STUDIES ON MICROSOMAL ELECTRON TRANSPORT

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ABSTRACT

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As the hepatic flavoprotein, NADPH cytochrome c reductase is purified there is a loss of nitrobluetetrazolium (NBT) diaphorase activity. A factor, without intrinsic NBT diaphorase activity, has been found in goat and rat microsomes which will stimulate the diaphorase. The unequal purification of NBT diaphorase and cyt c reductase activity may be due to the loss of this stimulating factor. The stimulating factor is a heat-stabile, trypsin insensitive macromolecule. Precipitation with a solution of barium and zinc or extraction with chloroform:methanol will destroy the stimulating activity. The factor is most probably a lipoprotein but whether it acts as part of the microsomal electron transport system or as a structuring agent for NBT or the flavoprotein is unknown.

During the course of the investigation into NBT reduction several experiments were done which suggest that there is more than one cyt c reductase flavoprotein. The heat denaturation curves for the cyt c reductase, NBT diaphorase,

and NADPH oxidase activity of the solubilized flavoprotein were biphasic. Induction with phenobarbital or 3-methylcholanthrene increased the amount of the 'more' heat-resistant component. Disc gel electrophoresis showed that solubilized microsomal protein had two bands of NBT diaphorase activity. On the other hand induction and inhibition studies on the above three activities of the flavoprotein gave no evidence for multiple flavoproteins.

STUDIES ON MICROSOMAL ELECTRON TRANSPORT

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Leonard Mascaro, Jr.

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2'-AMP	Adenosine 2'-monophosphoric Acid
BSA	Bovine serum albumin
DCPIP	Dichlorophenolindophenol
DEAE	Diethylamino ethyl
DDT	1,1,1,-trichloro-2,2-tris(p-chlorophenyl)ethane
EDTA	Ethylene dinitrilotetraacetic acid
FAD	Flavin adenine dinucleotide
i.p.	intraperitoneally
K_3	Vitamin K_3 (menadione)
3-MC	3-Methylcholanthrene
NADH	Nicotine adenine dinucleotide, reduced
NADPH	Nicotine adenine dinucleotide phosphate, reduced
NBT	Nitrobluetetrazolium
NT	Neo-tetrazolium
PB	Phenobarbital
PCMB	Para-chloromercuribenzoate
TLC	Thin Layer Chromatography
Tris	Tris-hydroxymethylaminomethane

INTRODUCTION

The mechanism for the hepatic mixed-function oxidase system in mammals is unknown even though it has been the subject of intensive investigation by many laboratories. A flavoprotein and hemoprotein are believed to be involved in the system. The hemoprotein acts to activate oxygen and to bind substrate while the flavoprotein is the initial acceptor of electrons from NADPH. The mixed-function oxidase seems to be nonspecific and this may be explained by the existence of multiple forms of the hemoprotein. This research was begun in order to investigate the possible existence of multiple forms of the flavoprotein and to see whether the hemoprotein is reduced directly by the flavoprotein or through an electron carrying intermediate.

LITERATURE REVIEW

Xenobiotic Metabolism

Mammalian liver endoplasmic retimulum contains a mixed-function oxidase responsible for the metabolism of such xenobiotics as drugs, pesticides, and carcinogens (1,2,3). This xenobiotic metabolism is accomplished by a wide variety of reactions such as aromatic and aliphatic hydroxylation, N- and O-dealkylation, deamination, sulf-oxidation, and N-oxidation. Oxygen and NADPH are required in all of these reactions. This mixed-function oxidase is also responsible for the hydroxylation of steroids and the ω-oxidation of fatty acids (4,5,6). The xenobiotic metabolizing ability is believed to have evolved from this ability to metabolize these naturally occurring compounds.

This enzyme system has an important role in the detoxification of foreign compounds. Therefore, the ability to metabolize a wide variety of compounds by a variety of reactions is of special value. This apparent lack of substrate specificity allows the organism to be ready for the appearance of new toxic compounds. Detoxification of the lipophylic xenobiotics is possible because the mixed-function oxidative reactions generally result in a more water soluble compound which is not reabsorbed by the kidneys (7). Substrate nonspecificity is unique, however, because this does not agree intuitively with our present ideas about enzyme catalysis.

Liver endoplasmic reticulum, as isolated in the microsomal fraction, contains, among others, NADPH: cytochrome c reductase, NADH: cytochrome b_5 reductase, cytochrome b_5, and cytochrome P-450 (8,9,10,11). A NADH: cytochrome c reductase activity also exists in microsomes but this has been shown to consist of a multienzyme system where electrons are transferred from NADH to cyt b_5 reductase to cyt b_5 to cyt c (12). Henceforth, "cyt c reductase" will refer to the NADPH dependent enzyme. Cytochrome b_5 and P-450 are the only heme containing proteins known in liver microsomes (13). Cyt c reductase and cyt b_5 reductase are both flavoproteins and are responsible for half the microsomal flavin (14). P-450 and cyt c reductase are believed to be part of the xenobiotic metabolism system (15).

Cyt P-450 received its name because the binding of CO to the reduced heme causes a shift in the absorption with the maximum difference at 450 nm (16). This protein is believed to be important in xenobiotic metabolism because of the observation that the phenobarbital (PB) induced increase in drug metabolism activity is mirrored by a similar increase in the level of P-450 (15). Also, Estabrook has shown that the action spectrum used to reverse the CO inhibition of xenobiotic metabolism is identical to the absorption spectrum of CO bound reduced P-450 (17). P-450 is believed to function by binding with molecular oxygen and substrate to form an "activated oxygen complex" which is then reduced (17,18). P-450 seems to be tightly bound to the membrane and most attempts at solubilization have

resulted in its conversion to an inactive form called P-420 (11).

Kupfer and Orrenius have concluded from studying a similar mixed-function oxidase from adrenal cortex that cyt c reductase does not reduce P-450 directly (19). Their approach was to compare the levels of cyt c reductase, P-450, and aminopyrine demethylase activity in hepatic and adrenal microsomes. The rate of oxidative demethylation of aminopyrine was seen to be faster in liver than in adrenal microsomes even though the adrenal system has twice the amount of P-450. This would indicate that the reduction of P-450 is the rate limiting step. If cyt c reductase acted directly on P-450 then one would expect to see significantly more cyt c reductase activity in liver than adrenal microsomes. However, both systems have nearly identical levels of cyt c reductase activity. This observation may be explained by the presence of an unknown factor which mediates electron flow from the flavoprotein to P-450.

Besides the microsomal system the adrenal cortex also contains a mitochondrial mixed-function oxidase system responsible for steroid metabolism (20). Although this mitochondrial system requires O_2 and NADPH it is different from the hepatic microsomal system. Cyt P-450, a NADPH dependent flavoprotein distinct from cyt c reductase, and a nonheme iron protein are the components of the adrenal mitochondrial system (17). It is possible to disassociate these three proteins from the membrane and then to reassociate them with activity. The flavoprotein will not

reduce cyt c and can not be replaced by hepatic cytochrome c reductase. The nonheme iron protein acts as an intermediate in the electron shuttle between the flavoprotein and P-450. Nonheme iron has not been detected in hepatic microsomes. However, the fact that the adrenal system uses an electron transport chain to reduce P-450 does suggest that a similar electron transport chain may exist in liver.

Lu and Coon have reported a method to resolve the hepatic microsomal ω-hydroxylation system into three components (21, 22). These are P-450 fraction, a P-450 reductase fraction, and a lipid factor. These three components can be reconstituted and do have some ability to metabolize xenobiotics. The P-450 reductase fraction can act as a cyt c reductase but there is evidence that these two activities are not identical. For instance, pure lipase-solubilized cyt c reductase can not replace the P-450 reductase fraction when reconstituting the system. Also, cyt c reductase activity can be purified 10 fold greater than the P-450 reductase activity. Furthermore, the two reductase activities do not show the same stability on storage.

Some researchers have questioned the techniques used by Lu and Coon (23). These critics feel that treatment with deoxycholate does not result in a true soluble enzyme preparation. Lu and Coon base their claim of solubilizing the system on the fact that the mixed-function oxidase components will not sediment when centrifuged at 105,000 x g for 90 minutes, although a higher g-force will sediment the P-450 component. This, the critics say, shows that

deoxycholate is breaking the membrane up and creating a heterogeneous collection of P-450 particles which then bind deoxycholate. This detergent binding then keeps the particles from sedimenting at 105,000 x g.

Induction

The pretreatment of mammals with certain chemicals causes an increase in activity of the hepatic xenobiotic metabolism system. This inductive effect was first noticed with the polycyclic hydrocarbons 3-methylcholanthrene (3-MC) and benzpyrene (24). Over 200 compounds, including PB, act as inducers (25). Almost any chemical capable of being metabolized by this system can act as an inducer. These inducers can be divided into two classes based on their range of effect. General inducers will increase metabolic activity towards all xenobiotic substrates while specific inducers will selectively increase activity towards particular substrates only (25). PB is a member of the former class while 3-MC and benzpyrene fall into the latter class.

Very little is known about the mechanism of induction although several changes in the liver are seen (15). On the gross level there is an increase in the size and redness of the liver. Biochemically, induction causes an increase in both the lipid content of the membrane and in the levels of P-450 and cyt c reductase but there is no effect on the levels of cyt b_5 and NADH: cyt b_5 reductase. Turnover studies have shown that the induction of P-450 heme results from both an increase in rate of synthesis and a decrease in rate of degradation (26). However, there is no idea as to how the

inductive agent causes these changes in turn-over rate.

Enzyme Multiplicity

The apparent nonspecificity of the xenobiotic metabolism system suggests that the hepatic microsomes may contain more than one mixed-function oxidase system. A growing number of experiments based on induction studies seem to point to the existence of more than one population of P-450 and more than one pathway for xenobiotic metabolism. For example, if only one pathway existed it would be hard to see why the K_m for the hydroxylation of benzpyrene is dependent on whether the animal has been pretreated with PB or 3-MC (27). Multiple mixed-function oxidase systems may also explain how such specific inducers as 3-MC and benzpyrene can induce activity towards one substrate and not another.

Inhibition studies of aminopyrine demethylase also suggest the possible existence of multiple mixed-function oxidases. The Lineweaver-Burke plot of demethylase activity is nonlinear. Pederson and Aust have postulated that this nonlinearality is the result of multiple aminopyrine activities with widely differing K_m's (28). Aust and Stevens have used DDT inhibition to validate this hypothesis (29). DDT is believed to act as an inhibitor of aminopyrine metabolism because it is an alternate substrate of microsomal oxidases. A plot of demethylase activity versus DDT concentration was seen to be made up of three linear segments which divided total activity into three components, one not inhibited by DDT, one moderately inhibited, and a third which is extremely sensitive to DDT inhibition. These three components are believed to represent

three separate mixed-function oxidases. The components also show different degrees of induction with PB and 3-MC.

Several experiments have been reported which suggest that more than one form of P-450 exist. These results lend support to the belief that there are multiple mixed-function oxidases in microsomes. However, none of these experiments are conclusive because it has been impossible to isolate pure P-450 and to directly analyze it for multiple forms.

Shifts of the CO bound reduced P-450 differences spectra caused by the binding of substrate has been used as a tool to investigate P-450 multiplicity. These shifts can be divided into two classes (30,31). "Type I" shifts are caused by such compounds as hexobarbital and aminopyrine and are characterized by a trough at 420 nm and a peak at 385 nm. On the other hand, "Type II" shifts, as caused by aniline and pyridine, have a peak at 430 nm and a trough at 390 nm. The possibility that these two shifts are the result of two different forms of P-450 is suggested by induction studies (32). PB induces an increase in intensity in both types of shifts but 3-MC causes an increase only in the "Type II" shift. The existence of two forms of P-450, of which only one form is inducible by 3-MC, would explain this observation. This theory also agrees with the fact that 3-MC induces the metabolism of the "Type II" substrate aniline but not of the "Type I" substrate hexobarbital.

Density subfractionation of rat liver microsomes also suggests that there are two forms of P-450 (33). In the case of uninduced microsomes, P-450 has a reproducible profile

as it is separated in a zonal sucrose gradient. PB and 3-MC pretreatment of rats will alter this distribution in different ways. PB causes an increase of P-450 in the lighter fragments while 3-MC increases the P-450 content of the heavier fragments. This suggests that these two inducers act to promote an increase in separate forms of P-450. Animals induced simultaneously with 3-MC and PB have an increase in the P-450 content of both the heavy and light fragments.

The turnover of P-450 heme also indicates that two pools of P-450 exist (34). Heme can be labeled by injecting animals with ^{3}H-levulinic acid. Since the loss of radioactive hemoprotein over time is biphasic this could indicate that two different P-450 hemes are being degraded. Interestingly, PB pretreatment results in an increase in 'fast' turnover heme in comparison to noninduced microsomes. 3-MC, however, increases the amount of 'slow' turnover heme.

Cyt c Reductase

Cyt c reductase is an important part of xenobiotic metabolism because it is believed to act as the acceptor of electrons from NADPH. The proof of this role is the observation that the PB induced increase in aminopyrine demethylase activity is mirrored by a similar increase in the content of cyt c reductase in the microsome (15). Because of the importance of this enzyme, its physical properties and mechanism of action have been studied extensively. Although this flavoprotein is called "cyt c reductase" several other compounds can act as electron acceptors for it. In fact, the

natural substrate for this enzyme is unknown since cyt c and the other known electron acceptors are not found in microsomes. There is some thought that cyt c reductase can reduce P-450 directly when both are intact in the membrane although this is not certain. Since this thesis will investigate microsomal electron transport and cyt c reductase is believed to have a key role, the balance of this literature review will briefly survey the knowledge about this enzyme.

Several investigators have been able to solubilize and purify cyt c reductase. Horecker was the first to do so by using rat liver acetone powder as the enzyme source (35). Next, Williams and Kamin were able to solubilize the enzyme from pork liver microsomes by digestion with lipase and then to purify it (10). Concurrently, Phillips and Langdon announced another purification method using trypsin to release the enzyme from the membrane (36). All of these methods subjected the flavoprotein to low pH and high ammonium sulfate concentrations during the purification process which could have caused some flavin to disassociate from the apoenzyme. Omura and Takesue have developed a gentler system using gel and ion-exchange chromatography to purify a trypsin solubilized enzyme (37). The enzyme seems to be the same whether it is solubilized by lipase or trypsin. The purified enzyme has a molecular weight of approximately 84,000 with two FAD prosthetic groups per enzyme molecule (37).

Cyt c reductase will also act as a tetrazolium diaphorase. In fact, Kamin and Williams isolated cyt c reductase

while studying NADPH: neotetrazolium diaphorase (NT diaphorase) in microsomes (10). They reported, however, that there was a loss of NT diaphorase activity as cyt c reductase was purified. The lost NT diaphorase was not found in any other fraction. This observation could be explained by the loss of some factor required for NT diaphorase activity. Kato has felt that such a factor could be an intermediate in an electron transport chain and proposed the following model for P-450 reduction (38).

NADPH flavoprotein [X] P-450

cyt c NT

No intermediate for NT reduction has been found, however, and such studies are complicated by the fact that such agents as bovine serum albumin (BSA) and iron: EDTA can stimulate NT reduction (39,40). NT is a four electron acceptor although the half reduced compound is also stabile.

Microsomes will oxidize NADPH when catalytic amounts of 2-methyl-1,4-naphthoquinone (vitamin K_3 or menadione) are present (41,42). The purification of this K_3-dependent NADPH oxidase has shown that it is identical with the flavoprotein, cyt c reductase (43). The mechanism consists of the enzymatic reduction of menadione to the semiquinone by a 1-electron transfer from the $FADH_2$ prosthetic group of the flavoprotein. The semiquinone is in turn nonenzymatically oxidized by O_2 with the formation of H_2O_2 (43,44). This cyclic reduction and oxidation of menadione is why only catalytic quantities of the naphthoquinone are required. Measurement of NADPH dissappearence is in fact actually measuring the rate of

reduction of menadione by the flavoprotein. Although a K_3-independent NADPH oxidase also exists in microsomes the rest of this thesis will always refer to the K_3-dependent system when talking about NADPH oxidase.

Besides the three electron acceptors already discussed, the flavoprotein, cyt c reductase, is capable of reducing several other compounds including ferricyanide and dichlorophenolindolphenol (DCPIP) (45). Although cyt c reductase can not reduce cyt b_5 at physiological ionic strength it can do so at high ionic strength (40). Any mechanism postulated for this enzyme must take into consideration this nonspecificity toward electron acceptors and the ability to reduce both 1-electron acceptors such as cyt c and ferricyanide and 2-electron acceptors such as DCPIP and menadione.

The elucidation of the mechanism for cyt c reductase has been possible through the use of stopped-flow spectrophotometry (45,46). The flavins of the enzyme are seen to alternate between the fully and semireduced states ($FADH_2 \rightarrow FADH$). The two flavins are believed to be close to one another on the protein. Thus, they can shuttle electrons between each other. This allows for identical mechanisms for the reduction of 1- and 2-electron acceptors. In both cases FADH is the product of electron transfer. The juxtaposition of the two flavin molecules is also required because the two FADH groups are reduced by a single NADPH molecule. The large number of electron acceptors indicates

that the flavins are probably exposed on the surface of the protein molecule where they can easily come into contact with the substrate.

MATERIALS AND METHODS

Chemicals

Nitrobluetetrazolium chloride (2,2'-Di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride) was purchased from the Aldrich Company, Milwaukee, Wis. Menadione (2-methyl-napthoquinone) was received from the Nutritional Biochemicals Company, Cleveland, Ohio. Aminopyrine (4-dimethylamino-1,5-dimethyl-2-phenyl-3-pyrazolone) was purchased from K and K laboratories, Inc., Plainview, N.Y.

Acrylamide, N,N'-methylenebisacrylamide, ammonium persulfate, and TEMED (N,N,N',N'-Tetramethylethylenediamine) were used in disc gel electrophoresis as purchased from the Canal Industrial Corporation, Rockville, Md. Carbon Monoxide was obtained from the Matheson Company, Inc., Joliet, Ill. Phenobarbital was obtained from Merck and Company, Inc., Rahway, N.J.

