MICROSOMAL ELECTION TRANSPORT WITH a-NADH AS DONOR

PETER M. MACALUSO


CENTRE FOR NFLD. STUDIES
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Peter M. Macaluso

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SYNOPSIS

α-NADH is neither active as an electron donor when tested with several β-NADH-linked dehydrogenases, nor as a competitive inhibitor of the β-isomer in these enzyme systems. Recently, however, an α-NADH-oxidoreductase activity was found in hepatic microsomes and the soluble fraction of rat liver.

The enzymatic oxidation of α-NADH by the hepatic microsomal electron transport system has been investigated. A comparative study of the factors responsible for the microsomal catalyzed oxidation of α- and β-NADH has implicated that the enzymatic activity of α-NADH is most likely a manifestation of isoenzyme reductases associated with the microsomal electron transport system, rather than a manifestation of a broad electron acceptor specificity of the β-NADH-linked microsomal reductases.

Substrate specificity studies revealed that the most active electron acceptors for the β-NADH-linked reductases are completely inactive as substrates in the α-NADH linked reductase system.

Using cytochrome c and DCPIP as final electron acceptors it was observed that: The enzymatic oxidation of -NADH is optimal at a more acidic pH than is β-NADH oxidation; the apparent Km values for α-NADH are somewhat higher than those for β-NADH; α-NADH oxidation is more readily inhibited by sulfhydryl group reagents and X-irradiation than

is the corresponding β-NADH oxidation; after acetone extraction the enzymatic oxidation of α- and β-NADH respond differently to the addition of phospholipid micelles; TTFA readily inhibits α-NADH oxidation whereas β-NADH oxidation is relatively resistant; trypsin digestion results in a dramatic increase in the Km for α-NADH with little effect on the rate of α-NADH oxidation. In contrast, the effect on the rate of β-NADH oxidation is inactivated by trypsin with the Km for β-NADH remaining unaltered.

DOC solubilization of microsomes followed by DEAE-cellulose chromatography results in the resolution of the α- and β-NADH-DCPIP reductases.

Evidence inconsistant with the existence of microsomal isoenzyme reductases has been described and discussed. It was observed that: α-NADH may serve as a competitive inhibitor of the β-isomer with the β-NADH-DCPIP and cytochrome c reductases; β-NAD^+ may protect both the α- and β-NADH-DCPIP and cytochrome c reductase activities against pCMB inhibition; the lysosomal solubilization of microsomes followed by DEAE-cellulose chromatography and isoelectric focusing revealed that α- and β-NADH-DCPIP reductases and β-NADH-$Fe(CN)_6^{-3}$ reductase possess identical isoelectric points.

Hypothetical model systems illustrating the possible heterogeneity of the microsomal electron transport system has also been presented.

ABBREVIATIONS

The following abbreviations have been used in this thesis:

NAD^+ - Nicotinamide adenine dinucleotide

α-NAD^+ - α-anomer of nicotinamide adenine dinucleotide

β-NAD^+ - β-anomer of nicotinamide adenine dinucleotide

$NADH^{(A)}$ - Reduced nicotinamide adenine dinucleotide

α-NADH - α-anomer of the reduced nicotinamide adenine nucleotide

β-NADH - β-anomer of the reduced nicotinamide adenine nucleotide

NADPH - Reduced nicotinamide adenine dinucleotide phosphate

α-NADPH - α-anomer of the reduced nicotinamide adenine dinucleotide phosphate

β-NADPH - β-anomer of the reduced nicotinamide adenine dinucleotide phosphate

$NAD(P)^+$ - Nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate

α-$NAD(P)^+$ - α-anomer of nicotinamide adenine dinucleotide and α-anomer of nicotinamide adenine dinucleotide phosphate

β-$NAD(P)^+$ - β-anomer of nicotinamide adenine dinucleotide and β-anomer of nicotinamide adenine dinucleotide phosphate

NAD(P)H - Reduced nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide phosphate

α-NAD(P)H	- α-anomer of the reduced nicotinamide adenine dinucleotide and α-anomer of the reduced nicotinamide adenine dinucleotide phosphate
NMR	- Nuclear magnetic resonance
ATP	- Adenosine triphosphate
DCPIP	- 2,6-dichlorophenolindophenol
EDTA	- (ethylenedinitrilo) tetraacetate, disodium salt
pCMB	- p-chloromercuribenzoate
NEM	- N-ethylmaleimide
DEAE	- Diethylamine ethyl cellulose
TTFA	- Thenoyltrifluoroacetone
Fp	- Flavoprotein
FAD	- Flavin adenine dinucleotide
TYR	- Tyrosine
DOC	- Deoxycholate, disodium salt
OD	- Optical density

CONTENTS

INTRODUCTION

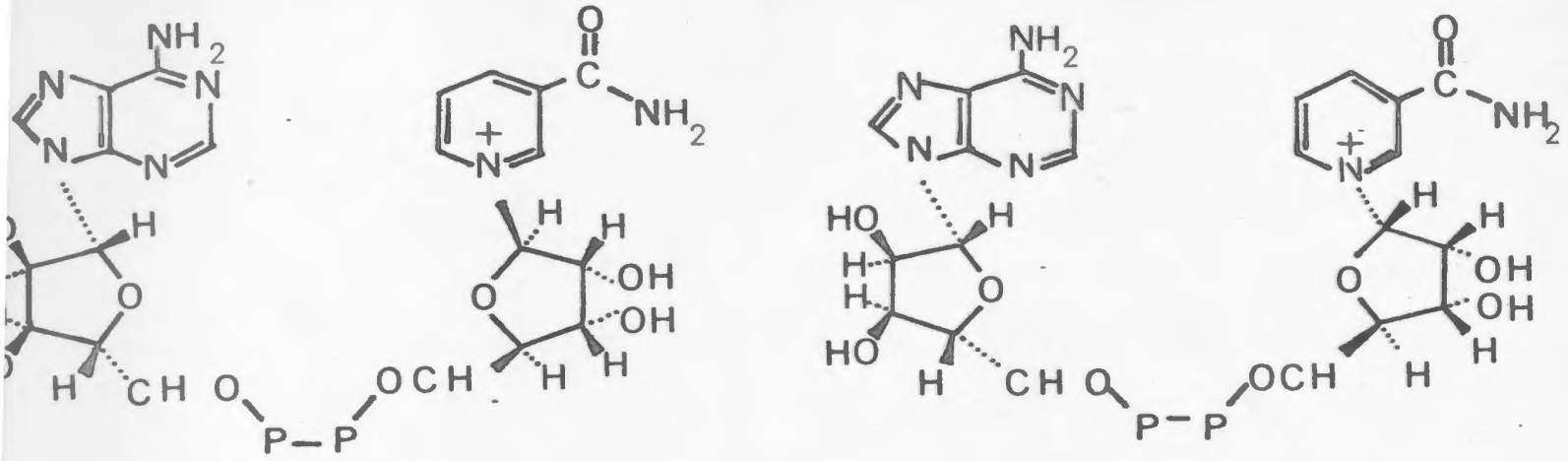
The treatment of nicotinamide adenine dinucleotide by NAD(P)$^+$glycohydrolase (EC 3.2.2.6) from Neurospora crassa results in the cleavage of the riboside bond[2] and the complete destruction of the NAD$^+$-linked yeast alcohol dehydrogenase activity[1]. However, there still remains material capable of forming the typical cyanide addition complex characteristic of pyridinium derivatives[3]. KAPLAN et al[1] were able to isolate this material from a number of highly purified commercial NAD$^+$ preparations, and illustrated that it was the oxidized form of nicotinamide adenine dinucleotide containing an α-riboside linkage.

