The plasmid harboring the largest insert was designated pDTD55 and utilized for further characterization. Hybrid select translation was utilized to positively identify the putative DT-diaphorase cDNA. pDTD55 was linearized with Bam HI and bound to nitrocellulose. Filter-bound pDTD55 was hybridized to 60 g of poly (A⁺)-RNA isolated from rats which had received a chronic dosage of 3MC. Selected mRNA was eluted from the filter, translated in vitro, immunoprecipitated and subjected to electrophoresis on a 10% SDS-polyacrylamide gel. The plasmid pDTD55 is shown (Fig. 12) to hybrid select DT-diaphorase mRNA and, therefore, contains an insert which is specific for DT-diaphorase.

**Sizing of pDTD55 and Construction of Restriction Map**

The size of the insert in pDTD55 was determined by restriction digestion of the plasmid with Pst I. The digests were fractionated on a 1% agarose gel. Five bands were obtained, at 4362 (pBr322), 1050, 600, 200, and 150 bp, yielding a total insert size of approximately 2000 bp (Fig. 13A). In addition, restriction sites for the enzymes Ava I, Ava II, Dde I, Hind III, Hinf I, and Nco I (see Fig. 13B,14,15) were detected as described in Methods. A partial restriction map of pDTD55 is shown in Fig. 16.
Fig. 11. In situ hybridization of transformed colonies. Colonies which were Tc$^R$ Ap$^S$ were lysed in situ and the DNA was denatured and fixed to nitrocellulose. The DNA was hybridized to a $^{32}$P-cDNA probe prepared by reverse transcription of poly (A$^+$)-RNA purified by polysome immunoselection. The arrow marks a strongly hybridizing colony.
Fig. 12. Autoradiograph of SDS/polyacrylamide gel of \textit{in vitro} synthesized DT-diaphorase from translation system programmed with poly (A$^+$)-RNA selected by pDTD55. Lane 1, total endogenous translation products; lane 2, total translation products from system programmed with selected mRNA; lane 3, DT-diaphorase immunoprecipitated from total translation products. Molecular weight standards are identical to those presented in Fig. 8A.
Pst I digestion of pDTD55 on 1% agarose stained with ethidium bromide. pDTD55 was digested to completion with Pst I and fractionated on a 1% agarose gel in the presence of 0.5 μg/ml ethidium bromide. The digestion yielded 5 bands (lane 1): 4362 (pBR322), 1050, 600, 200 and 150 bp. Lane 2 contains Hind III-digested λ DNA and Hae III-digested φX174 DNA fragments.

Fig. 13A. Autoradiograph of time course for Pst I digestion of 32P-pDTD55. The 32P-labeled Sca I-Cla I fragment, prepared as described in Methods, was subjected to limited digestion with Pst I. Aliquots were removed from the digest at 5 min (lane 1), 10 min (lane 2), 15 min (lane 3), 30 min (lane 4), and 60 min (lane 5) and fractionated on a 1% agarose gel in the presence of 0.5 μg/ml ethidium bromide.
Fig. 14. Autoradiograph of time course for Ava II digestion of $^{32}$P-pDTD55. The $^{32}$P-labeled Sca I-Cla I fragment was digested as above except that Ava II was utilized. Lane 1, undigested $^{32}$P-labeled Sca I-Cla I fragment; lane 2, 5 min; lane 3, 10 min; lane 4, 15 min; lane 5, 30 min; and lane 6, 60 min following the addition of the enzyme. Molecular weight standards are identical to those presented in Fig. 13A.
Fig. 15. Autoradiograph of time course for Dde I and Hinf I digestion of pDTD55. The $^{32}$P-labeled Sca I-Cla I fragment was digested as above except that Dde I or Hinf I was utilized. Lane 1 (5 min); lane 2 (10 min); lane 3 (15 min); lane 4 (30 min); lane 5 (60 min). Molecular weight standards are identical to those presented in Fig. 13A. Times for lanes 6-10 correspond to that of lanes 1-5.
Fig. 16. Partial restriction map of pDTD55.
Quantitation of In Vitro Synthesized DT-Diaphorase Following Induction by 3-Methylcholanthrene, Trans-Stilbene Oxide and Phenobarbital