D,L-Isocitrate, $NADP^+$-isocitrate dehydrogenase, $NADP^+$, NADPH, and cytochrome c were all purchased from the Sigma Chemical Company, St. Louis, Mo. 20-Methylcholanthrene (3-methylcholanthrene), steapsin (hog pancreatic lipase), and Crotalus atrox venom were also supplied by the Sigma Chemical Company as were Tris (hydroxymethyl) aminomethane, nicotinamide, and nitrobluetetrazolium diformazan.

Animals

Microsomes were isolated from the livers of male rats and goats. The rats were of the Sprague-Dawley strain and weighed from 200 to 250 g. Rats pretreated with PB were given 0.1% PB in their drinking water for at least 10 days prior to being sacrificed. PB pretreated goats were given daily i.p. injections of 100 mg/kg in water for 5 days. Rats injected with 3-MC were given an i.p. injection, of 20 mg/kg in corn oil, 36 hours prior to isolation.

Preparation of Microsomes

Rats were sacrificed by decapitation. The livers were immediately perfused *in situ* through the portal vein with 10 ml of ice cold 1.15% KCl containing 0.2% nicotinamide. The livers were then removed, placed in ice cold 1.15% KCl, rinsed clean of blood and hair, weighed, and minced with scissors. Goats were sacrificed either by electrocution or by severing the carotid artery. The livers were removed, rinsed in ice cold 1.15% KCl, weighed, and minced for 10 seconds in a Waring Blender.

The minced rat or goat liver was then homogenized in four volumes of 1.15% KCl containing 0.2% nicotinamide with four strokes of the teflon pestle of a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 20 minutes at 10,000 x g (8,500 rpm, GSA rotor) in a Sorval RC2-B centrifuge in order to remove mitochondria, nuclei, and other organelles. The supernatant was decanted and centrifuged for 90 minutes at 105,000 x g (30,000 rpm, 30 rotor) in a Spinco Model L ultracentrifuge. The resulting microsomal

pellet was then resuspended in Tris-HCl buffer (0.05 M, pH 7.5) containing 50% glycerol. All of the above operations were done at 0-5° C. The microsomes were stored at -20° C under N_2 until they were used. All determinations of protein concentration were done by the Lowry Method (47).

Lipase Solubilization of Microsomes

Thawed microsomes (40 to 80 mg protein/ml) were diluted with four volumes of sodium phosphate buffer (0.05 M, pH 7.3). The diluted microsomes were incubated with lipase (0.12 mg/ml) for thirty minutes at 37°. The incubate was then centrifuged for 90 minutes in a Spinco Model L ultracentrifuge at 105,000 x g (30,000 rpm, 30 rotor) or 140,000 x g (39,000 rpm, 40.2 rotor).

Purification of Cyt c Reductase

Kamin and Williams Method (10). Lipase solubilized protein was first fractionated by adjusting the pH with 0.1 N HCl. The protein which precipitated between pH 5.4 and 4.6 was then fractionated with ammonium sulfate. Solid ammonium sulfate was added and the protein which precipitated between 50 and 70% of saturation was collected, resuspended in buffer, and dialyzed overnight. All steps were done at 0-5° C and precipitates were collected by centrifuging for 10 minutes at 12,100 x g (10,000 rpm, SS34 rotor) in a Sorval RC-2B centrifuge.

Omura and Takesue Method (37). The whole microsomes were first washed free of glycerol by suspending them in four volumes of sodium phosphate buffer (0.05 M, pH 7.3) and centrifuging for 60 minutes at 105,000 x g (30,000 rpm,

30 rotor) in a Spinco Model L ultracentrifuge. The microsomal pellets were resuspended in the same volume of buffer as when washed and solubilized by incubating with lipase. The solubilized supernatant was freeze-dried, resuspended in 10 ml of distilled water, and applied to a Sephadex G-100 column (76 x 1.6 cm) equilibrated with 0.1 M sodium phosphate buffer (pH 7.5). The column was eluted with this buffer and 5 ml fractions were collected. The fractions containing cyt c reductase (12-17) were pooled and placed on a DEAE-cellulose column (1 x 13 cm) which had been equilibrated with 0.1 M phosphate buffer (pH 7.5). The column was washed with 15 ml of 0.05 M phosphate buffer (pH 7.5) and then eluted with 60 ml of a linear gradient of KCl (0 to 0.35 M) in 0.05 M phosphate buffer (pH 7.5). At the completion of the gradient the column was washed with 8 ml of 0.35 M KCl in 0.05 M phosphate buffer (pH 7.5). The elutant was collected in 2 ml fractions and those (26-34) with cyt c reductase activity were pooled and frozen at -20° C. All of the above operations were done at 0-5° C.

Preparation of H-Factor

Method A. The material which precipitated between pH 7.5 and 5.4 from lipase solubilized microsomes during the Kamin and Williams purification method was resuspended in buffer.

Method B. Microsomes were diluted in four volumes of 0.05 M phosphate buffer (pH 7.3) and digested with lipase (0.2 mg/ml) for one hour at 37°. NaCl was added to give a 12.5% solution which was then placed in a boiling water bath

until protein coagulation was seen (there is no coagulation without NaCl). The precipitate was removed by centrifuging in an I.E.C. clinical centrifuge. The supernatant contained H-Factor.

Aminopyrine Demethylase Assay

All assays contained: 0.05 M aminopyrine, 7 mM $MgCl_2$, Tris-HCl (0.05 M pH 7.5) and a NADPH generating system made up of 0.1 mM $NADP^+$, 2 mM D,L-isocitrate, and isocitric dehydrogenase (0.16 µmole units/ml). The reaction mixtures were incubated in a Dubmoff metabolic shaker for 15 minutes at 37°. The reaction was stopped by placing the incubate in a test tube containing an equal volume of 10% trichloroacetic acid and allowed to stand for 20 minutes to allow for total protein precipitation. After this time an equal volume of Nash reagent (2 M $NH_4C_2H_3O_2$, 0.5 M CH_3COOH, and 0.02 M 2,4-pentanedione) was added to the denatured assay mixture and heated for 15 minutes at 50°. The reaction product (extinction coefficient = 7.08 cm^{-1}, uM^{-1}, HCHO) was read spectrophotometrically at 412 nm in a Coleman Jr. Spectrophotometer.

Cytochrome c Reductase Assay

The 1.0 ml assays contained protein, 7.2 nM cytochrome c, and 0.05 M sodium phosphate buffer (pH 7.3). The reaction was initiated with 10 µl of 10 mM NADPH and followed by reading the absorbance increase at 550 nm on a Beckman DB spectrophotometer with Sargent SRL recorder. Reduced cytochrome c has an extinction coefficient of 27.7 cm^{-1}, mM^{-1} although the activity was generally reported as

ΔA_{550}/minute.

NBT Diaphorase Assay

The 1.0 ml assays contained protein, 0.5 mM NBT, and 0.05 M sodium phosphate buffer (pH 7.3). The reaction was initiated with 10 µl of 10 mM NADPH and followed by reading the absorbance increase at 550 nm on a Beckman DB spectrophotometer with Sargent SRL recorder. The activity was generally expressed as ΔA_{550}/minute.

K_3-Dependent NADPH Oxidase Assay

The 1.0 ml assays contained protein, 58 µM menadione, and 0.5 M potassium phosphate buffer (pH 6.5). The reaction was initiated with 10 µl of 10 mM NADPH and followed by reading the absorbance decrease at 340 nm on a Beckman DB spectrophotometer with Sargent SRL recorder. NADPH has an extinction coefficient of 6.22 mM^{-1}, cm^{-1} although activity has generally expressed as ΔA_{340}/min.

CO-Difference Spectroscopy

Protein was resuspended in 0.05 M sodium phosphate buffer (pH 7.3), placed in two cuvettes, and reduced with a few grains of sodium dithionite. The solution in the sample cuvette was then saturated with CO. Spectra were recorded between 500 nm and 400 nm on a Beckman DB spectrophotometer with Sargent SRL recorder. The difference in absorbance between 450 nm and 419 nm represents the amount of P-450 in the assay. The extinction coefficient of P-450 is 91 cm^{-1}, mM^{-1}.

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis was done using the method of Ornstein and Davis (48,49). Four stock solutions of 100 ml each were made: Solution A consisting of 48 ml 1 N HCl, 36.6 g Tris, and 0.23 ml TEMED; Solution B consisting of 28 g acrylamide and 0.735 g N,N'-methylenebisacrylamide; Solution C consisting of 0.14 g ammonium persulfate; Solution D consisting of 48 ml 1 N HCl and 5.98 g Tris. A 6% solution of acrylamide was made by mixing 2.5 ml of Solution A, 4.3 ml of solution B, 10 ml of solution C, and 3.2 ml of water. Polymerization of the gels (7.0 x 0.5 cm) took 30 minutes at room temperature. A thin layer of water had been placed on each gel during polymerization to insure a flat surface.

Protein solutions were made in a 1/8 dilution of Solution D which contained 20% glycerol. The tray buffer consisted of 0.6 g Tris and 2.88 g glycine per liter. Samples (10-50 µg of protein) were electrophoresed for 80 minutes (cathode in the bottom) at 3 mamps/tube. After electrophoresis the gels were stained for protein overnight in Coomassie Blue (0.05% in 12.5% trichloroacetic acid) and destained in 10% trichloroacetic acid. A specific stain for NBT diaphorase was made by soaking an acrylamide gel in 0.083 mM NBT, 7 mM $MgCl_2$, 0.05 M Tris-HCl (pH 7.5) and a NADPH generating system made up of 0.1 mM $NADP^+$, 2 mM D,L-isocitrate, and isocitric dehydrogenase (17 µg/ml).

EXPERIMENTAL

The NBT Diaphorase Assay

This section describes the investigation of the parameters of the NBT diaphorase assay. This was accomplished by varying the concentrations of NADPH, NBT, and enzyme as well as the pH and ionic strength. The absorption curve and absorbtivity of reduced NBT were also studied. This information was used to pick the best conditions for running the assay.

The discovery of the wavelength where enzymatically reduced NBT has its maximum absorption was the goal of the next experiment (nonreduced NBT was used as the reference). The difference spectrum of this material has a broad absorption band in the visible region and the shape of this band seems to be dependent on the source of enzyme (Fig 1 and 2). For whole goat or rat microsomes the absorption maximum occurs at approximately 580 nm but for solubilized rat microsomal protein the maximum shifts to approximately 550 nm. This probably shows that whole microsomes are capable of other reactions with NBT besides reduction. In subsequent experiments NBT reduction was followed by measuring the change in absorbance at 550 nm.

In order to see the number of products of NBT after enzymatic reduction the chloroform extract of the reduced material was separated by thin layer chromatography (TLC)

Figure 1. -- The absorption scan of reduced NBT. Goat microsomes and 150 uM NADPH were used to reduce 20 uM NBT. A difference scan was done versus nonreduced 20 μM NBT.

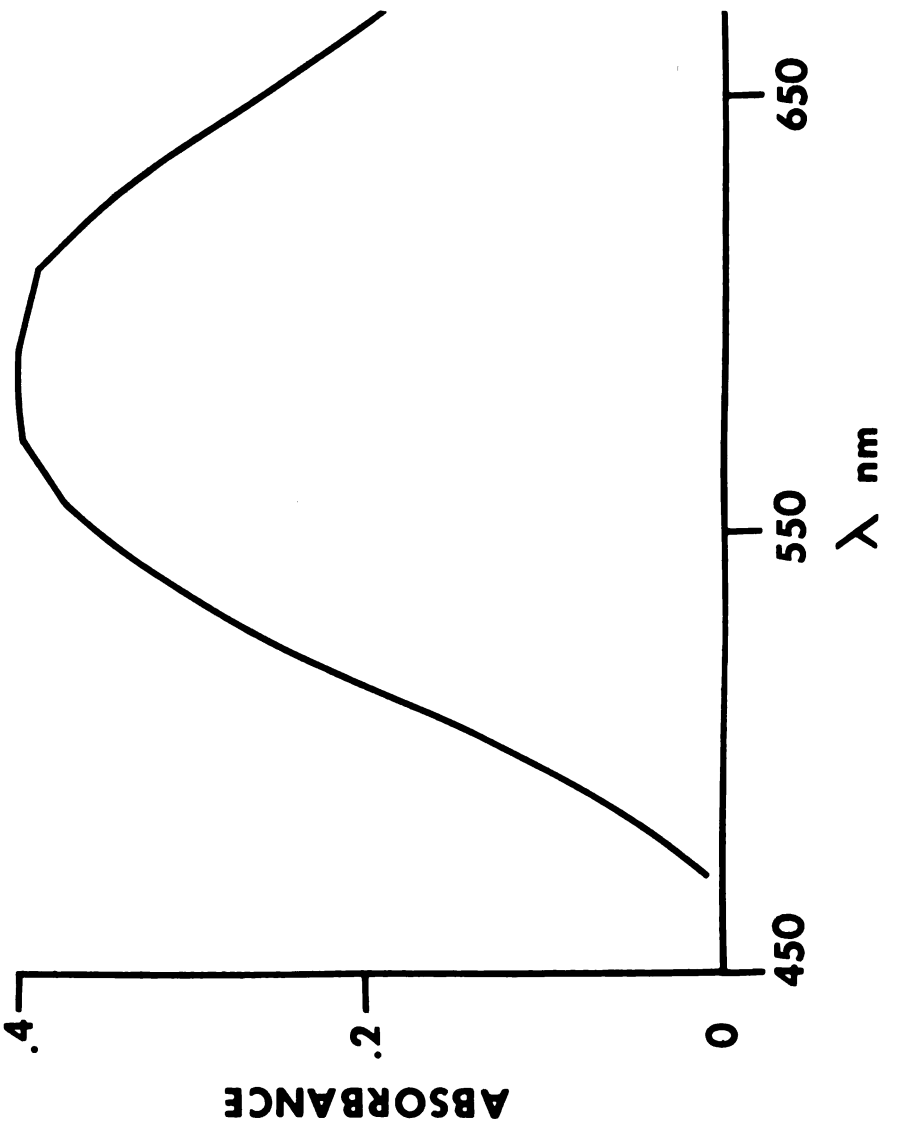
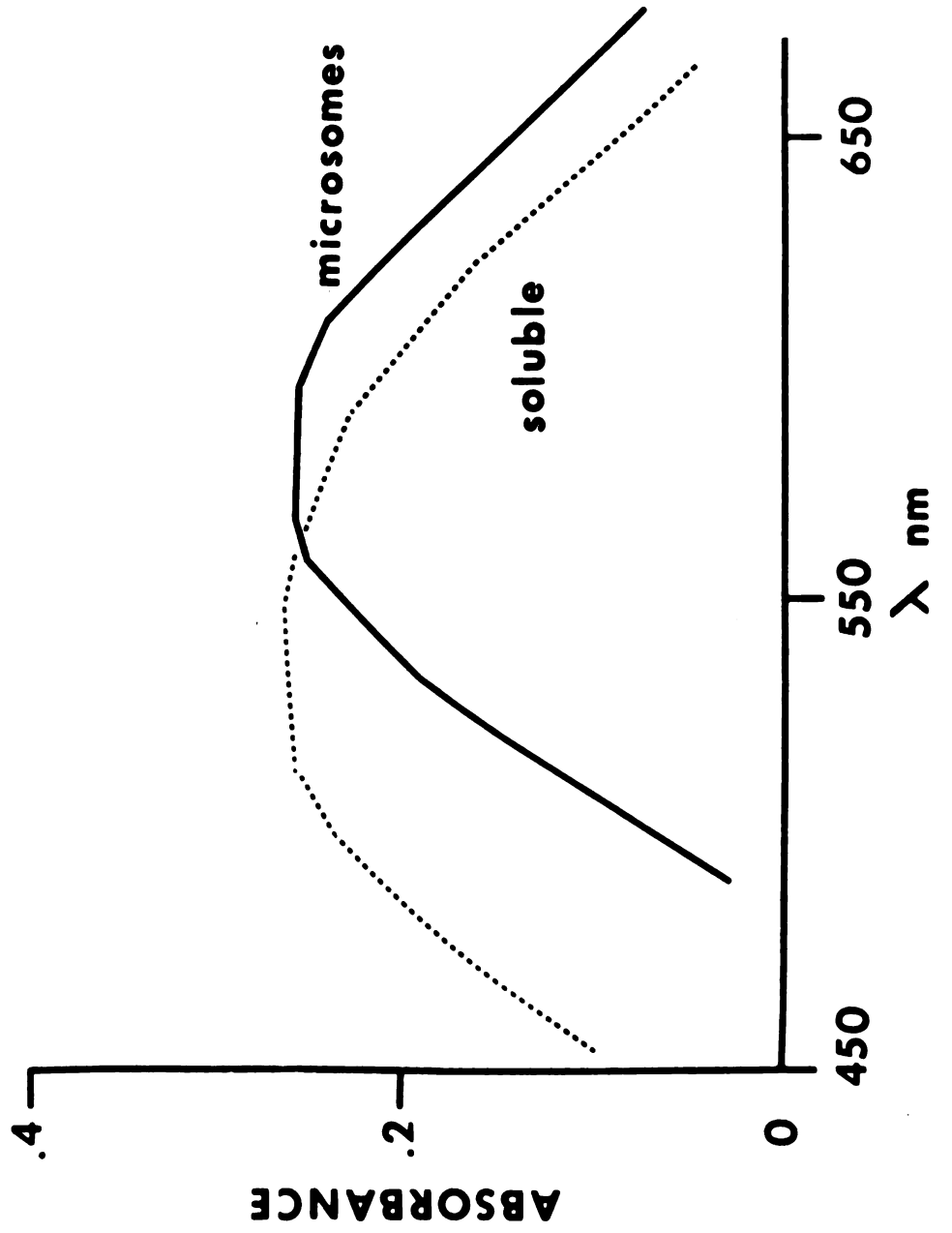

ABSORBANCE
.4
.2
0
450
550
650
λ nm

Figure 2. -- The absorption scan of reduced NBT. Soluble rat protein and whole rat microsomes were used to reduce 20 mM NBT with 150 mM NADPH. The soluble protein had been "purified" through DEAE-cellulose purification according to the Omura and Takesue method.