The scientific inquiry which followed the identification of α-NAD$^+$ has yet to be reviewed in the literature. In the following the chemical and biochemical properties, the biosynthesis, and the enzymatic activity of α-NAD$^+$ and α-NADH are reviewed.

CHEMICAL AND BIOCHEMICAL PROPERTIES

1. Structure of α-NAD$^+$ and β-NAD$^+$ -

Figure 1 shows the currently accepted structure of α- and β-NAD$^+$,[1] the evidence for which has been obtained from a number of divergent sources[6,45] and subsequently proven by synthesis[7,46].



β−NAD+ α−NAD+

Fig. 1. Structures of NAD^+ illustrating α- and β- configuration of the nicotinamide-ribosidic linkage, from SARMA et al[4]

Both the α- and β- forms of NAD^+ have been found to contain a β-adenylic acid moiety[5]. However, the glycosidic linkage of α-NAD^+ results in the molecule having a configuration in which the nicotinamide moiety is on the same side of the ribose ring as the 2'- and 3'-hydroxyls. The ribose ring of β-NAD^+, on the other hand, contains the nicotinamide on the same side of the ring as the 5'-carbon.

2. Optical Rotatory Dispersion -

In accordance with the rotational trends found by LeMIEUX et al[15] for the α-and β-nicotinamide D-glycosides, KAPLAN et al[1,5] and SUZUKI et al[9] have found that the α-isomer possesses positive rotation at all wavelengths from 300 mu to

700 mu; whereas the corresponding β-anomers possess negative rotation. More recently MILES *et al*[6] have used the characteristic optical rotatory and circular dichroism measurements of the α and β-isomers to indicate that α and β-NADH undergo an α,β, epimerization (an inversion of the configuration at C-1'), when the anomers undergo and addition reaction (i.e. hydration, across the 5,6-double bond of the dihydropyridine moiety), and are in the presence of acids or polybasic anions. Moreover, these workers have reported that the acid-catalyzed epimerization appears to favor closure in the α configuration.

3. Acid Hydrolysis -

Complementary to these epimerization studies of MILES and coworkers, SUZUKI *et al*[9] have investigated the relative susceptibility of the α and β-NAD^+ isomers to acid hydrolysis. Accordingly, it has been found that after 60 min in 4N HCl at 100 C only 53% of the nicotinamide-ribosidic bond of α-NAD^+ is cleaved, while nearly 100% of the bond of β-NAD has been hydrolyzed.

4. Spectrophotometric constants -

KAPLAN *et al*[1] and SUZUKI *et al*[9] have shown that the α-isomer of nicotinamide adenine dinucleotide possesses characteristic absorption maxima and extinction coefficients for both its reduced and cyanide addition product. In all cases studied, it has been found that in contrast to the

β-isomer, the spectrophotometric constants for the oxidized, reduced, and cyanide addition product of the α-anomer has the tendency to shift to longer wavelengths.

5. Fluorescence Excitation Spectra -

Both WEBER[16] and SHIFRIN and KAPLAN[17,29] have pointed out that the fluorescence spectra of β-NADH shows a 462 mu band not only when the dihydronicotinamide part of the molecule is excited by 340 mu irradiation, but also when the molecule is activated at 260 mu, a region where the absorption is primarily due to the adenine moiety. SHIFRIN and KAPLAN have also shown that α-NADH exhibits the same fluorescence as the β-isomer when excited at 340 mu; but only slight fluorexcence when excited at 260 mu. This results indicated that the excitation of fluorescence with 260 mu light was dependent not only on the presence of adenine in the molecule, but also on the proper spatial relationship between the adenine and the dihydropyridine ring.

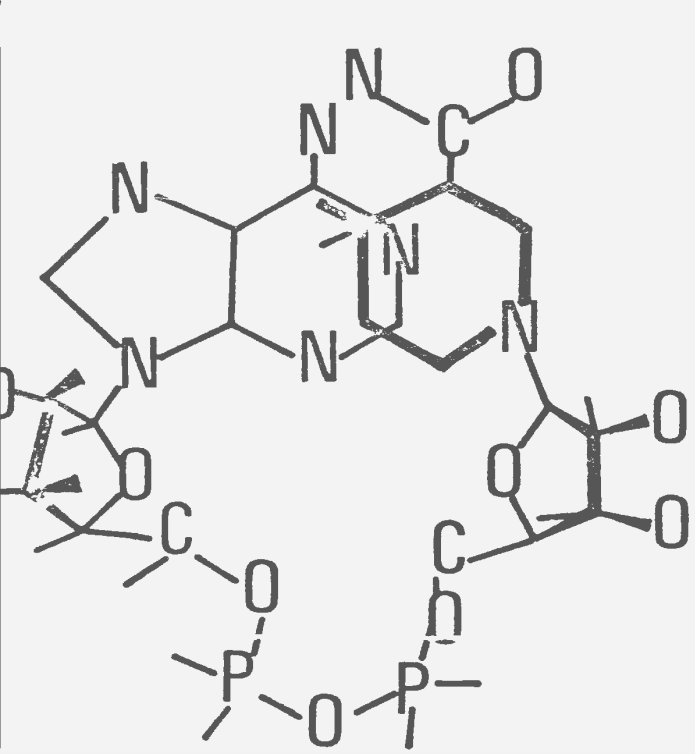
6. Conformation -

Several pertinent suggestions have been made regarding the conformation of pyridine dinucleotides in aqueous solution. Evidence for the intramolecular transfer of electronic energy between the adenine and pyridine rings of NADH has been provided from a number of sources[16, 18-20]. The fluorescence transfer studies of SHIFRIN and KAPLAN[17, 29] led them to propose

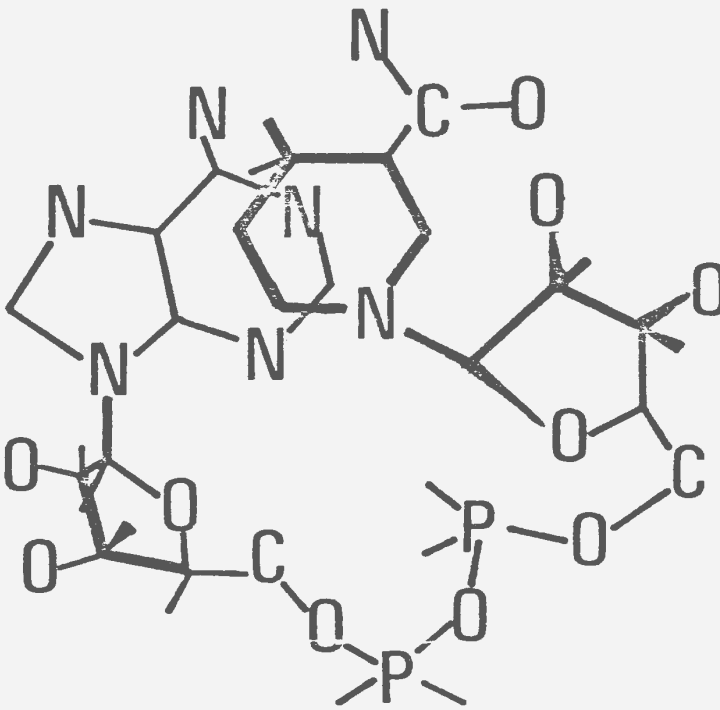
a hydrogen-bonded structure in which the pyridine and adenine rings lie co-planar for effective energy transfer[5]. On the other hand, fluorescence data led VELICK[21,22] to infer a folded conformation for NADH in which the pyridine and adenine rings are stacked in parallel planes, the nicotinamide ring lying above the plane of the adenine ring. Furthermore, both VELICK[22] and KAPLAN[23] have constructed molecular models which show that the β-isomer allows juxtaposition of the adenine and dihydropyridine rings more readily than does α-NADH, (i.e. Figure 2).