In order to quantitate the levels of DT-diaphorase mRNA at various time points, total poly (A⁺)-RNA was isolated from 3MC, TSO and PB-treated rat liver. The mRNAs were translated in vitro and DT-diaphorase was quantitated as described above. The data obtained reveal that a single injection of 3MC eight hours prior to sacrifice induces functional DT-diaphorase mRNA by 8-fold (Fig. 17 & 18). In contrast, the administration of trans-stilbene oxide results in a slight (3-fold) increase following an initial lag of sixteen hours (Fig. 18 & 19). This response is consistent with earlier findings by Lind et al., who detected no increase in enzyme activity twenty-four hours following administration of TSO, but recorded elevated DT-diaphorase activity twelve hours after 3MC administration. Additionally, in correlation with what was previously reported, PB yielded only a 2 to 2.5 fold increase in DT-diaphorase mRNA (Fig. 18 & 20).

Quantitation of In Vitro Synthesized DT-Diaphorase in Persistent Hepatocyte Nodules

DT-diaphorase mRNA levels were determined by in vitro translation of poly (A⁺)-RNA isolated from control liver tissue, nodular tissue, and surrounding liver tissue. Total translation products were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and autoradiographed. In Fig. 21, differences in the mRNA content are shown to be reflected by the appearance of decreased synthesis of some proteins (arrows) and elevated synthesis of other proteins (circles) in the nodules. Relative DT-diaphorase levels were determined as described previously.
Fluorograms of in vitro synthesized DT-diaphorase immunoprecipitated from translation systems programmed with liver mRNA isolated from rats at various times after 3-methylcholanthrene administration. Total liver poly (A\(^+\))-RNA was isolated from rats at various times after a single administration of 3-methylcholanthrene and translated in the rabbit reticulocyte cell-free translation system. The translations were subjected to immunoprecipitation using DT-diaphorase IgG and an equal aliquot of each immunoprecipitation was subjected to SDS-polyacrylamide gel electrophoresis using a 10% gel. The arrow corresponds to the position of purified DT-diaphorase in the polyacrylamide gel. The molecular weight markers are identical to those presented in Figure 8A.
Fig. 18. Effect of 3-methylcholanthrene, trans-stilbene oxide and phenobarbital on the level of translatable DT-diaphorase mRNA. The procedure for quantitating DT-diaphorase mRNA levels is described in the Methods section. For all experiments an equal number of input cpm was utilized for the immuno-precipitation reactions. For each mRNA isolation the livers from four rats were pooled. The relative mRNA level for untreated rats was arbitrarily given a value of one.
Fluorograms of *in vitro* synthesized DT-diaphorase immunoprecipitated from translation systems programmed with liver mRNA isolated from rats at various times after trans-stilbene oxide administration. The experiments presented in this figure are identical to those in Figure 17 except trans-stilbene oxide was used as the inducing agent instead of 3-methylcholanthrene.
Fig. 20. Fluorograms of in vitro synthesized DT-diaphorase immunoprecipitated from translation systems programmed with liver mRNA isolated from rats at various times after phenobarbital administration. The experiments presented in this figure are identical to those described in Figure 17 except phenobarbital was used as the inducing agent instead of 3-methylcholanthrene.
Fig. 21. $[^{35}S]$methionine-labeled translation products directed by poly (A$^+$)-RNA isolated from normal rat, liver tissue surrounding persistent hepatocyte nodules, and nodular tissue. Lane 1 represents endogenous protein synthesis in the absence of added mRNA, lane 2, normal liver, lane 3, surrounding liver, and lane 4, nodular tissue. The arrowheads represent polypeptide(s) whose levels are decreased in the translations programmed with mRNA isolated from persistent hepatocyte nodules whereas the closed circles represent those polypeptides whose levels are elevated.
Functional DT-diaphorase mRNA was elevated 5- to 7-fold in the nodules, whereas only a very slight increase was detected in the surrounding liver tissue (Fig. 22 and Table 1). This elevation corresponds to the increased activity of DT-diaphorase previously reported. The modest elevation in the surrounding tissue may be attributed to the existence of microscopic nodules present in the tissue.