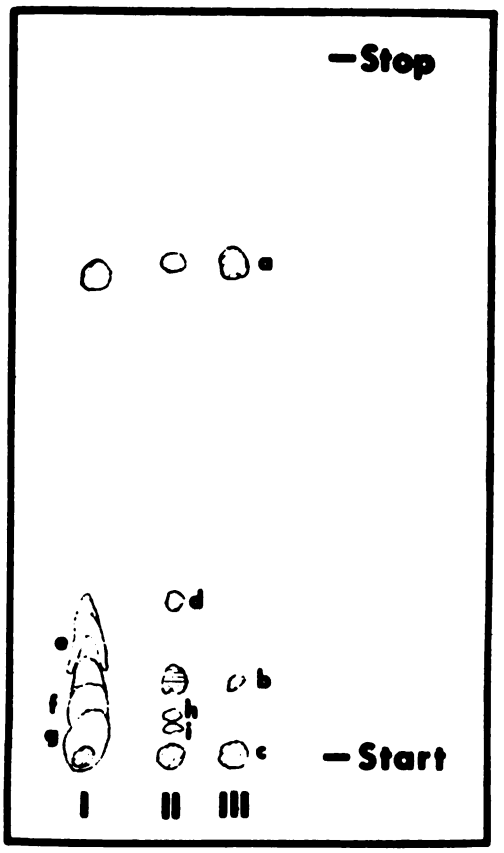

microsomes
soluble
.4
.2
0
ABSORBANCE
450
550
650
λ nm

on silica gel (solvent was benzene-methanol 5:1). Both whole microsomes and solubilized, partially purified cytochrome c reductase were used to reduce NBT. Thin layer chromatography of these two preparations along with a commercial NBT diformazone standard showed that enzymatic reduction gave other products besides diformazan (Fig 3). The diformazan standard had three spots, the microsomal reduced material had seven spots, and the cyt c reductase reduced material had six spots. Spot c is probably the diformazan.

The extinction coefficient of reduced NBT was calculated by measuring the absorbance of known concentrations of reduced NBT. Dithionite reduced dye had an extinction coefficient of 1.82×10^{-2} nmoles^{-1} ml cm^{-1} but this value gave impossibly high rates of reduction when used in later experiments. When the extinction coefficient was recalculated using enzymatically reduced NBT a new value of 2.86×10^{-2} cm^{-1} nmoles^{-1} ml was obtained (Fig 4).

The effects of varying enzyme and substrate concentrations were tested next with goat microsomes. As expected, the rate of reduction of dye was linear with enzyme concentration (Fig 5). 100 μM NADPH will saturate the enzyme (Fig 6), as will 10 μM NBT (Fig 7). However, concentrations of NBT greater than 10 μM caused a decrease in activity. This substrate inhibition may be related to the observation that higher concentrations of NBT give increasingly turbid solutions which may result from a NBT-protein interaction.

Figure 3. -- Thin Layer Chromatography of reduced NBT. NBT was reduced by whole PB rat microsomes and solubilized, partially purified cyt c reductase. The diformazan product was extracted into chloroform: methanol (5:1) as solvent. Commercially prepared NBT diformazan was also chromatographed as a standard. Spots e, g, and i were fluorescent.



I Whole microsomes

II Solubilized enzyme

III Commercial Diformazan

Figure 4. -- The extinction coefficient of reduced NBT at 550 nm. Both enzyme and dithionite were used to reduce NBT. A NADPH generator was used with the whole goat microsomes.

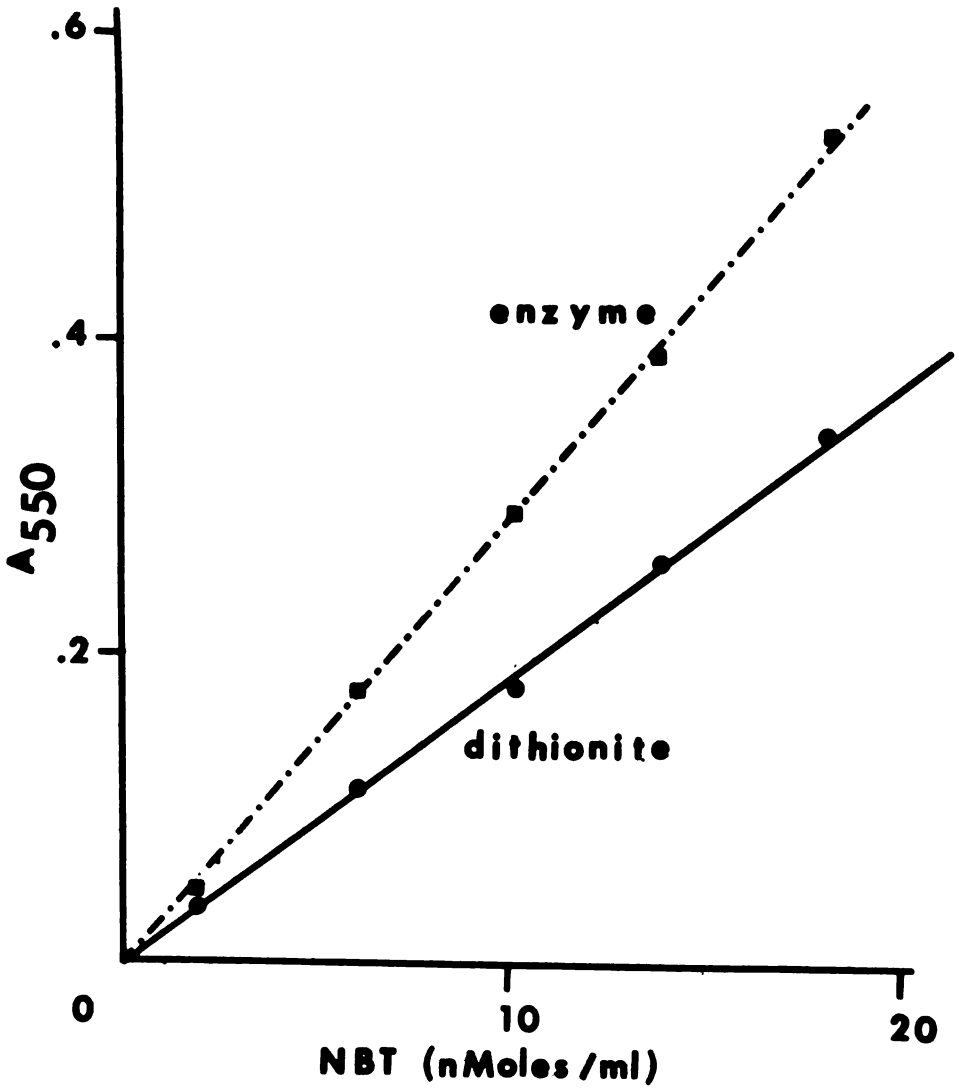

.6
.4
.2
A550
enzyme
dithionite
0
10
20
NBT (nMoles/ml)

Figure 5. -- NBT diaphorase activity versus protein concentration. Whole goat microsomes were used. The assays were done in 0.05 M Tris-HCl (pH 7.5) with a NBT concentration of 1 mM. Activity is expressed as ΔA_{550}/min.

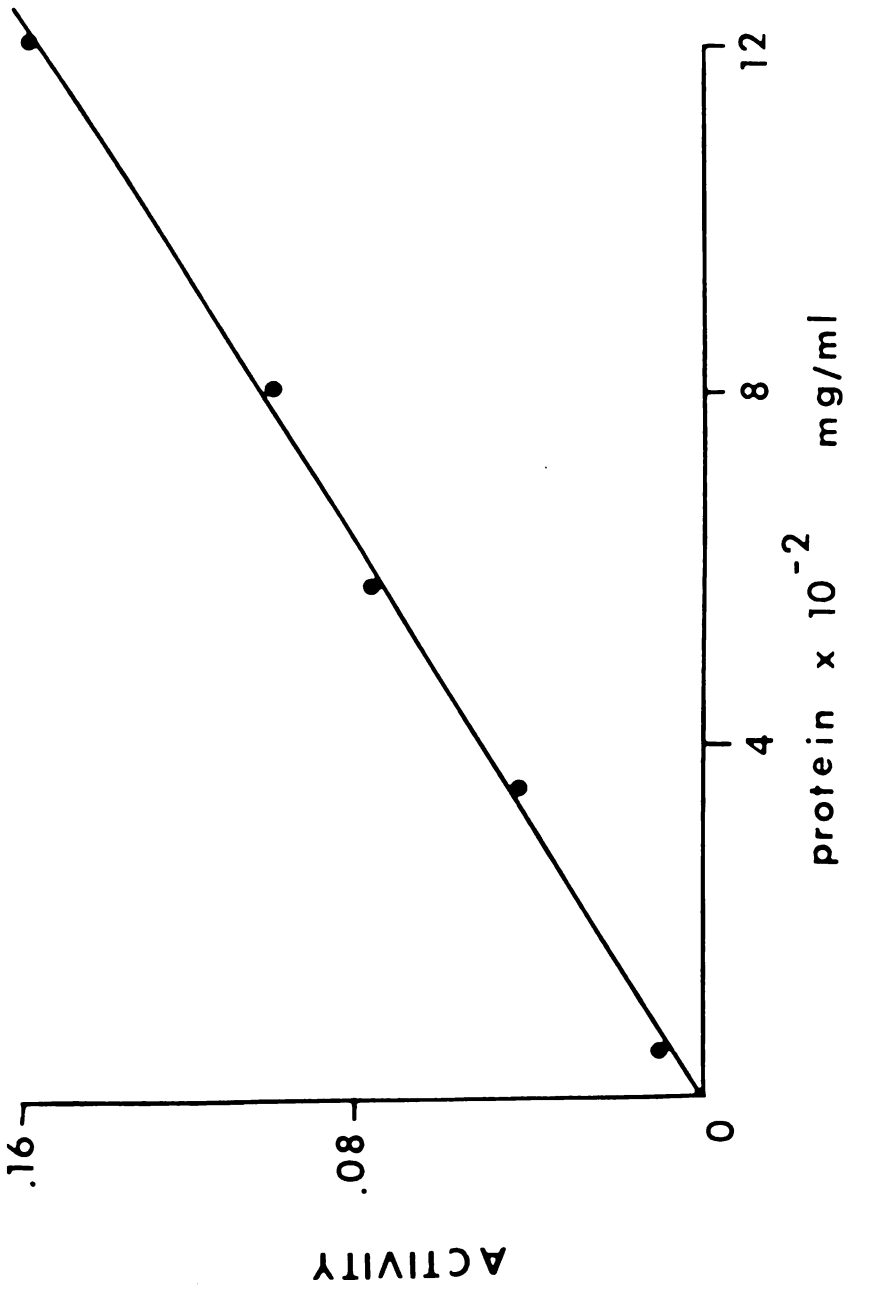

ACTIVITY
.16
.08
0
4
8
12
protein x 10^{-2} mg/ml

Figure 6. -- NBT diaphorase activity versus NADPH concentration. Whole goat microsomes were used. The assays were done in 0.05 M Tris-HCl (pH 7.5) with a NBT concentration of 1.0 mM. Activity is expressed as ΔA/min.

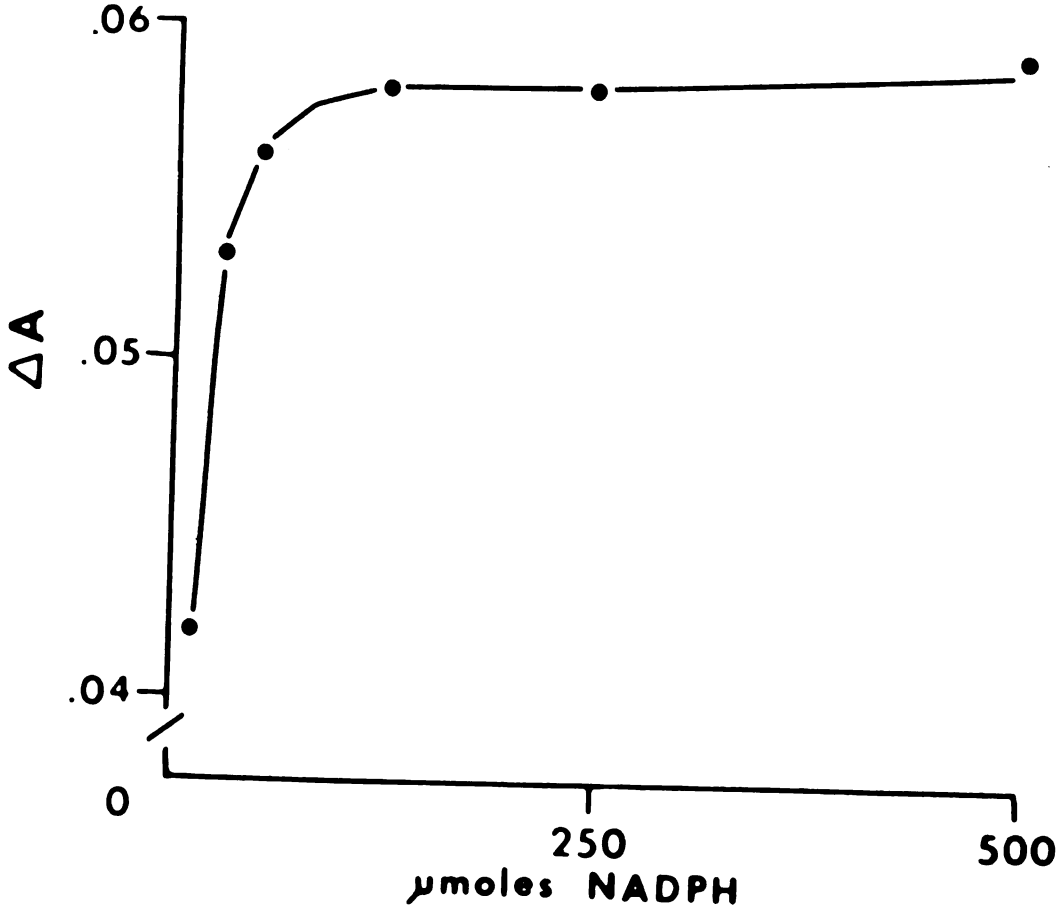
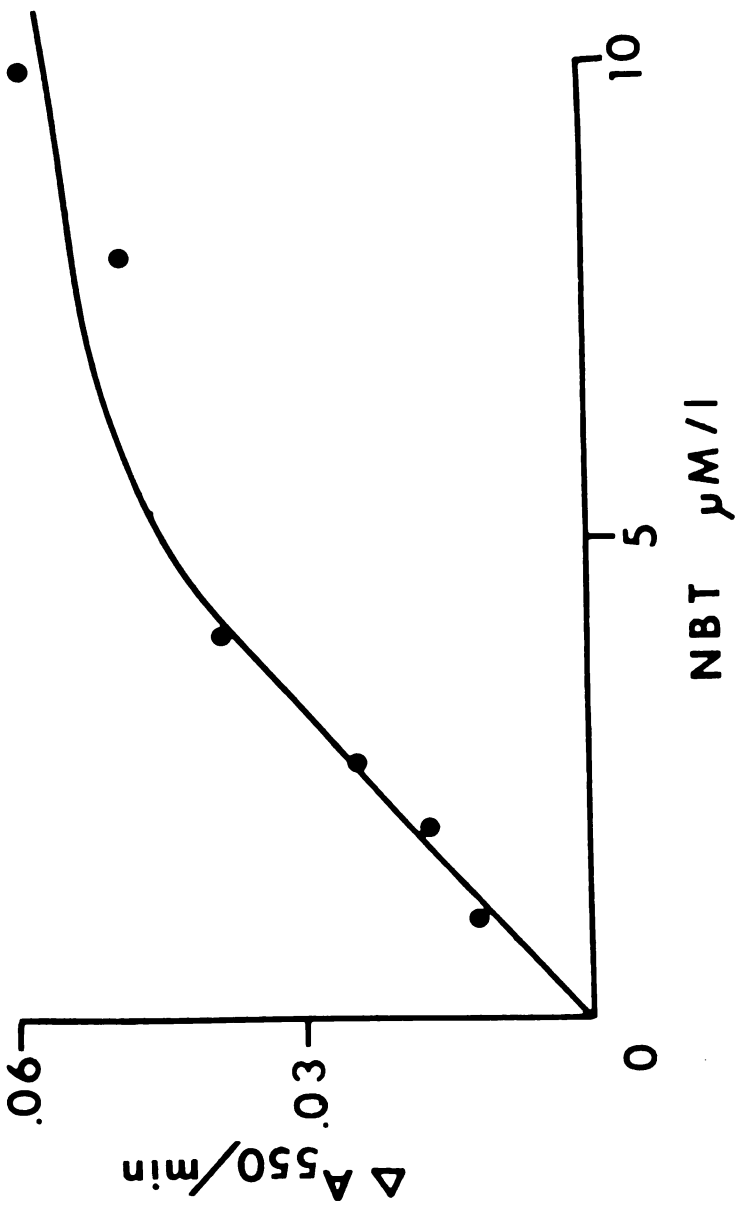

.06
.05
.04
ΔA
0
250
500
μmoles NADPH

Figure 7. -- NBT diaphorase activity versus NBT concentration. The assays were done with whole goat microsomes in 0.05 M Tris-HCl (pH 7.5).


.06
.03
0
$\Delta A_{550}/min$
0
5
10
NBT μM/l

A study of the pH dependence of the diaphorase activity showed that it was atypical. The enzyme is active over a wide range of pH and is without a sharp pH optima (Fig 8). Maximum activity seems to occur at pH 8.5 but the sizable amount of activity up to pH 10 is quite unusual. This pH curve can be explained by assuming that reduced NBT, itself, is an efficient pH indicator. Future assays were done at pH 7.3 because this value is midway on a plateau where there is little change of activity with small changes of pH.