(a) β-NADH - Again using molecular models, SARMA *et al*[4] have demonstrated that the molecule of the β-isomer is flexible enough to have two folded conformations, one in which the pyridine ring lies above the plane of the adenine ring, termed right-handed folding (P-helix); and the other in which the pyridine ring lies below the plane of the adenine ring, termed left-handed folding (or M-Helix)[24]. Furthermore, in investigating the conformation of pyridine dinucleotides in aqueous solutions, MEYER[18], JARDETZKY[19], and CATTERALL[25], employing NMR, SUZUKI *et al*[9], using fluorescence spectral data, CZERLINSKI[26], working with temperature jump experiments, and MILES and URRY[20] employing circular dichorism, all conclude that β-NADH probably exists as a mixture of two folded conformational forms.

(b) α-NADH - Fluorescence data and molecular models reveal that the juxtaposition of the adenine and dihydropyridine moieties



BETA



ALPHA

Fig. 2. Folded conformations of NADH with α- and β-isomerism at the pyridine-n-riboside bond. These are projections of skeletal models in which the planes of the nitrogen rings are parallel to the plane of the paper, nicotinamide above and adenine below. Other bonds are out of plane and hence appear at varying length in projection, From VFLICK[22].

of α-NADH (i.e., Fig. 2) allows less intramolecular interaction between these chromophors than does the corresponding β-anomer [17,21,22,28,29]. Furthermore, MILES and URRY[20], investigating the circular dichrosim spectra of α-NADH, and SARMA et al[4], investigating NMR chemical shift data, have also reached this conclusion. Moreover, the reports of SARMA and MILES[20], together with that of JARDETZKY et al[19], have concluded that α-NADH, like β-NADH, exists in a folded conformation. On the other hand, however, unlike β-NADH which exists primarily as P and M helices, MILES[20] and JARDETZKY[19] concluded that α-NADH may exist in several possible conformations. Indeed, the unique geometrical relationship which exists between the two chromophors of α-NADH is revealed in molecular models which illustrate that this molecule may possess several conformational configurations[20].

BIOSYNTHESIS OF α-NAD^+

KAPLAN et al[1] originally found α-NAD^+ to comprise 10-15 percent of purified commercial NAD^+ preparations (i.e. PABST NAD^+), which were prepared from either liver or yeast. Subsequently, the α-isomer has been found to exist in other tissues and organisms.

Two hypotheses have been formulated to account for the origin of the two isomers. Either the two molecules could originate from a partially asymmetric synthesis, or on the other hand, only one isomer could be synthesized and this

isomer could subsequently undergo an isomerization. At present there exists preliminary evidence to support both hypotheses.

Experiments in the laboratory of SUZUKI et al[8,9] have pointed to the existence of α-NAD^+ in Azotobacter vinelandii together with several other forms of the α-pyridine nucleotides. These are (a) α-nicotinic acid mononucleotide (α-N_aMN); (b) α-nicotinic acid adenine dinucleotide (α-N_aAD); and (c) α-nicotinamide adenine dinucleotide phosphate (α-NADP). Enzymes found in cell free extracts of A. vinelandii indicate that the biosynthesis of the α-pyridine nucleotides proceeds by the following scheme.

$$\alpha\text{-}N_aMN \xrightarrow{ATP} \alpha\text{-}N_aAD \xrightarrow{\text{glutamine ATP}} \alpha\text{-}NAD^+ \xrightarrow{ATP} \alpha\text{-}NADP^+$$

Using a combination of selective cleavage of the β-isomers by Neurospora NAD(P)ase and anion exchange chromatography, SUZUKI has been successful in separating the individual α-isomers and has gathered information to indicate that α-NAD^+ synthesis is accomplished by an enzyme system distinct from that responsible for the synthesis of β-NAD^+

Although the conditions suitable for the formation of α-N_aAD and α-NAD^+ are analogous to those reported by PREISS et al[10] and by IMSANDE[11] in their respective systems for the synthesis of the β-isomers in yeast and E. coli, the results obtained by SUZUKI are in accord with the view that

both α- and β-anomers are present in the original α-nicotinic acid mononucleotide sample. However, as of yet no quantitative data has been obtained.

In addition to the α-$NADP^+$ occurring in Azotobacter vinelandii, NAKANISHI et al[12] have reported the presence of this α-nucleotide in the isthmus of hen oviduct. Although further investigation is needed to elucidate the function of α-$NADP^+$ in the oviduct, it has been interestingly pointed out that in microorganisms the relative amount of α-$NADP^+$ and β-$NADP^+$ occur in a molar ratio of 5:1 while in the oviduct they occur in nearly equal concentrations[12].

In contrast to the data reported by SUZUKI and NAKANISHI indicating the possible asymmetrical synthesis of the α-nucleotide PICCI et al[13] have proposed that the only product of NAD^+ synthesis in vivo and in vitro is the β-isomer. Using the classical conditions of PREISS and HANDLER[14], RICCI synthesized NAD^+ from nicotinic acid with human erythrocytes and reported that after 24 hrs of incubation NAD^+ increases 7-fold; with the α-NAD^+ level decreasing from 11% of the total before incubation to only 2% of the total NAD^+ after incubation[13], strongly suggesting therefore, that only β-NAD^+ is synthesized from the nicotinic acid.

Likewise, it was noticed that the percentage of the α-isomer decreases during the initial period of enhanced NAD^+ synthesis within the rat liver. Similarly, RICCI et al[13]

have proposed that only β-NAD^+ is synthesized *in vivo*. However, these workers have also reported that the concentration of the α-isomer shows variationswhich are not within experimental error. For example, an increase in α-NAD^+, with a concomitant decrease in the β-NAD^+ concentration, was noted after the total level of NAD^+ synthesis was reached. It may be probable therefore that a conversion of the β-isomer to the α-isomer takes place; indeed, in microorganisms the enzyme, mutarotase, is presumably responsible for such an activity[49].

ENZYMATIC ACTIVITY OF THE α-ISOMER

It was first reported by KAPLAN[1] that the nicotinamide riboside bond of α-NAD^+ is resistant to cleavage by *Neurospora* NAD(P) glycohydrolase (EC 3.2.2.6.), an enzyme which cleaves the β-isomer. Likewise, SUZUKI *et al*[8,9] demonstrated that α-$NADP^+$, isolated from *Azotobater vinelandii* or synthesized *in vitro* is also not attacked by the *Neurospora* NAD(P) glycohydrolase enzyme. Furthermore, α-$NAD^+$is neither active as a cofactor with many β-NAD^+-linked dehydrogenases including yeast or liver alcohol dehydrogenase, muscle lactate dehydrogenase, muscle triosephosphate dehydrogenase[1], nor as a competitive inhibitor of the β-isomer in these enzyme systems[32]. Likewise, α-$NADP^+$ is not active with yeast glucose-6-phosphate dehydrogenase[9]. It has also been found that in contrast to β-NAD^+, α-NAD^+ is unable to serve as growth factor for *Hemophilus*

parainfluenzae[+].