Induction of DT-Diaphorase mRNA in Hepatocyte Nodules by 3-Methylcholanthrene

In order to determine whether DT-diaphorase mRNA retained the capability to be regulated by 3MC, rats were administered the polycyclic aromatic hydrocarbon following nodule induction as described in Methods. DT-diaphorase mRNA levels were quantitated as before. After 3MC administration, the level of DT-diaphorase mRNA was elevated 13- to 21-fold in the nodular tissue as compared to the control (Fig. 23 and Table 2). This represents a 3-fold increase over control nodular tissue, 3MC-induced liver tissue, or 3MC-induced surrounding liver tissue indicating that DT-diaphorase mRNA is further induced following nodule formation and, suggesting that the regulatory regions of the gene have probably not been altered by nodule induction.

RNA Blot Hybridization

The plasmid pDTD55 was utilized to quantitate DT-diaphorase mRNA by RNA blot hybridization in preparations isolated from 3MC-treated rat liver at various time points. The mRNAs were fractionated on a denaturing gel in the presence of methylmercuryhydroxide. The RNA was transferred to DBM paper and hybridized to a $^{32}$P probe prepared by nick translation of pDTD55.
**Fig. 22.** Fluorograms of SDS/polyacrylamide gel electrophoresis of DT-diaphorase immunoprecipitated from translation mixtures programmed with poly (A⁺)-RNA isolated from normal liver, surrounding liver tissue and nodular tissue. Lane 1 represents normal liver, lane 2, surrounding liver and lane 3, nodular tissue. The arrow represents the migration of purified DT-diaphorase on the 10% Na DodeSO₄ polyacrylamide gel. The molecular weight markers are identical to those presented in Figure 8A.
Table 1. DT-Diaphorase mRNA Levels in Normal Liver, Surrounding Liver Tissue and Nodular Tissue Isolated from Untreated Rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td>1161 (1.0)</td>
<td>788 (1.0)</td>
</tr>
<tr>
<td>Surrounding liver</td>
<td>1825 (1.6)</td>
<td>1476 (1.9)</td>
</tr>
<tr>
<td>Nodules</td>
<td>6153 (5.3)</td>
<td>5769 (7.3)</td>
</tr>
</tbody>
</table>

For experiment #1 the total input cpm used in the immunoprecipitation reaction was $28 \times 10^6$ whereas in experiment #2 the total input cpm was $31 \times 10^6$. The values in parentheses represent the fold increase over normal liver tissue. Liver tissue from three to five animals was pooled for each experiment.
Fig. 23. Fluorograms of SDS/polyacrylamide gel electrophoresis of DT-diaphorase immunoprecipitated from translation mixtures programmed with poly (A⁺)-RNA isolated from normal liver, liver isolated from 3-methylcholanthrene-treated rats, surrounding liver tissue from 3-methylcholanthrene-treated rats, and nodular tissue from 3-methylcholanthrene-treated rats. Lane 1 represents normal liver, lane 2, liver from 3-methylcholanthrene-treated rats, lane 3 surrounding liver from 3-methylcholanthrene-treated rats and lane 4, nodular tissue from 3-methylcholanthrene-treated rats. The arrow represents the migration of purified DT-diaphorase on the 10% NaDodSO₄ polyacrylamide gel. The molecular weight markers are identical to those presented in Figure 8A.
Table 2. DT-Diaphorase mRNA Levels in Normal Liver Tissue, Surrounding Liver Tissue and Nodular Tissue Isolated from 3-Methylcholanthrene-Treated Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Untreated liver</td>
<td>742 (1.0)</td>
<td>552 (1.0)</td>
</tr>
<tr>
<td>3MC</td>
<td>Normal liver</td>
<td>4569 (6.2)</td>
<td>2907 (5.3)</td>
</tr>
<tr>
<td>3MC plus nodule induction</td>
<td>Surrounding liver</td>
<td>4292 (5.8)</td>
<td>3358 (6.1)</td>
</tr>
<tr>
<td>3MC plus nodule induction</td>
<td>Nodules</td>
<td>10002 (13.5)</td>
<td>11801 (21.4)</td>
</tr>
</tbody>
</table>