The diaphorase ionic strength dependence was investigated next. The rate of NBT reduction in phosphate buffer was greater than in the same concentration of Tris-HCl. This could be explained by an ionic-strength dependence which we investigated by assaying NBT diaphorase in several concentrations of Tris-HCl and sodium phosphate buffer (Fig 9). The results were as expected and it was decided to use 0.05 M sodium phosphate buffer in future assays.

K_3-Dependence of NADPH Oxidase

The parameters of the K_3-dependent NADPH oxidase assay have been reported in the literature so a detailed investigation was not necessary (43). However, a check of the menadione dependency of the enzyme showed that a concentration of 20 μM menadione was necessary to saturate the enzyme. This is higher than has been reported previously.

Co-Purification of Cyt c Reductase and NBT Diaphorase by Kamin and Williams Method

This experiment was designed to purify cyt c reductase by the method of Kamin and Williams and to see whether NBT

Figure 8. -- The pH dependency of NBT diaphorase. Assays were done in 0.05 M sodium phosphate buffer. The pH of each assay was adjusted with 1 M KOH. The assays were done with whole goat microsome and a NBT concentration of 1.0 mM. Activity is expressed as ΔA/min.

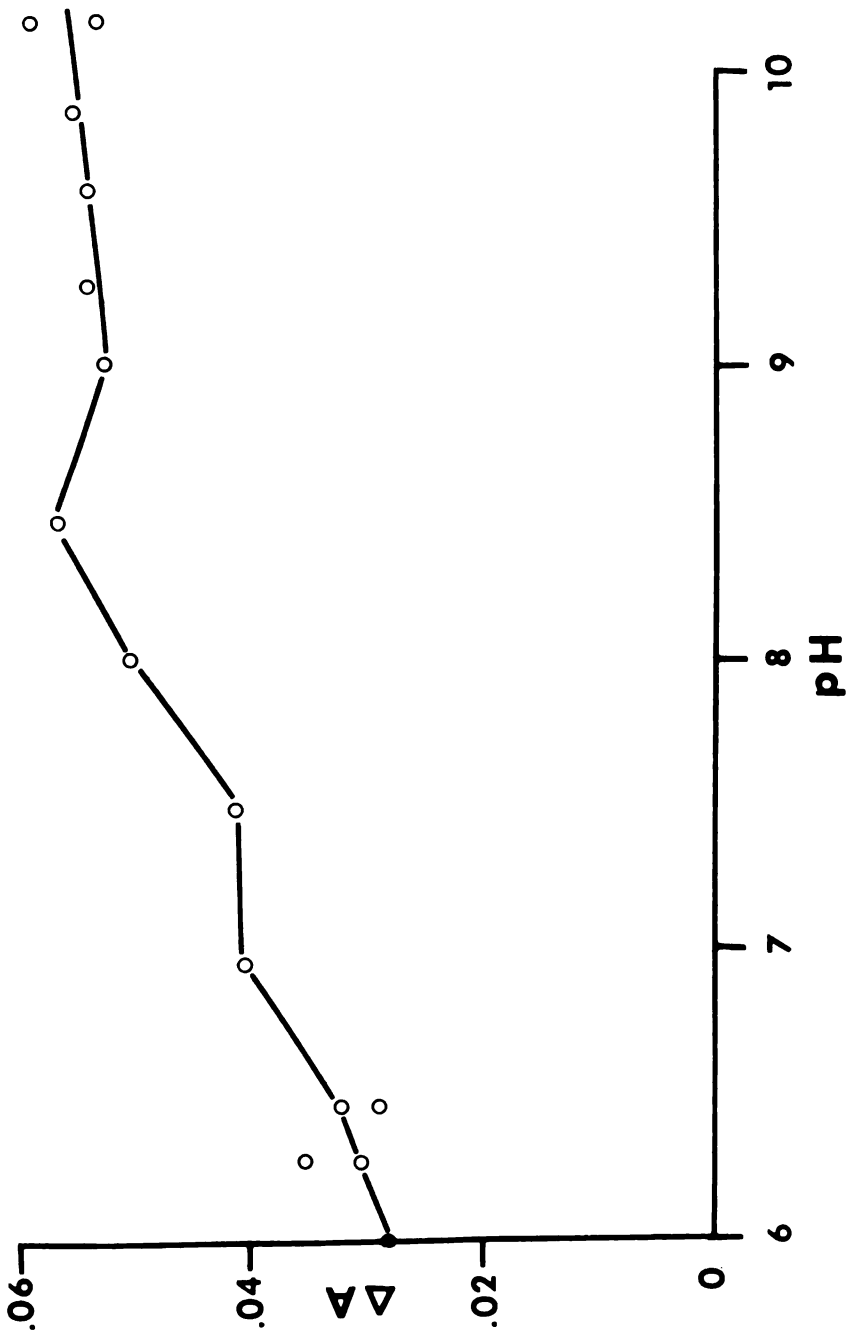
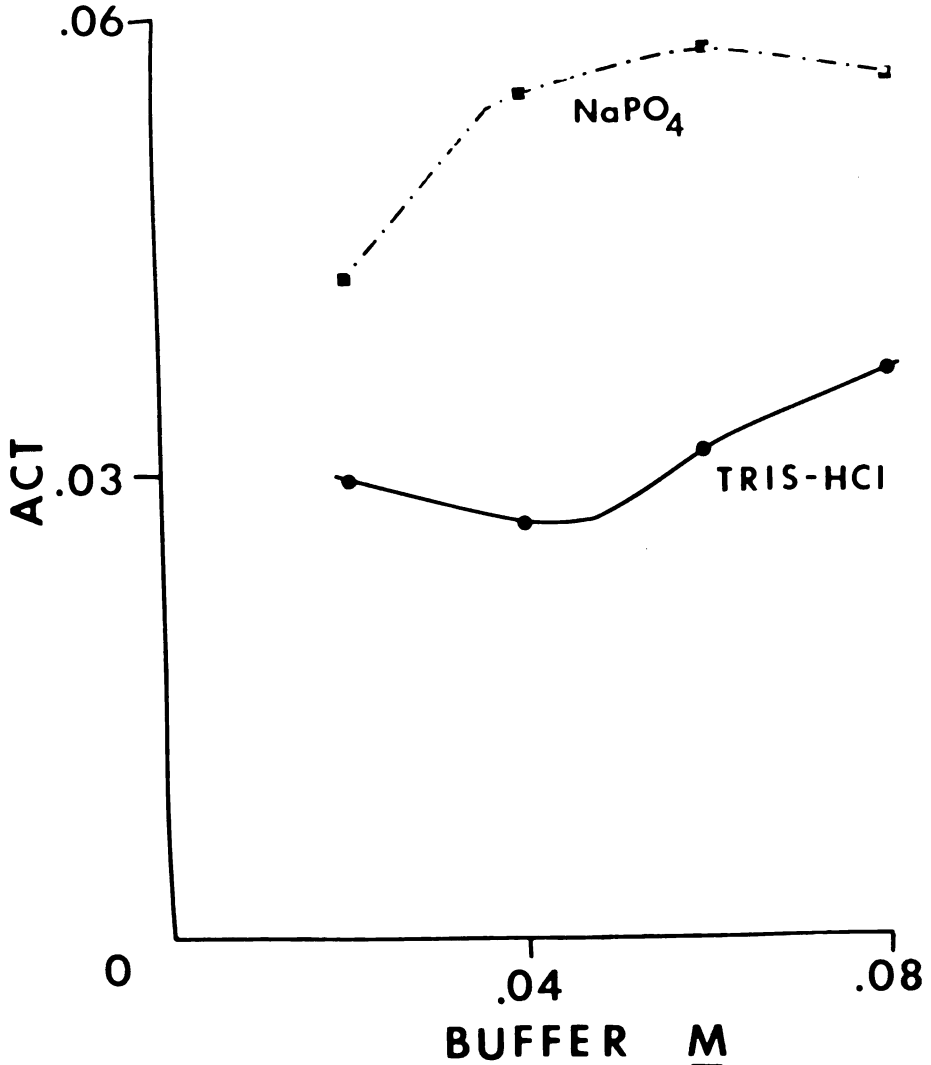

.06
.04
.02
0
ΔA
6
7
8
9
10
pH

Figure 9. -- Effect of Tris-HCl and sodium phosphate buffer on NBT diaphorase. The assays were done at pH 7.2 with whole goat microsomes and 0.5 m$\underline{M}$ NBT. Activity is expressed as ΔA/min.


.06
.03
0
ACT
NaPO4
TRIS-HCl
.04
.08
BUFFER M

diaphorase and cyt c reductase would co-purify. PB induced goat microsomes were solubilized and fractionated as described in the methods section and each fraction was assayed for its content of cyt c reductase and NBT diaphorase (Table 1). As had been previously reported by Kamin and Williams the purification did not proceed equally for the two activities (10). The missing NBT diaphorase activity was not in any other fraction. This data can be interpreted to mean that a component necessary for NBT diaphorase activity is lost in the purification procedure. Next, a fraction was sought which would stimulate the diaphorase. This was found by combining the protein which precipitates between pH 7.5 and 5.4 with the reductase in the pH 5.4 to 4.4 cut which resulted in a 3.5 fold stimulation of NBT activity. Interestingly, the combination of these two fractions gave only a 1.2 fold stimulation of cyt c reductase.

Characterization of H-Factor

A series of experiments were performed in order to partially characterize this diaphorase stimulating factor. The factor, when used to stimulate diaphorase isolated by the Williams and Kamin method, will be referred to as H-Factor. The following experiments were done using an assay mixture containing 1.0 m$\underline{M}$ NBT in 0.05 $\underline{M}$ Tris-HCl buffer (pH 7.5). The protein fraction containing NBT diaphorase activity was solubilized from goat microsomes and partially purified by pH fractionation as described in Methods and Materials.

Table 1. -- Purification of goat cyt c reductase by the method of Kamin and Williams. (1500 mg of microsomes was solubilized for 30 minutes with 12 mg of lipase and 6 mg of trypsin inhibitor and purified for cyt c reductase by the Williams and Kamin method (Methods and Materials). Each fraction was analyzed for cyt c reductase and NBT diaphorase activity. The NBT diaphorase assays were done in 1.0 mM NBT at pH 7.5 in 0.05 M Tris-HCl buffer. Specific activities (S.A.) are expressed in ΔA/min/mg protein. PB induced goat microsomes were used).

Fraction	Protein (mg/ml)	Cyt c reductase		NBT diaphorase	
		S.A.	Fold purification	S.A.	Fold purification
Whole microsomes	48	0.24		1.02	
Solubilized protein	13	1.37	5.6	1.52	1.48
pH 5.4 to 4.6	2.5	5.35	22.6	1.86	1.83
Ammonium sulfate (50 to 70%	1.0	7.50	31.8	1.40	1.37

Heat treatment. The heat stability of the factor was tested by placing it in a boiling water bath for 25 minutes. This heat treatment would be expected to destroy the stimulatory ability of H-Factor if it were a protein. However, H-Factor stimulated NBT diaphorase 3.2 fold before treatment and 3.1 fold after treatment. Unless H-Factor is a protein particularly resistant to heat denaturation this seemed, at the time, to rule out the protein nature of the diaphorase stimulator.

Dialysis. A quantity of H-Factor was dialyzed for 30 hours versus Tris-HCl with several changes of buffer. This experiment was performed in order to see whether H-Factor is a macromolecule. Undialyzed H-Factor stimulated NBT diaphorase activity 1.6 fold while after dialysis there was a 1.3 fold stimulation. Since part of the stimulatory activity remains after dialysis, this indicates that some of the H-Factor is relatively large. The loss of stimulation may be the result of dilution of the factor during dialysis and loss of ionic strength (the factor was prepared by method B and contained some NaCl).

Gel chromatography. H-Factor was run through columns of Sephadex G-10 and G-100 in order to see if the stimulatory activity would be excluded by the gels. A rough estimate of H-Factors molecular weight may be obtained by this method. A 2.5 x 30 cm column of Sephadex G-10 was equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) and a 3.0 x 40 cm column of Sephadex G-100 was equilibrated with 0.05 M sodium phosphate buffer (pH 7.3). The void volume was 64 ml for the G-10

column and 52 ml for the G-100 column as determined with Blue Dextran 2000. H-Factor was run through both columns. The stimulatory activity passed out of the G-10 column in the void volume which indicates a molecular weight of at least 700. Stimulatory activity was lost on the G-100 column and could not be recovered. This could be the result of band spreading in the column which would dilute the activity too much to be assayed for.

Extraction with organic solvent. Ether and chloroform: methanol (5:1) were used to see if they could extract H-Factor from aqueous solution. This would investigate the lipid nature of the factor. Heat treated H-Factor was extracted with the organic solvent and the two phases were separated. The organic phase was blown to dryness with N_2 and the residue was resuspended in buffer. A stream of N_2 was blown across the aqueous phase until the smell of organic solvent was removed. After extraction with ether all of the stimulatory activity remained in the aqueous phase. There was a 3.1 fold stimulation of diaphorase before extraction and a 3.5 fold stimulation after ether extraction. This increase in stimulatory ability is probably due to a volume decrease caused by water saturation of the ether phase and loss of water during the process of blowing ether from the aqueous phase. Extraction with chloroform:methanol resulted in the denaturation of protein which collected at the interphase of the two solvents. Since a small amount of salt had been used during the extraction both phases were dialyzed overnight in buffer. An assay for NBT diaphorase stimulation

in phosphate buffer according to the Methods and Materials section showed that both phases had no stimulatory ability. Presumably, the H-Factor was precipitated in the interphase or an essential lipid was removed by chloroform:methanol and lost during dialysis. Both results would suggest that H-Factor is lipoprotein.

Trypsin digestion. H-Factor was digested for 20 minutes at 25° in a 2 mg/ml trypsin solution. This was another test to see if the factor might be a protein. There was a 1.9 fold stimulation of NBT diaphorase before and after trypsin digestion. Again, this is evidence against H-Factor being a protein.

Mechanism of stimulation. A series of experiments were begun to study the mechanism by which H-Factor stimulates NBT diaphorase. Since the literature had reported that BSA could stimulate NT diaphorase it was thought that H-Factor might be a similar mechanism. This possibility was tested by adding 200 µg of boiled BSA to a NBT diaphorase assay, but this resulted in only a 1.19 fold stimulation. Boiled lipase, on the other hand, did have some stimulatory power since 600 µg of it gave a 2 fold stimulation of NBT diaphorase. Lipase can not be the sole cause of stimulation in the H-Factor fraction, however, since there can be a maximum of 60 µg/ml of lipase in this fraction. Boiled microsomes, alone, could stimulate diaphorase activity since 980 µg of such a preparation gave a 3.2 fold stimulation. In the last experiment of this series the effect of varying the H-Factor concentration on a constant level of diaphorase was tested. With increasing

H-Factor there was initially a rise in diaphorase activity which eventually leveled off (Fig 10).

The studies were switched from goat to rat microsomes and here a problem developed. A large quantity of rat microsomes was solubilized by lipase digestion and purified for cyt c reductase (Table 2). Once again the activities for cyt c and NBT did not co-purify and the data suggested that a cofactor for NBT diaphorase was lost during purification. However, the heat treated pH 7.5 to pH 5.4 precipitable material from rat, which should have stimulatory ability, did not stimulate rat NBT diaphorase. This was unexpected so fresh preparations of rat reductase, rat H-Factor, and goat H-Factor were made and the stimulatory ability of the two H-Factors were assayed on rat reductase (Table 3). Although goat H-Factor could stimulate rat NBT diaphorase there was much less stimulation with material simularly prepared from rat microsomes.

Purification of Cyt c Reductase by Omura and Takesue Method

A series of experiments were begun where cyt c reductase was purified from rat microsomes by the method of Omura and Takesue. There were two reasons for switching from the Williams and Kamin method of purification: with the Omura and Takesue method there is less chance of removing the flavin from the protein and maybe this method would give a clearer indication of whether rat microsomes contain a factor necessary for NBT diaphorase activity. The enzyme was solubilized and purified as described in Methods and

Figure 10. -- Activity of NBT diaphorase versus concentration of H-Factor. The diaphorase enzyme was obtained from the pH 5.4 to 4.5 precipitate of lipase solubilized goat microsomes and the H-Factor was obtained by method A. A constant level of diaphorase was used in all assays.