The reduction of cytochrome c in the presence of α-NADH by hamster liver mitochondria was briefly described in a review paper by CLARK et al[31], but experimental details have not as yet been published. Likewise, SUZUKI et al[9] have reported that α-$NADP^+$ when incubated with the supernatant fraction from the isthmus of hen oviducts results in a slow increase in the absorption at 345 mu; however, the factors responsible for this reaction were not investigated.

Recently OKAMATO et al[32] have reported on the enzymatic oxidation of α-NADH by liver mitochondria which is resistant to inhibition by antimycin A, rotenone, and amytal. These workers have also detected the enzymatic reduction of DCPIP via α-NADH and a purified "diaphorase" from pig heart; while in rat liver microsomes, α-NADH has been reported to reduce cytochrome c. Moreover, OKAMATO has found that addition of α-NADH to a sample of rat liver microsomes gives essentially the same spectral change of cytochrome b_5 to reduced form, than if β-NADH were used; however, the significance and factors responsible for this reaction were not investigated.

To date therefore, the biological significance of α-NAD^+ and α-NADH is so far completely unknown. Since the discovery by STRITTMATTER and BALL[33] of a microsomal electron transport chain, considerable work has been carried out to

elucidate the nature of the electron flow through the system. However, as yet no work has been published that characterizes the role which α-NADH may play in this electron transport chain.

The following report investigates the factors responsible for the microsomal enzymatic oxidation of α-NADH in order to: (a) compare these factors to those responsible for the β-NADH microsomal enzymatic activity; (b) to elucidate the path of electron flow from α-NADH through the microsomal electron transport system; (c) and to determine whether the reductase ability of α-NADH is associated with any function of some hitherto unknown enzyme(s); or is a mere manifestation of broad specificity of the β-NADH microsomal reductase system.

MATERIALS AND METHODS

MATERIALS

Chemicals and Biochemicals -

Thenolytrifluoracetone, indigo carmine, 2,6-dimethyl-benzoquinone, and tetramethyl-para-benzoquinone were obtained from Aldrich Chemical Co.

2-methyl-1,4-napthoquinone, 1,4-napthoquinone, and 1,4-napthoquinone-2-sulfonic acid were supplied by Eastman Organic Chemicals.

Para-benzoquinone was from the J.T. Baker Chemical Co.

Sodium deoxycholate was obtained from the Matheson Coleman and Bell Co.

LKB Instruments, Inc. supplied the ampholine carrier ampholytes.

Cytochrome b_5 was prepared and purified from pig liver microsomes by an identical procedure originally described by OMURA and TAKESUE for the purification of microsomal b_5 from rat liver[50].

All other biochemicals, including α-NADH, were obtained from Sigma Chemical Co. The α-NADH obtained was tested with purified (Sigma Type III) muscle lactate dehydrogenase (EC 1.1.1.27) as assayed according to the method of KORNBERG[42]. Under the assay conditions no enzymatic oxidation of the α-NADH preparation was observed to exceed the lower limit of enzymatic detection (0.274 nmoles/min/mg protein). It was concluded therefore that the α-NADH preparation was essentially free of

the β-isomer[1,32].

METHODS

Preparation of Microsomes -

Hepatic microsomes from adult male SPRAGUE-DAWLEY rats were prepared by the method of SEDGWICK and HÜBSCHER[44], using 0.25 M sucrose (pH 7.4) containing 2 mM EDTA. The resulting microsomal pellet was resuspended in the sucrose-EDTA medium and sonicated at 50 watts for 1 minute prior to centrifuging at 105,000 x g for 60 min to precipitate the final microsomal fraction.

Analytical Procedures -

Cytochrome b_5 was determined from the reduced minus oxidized difference spectrum assuming the extinction difference of the cytochrome between 424 mu and 409 mu to be 185 $cm^{-1}mM^{-1}$ [43]. Microsomal suspensions were reduced by 0.1 mM α- or β-NADH; purified cytochrome b_5 via 0.1 mM α- or β-NADH and a suitable amount of purified NADH - cytochrome b_5 reductase (EC 1.6.22).

Isoelectric fractionation was performed with a 110 ml (LKB Instruments Inc.) electrofocusing column according to the methods of VESTERBURG and SVENSSON[54]. The electrolysis was carried out at 0.4°C for 48 hrs in the presence of 1.5% ampholine carrier electrolytes (pH 6-8). The column was eluted in 1 ml fractions and the pH measured at 4°C with a Radiometer-Copenhagen Type PHM26 pH meter.

Protein was estimated by the method of LOWRY et al[34] using bovine serum albumin as the standard.

Enzyme Assays -

All enzyme determinations were assayed at 22°C in the presence of 1.0 mM EDTA and either 0.1 mM α- or β-NADH in a total reaction mixture of 1 ml.

The enzymatic reduction of cytochrome c (Sigma, Type III) was assayed in a modification of the procedure of MAHLER[35]: The reduction of 50 µM cytochrome c in 0.05 M Tris-HCl buffer (pH 7.4) was followed at 550 mu; when indicated, 1 mM KCN was included in the reaction mixture to prevent the oxidation of reduced cytochrome c by mitochondria. The reduction of DCPIP and $Fe(CN)_6^{-3}$ was measured in 0.05 M Tris-HCl buffer (pH 7.4); the concentrations of DCPIP and $Fe(CN)_6^{-3}$ being 0.025 mM and 0.50 mM respectively. The enzymatic reduction of purified cytochrome b_5 was assayed by the method of TAKESUE and OMURA[36].

The reductase activities were calculated from the initial rate of reduction of the electron acceptor and are expressed in n moles of electron acceptor reduced per minute under the assay conditions. When indicated, non-enzymatic rates were subtracted from the initial rate of reduction to obtain the true enzymatic activity. The millimolar extinction differences (cm^{-1} mM^{-1}) between reduced and oxidized

electron acceptors used in calculating enzyme activity were 21.1 at 550 mu for cytochrome c[37], 21.0 at 600 mu for DCPIP[38], 1.02 at 420 mu for $Fe(CN)_6^{-3}$ [39], and 100 at 424 mu for cytochrome b_5 [40].

The enzymatic reduction of the various quinones and dyes used in specificity studies were assayed by a slightly modified procedure of the method described by KOLI et al[41] for the measurement of NAD(P)H menadione reductase. In addition to 0.1 mM α- or β-NADH and 1.0 mM EDTA, the reaction mixture contained: 0.1 M phosphate buffer (pH 7.4) and 0.25 mM quinone or dye in a total volume of 1.0 ml. The reaction was followed spectrophotometrically by measuring the oxidation of either α- or β-NADH at 340 mu. Nanomoles of electron donor oxidized per minute under the assay conditions were calculated from the true enzymatic oxidation (initial minus non-enzymatic rate of oxidation) and using a millimolar extinction coefficient of 5.4 and 6.2 respectively for α- and β-NADH[9].