The input cpm used in each immunoprecipitation reaction were $14 \times 10^6$. The values in parentheses represent the fold elevation over the level of mRNA in untreated rats.
The paper was exposed for autoradiography (Fig. 24A). It was not possible to quantitate the mRNA levels by densitometry because the control DT-diaphorase mRNA was barely detectable by this method. However, an induction pattern exists which corresponds to that obtained by in vitro translational analysis of mRNA isolated from 3MC-treated rat liver. Thus, the induction of DT-diaphorase in response to the polycyclic hydrocarbon 3MC appears to be solely due to an accumulation of mRNA specific for DT-diaphorase. The mRNA species, to which pDTD55 hybridizes, migrates at approximately 2000 bp. Thus, DT-diaphorase is encoded by an mRNA which is considerably larger than would be expected for a protein of 32,000 daltons. The presence of long untranslated regions presumably accounts for this discrepancy.

**RNA Dot Blot Hybridization of 3-Methylcholanthrene, Trans-Stilbene Oxide, Nodule and 3-Methylcholanthrene-Induced Nodule mRNAs**

To determine whether the increased translational activity of DT-diaphorase mRNA was due to an accumulation of mRNA specific for DT-diaphorase, mRNA levels were quantitated by hybridization analysis of 3MC, TSO, nodular, and 3MC-induced nodular mRNAs to pDTD55. The mRNAs were bound covalently to DBM paper and hybridized to a 32P probe prepared by nick translation of pDTD55. The signal obtained for the control mRNA was very weak, thus precluding the opportunity to quantitate the mRNA by densitometry (Fig. 24B). The patterns obtained, however, appear to be identical to those of the in vitro translational assays. Induction of the xenobiotic-induced mRNAs, therefore, appears to be regulated at the transcriptional or post-transcriptional levels. This transient elevation is a typical response for drug metabolizing enzymes when challenged by
RNA gel blot hybridization analysis of $^{32}$P-pDTD55 to denatured poly (A$^+$)-RNA isolated from rat liver at various times after methylcholanthrene administration. Poly (A$^+$)-RNA (5 µg) was fractionated on a 1.5% agarose gel containing 10 mM CH$_3$HgOH and blotted onto DBM paper. The poly (A$^+$)-RNA was hybridized to $^{32}$P-pDTD55 and the blot was autoradiographed. Lane 1, poly (A$^+$)-RNA from control rat liver; lane 2, poly (A$^+$)-RNA following a single injection of 3MC at 8 hr; lane 3, 16 hr and lane 4, 24 hr. Molecular weight markers are identical to those in Fig. 13A.
**Fig. 24B.** 3-methylcholanthrene and nodular poly (A⁺)-RNA dot blots. Poly (A⁺)-RNA (5 µg) was pipetted onto DBM paper and hybridized to ⁳²P-pDTD55. The blot was subjected to autoradiography. Row a, poly (A⁺)-RNA isolated from: 2, chronic 3MC-treated rat liver; 3, liver tissue surrounding hyperplastic nodule from 3MC-treated rat liver; 4, hyperplastic nodule from 3MC-treated rat liver; Row b, 2, liver tissue surrounding hyperplastic nodule from "control" rat liver; 4, hyperplastic nodule from "control" rat liver; Row c, 1, control rat liver; 2, 3MC-treated rat liver 8 hr following a single injection of 3MC; 3, 16 hr; 4, 24 hr.
acute xenobiotic administration as is seen in the glutathione transferases, \textsuperscript{40,41} cytochrome P-450s, \textsuperscript{41,45,61,62} epoxide hydrolase, \textsuperscript{39} and NADPH cytochrome P-450 reductase.\textsuperscript{38} The increase of the mRNAs in the nodular and nodular 3MC-induced tissues appears to be regulated at an alternate site due to the more sustained response elicited. (Animals used in these experiments entered the nodule induction regimen approximately 5 months preceding sacrifice and mRNA isolation). Possible mechanisms involved will be addressed in greater detail in the next section.