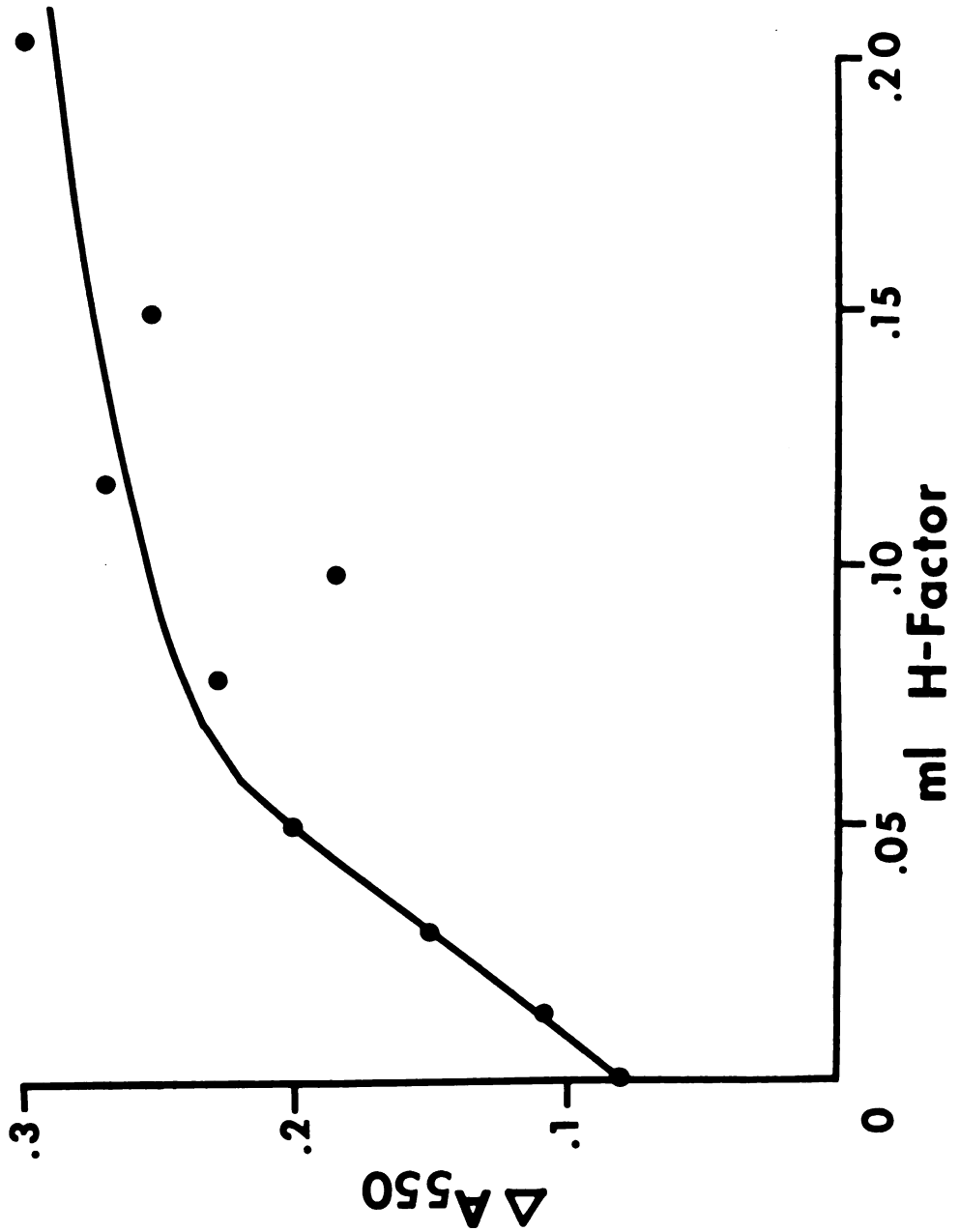

.3
.2
.1
0
ΔA550
.05
.10
.15
.20
ml H-Factor

Table 2. -- Purification of rat cyt c reductase by the Kamin and Williams method. (782 mg of PB microsomes was solubilized for 45 minutes with 24 mg of lipase and 6 mg of trypsin inhibitor. Cyt c reductase was purified by the Williams and Kamin method (Methods and Materials). NBT assays were done with 1 mM NBT at pH 7.5 in 0.05 M Tris-HCl buffer. Specific activities are expressed in ΔA/min/mg protein).

Fraction	Units		S.A.		Fold purification		% recovery	
	Cyt c	NBT	Cyt c	NBT	Cyt c	NBT	Cyt c	NBT
Whole microsomes	595	610	.76	.78	-	-	-	-
Solubilized protein	512	288	1.87	1.05	2.46	1.34	86.1	47.2
pH 5.4 to 4.6	325	199	5.56	3.40	7.31	4.34	54.6	32.6
Ammonium sulfate (50 to 70%)	121	54	6.53	2.90	8.58	3.71	20.3	8.8

Table 3. -- Stimulation of rat NBT diaphorase by H-Factor from goat and rat microsomes. (Rat H-Factor was isolated by Method A and goat H-Factor was isolated by Method B. The goat H-Factor was not dialyzed so a 20% NaCl solution was used as a control.)

	Activity (ΔA/min)	Fold stimulation
Reductase alone	.020	-
Reductase + rat H-Factor	.027	1.4
Reductase + goat H-Factor	.205	10
Reductase + 20% NaCl	.064	3.2

Materials. Cyt c reductase, NBT diaphorase, and NADPH oxidase activities were measured for the various fractions. Although the final step of purification on a DEAE-cellulose column gave a 84 fold purification for cyt c reductase there was only a 20 fold purification for K_3-dependent NADPH oxidase (Table 4). Disc gel electrophoresis showed that DEAE-cellulose chromatography had purified the protein to about 10 bands. A final determination for the purification of NBT diaphorase could not be made because once the protein had been purified on a G-100 column it was impossible to get a linear diaphorase assay over time (Fig 11).

Factor 10

This loss of linearality in the NBT diaphorase assay after gel chromatography again suggested that a necessary factor for the reaction was being separated from the enzyme. In order to find this factor the effect of adding various fractions of the G-100 column to the reductase fraction was investigated. The void volumn fraction stimulated the diaphorase assay but had no effect on the reduction of cyt c (Fig 11). Since this stimulatory factor was excluded from G-100 Sephadex it must have a molecular weight greater than 150,000. We named this stimulatory agent "Factor 10" in order to distinguish it from the stimulatory factor obtained from goat microsomes.

The next study on Factor 10 was designed to see if lipase digestion released all of it from the microsome. Since Factor 10 seemed to be a large molecule there was the possibility that there might be some stimulatory activity

Figure 11. -- Stimulation of NBT diaphorase by Factor 10. Lipase solubilized PB induced cyt c reductase was purified by gel chromatography on a Sephadex G-100 column. The NBT diaphorase activity of this enzyme was assayed for with and without Factor 10.

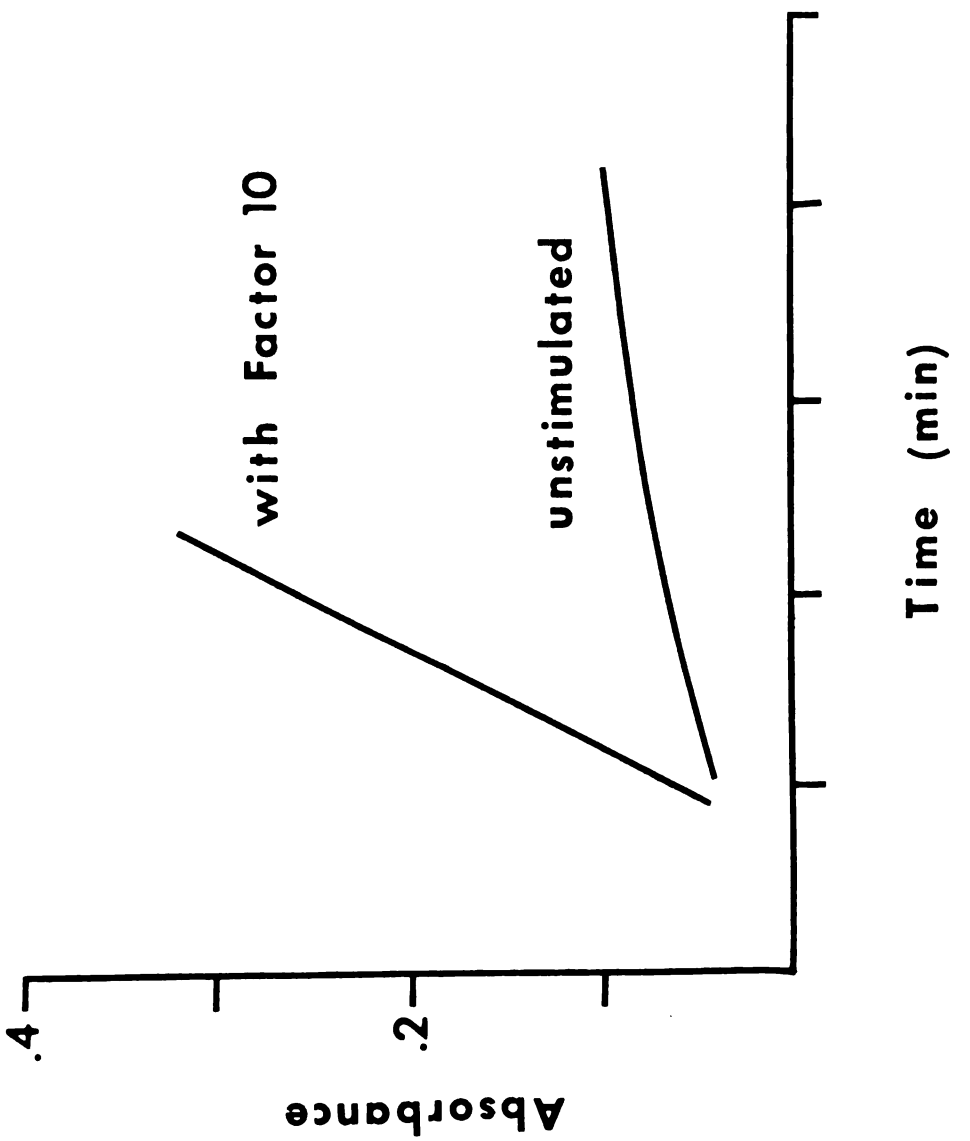

with Factor 10
unstimulated
Absorbance
.4
.2
Time (min)

Table 4. -- Isolation of cyt c reductase by Omura and Takesue method. (The enzyme was solubilized and isolated as described in 'Methods and Materials'. Specific activities are in ΔA/min/mg protein. NADPH oxidase assays were done with 0.56 ug/ml menadione in 0.05 M phosphate.)

Step	NBT diaphorase		cyt c reductase			NADPH oxidase		
	S.A.	units recovered	S.A.	units recovered	fold purification	S.A.	units recovered	fold purification
Washed microsomes	.458	953	1.8	3720		.11	236	
Solubilized protein	8.8	405	15.6	3240	8.9	.70	144	6.3
Sephadex fraction	*	*	66	2280	37.8	2.20	76	20
DEAE-cellulose fraction	*	*	147	684	84.0	2.25	11	20

*Assays were non-linear.

remaining in the unsolubilized protein of the 105,000 x g pellet. This hypothesis was tested by adding varying quantities of void volumn material and lipase digested pellet protein to G-100 purified reductase and indeed the pellet protein could stimulate NBT diaphorase (Fig 12).

Next, a procedure to solubilize Factor 10 from the lipase digested pellet was sought. This was complicated by the fact that lipase digestion resulted in the formation of a syrupy layer of protein between the supernatant and pellet which was contaminated with solubilized reductase. Both the syrupy layer and pellet contained Factor 10; with the syrupy layer predominating (Table 5). We attempted to solubilize the Factor 10 from the syrupy layer with Triton X-100 at 0^{o} and by enzymatic digestion with Crotalus atrox venom at 37^{o} (the venom is a good source of phospholipases). As a control of the actual solubilizing power of these agents some of the syrupy layer was untreated except for suspending it in buffer. This control was deemed necessary because lipase in the pellet would continue to solubilize the pellet while it was stored overnight in the freezer. After treatment the protein was centrifuged for 90 min at 140,000 x g. The supernatants were then assayed for their ability to stimulate the NBT diaphorase activity of DEAE-cellulose "purified" protein (Table 6). All three supernatants could stimulate the reductase and the amount of stimulation seems to depend on the amount of protein solubilized.

The heat stability of Factor 10 was studied next. The

Figure 12. -- The location of Factor 10 in microsomes. Pellet material was prepared by resuspending in buffer the protein collected in the 105,000 x g pellet after lipase solubilization of microsomes (1.05 mg/ml). Factor 10 material consists of the protein collected in the void volume of a Sephadex G-100 column during the purification of cyt c reductase from lipase solubilized microsomes (1.25 mg/ml). The ability of varying concentrations of these two preparations to stimulate NBT diaphorase was assayed. PB microsomes supplied diaphorase and stimulator. Activity is expressed as $\Delta A/min$.

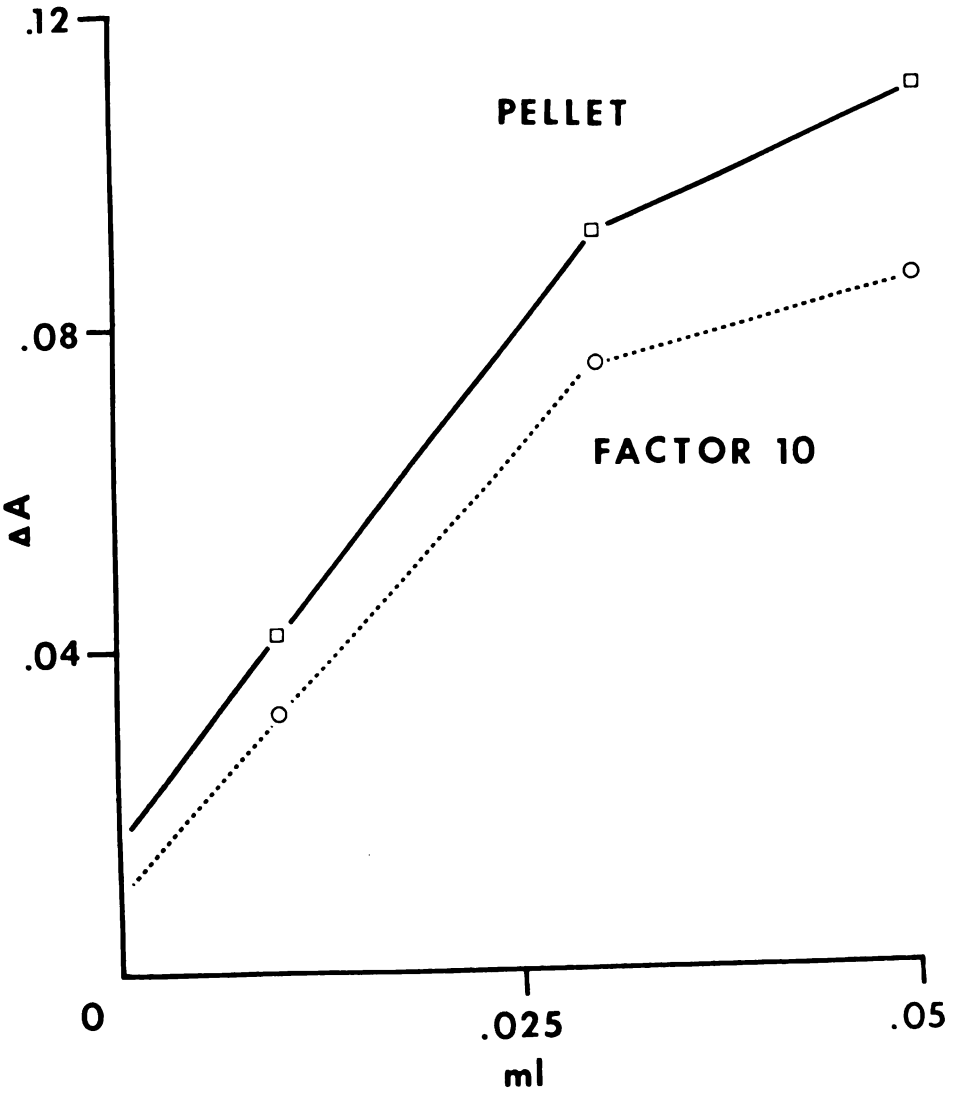

.12
.08
.04
ΔA
PELLET
FACTOR 10
0
.025
.05
ml

Table 5. -- Location of Factor 10 in rat microsomes. (The following three sources were assayed for their ability to stimulate NBT diaphorase activity. The reductase (NBT activity = 0.011 ΔA/min) was partially purified through DEAE-cellulose chromatography. The lipase pellet and syrupy layer were obtained by centrifuging lipase digested microsomes for 90 minutes at 105,000 x g (see text). Void volume material consists of the excluded protein from a Sephadex G-100 column during the purification of the reductase (Methods and Materials).

Stimulator added	Protein (μg)	Activity (ΔA/min)	Fold Stimulation
Lipase pellet	130	.059	5.4
Syrupy layer	32	.114	10.4
Void volume	?	.120	10.9

three soluble preparations with diaphorase stimulating activity, prepared in the above experiment, were placed in a boiling water bath for 15 minutes. The stimulatory factor was seen to be essentially heat stabile (Table 6). This result would rule out the possibility of Factor 10 being a simple protein although it may be a lipoprotein since they are theoretically resistant to heat denaturation.

In the next experiment the effect of a solution of $Ba(OH)_2$ and $ZnSO_4$ on Factor 10 was investigated. A barium and zinc solution acts as a denaturant and precipitant of protein and if such treatment would eliminate the stimulation by Factor 10 then this would indicate that Factor 10 may be a protein. The heat stability of Factor 10 could then be explained by assuming that it is a lipoprotein or that it acts nonenzymatically and may be important, for example, as a structural component. Equal volumes of 5% $ZnSO_4$ and 7.5% $Ba(OH)_2$ were added to an equal volume of Factor 10 and the resulting precipitate was removed by centrifuging. The supernatant was then dialyzed against buffer in order to remove barium and zinc ions. The resulting dialysate had no diaphorase stimulating ability.

By varying the concentrations of reductase and Factor 10 it was hoped that the changes in NBT diaphorase activity would give a clue for the mechanism of stimulation. Two separate experiments were carried out; in the first the levels of reductase were varied while the level of Factor 10 was kept constant, while in the second the inverse was

Table 6. -- The solubilization and heat stability of Factor 10. (Three 6.5 ml aliquots of the syrupy layer of unsolubilized goat microsomes (7.4 mg/ml) were treated as follows: incubation for 30 minutes at 37° with 0.7 mg of Crotalus atrox venom, digestion for 30 minutes at 0° with Triton X-100 (0.1%) and untreated. The 105,000 x g supernatants after these treatments were tested for their ability to stimulate NBT diaphorase and to retain this ability after being heated in a boiling water bath for 15 minutes. The activity of the diaphorase alone is .008 ΔA/min. Activity is expressed as ΔA/min).

			untreated			heat treated		
	protein (μg/ml)	% protein solubil.	act.	fold stim.	A/min protein	act.	fold stim.	% activity after heat
Crotalus atrox	32	43	.074	9.2	2.3	.069	8.6	93
Triton X-100	27	36	.052	6.5	1.9	.047	5.9	91
untreated	16	22	.052	6.5	3.2	.041	5.1	79

done (Fig 13 and 14). The results show that stimulation is proportional to concentration of Factor 10 although the reductase can be saturated by high levels of Factor 10. Also of interest was the observation that an assay with a high concentration of Factor 10 and a low concentration of reductase exhibited a lag at the start of the reaction before the maximal rate was obtained. This lag was not seen in assays where the converse conditions were true. Such a lag could be explained by a mechanism where the flavoprotein passes electrons to Factor 10 which then reduces NBT. The reduction of NBT would be the rate limiting step. In such a scheme a time lag would exist before enough Factor 10 is reduced to reach the maximum rate of NBT reduction.