Experimental Procedures -

Preparation of subcellular fractions from rat liver,heart and kidney

Adult male SPRAGUE-DAWLEY rats, which had been fasted for about 24 hrs were sacrificed and the appropriate organs fractionated by a modification of the method reported by SEDGWICK and HÜBSCHER[44]. The organs under investigation were quickly excised, chilled with ice-cold 0.25 M sucrose (pH 7.4) containing 2 mM EDTA, and homogenized with 9 volumes of the

cold sucrose-EDTA medium using a Potter glass homogenizer with a teflon pestle.

The homogenate was centrifuged at 1,000 x g for 10 min to remove cellular debris and nuclei. The supernatant was then successively centrifuged at 5,000 x g for 10 min, at 12,000 x g for 30 min, and finally at 105,000 x g for 60 min to precipitate: mitochondrial (Mt), heavy microsomal (HMs), and light microsomal (LMs) fractions, respectively, together with a post-microsomal supernatant fraction (Spt).

Preparation of subcellular fractions from hog adrenals

Freshly excised hog adrenal glands were trimmed of adhering fat and removed from their fibrous capsule. No attempt was made to separate cortical from medullary tissue and the intact adrenal minus its capsule was subjected to an identical fractionation procedure as described for rat tissue.

Treatment of microsomes with sulfhydryl reagents

Sonicated liver microsomes at a protein concentration of 1 mg/ml were preincubated at $4^{o}C$ in a reaction mixture containing 125 mM sucrose, 1 mM EDTA, 25 mM Tris-HCl buffer (pH 7.4) and varying concentrations of either pCMB or NEM. After 10 min of incubation the reaction mixture was assayed for its enzymatic activity.

In studying the protection of enzymatic activity against the inhibitory effects of pCMB, an identical procedure was followed with the exception that the microsomal suspension was first pre-exposed to various nucleotides (in a concentration of 0.25 mM) for 5 min prior to the addition of 0.025 mM pCMB.

Effects of x-irradiation on microsomal enzymatic activity

Fresh sonicated liver microsomes at a protein concentration of 0.2 mg/ml were irradiated in a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 1 mM EDTA. The microsomal suspension at 4°C was irradiated aerobically in glass vials with a Philips MG100 x-ray machine. The irradiation parameters were 100KV, 10 mA, with 0.5 mm Be filtration. A dose rate of 2.5 KR/min was calculated by Friske dosimetry.

Preincubation of microsomes with trypsin

To investigate the proteolytic effects of trypsin upon the NADH-oxidizing system of microsomal membranes, sonicated microsomes were prepared as previously described, and preincubated at 4°C for 10 min in a final protein concentration of 1 mg/ml in the presence of: 5 mM phosphate buffer (pH 7.4), 0.25 M sucrose, 2 mM EDTA, and varying quantities of freshly made up trypsin solution (Sigma Type III). At the end of the incubation period the reaction was stopped by the addition of soyabean trypsin inhibitor (Sigma Type I-S) in a four-fold weight excess to the amount of trypsin present. Suitable aliquots of the preincubation mixture were immediately taken and assayed for the enzymatic activity under investigation.

Incubation of sonicated microsomes in the presence of trypsin inhibitor, in the presence of trypsin and trypsin inhibitor, and in the absence of trypsin, served as controls.

Effects of solubilized phospholipid micelles on the enzymatic activity of acetone-extracted microsomes.

The lipid component of freshly prepared sonicated liver microsomes were removed by the 10% aqueous acetone extraction method of LESTER and FLEISCHER[47], a procedure reported to result in the loss of at least 80% of the microsomal lipid as determined by lipid phosphorus analysis[48].

Lipids consisting of a mixture of lecithin (Sigma Type III-E) and lysolecithin (Sigma Grade II) in a 70% to 30% weight proportion ratio respectively, were solubilized by sonic oscillation in a procedure identical to that described by JONES and WAKIL[48]. After formation of the lipid micelles various concentrations were immediately taken and preincubated for 10 min at 4°C with the acetone extracted microsomes in a reaction medium consisting of: 0.44 M sucrose, 15 mM Tris-HCl buffer (pH 8.0), 2 mM EDTA and 0.5 mgs/ml of microsomal protein.

Without further treatment suitable aliquots of the preincubation mixture were tested for the enzymatic activities under investigation. Simultaneously following an identical procedure, unextracted sonicated microsomes were incubated with the appropriate concentration of lipid micelles and used as controls.

DEAE-cellulose chromatography of solubilized microsomal enzymes

A. Lysosomal solubilization - Purification of NADH cytochrome b_5 reductase -

Rat liver microsomes were solubilized by lysosomal digestion and the resulting digest purified in an analogous manner to that originally described by TAKASUE and OMURA for the purification of microsomal NADH-cytochrome b_5 reductase[52]. The solubilization procedure consisted of incubating microsomes (7 mg protein/ml) with lysosomes (3 mg protein/ml) at 35°C in the presence of 0.1 M Tris-malate buffer (pH 5.7) and 1 mM EDTA. The reaction mixture was kept anaerobic under a stream of nitrogen to minimize lipid peroxidation, and at the end of a 6 hr incubation period the digest was centrfigued at 105,000 x g for 90 minutes. Without employing gel-filtration[52], the solubilized enzyme extract was purified by ammonium sulfate fractionation, desalted, and placed directly on a DEAE-cellulose column (2.5 x 45 cm). Elution was achieved with a continuous linear concentration gradient of KCl prepared with 500 ml of 50 mM Tris-HCl buffer (pH 8.5) and 500 ml of the same buffer containing 0.1 M KCl; the eluate was collected in 5 ml fractions at a rate of 0.6 ml/min.

B. Deoxycholate solubilization of microsomal membrane-bound components -

Rat liver microsomes were solubilized with deoxycholate by a modification of the method reported by LU and

COON for the isolation of hepatic microsomal P-450 and cytochrome b_5 [53]. The membrane-bound components were solubilized by incubating a microsomal suspension (30 mgs protein/ml) at 4°C, in a solubilizing mixture containing: 0.1 M potassium citrate buffer (pH 7.6), 25% glycerol, and 0.5% sodium deoxycholate. At the end of 20 min of incubation the reaction mixture was centrifuged at 105,000 x g for 2 hrs and the sediment discarded. The supernatant solution was diluted with 1.5 volumes of cold deionized water and the pH adjusted to 8.5 before charging into a DEAE-cellulose column (2.5 x 45 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 8.5). After washing with 50 ml of the Tris-HCl buffer (50 mM at pH 8.5), the column was re-equilibrated with 500 ml of a buffer consisting of 50 mM Tris-HCl (pH 8.5) and 0.1% DOC. Elution of the column was achieved with a continuous linear concentration gradient of KCl prepared with 600 ml of the 50 mM Tris-HCl (pH 8.5 and 0.1% DOC) buffer and 600 ml of the same buffer containing 0.3 M KCl. The eluate was collected in 5ml fractions at a rate of 0.5 ml/min.

RESULTS

Substrate Specificity for the Enzymatic Oxidation of α- and β-NADH by Hepatic Microsomes

In order to differentiate the relative enzymatic oxidation rates for α- and β-NADH, the two isomers were used as cofactor with hepatic microsomes and a range of electron acceptors.

Table I summarizes the reductase activity found in sonicated rat liver microsomes for a number of electron acceptors with either α- or β-NADH as primary reductant.(B) Under the assay conditions $Fe(CN)_6^{-3}$ is the most actively reduced by β-NADH; however, it is characterized by being unable to accept electrons from α-NADH. In contrast,DCPIP is readily reducible by α-NADH, with the activity ratio for the α and β isomers being approximately 0.50. Both cytochrome b_5 and cytochrome c are also reduced by α-NADH; furthermore, the α/β ratio for these cytochromes are approximately the same (0.68 vs 0.67). This may indicate that, if the microsomal NADH-cytochrome c reductase system involves a cytochrome to cytochrome electron transfer as previously postulated[55,56], then α-NADH probably feeds into the microsomal electron transport chain prior to cytochrome b_5.