\textbf{Organization of the DT-Diaphorase Gene}

As mentioned in the previous section, the induction of DT-diaphorase mRNA in the nodules does not appear to involve the more transient transcriptional activation that is seen in the xenobiotic-induced state; rather, the alteration must be of a more permanent nature. Several investigators have proposed mechanisms which involve alterations at the genomic site, including gene amplification,\textsuperscript{42} gene rearrangement,\textsuperscript{43} and differences in methylation.\textsuperscript{44} In order to determine if any of these mechanisms are responsible for the induction of DT-diaphorase mRNA, the genomic organization of DT-diaphorase was probed by restriction endonuclease analysis with radiolabeled pDTD55. Genomic DNA was isolated from control rats and characterized by hybridization of restriction digests to a $^{32}$P probe prepared by nick translation of pDTD55. As is seen in Fig. 25, lane 2, a single gene spanning 7,200 bp encodes DT-diaphorase as evidenced by the migration of a single band at 7,200 bp in the Bgl II digest. Since there were no Bam HI sites in the cDNA insert of pDTD55, the hybridization of $^{32}$P-pDTD55 to four bands in the Bam HI digest of genomic
DNA (see Fig. 25, lane 1) represents the presence of a minimum of three introns in the gene.

Rearrangement of the gene within the nodules was probed by hybridization of Bam HI- and Bgl II-restricted genomic DNA from control, surrounding, and nodular liver tissue to the \textsuperscript{32}P-pDTD55 probe. A restriction pattern of the nodular DNA was obtained which was identical to that of the control and surrounding DNAs. Thus, no apparent rearrangement of the gene has occurred in the nodules.

The possibility that induction of the mRNA was a result of amplification was approached by slot blot analysis of the genomic DNA from control, surrounding and nodular tissue bound to nitrocellulose. Utilizing a Slot Blot apparatus (Schleicher and Schuell), equivalent amounts of genomic DNA from each of the tissues were applied to nitrocellulose paper. The paper was hybridized to \textsuperscript{32}P-pDTD55 and autoradiographed. If amplification had occurred, its detection should be possible by an increased autoradiographic signal intensity in the nodules. In Fig. 26, it can be seen that the signal intensities obtained for the control, surrounding, and nodular DNAs are identical. Thus, amplification did not occur in this system.