Another clue for the mechanism of diaphorase stimulation may be obtained by studying the dependency of diaphorase activity on protein concentration. A plot of NBT diaphorase activity versus protein concentration for solubilized rat reductase will not extrapolate through zero (Fig 15). This observation was also seen for whole rat microsomes but not for whole goat microsomes. Factor 10 was tested next to see if the addition of it to the reductase would transpose the plot through zero. This was done by adding 0.1 ml of heat treated Factor 10 to 0.5 ml of NBT and allowed them to sit for approximately 30 minutes. This preincubation was done because NBT and Factor 10 together form a turbid solution and time is necessary to complete agglutination. A plot of activity versus enzyme concentration did pass through zero

Figure 13. -- Activity of Factor 10 stimulated NBT diaphorase versus enzyme concentration. The amount of Factor 10 was held constant while the concentration of diaphorase was varied. 1 ml of enzyme solution would reduce 74 uM of cyt c/min. Factor 10 was prepared by method A. Activity is expressed as $\Delta A/min$.

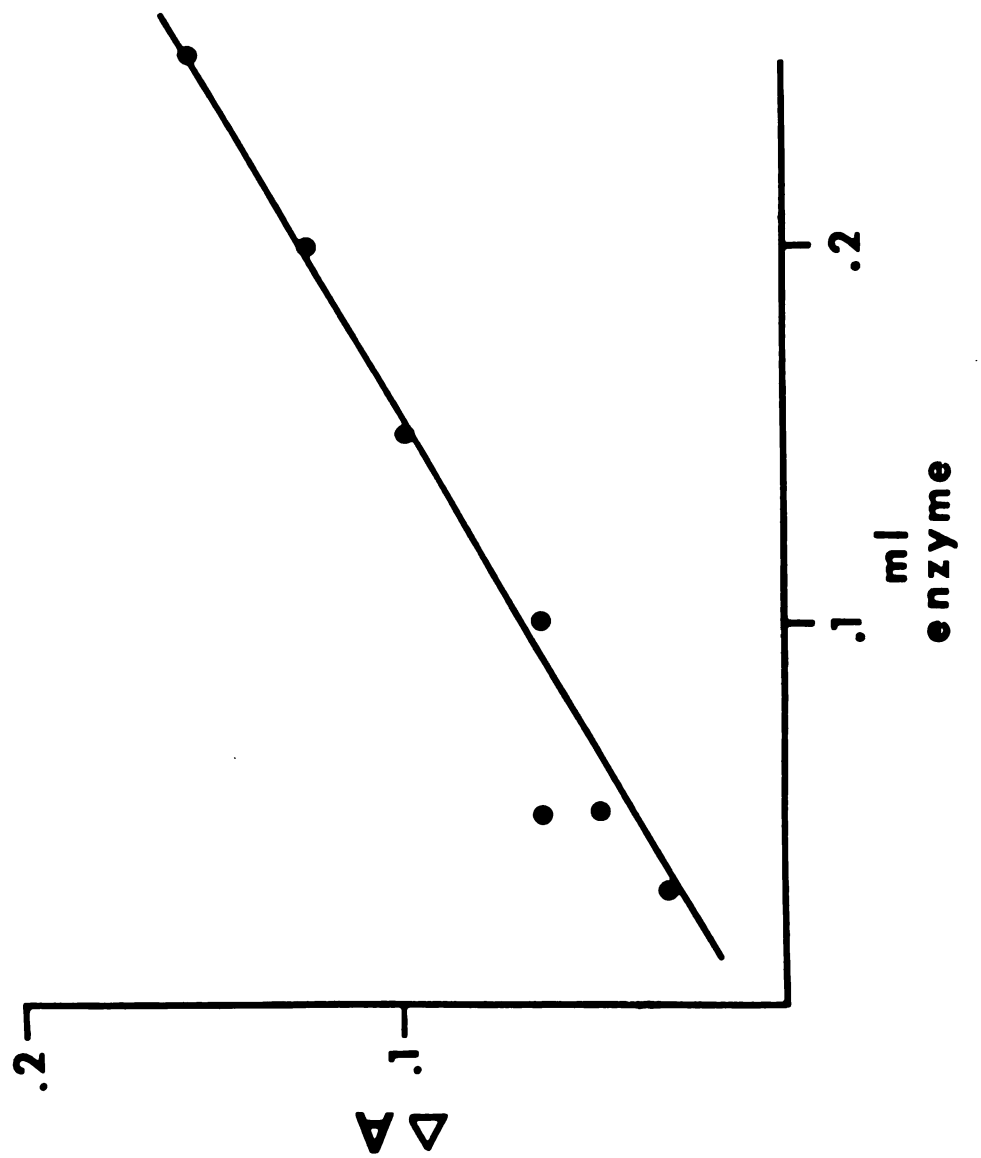
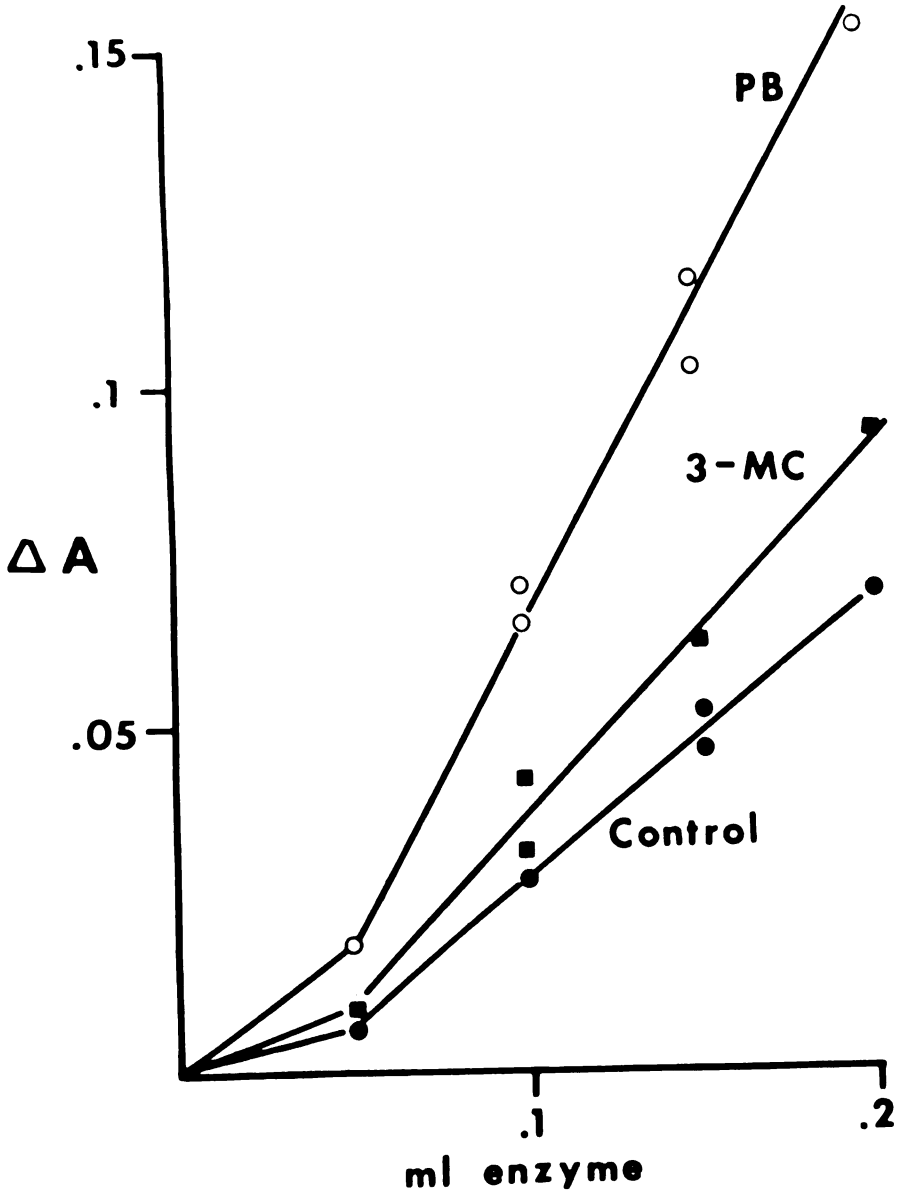

.2
.1
ΔA
.1
.2
ml
enzyme

Figure 14. -- Activity of NBT diaphorase versus Factor 10 concentration. The concentration of diaphorase (cyt c reductase activity = 74 u$\underline{M}$/min/ml) was held constant while the concentration of Factor 10 (prepared by method A) was varied. Activity is expressed as ΔA/min.

Figure 15. -- Activity of NBT diaphorase versus concentration of solubilized rat diaphorase. Control, PB, and 3-MC induced microsomes were solubilized with lipase. Activity is expressed as $\Delta A/min$.


.15
.1
.05
ΔA
PB
3-MC
Control
.1
.2
ml enzyme

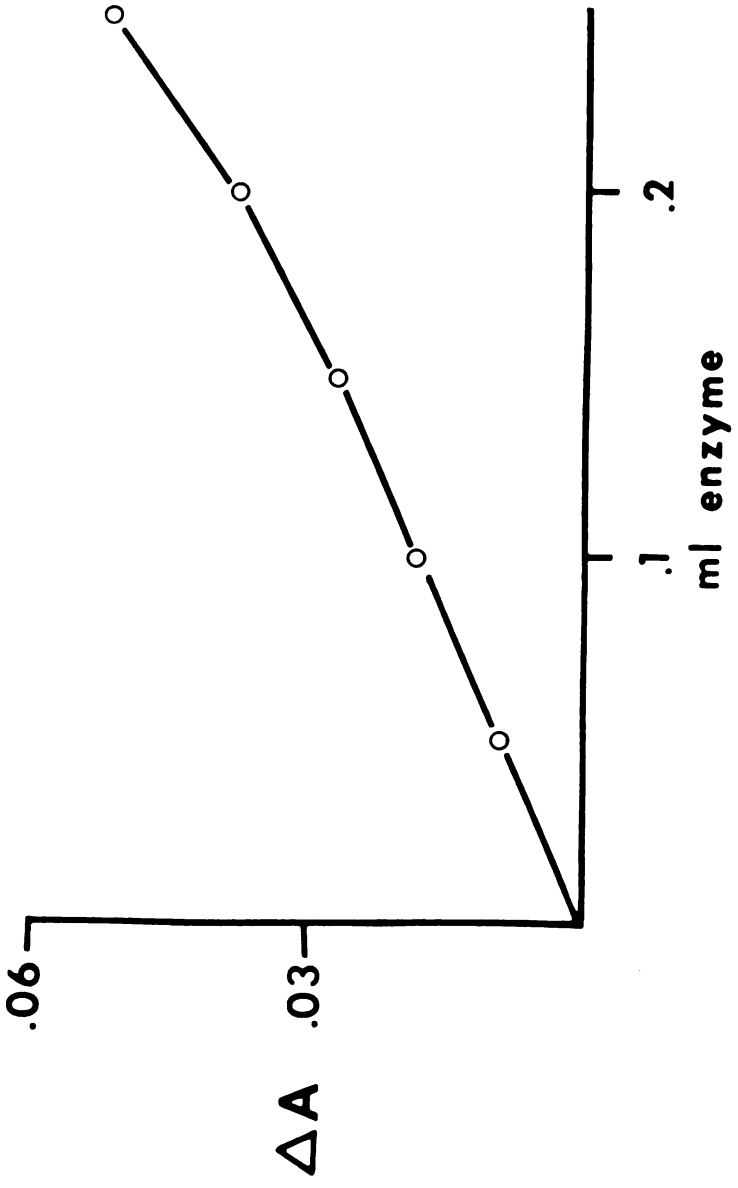
although the plot is not linear over the entire range of concentrations (Fig 16).

The turbidity seen when Factor 10 or H-Factor was added to an NBT solution suggested that NBT and the stimulating factors may form an insoluble complex. The idea was tested by seeing if the removal of the precipitate would alter the diaphorase stimulation. The experiment was done using goat H-Factor and soluble goat reductase which had been partially purified by pH fractionation. A fresh solution of H-Factor and NBT stimulated diaphorase activity 1.8 fold. After incubating together at 25° the solution was noticeably cloudy but still stimulated diaphorase activity 1.7 fold. The NBT and H-Factor solution was centrifuged for 10 minutes at 12,100 x g in order to remove the precipitate. This treatment reduced the supernatant's stimulating ability to 1.3 fold. The precipitate was resuspended in a NBT solution but had no stimulatory ability. This experiment shows that H-Factor is precipitated in a NBT solution.

Multiple Microsomal NADPH Reductase

Experiments in this area began as a study to see whether NBT and cyt c were reduced by the same enzymatic mechanism. If NBT reduction requires a factor in addition to the cyt c reductase flavoprotein then the induction and inhibition of these two activities should be different. During this study it was realized that these experiments might also indicate if there were more then one enzyme capable of reducing NBT or cyt c. The NADPH oxidase activity of the flavoprotein

Figure 16. -- Activity of Factor 10 stimulated NBT diaphorase versus concentration of solubilized protein. Lipase solubilized, control rat enzyme (ΔA = 0.06/min/ml was used. A constant level of Factor 10, which had been shown to saturate NBT diaphorase stimulation, was added to each assay.


.06
.03
ΔA
.1
.2
ml enzyme

was also studied.

In the first experiment the solubilization of cyt c reductase and NBT diaphorase activity from the membrane was followed with time. If a flavoprotein and electron carrying intermediate was needed for NBT reduction it was expected that cyt c reductase and NBT diaphorase would not solubilize together. The experiment was performed by removing aliquots of solubilizing enzyme at various times and placing them in ice cold buffer and centrifuging at 105,000 x g. The amount of NBT diaphorase and cyt c reductase in the supernatants were then assayed and expressed as the percent of final solubilization. The results show that the two activities solubilized together (Fig 17).

Disc gel electrophoresis was used during the purification of the cyt c reductase in order to check each step. The diaphorase activity in each step can be stained for directly in the acrylamide gel by incubating in a solution of NBT and NADPH. With this method, two closely spaced bands of NBT diaphorase activity were seen in lipase digested microsomes (Fig 18). These two bands were also seen during the purification steps of the Omura and Takesue method. This was the first evidence obtained which might indicate the existence of two diaphorase enzymes. Unfortunately, cyt c reductase activity can not be stained for directly in a gel.

In the next experiment the effect of PB induction on cyt c reductase and NBT diaphorase was investigated. If these two activities are induced equally this could indicate

Figure 17. -- Co-solubilization of NBT diaphorase and cyt c reductase activity. 400 mg of PB induced microsomes was solubilized with 5 mg of lipase and aliquots were removed at various times during solubilization. 100% solubilization refers to the amount of activity released after 26 minutes.

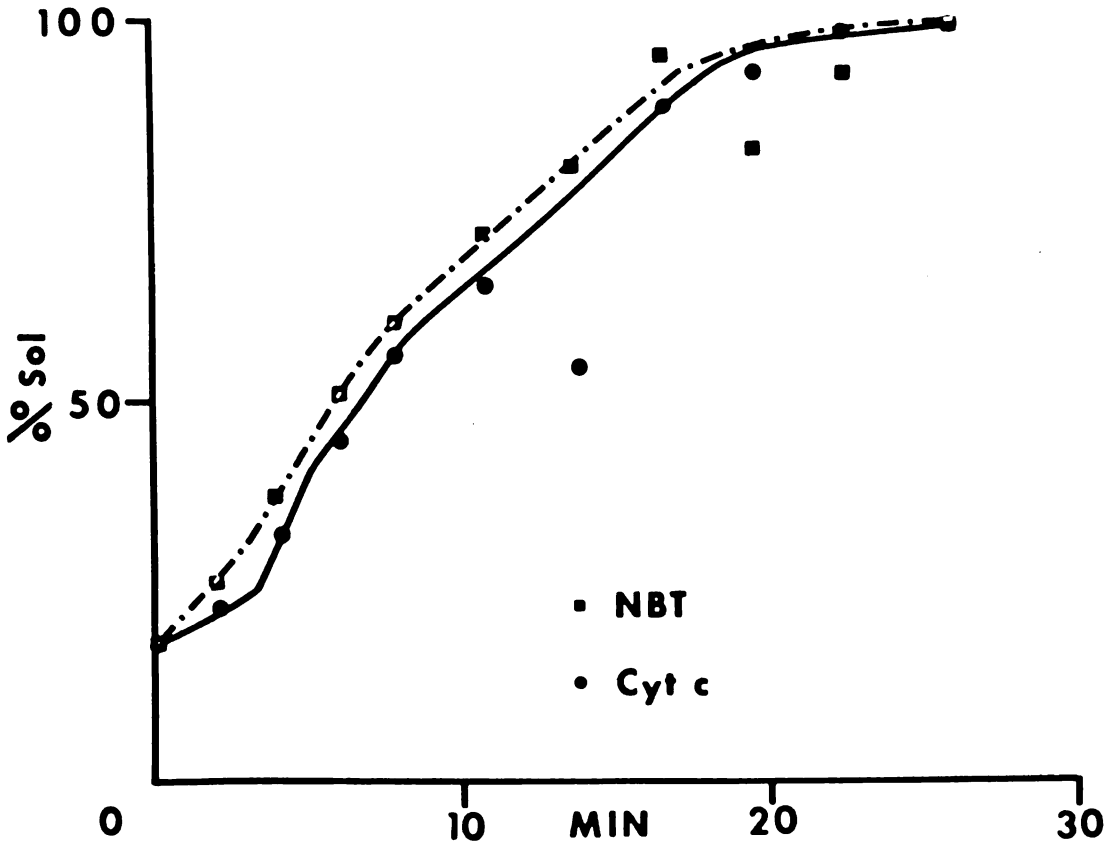

100
% Sol
50
0
10
MIN
20
30
NBT
Cyt c

Figure 18. -- Disc gel electrophoresis of NBT diaphorase. PB induced rat microsomes were solubilized with lipase and electrophoresed in acrylamide gel. The bottom of the tubes corresponds to the cathode. The left tube was stained with Coomassie Blue and the right tube by the NBT activity stain method.

that cyt c reductase and NBT diaphorase are probably activities of the same enzyme. Special care was taken to insure that the control and PB induced animals were similar in age and weight. A slight difference in the induction of cyt c reductase and NBT diaphorase was seen. However, in contrast to the findings of Orrenius, these increases were much smaller than the ones seen for aminopyrine demethylase and P-450 (Table 7).