Among the quinones, the benzoquinones showed higher activities with both α-and β-NADH, than did the naphthoquinones. The addition of a methyl group in the 2-position

TABLE I *

ELECTRON ACCEPTORS FOR THE MICROSOMAL α- AND β-NADH-LINKED REDUCTASES

Electron Acceptor	n moles/min/mg protein			
	α-NADH[a]	β-NADH[b]	α/β[e]	% Relative α/β
DCPIP	47	100	0.47	100
$Fe(CN)_6^{-3}$	< 0.166 [c]	3700	0	0
Cytochrome b_5	15	22	0.68	144
Cytochrome c	147	216	0.67	143
2-Methyl-1,4-napthoquinone	22	64	0.34	72
1,4-Napthoquinone	29	340	0.09	19
1,4-Napthoquinone-2-sulfonic acid	< 0.315 [d]	333	0	0%
Para-benzoquinone	< 0.315	1257	0	0
Methyl-para-benzoquinone	143	1206	0.12	26
2,6-Dimethylbenzoquinone	175	810	0.22	47
Tetramethyl-para-benzoquinone	30	37	0.81	172
Indigo camine	29	19	1.52	323
Methylene blue	< 0.315	338	0	0

Table I. Sonicated hepatic microsomes were prepared as described in Methods and tested for their reductase activity with the indicated electron acceptors and either α- or β-NADH as primary reductant. The various enzymatic activities were measured as described in Methods and are expressed as n moles of substrate oxidized or reduced per min per mg of microsomal protein.

* Figures represent the average value of three experiments; the mean deviations being less than 10%.

[a] Enzymatic activity of the microsomal α-NADH-linked reductases

[b] Enzymatic activity of the microsomal β-NADH-linked reductases

[c] The lower limit of detection under the experimental conditions

[d] The lower limit of detection under the experimental conditions

of 1-4 napthoquinone greatly decreases the β-NADH oxidation while having little effect on the α-NADH activity. On the other hand, the substitution of a sulfonic acid group at the same position has the opposite effect of abolishing the α-NADH oxidation while having little effect on the β-NADH activity. With the benzoquinones increased methylation results in a decrease of the β-NADH reductase activity with a concomitant increase in their α-NADH activity. Although indigo carmine is reduced less rapidly than most of the other electron acceptors it was the only acceptor used where a preference was shown for the α-isomer.

The enzymatic reduction of these electron acceptors was found to exhibit a linear relationship with microsomal protein and upon boiling the latter for 10 minutes, the enzymatic oxidation of α- and β-NADH was obliterated. It was concluded therefore that the oxidation of α- and β-NADH, as observed under the assay conditions, was representative of a genuine enzymatic activity associated with the hepatic microsomal membrane.

Tissue Distribution and Subcellular Localization of α-NADH-DCPIP Reductase; α-NADH-CYTOCHROME c Reductase; and α-NADH-$Fe(CN)_6^{-3}$ Reductase

Subcellular components of rat liver, heart, kidney, together with hog adrenals, were obtained and the activities for α-NADH-DCPIP, cytochrome c, and ferricyanide reductases

TABLE II *

TISSUE DISTRIBUTION OF THE α-NADH-DCPIP REDUCTASE, α-NADH-CYTOCHROME c REDUCTASE, and α-NADH-$Fe(CN)_6^{-3}$ REDUCTASE

Tissue Fraction	α-NADH-DCPIP reductase		α-NADH-cytochrome c reductase		α-NADH-$Fe(CN)_6^{-3}$ reductase	
	Specific[a] Activity	% Total Activity	Specific[a] Activity	% Total Activity	Specific[a] Activity	% Total Activity
Liver Mt	28	19	59	26	<0.166[b]	<0.166
Liver HMs	19	6	89	25	<0.166	<0.166
Liver LMs	22	10	75	28	<0.166	<0.166
Liver SPT	50	64	15	16	<0.166	<0.166
Adrenal Mt	16	3	26	4	115	7
Adrenal HMs	24	15	34	16	78	16
Adrenal LMs	20	5	53	11	227	19
Adrenal SPT	89	67	108	69	200	49

Table II. Subcellular fractions of hog adrenal and rat liver were prepared as described in Methods and the mitochondrial (Mt), heavy microsomal (HMs), light microsomal (LMs) and the post-microsomal supernatant fraction (SPT) were assayed for α-NADH-linked reductase activity. Protein and the α-NADH-DCPIP, cytochrome c, and $Fe(CN)_6^{-3}$ reductase activities were measured as described in Methods.

* Figures represent the average value of two experiments. Mean deviations were in the order of 15% or less.

[a] μmoles of either DCPIP, cytochrome c or $Fe(CN)_6^{-3}$ reduced per min per mg of protein.

[b] Lower limit of detection under the experimental conditions.

TABLE IV

APPARENT SUBCELLULAR LOCALIZATION OF THE α-NADH-DCPIP REDUCTASE α-NADH-CYTOCHROME c REDUCTASE, AND α-NADH-$Fe(CN)_6^{-3}$ REDUCTASE

Tissue	α-NADH-DCPIP Reductase		α-NADH-cytochrome c Reductase		α-NADH-$Fe(CN)_6^{-3}$ Reducta	
	Relative Tissue Activity	Localization	Relative Tissue Activity	Localization	Relative Tissue Activity	Localizati
Adrenal	1	SPT	1	SPT	1	SPT
Liver	0.58	SPT	0.70	HMs-LMs	0	0
Kidney	0.27	SPT	0.18	Mt	0	0
Heart	0.18	SPT	0.07	Mt	0	0

* Summarizes the data of Tables II and III giving the apparent subcellular localization of the α-NADH-linked reductases.

[a] Relative tissue activity is equal to the specific activity (n moles substrate reduced/min/mg protein) of the post-nuclear homogenate, taking the adrenal activity as 1. The α-NADH-DCPIP, cytochrome c and $Fe(CN)_6^{-3}$ reductase of the post-nuclear homogenate was assayed as described in Methods.

were determined as described under Methods. Tables II and III summarize the data of the fractionations from which the apparent localization of the particular α-NADH-linked reductases within the cell was determined (Table IV).

As seen from Table II, with α-NADH reducing 89 and 108 n moles (per min/mg protein) of DCPIP and cytochrome c respectively in the adrenals supernatant fraction, this tissue exhibits the highest specific activity of the four tissues investigated for the enzymatic oxidation of α-NADH. Indeed, it was the only tissue that was found to possess α-NADH-$Fe(CN)_6^{-3}$ activity. In comparison to adrenal, heart tissue possesses only nominal α-NADH-linked reductase activity (Tables III and IV). While α-NADH-DCPIP reductase was found to be localized in the cytosol of all four tissues, α-NADH-cytochrome c reductase was localized in the hepatic microsomal, heart and kidney mitochondrial, and adrenal supernatant fractions (Table IV).

Generally, with the exception of the post-microsomal supernatant fractions of liver, heart, and kidney, α-NADH-cytochrome c reductase exhibits somewhat higher specific activity than α-NADH-DCPIP reductase in all the tissues and subcellular components analyzed.