Methylation patterns of the enzyme were, therefore, probed by comparison of the restriction fragments resulting when genomic DNA from control, surrounding, and nodular tissue was digested with Hpa II and Msp I. These enzymes are isoschizomers which cleave the sequence $\text{CGGG} \quad \text{GGCC}$. However, Hpa II is only active at this site if 5-methylcytosine is not present, whereas Msp I is insensitive to methylation of cytosine residues. The fragments were transferred to nitrocellulose and hybridized to the \textsuperscript{32}P
Fig. 25. Hybridization of $^{32}$P-pDTD55 to restriction digests of rat liver genomic DNA. Genomic DNA (15 μg) was digested to completion with one of several restriction endonuclease and the digests were fractionated on 1% agarose. The DNA was transferred to nitrocellulose and hybridized to $^{32}$P-pDTD55. The blot was autoradiographed. Lane 1, Bam HI; lane 2, Bgl II; lane 3, Eco RI; lane 4, Eco RV; lane 5, Kpn I; lane 6, Pvu II; and lane 7, Sma I. Molecular weight markers are Hind III-digested λ DNA fragments.
Fig. 26. Slot blot hybridization analysis of $^{32}\text{P}-\text{pDTD55}$ to rat liver genomic DNA isolated from control, surrounding and nodular tissue. Genomic DNA (1-12 $\mu$g) was applied directly to nitrocellulose and hybridized to $^{32}\text{P}-\text{pDTD55}$. Column A, genomic DNA from control rat liver; column B, genomic DNA from tissue surrounding hyperplastic nodules and column C, genomic DNA from nodular tissue.
probe. The digestion patterns obtained for the control and surrounding liver DNAs appear identical in the autoradiograph, (compare lanes 1 and 3, and lanes 2 and 4 in Fig. 27), indicating no difference in methylation in the DNA. However, in the Hpa II digest of the nodular DNA (lane 5) a fragment which migrates at ~4000 bp is generated which is absent from the control and surrounding DNA digests. This fragment corresponds to a fragment of identical size generated by the Msp I digestion. Therefore, it appears that the nodular genomic DNA may be hypomethylated. It has been previously demonstrated that genes that are being actively expressed are undermethylated or unmethylated.63 Thus, the mechanism of induction of DT-diaphorase in nodules induced by chemical carcinogens may involve, at least in part, hypomethylation of the DNA.
Fig. 27. Hybridization of $^{32}$P-pDTD55 to Hpa II and Msp I digests of rat liver genomic DNA isolated from control, surrounding and nodular tissue. Genomic DNA was isolated from control, surrounding and nodular rat liver tissue. Equivalent amounts of each were digested to completion with Hpa II and with Msp I and the digests were electrophoresed on 1% agarose. The DNA was blotted onto nitrocellulose and hybridized to $^{32}$P-pDTD55. The blot was autoradiographed. Lane 1, control DNA (Hpa II); lane 2, control DNA (Msp I); lane 3, surrounding liver DNA (Hpa II); lane 4, surrounding liver DNA (Msp I); lane 5, nodular DNA (Hpa II) and lane 6, nodular DNA (Msp I).
CONCLUSION

Quinones and quinonoid compounds are encountered in the environment in automobile exhaust, dyes, cigarette smoke, antitumor agents, and many foods. The metabolism of these compounds is accomplished primarily by two pathways in the rat liver. DT-diaphorase, a cytosolic flavoenzyme, utilizes either NADPH or NADH to metabolize a wide variety of quinones to hydroquinones by the transfer of two electrons. An alternate pathway involves the microsomal enzyme NADPH cytochrome P-450 reductase. This enzyme converts quinones to semiquinone radical intermediates by a one electron pathway resulting in redox cycling of the semiquinone and the generation of oxygen anion radical and singlet oxygen. Recently, evidence was presented to support the hypothesis that DT-diaphorase exerts a role in preventing semiquinone radical-mediated cytotoxicity by acting in competition with NADPH cytochrome P-450 reductase.