As a further study of the co-identity of these reductases the effect of induction and solubilization was investigated. Freshly washed microsomes from control, PB induced, and 3-MC induced rats were digested with lipase and the solubilized protein was separated by ultracentrifugation. Cyt c reductase, NBT diaphorase, and NADPH oxidase activities were then assayed in the whole microsomal, lipase supernatant, and pellet fractions. The microsomes had been previously prepared and stored in the freezer so it is not definite that all three sets of rats were similar in age, weight and care. This experiment has been done several times and Table 8 gives the most reliable data obtained. 3-MC pretreatment seems to cause little change in comparison to control microsomes. In two previous experiments with unwashed microsomes, however, 3-MC pretreated microsomes had a 3.5 fold increase in NADPH oxidase activity. Since this increase could not be duplicated with other preparations of 3-MC microsomes the increase was assumed to be a special case. PB microsomes had an increase in NBT diaphorase, cyt c reductase, and NADPH oxidase activity of 2.3, 1.9, and 1.8 respectively. The solubilization seemed to have

Table 7. -- The PB induction of rat microsomes. (Four uninduced and 29 PB treated rats were used in this experiment. PB rats were starved overnight while control rats were not).

	control	PB	Fold stimulation
Protein (mg/ml)	43	84	
NBT diaphorase (ΔA/min/mg protein)	.413	.629	1.52
Cyt c reductase (ΔA/min/mg protein)	.542	1.00	1.85
P-450 (ΔA/mg protein)	.114	.381	3.35
Aminopyrine demethylase (nmoles/min/mg)	2.18	8.4	3.86

Table 8. -- Induction of microsomal NADPH reductases and effect of solubilization. (Control, PB, and 3-MC microsomes were solubilized with lipase (Methods and Materials). The whole microsomes, solubilized protein, and unsolubilized pellet were assayed for cyt c reductase, NBT diaphorase, and NADPH oxidase. 67%, 86%, and 88% of the protein was recovered after lipase digestion for control, PB, and 3-MC microsomes, respectively. Values reported are in nmoles/min/mg protein.

	Whole microsomes			Solubilized protein			Unsolubilized protein		
Microsome	NBT*	cyt c	NADPH	NBT*	cyt c	NADPH	NBT*	cyt c	NADPH
Control	90	52	93	156	182	264	0	0	30
PB	206	100	172	413	422	480	63	12	47
3-MC	90	56	108	195	187	273	20	6	29

*Values multiplied by 4 because NBT is a four electron acceptor.

Table 8a. -- Percent units recovered after solubilization. (Activity in supernatant + activity in pellet)

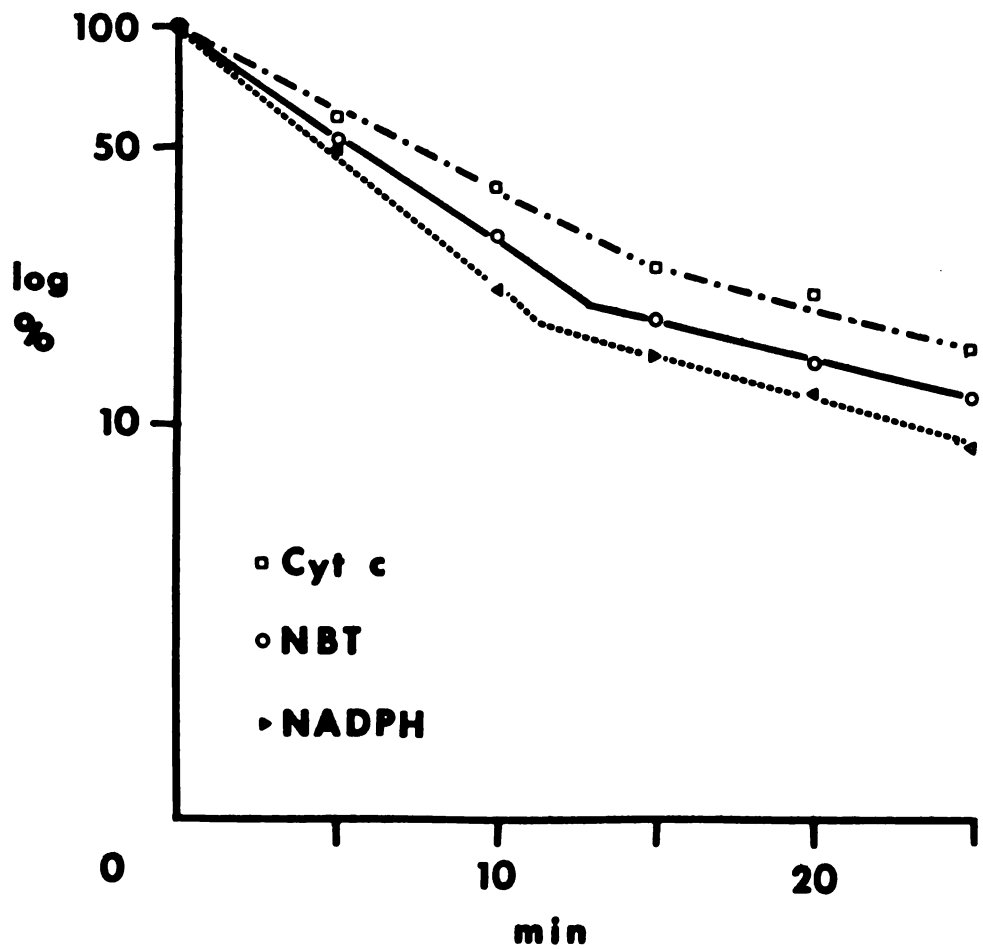
	NBT	Cyt c	NADPH
Control	49%	98%	95%
PB	73	128	97
3-MC	81	124	97

the same effect on all three kinds of microsomes except that there was an increase in the amount of cyt c reductase in PB and 3-MC microsomes. The lack of activation of cyt c reductase by solubilization in control microsomes, however, may be caused by the fact that only 67% of the protein was recovered. Except for the activation of cyt c reductase this experiment does not seem to have told much about the existence of multiple reductases.

A heat denaturation study was next done in order to look for multiple reductases. Since heat denaturation of a protein is a first order reaction and assuming that only one enzyme is responsible for the three enzymatic reactions being studied then all three activities should have identical half-lives. When doing this experiment on lipase solubilized microsomal protein it was found, however, that first order plots of loss of activity were biphasic and that the plots for the three activities were not superimposable (Fig 19). The half-lives for the second, slower decay are roughly equal for all three activities but the first, faster decay rates are different.

These biphasic curves do seem to indicate that there are two separate diaphorases. One has to be careful, however, since there may be only one enzyme in the intact microsome and lipase digestion could degrade the flavoprotein unequally and result in two slightly different, soluble enzymes. The position of the breaks in these curves varied between preparations and differences in the solubilization procedure

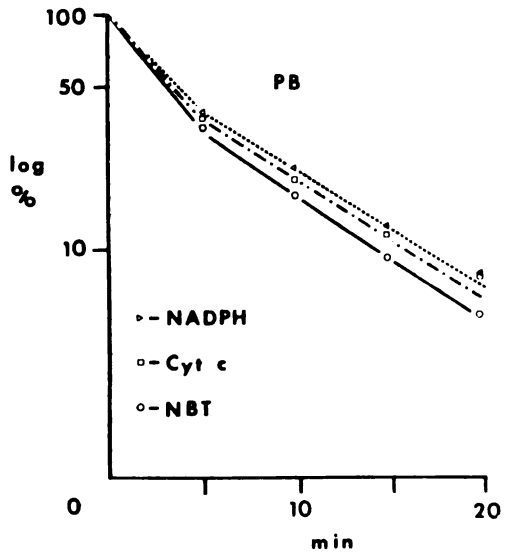
Figure 19. -- Heat denaturation of the microsomal NADPH reductase activities. Lipase solubilized protein from control rat microsomes was denatured at 50°. The cyt c reductase, NBT diaphorase, and NADPH oxidase activities were assayed at various times during the denaturation.

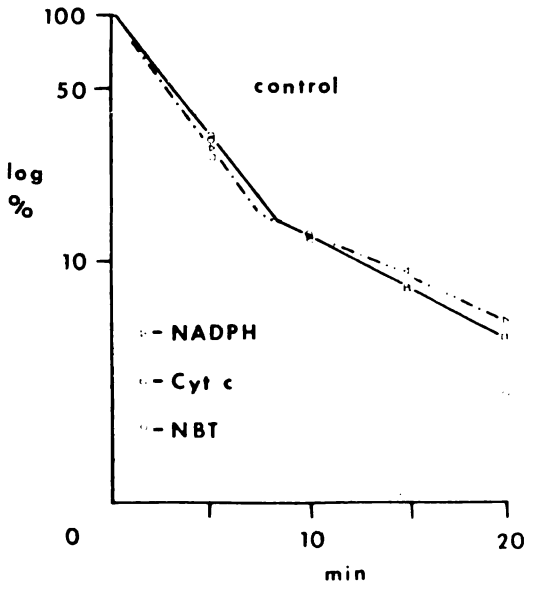

100
50
10
0
log
%
Cyt c
NBT
NADPH
10
20
min

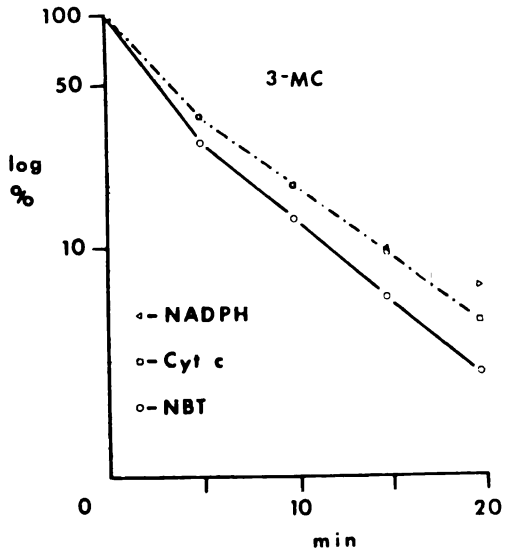
from day to day may account for this. If there are two reductase enzymes they might be expected to be induced differently by PB and 3-MC. The biphasic turnover of P-450, for example, was seen to be induced differently by PB and 3-MC when studied by induction. In this thesis, control, PB, and 3-MC microsomes were solubilized by lipase treatment and heat denatured at 50° (Fig 20). For control microsomes the slowly denatured component had an extrapolated concentration of 32% for all three activities. PB and 3-MC microsomes, on the other hand, had extrapolated initial concentrations between 55% and 65% for the slowly denatured component. Induction does increase the amount of one component over another and this supports the hypothesis of two different reductases.

If there are two separate reductase enzymes with activities for the reduction of cyt c, NBT, and menadione one would expect that the two enzymes would not show identical activities for all three substrates. Therefore, if one of the reductase enzymes could be inhibited differently from the other the inhibition of the three substrates would not be equal. This experiment was first attempted with p-chloromercuribenzoate (PCMB) which has been used previously to show that NADPH oxidase and cyt c reductase were identical enzymes (43). However, this inhibitor was difficult to work with because reproducible rates were impossible to obtain unless careful timing of all steps was carried out. Because of this a search was begun for another inhibitor.

Figure 20. -- Effect of induction on heat denaturation of microsomal NADPH reductases. Lipase solubilized protein from control, PB, and 3-MC induced rat microsomes were heat denatured at 51°.


100
50
10
0
log
%
PB
- NADPH
- Cyt c
- NBT
10
20
min


100
50
10
0
log
%
control
- NADPH
- Cyt c
- NBT
10
20
min


100
50
10
0
log
%
3-MC
- NADPH
- Cyt c
- NBT
10
20
min

2'-AMP, a competitor for the NADPH binding site, was the choice for this inhibitor study. In this experiment it was hoped that 2'-AMP would inhibit the three activities differently and that the shape of the inhibition curves would vary depending on whether the microsomes had been untreated or induced with PB or 3-MC. Lipase solubilized microsomal protein was used as the source of reductase and 2'-AMP inhibition gave a smooth curve for all three activities (Fig 21). These results were disappointing since one could not show different components of inhibition or that the inhibition of cyt c reductase, NBT diaphorase, and NADPH oxidase was different. Induction with 3-MC, however, did alter the shape of the inhibition curves although PB did not (Fig 22). Cyt c reductase and NBT diaphorase were less susceptible to 2'-AMP inhibition after 3-MC induction while NADPH oxidase was more susceptible.

Figure 21. -- 2'-AMP inhibition of microsomal NADPH reductases. The cyt c reductase, NBT diaphorase, and NADPH oxidase activities of lipase solubilized control, PB, and 3-MC induced rat microsomes were inhibited with varying concentrations of 2'-AMP.

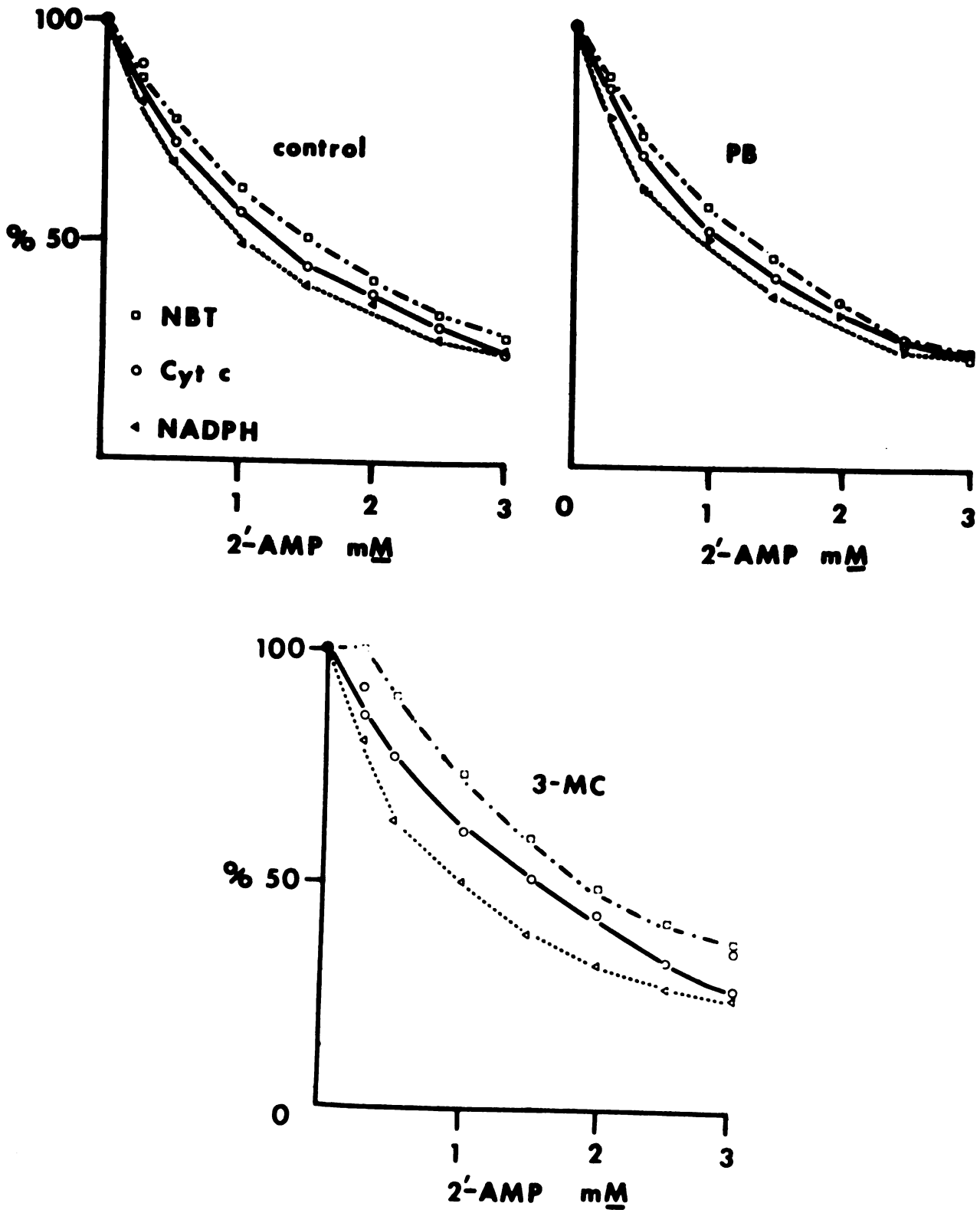
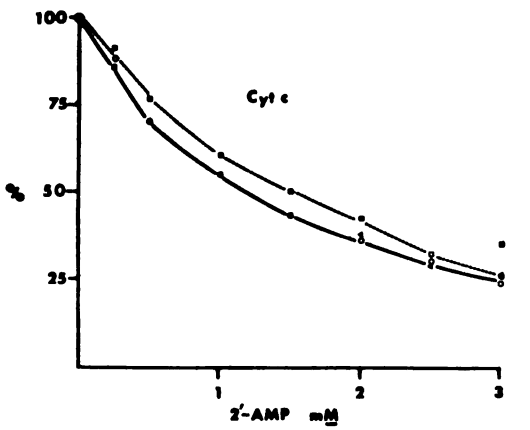
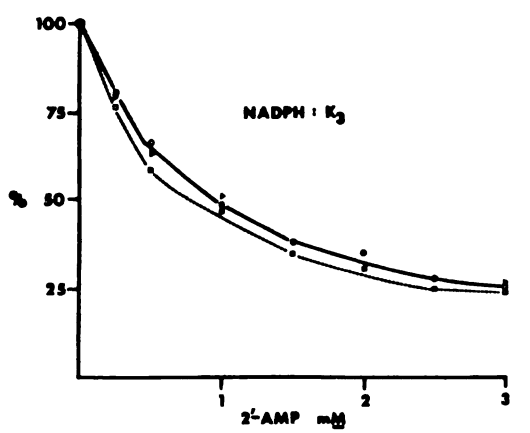
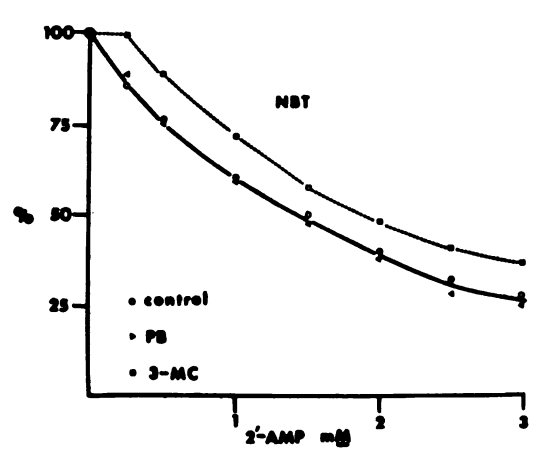

100
% 50
control
NBT
Cyt c
NADPH
1
2
3
2'-AMP mM
PB
0
1
2
3
2'-AMP mM
100
% 50
3-MC
0
1
2
3
2'-AMP mM

Figure 22. -- Induction effect on the 2'-AMP inhibition of microsomal NADPH reductase activities. Control, PB, and 3-MC induced rat microsomes were solubilized and the 2'-AMP inhibition of cyt c reductase, NBT diaphorase, and NADPH oxidase was assayed.