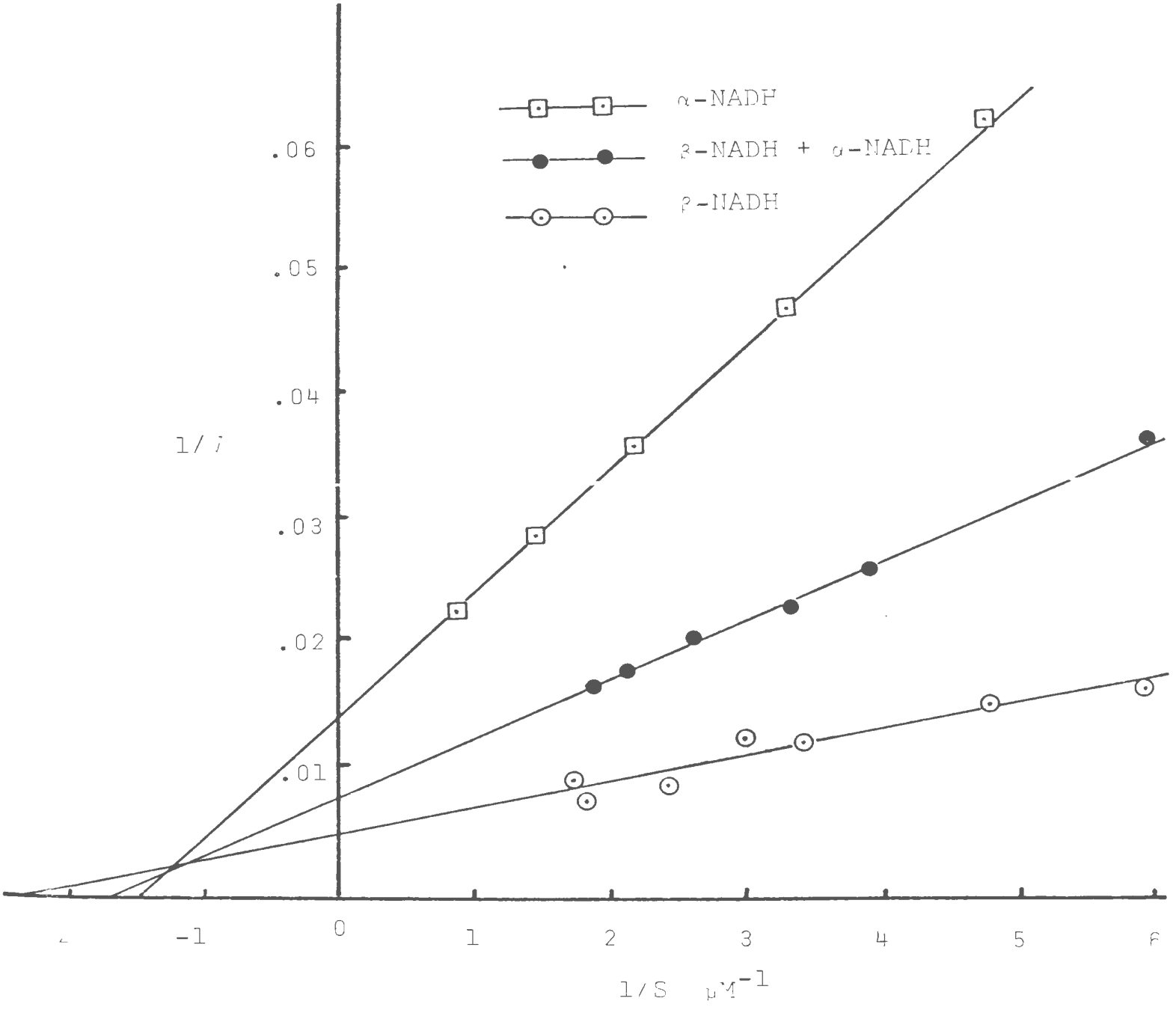
Kinetic Properties of the α- and β-NADH-DCPIP and cytochrome c reductases

Previously it has been reported that in those enzyme systems in which the α-isomer is inactive as cofactor,

Fig. 1. Effect of β-NADH, α-NADH, and α- + β-NADH on the microsomal enzymatic rate of DCPIP reductase.

Lineweaver-Burk plots illustrating the effect of β-NADH, α-NADH, and an equimolar mixture of α- + β-NADH on the reaction rate of DCPIP reduction by sonicated hepatic microsomes. The rate of reaction was followed at a nucleotide concentration of 5 μM or less, by employing a Coleman 124 spectrophotometer and a 0 - 0.1 A expanded scale on a Coleman 165 external recorder.

Figure 1



it is also inactive as a competitive inhibitor of the β-isomer[32]. Although this was found to be true for β-NADH-$Fe(CN)_6^{-3}$ reductase, the α-isomer may however serve as a competitive inhibitor of β-NADH in the β-NADH-DCPIP and cytochrome c reductase systems (see Fig. 1). Consequently the enzymatic rates of oxidation for α-NADH are not additive with the enzymatic rates of oxidation for β-NADH, when either DCPIP or cytochrome c is used as the final electron acceptor. Furthermore, the apparent Km values for α-NADH at infinite DCPIP and cytochrome c concentrations (0.67 μM and 13.0 μM respectively) are found to be somewhat higher than the corresponding Km's for β-NADH; for example, 0.40 μM and 1.5 μM respectively at infinite DCPIP and cytochrome c levels.

Influence of pH on the Microsomal α- and β-NADH-DCPIP and Cytochrome c Reductases

The pH optima in sonicated microsomes for the oxidation of α- and β-NADH with either DCPIP or cytochrome c as the final electron acceptor is seen in Figs. 2 and 3. With either acceptor, α-NADH oxidation is maximal at a comparatively more acidic pH than is the β-NADH oxidation. α-NADH-DCPIP reductase activity is maximal at a pH of approximately 5.7; while on the other hand, the β-NADH activity is optimum at pH 6.5. With cytochrome c as the final electron acceptor, α-NADH oxidation likewise shows a pH optimum in the region of 5.7. In contrast, cytochrome c reduction by β-NADH is maximal at a quite alkaline pH, approximately 8.5, but also shows a rather broad optimal

Fig. 2. Effect of pH on the microsomal α- and β-NADH-DCPIP reductase activity.