Increased enzyme activity of DT-diaphorase in response to the administration of xenobiotics has been previously reported. In this paper, the levels of functional or translatable DT-diaphorase mRNA have been quantitated by in vitro translation of poly (A')-RNA isolated from rats at various time points following xenobiotic induction. The level of functional DT-diaphorase mRNA is elevated 8-fold by 3MC, 3-fold by TSO, and 2- to 2.5-fold by PB. These data suggest that an accumulation of mRNA is responsible for the induction of activity of DT-diaphorase, whether by transcriptional or post-transcriptional alterations of the mRNA. Further evidence to suggest that regulation exists at the transcriptional or post-transcriptional level was compiled by quantitation of the level of mRNA in
the livers of rats which had been administered xenobiotics. Based on quantitation via blot hybridization analysis of the mRNAs to a cDNA complementary to DT-diaphorase mRNA, the increase seen in DT-diaphorase following induction by xenobiotics can be accounted for by an increase in the mRNA level. The increase in mRNA in response to xenobiotics is typical for drug metabolizing enzymes including the glutathione transferases, NADPH cytochrome P-450 reductase, cytochrome P-450s, and epoxide hydrolase. The induction of DT-diaphorase by the polycyclic aromatic hydrocarbon, 3MC, follows a pathway similar to the induction of cytochrome P-450 by 3MC. A mechanism for this induction was proposed by Nebert et al.\textsuperscript{64}, who reported the presence of a "cytosolic Ah receptor" which was able to bind to polycyclic aromatic hydrocarbons, such as 3MC and TCDD, and translocate across the nuclear membrane. Following translocation, the inducer-receptor complex was thought to bind to regulatory regions of the gene, or possibly temporal genes, to activate structural genes encoding the enzyme. In fact, Kumaki et al.\textsuperscript{65} reported that the induction of activity of DT-diaphorase by 3MC in the mouse is associated with the Ah locus, at least in part. Whether this relation exists in the rat has not yet been determined. Additionally, Tukey et al.\textsuperscript{66} have demonstrated that induction of cytochrome P-450\textsubscript{1} is due to a transcriptional activation of the gene. However, sufficient evidence does not yet exist to speculate as to whether this mechanism is operable in the case of DT-diaphorase.

In contrast to the immediate and substantial increase in DT-diaphorase mRNAs at early time points following 3MC induction, TSO elicits only a modest increase in the mRNA at later time points and the decline is much
more gradual. Whether this represents a difference in the mechanism of induction or biotransformation and distribution of metabolites is not yet known.

The activity of DT-diaphorase is increased in persistent hepatocyte nodules induced by chemical carcinogens. Evidence that the level of the mRNA is elevated correspondingly (5- to 7-fold) has been presented above. In contrast to the transient increase in mRNA levels following xenobiotic induction, the increase in DT-diaphorase mRNA in the nodules is sustained over a 5-month period. Thus, the mechanism of induction of DT-diaphorase mRNA in the nodules appears to involve a more permanent alteration, perhaps existing at the genomic level rather than the transcriptional or post-transcriptional level.

DT-diaphorase mRNA retains the ability to be induced further by 3MC following nodular induction, as evidenced by a 13- to 21-fold increase in translatable DT-diaphorase mRNA isolated from the tissue, suggesting that the regulatory mechanism of the DT-diaphorase gene remains intact. No rearrangements or amplification were detected at the genomic level.

Hypomethylation has been proposed to be involved in gene expression.63 β-globin genes are usually undermethylated in tissues where the genes are being actively transcribed, while inactive genes generally exhibit higher degrees of methylation.44,63 Additionally, the in vitro methylation of cloned genes by bacterial methyltransferases has been demonstrated to inhibit transcriptional activity.67

The onset of oncogenesis is thought to involve alterations of gene expression due to changes in methylation.68 The loss of methylation in DNA which had been damaged by chemical carcinogens was reported by Holliday68 and postulated to be involved in the altered expression of the genes.
Lapeyre and Becker later documented a significant decrease in methylation in DNAs isolated from premalignant nodules and hepatocellular carcinomas induced by 2-AAF, suggesting that neoplastic transformation elicited an aberration in endogenous methylation. More recently, Pfohl-Leszkowicz et al. detected binding of 2-AAF to the C-8 of guanine inducing a local destabilization of the chicken erythrocyte DNA helix. The binding of 2-AAF to DNA inhibited the activity of rat brain DNA methyltransferase and mouse spleen DNA methyltransferase. Thus, a correlation of methylation and altered gene expression due to chemically-induced DNA aberrations apparently exists, but the nature of this relation is not well understood.

Hypomethylation has been detected in genomic DNA isolated from persistent hepatocyte nodules induced by DEN and 2-AAF. Sufficient evidence is not yet available to speculate as to whether this hypomethylation will represent a general phenomenon in hyperplastic nodules induced by other systems or even if the hypomethylation in the DEN-2-AAF induced nodules is representative of the situation existing in other drug metabolizing enzymes that are induced in the nodules.
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