100
75
50
25
%
Cyt c
1
2
3
2'-AMP mM


100
75
50
25
%
NADPH : K3
1
2
3
2'-AMP mM

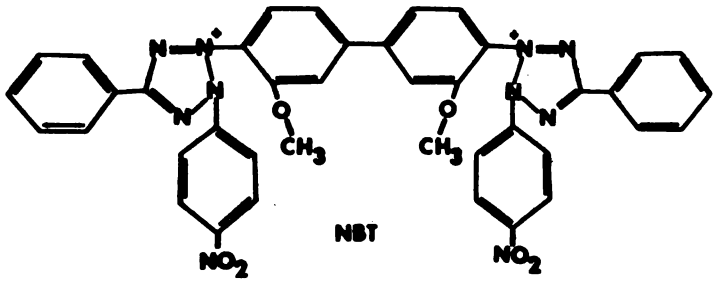

100
75
50
25
%
NBT
• control
• PB
• 3-MC
1
2
3
2'-AMP mM

DISCUSSION

Interest in the microsomal reduction of tetrazolium dyes grew from research carried out by Williams and Kamin and Lu and Coon. Williams and Kamin had reported that neotetrazolium (NT) diaphorase activity disappeared during the purification of cyt c reductase although the same flavoprotein was believed responsible for both activities. Lu and Coon's work on the solubilization of the microsomal fatty acid ω-hydroxylation system seemed to indicate that the cyt c reductase and P-450 reductase activities were not identical. The disappearance of NT diaphorase activity during cyt c reductase purification could indicate that an electron carrier between the flavoprotein and NT was being purified away. This research was started in the hope of isolating this unknown electron carrier and seeing if it would serve a similar role in shuttling electrons between the flavoprotein and P-450 during xenobiotic metabolism.

The NBT Diaphorase Assay

NBT was used instead of NT to measure tetrazolium disphorase activity. These two dyes are closely related structurally but the higher aqueous solubility of NBT make it easier to work with.



Reduction of NBT can be followed by a kinetic assay while NT reduction could only be followed by an end point assay. Since the diaphorase reactions were linear over time for only a short duration the kinetic assay has a definite advantage. The loss of linearity is presumably due to the low solubility of the diformazan product in buffer.

Despite the short duration of the linear phase of the kinetic assay, however, rates of NBT reduction obtained by this method were reproducible. A second problem seen was that NBT diaphorase activity was not proportional to rat enzyme concentration, although it was proportional to whole goat microsomal concentration. Therefore, one must be careful when studying the purification of rat NBT diaphorase since a two-fold increase in NBT activity does not necessarily mean that there was a two-fold increase in the concentration of diaphorase enzyme. No theory has been proposed as to why this nonproportionality exists or why it is eliminated by adding H-Factor.

The TLC experiment on enzymatically reduced NBT suggests that NBT can undergo other reactions besides reduction. This

conclusion also may explain why the NBT absorption scans are different when reduced by whole microsomes versus solubilized protein. This means that the extinction coefficient experimentally calculated for whole goat microsomes may not be appropriate for other enzyme preparations. Goat and rat microsomes may have different side reactions for NBT and as the enzyme is purified the total extinction coefficient may be changed.

Solubilization and Purification of Cyt c Reductase

The mechanism by which lipase digestion releases cyt c reductase is unknown. The relative ease of releasing the flavoprotein from the membrane in comparison to P-450 suggests that the reductase is loosely bound at the surface. The lipase used in these experiments may have some proteolytic contamination so the solubilization may be dependent on more than the hydrolysis of lipids. It is quite possible that the enzyme as solubilized is only a degraded form of the naturally occurring enzyme. Solubilization of cyt b_5 by trypsin, for example, has been shown to release only a core peptide with the prosthetic group attached (50).

A careful look at the specific activities of cyt c reductase, NBT diaphorase, and K_3-dependent NADPH oxidase before and after solubilization may give a clue as to why lipase treatment activates the level of cyt c reductase activity (Table 8). This data shows that in whole microsomes the specific activities for NBT diaphorase and NADPH oxidase are approximately equal while the cyt c reductase

specific activity is approximately one-half. However, after solubilization the specific activities of cyt c reductase and NBT diaphorase are roughly equal although less then the NADPH oxidase specific activity. These results can be interpreted to mean that in the intact membrane the large cyt c molecules can not reach all of the available reduction sites but that the smaller menadione and NBT molecules can. After solubilization the size limitation on cyt c molecules is removed. Some caution must be exercised in this interpretation because the NADPH oxidase assays were done at a lower pH and higher ionic strength than the other substrates and there is some question as to the validity of the absorptivity used to calculate the NBT specific activities. This may explain why the specific activity of NADPH oxidase is higher than that of NBT diaphorase after solubilization. By following the specific activity of cyt c reductase in the intact membrane in comparison to NBT diaphorase one may have a tool to monitor the effect of different perturbations on membrane structure.

NBT diaphorase did not purify equally with cyt c reductase and the missing NBT activity was not found in any other fractions during purification. The maximum loss of NBT diaphorase activity usually occurred during the pH fractionation of the Williams and Kamin purification and always during the gel chromatography step in the Omura and Takesue purification. Interestingly, after the loss of diaphorase activity, fractions were found in each purification method

which would stimulate NBT diaphorase but not cyt c reductase. These results suggest that for NBT reduction a factor is necessary in addition to the flavoprotein, cyt c reductase. Whether cyt c reductase can reduce NBT without the factor is not certain since the flavoprotein has not been purified to homogeneity.

Stimulation of NBT Diaphorase

There is some question as to whether the stimulatory agents contained in the H-Factor and Factor 10 preparations are identical. However, since the procedure used to isolate H-Factor from goat microsomes will not work for rat microsomes, this may indicate that there is a species difference in the NBT reduction of these two animals. No attempt was made to see if the Factor 10 isolation procedure on goat microsomes would result in a diaphorase stimulator or whether goat H-Factor would stimulate rat NBT diaphorase purified by gel chromatography according to the Omura and Takesue method. It was seen that goat H-Factor would stimulate Williams and Kamin prepared rat reductase. Therefore, this shows that there is no major species difference in the stimulation mechanism. Although there is no definite experiments to show that the two factors are identical they both seem to act in the same manner.

The identity of these two stimulators has not been elucidated. They are presumably macromolecules since both factors will not pass through a dialysis membrane and Factor 10 is excluded from Sephadex G-100. The heat stability of the

factors would seem to argue against them being proteins. However, lipoproteins with their large number of hydrophobic bonds would be expected to be resistant to heat denaturation and this could explain the heat stability of the factors (51). The protein nature of the factors is also supported by the observations that a solution of $Ba(OH)_2$ and $ZnSO_4$ will precipitate Factor 10 and that both factors give a positive Lowry's protein determination. One experiment which did cast some doubt on the protein nature of H-Factor was that trypsin digestion did not destroy stimulatory activity.

A paper has recently appeared describing a liver protein which inhibited NBT reduction by xanthine oxidase (52). This inhibitor has been purified to a single protein band in electrophoresis on cellulose acetate strips although this is not proof that the protein is homogeneous. The inhibitory activity was seen to be relatively heat-resistant and insensitive to trypsin digestion as is the NBT stimulating factor reported in this thesis.

The nature of the stimulatory mechanism will be discussed next. This thesis work was originally carried out in order to find an intermediate in the electron transport chain between the flavoprotein and NBT. The discovery of the stimulatory factors was first thought to be the answer to this problem and the results of several experiments seemed to agree with this hypothesis. As discussed in the experimental section, the lag seen at the start of assays with a low concentration of flavoprotein and high concentrations of Factor 10 was

thought to indicate that the flavoprotein was reducing the factor. Also supporting the belief that Factor 10 had a direct role in NBT reduction was the fact that it does not stimulate cyt c reduction.

Several other experiments, on the other hand, seem to cast some doubt on whether these factors are acting directly as electron shuttles. For instance, there was no reduced versus oxidized absorption spectrum for H-Factor which is highly unlikely if it were capable of shuttling electrons. Of course it may also be true that H-Factor has not been purified sufficiently to see such an absorption. The obvious resistance of these factors to heating and trypsin digestion also make it hard to see how they could have direct enzymatic function. Also, the observation that cyt c reductase and NBT diaphorase activity solubilize simultaneously from the membrane would not be expected if NBT diaphorase were a two component system, especially since the unsolubilized pellet protein is known to have the majority of the Factor 10. For these reasons, the hypothesis has been developed that the factors may act only as structural components for NBT reduction. This would agree with the observation that NBT and H-Factor seem to form an insoluble complex. The factors might act directly on the flavoprotein by supplying an environment similar to the one seen in the membrane or act by binding NBT and therefore making it easier for the dye to reach the active site of the flavoprotein. If these factors were lipoprotein structuring agents another ramification

would be that they would not have to be homogeneous but could be a collection of proteins. This heterogeneity could explain the resistance of the factors to denaturing agents. The data presented in this thesis can neither prove or disprove these two theories of stimulatory action.

The xanthine oxidase study on the inhibition of NBT reduction seems to show that the inhibitor protein works on NBT rather then the enzyme (52). This was concluded because the phenazine methosulfate coupled reduction of NBT by alcohol dehydrogenase is also inhibited by this protein. The non-specificity of the inhibitor protein shows that the inhibitor is interacting with NBT rather than the enzymes. Xanthine oxidase is believed to catalyze the reduction of oxygen to the unstable superoxide anion which can in turn reduce NBT (53). The inhibitor protein has been suggested to act as a superoxide dismutase and therefore inhibit tetrazolium reduction by destroying the superoxide anions. It should be noted that the formation of superoxide anion by cyt c reductase has been looked for in the course of this research and was not seen.

The importance of these factors cannot be ascertained now without knowing their mechanism for NBT diaphorase stimulation. If the factors are part of the electron transport chain then their importance is self evident; for instance in P-450 reduction. However, if they act as structuring agents their importance is harder to assess. For instance the binding of NBT in free solution may have no connection

whatever with events in the membrane. If, on the other hand, the factors act to give the flavoprotein the correct conformation for NBT reduction, these same lipoprotein factors may have the same function in the membrane. But it is also possible that in the natural setting of the intact endoplasmic reticulum there is no need for specific structuring factors. H-Factor, it should be noted, will stimulate the diaphorase activity of whole microsomes so this may give greater strength to the belief that the factor acts by binding NBT but, on the other hand, Factor 10 will also stimulate NADPH oxidase activity.

Study of Multiple Reductases

Research in this area grew out of the investigation into whether NBT diaphorase was a multicomponent enzyme system consisting of the flavoprotein, cyt c reductase, and Factor 10. If this were so then the inhibition and induction of NBT diaphorase and cyt c reductase should be different. K_3-dependent NADPH oxidase was also studied because this activity of the flavoprotein is also stimulated by Factor 10. Any differences in these three activities might also give some clue as to whether there is more than one flavoprotein in microsomes. However, no easily interpreted differences were seen.

Careful thought should be given to the experiment where the increase in protein levels after PB induction was measured (Table 7). The goal in this experiment was to see whether NBT diaphorase and cyt c are induced equally. The

logic behind this may be faulty, however. As has been previously discussed, there is evidence that in intact microsomes cyt c molecules can not reach all of the reductase while NBT molecules can. Therefore, even if a single flavoprotein was responsible for both activities the induction of the enzyme would not necessitate an equal increase for cyt c and NBT reduction. The observation that cyt c reductase concentration did not increase as much as P-450 and aminopyrine demethylase activity is interesting since Orrenius has said that the reason cyt c reductase is implicated in xenobiotic metabolism is that it is induced equally with aminopyrine demethylase activity.

Induction with PB and 3-MC did not induce NBT diaphorase, cyt c reductase, or K_3-dependent NADPH oxidase differently (Table 8). If Factor 10 was part of the diaphorase electron transport chain this would mean that the factor is induced at least equally with the flavoprotein or that is is already in the membrane at saturating levels. However, if Factor 10 is only a structuring agent then the experimental results are expected. This experiment would also seem to cast doubt on the existence of multiple reductases.

The heat denaturation study of lipase solubilized cyt c reductase, NBT diaphorase, and NADPH oxidase activity was done to look for evidence of multiple reductases. The denaturation curves seem to agree with the above hypothesis because of their biphasic nature. Before greater confidence is taken in these results the enzymes need to be further

purified to see if the biphasic components can be separated. Interpretation of this experiment has been difficult because the shape of the denaturation curves vary between preparations. This may be the result of the variability of the lipase solubilization. Another problem with this study is the fact that very little theoretical or experimental work has been done on the heat denaturation of membraneous proteins. Since they normally exist in a hydrophobic environment their heat stability properties may be different from soluble proteins. In fact, the biphasic nature of the heat denaturation may be an artifact.

Disc gel electrophoresis has given the best evidence that there is more then one reductase enzyme. There are two bands of protein, after lipase solubilization, which can reduce NBT. The resolution of these two bands on the acrylamide gel is not good even though several experiments at different gel concentrations and pH were done to see if a better separation could be obtained. Diffusion of the diformazan product makes it impossible to stain long enough to get dark bands. When solubilizing an enzyme by enzymatic digestion there is always the question of whether this treatment is degrading the enzyme. This is especially important in this work because there is so little difference in the electrophoretic mobilities of the two diaphorase bands. Such a difference may be the result of just a few amino acids being clipped off the protein during solubilization. These two bands, shown in Fig. 19, were seen in all preparations. If the

acrylamide gels were allowed to incubate for several hours in NBT and NADPH occasionally other bands of diaphorase were seen. These were a 'fast' moving band located with the tracking dye and a 'slow' moving band approximately 15 mm from the top of the gels. These bands did not appear in every enzyme preparation and may be the result of a contaminating protein from the mitochondria, for example. An experiment was performed where microsomes were solubilized for varying lengths of time. The solubilized protein was electrophoresed and the gels were developed for NBT diaphorase activity. It was hoped that this might show if the protein bands were interconverted but the results did not allow any conclusions to be drawn because of the difficulties of quantitating the intensities of the bands. This particular preparation contained the 'slow' and 'fast' moving diaphorases besides the predominant double bands of diaphorase.

Inhibition studies with 2'-AMP were done to look for multiple reductases but only negative results were obtained. It is still possible, however, that there are more then one reductase because 2'-AMP is a competitive inhibitor of NADPH and all of the reductases may have the same affinity for NADPH, in which case inhibition could be the same. Another interesting sidelight of this experiment is the observation that the inhibition of cyt c and NBT reduction and NADPH oxidation by 3-MC microsomes is different from control and PB microsomes. There is a growing amount of evidence in this laboratory that 3-MC induction causes profound changes in

xenobiotic activity although it has little effect on the amount of P-450 or smooth endoplasmic reticulum. Since 3-MC is known to increase the phospholipid content of microsomes the change in 2'-AMP inhibition may be the result of an alteration in lipid-membrane structure.

Whether these experiments show that there is more then one reductase enzyme in microsomal electron transport is a difficult question to answer. The disc gel electrophoresis and heat denaturing experiments suggest that there are two separate enzymes which can reduce NBT, cyt c and menadione. One would feel more confident of the existence of these multiple reductases if 2'-AMP had inhibited NBT diaphorase, cyt c reductase, and NADPH oxidase to different extents. Possibly, 2'-AMP may have been a poor choice for an inhibitor and a different choice should have been made. Another unexpected result, if indeed there are multiple reductase enzymes, was the observation that induction did not selectively induce one substrate activity over another. Induction did, however, in heat denaturation studies seem to increase the amount of the more heat-resistant enzyme in microsomes. The possibility of only one enzyme in microsomes which is somehow degraded into two forms during solubilization can not be disregarded. The only way to decide whether there is indeed two separate forms of the reductase enzyme in microsomes will be to see if they can be separated from one another.

Summary

Two results of this thesis may be of future importance. The discovery of a factor which greatly stimulates the NBT diaphorase activity but not the cyt c reductase activity of a microsomal flavoprotein may be a clue for the mechanism of electron transport in xenobiotic metabolism. Whether this factor is important in the reduction of P-450 is unknown but will warrant further investigation. The nature of this factor is unknown but it may be a lipoprotein which acts as a structuring agent for NBT reduction although a direct role in electron transport can not be ruled out. The second interesting point is that heat denaturation studies and disc gel electrophoresis both seem to indicate that there is two different reductase enzymes.

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