The enzymatic reduction of DCPIP was measured as described in Methods using either 50 mM potassium phosphate buffer or 50 mM Tris-HCl buffer. The pH was measured at the end of each assay with a radiometer - Copenhagen type pHM26 pH met

Figure 2

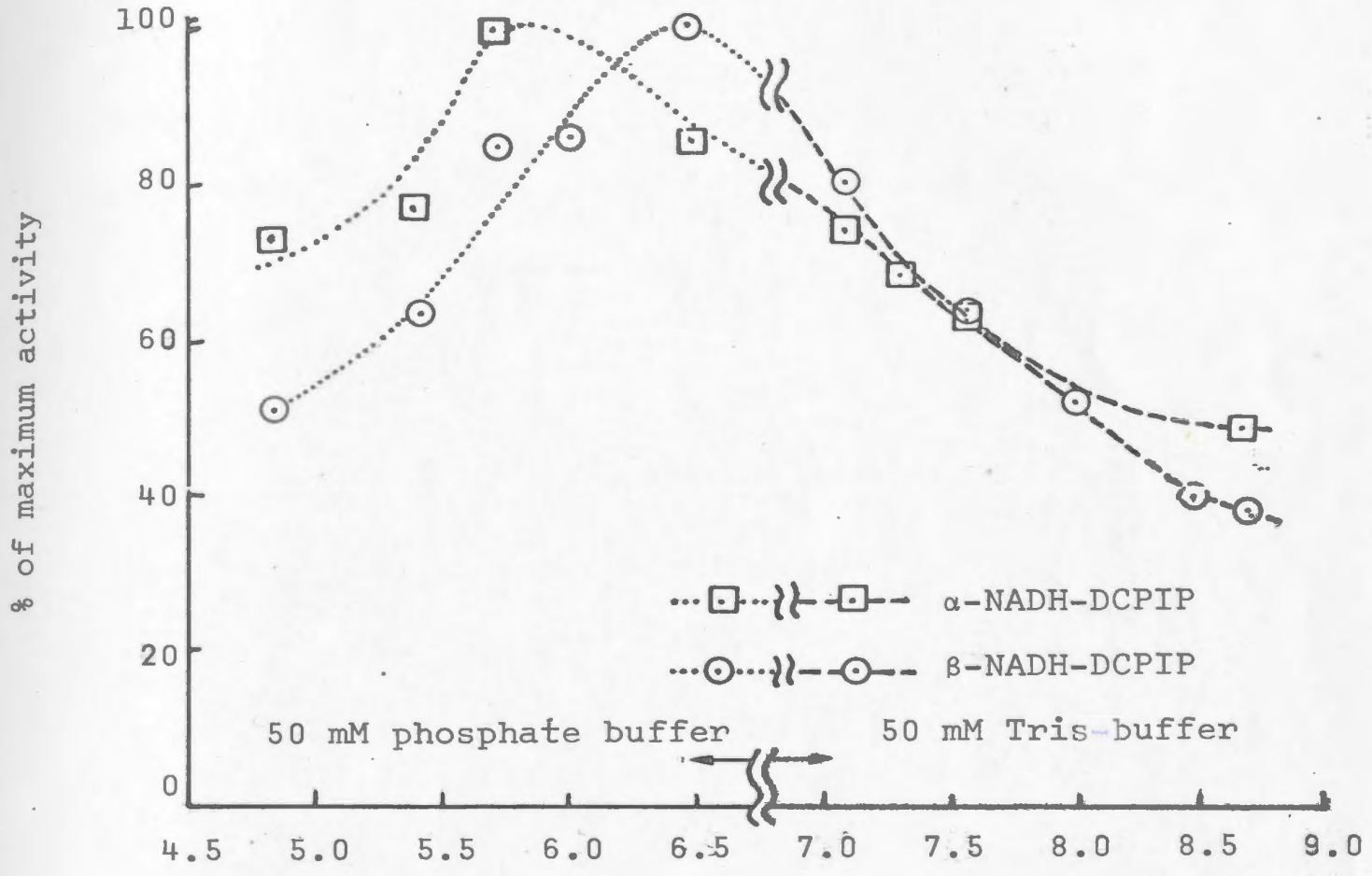
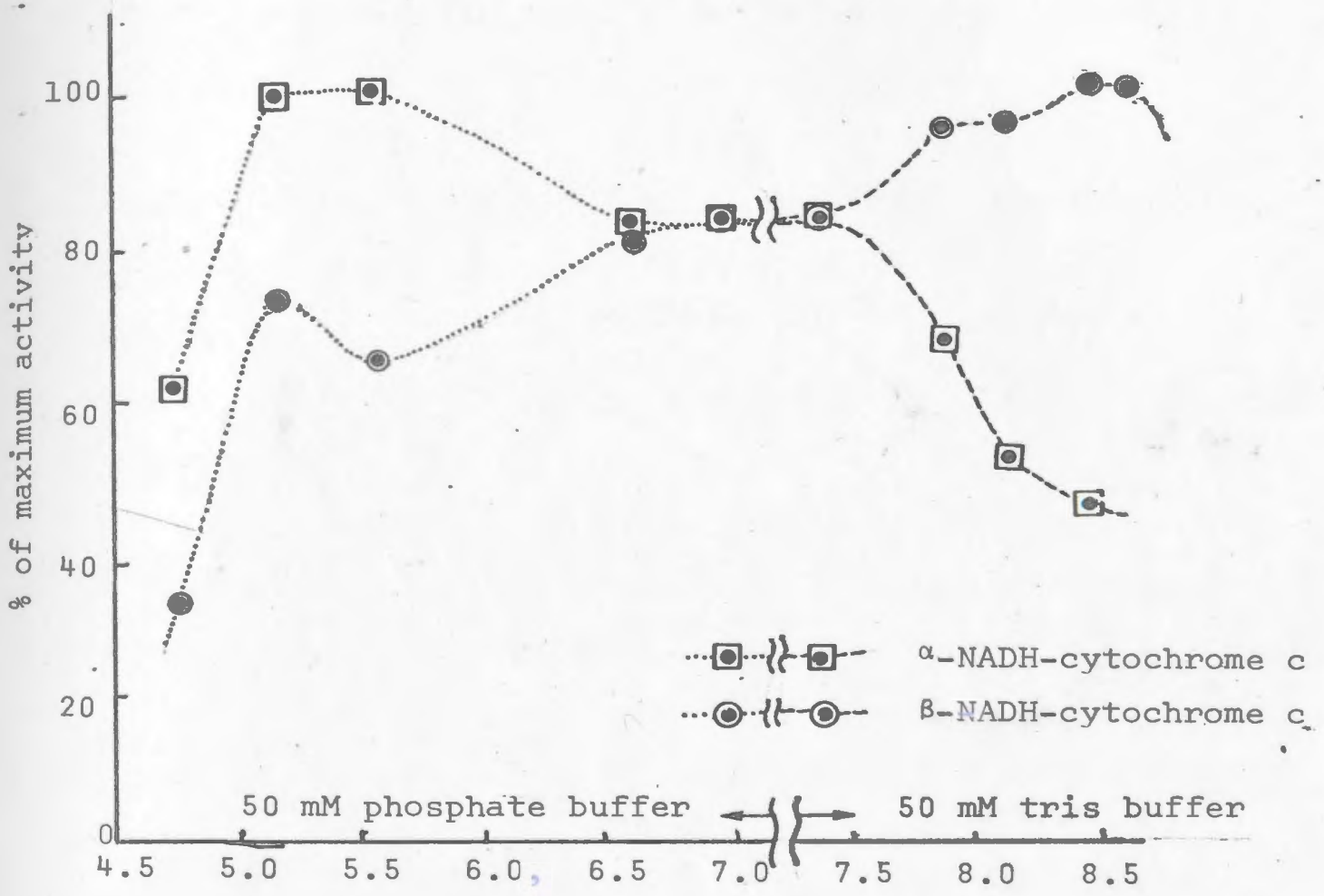

% of maximum activity
100
80
60
40
20
0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
α-NADH-DCPIP
β-NADH-DCPIP
50 mM phosphate buffer
50 mM Tris-buffer

Fig. 3. Effect of pH on the microsomal α- and β-NADH-cytochrome c reductase activity

The enzymatic reduction of cytochrome c was measured as described in Methods using either 50 mM potassium phosphate buffer or 50 mM Tris-HCl buffer. The pH was measured at the end of each assay with a radiometer - Copenhagen type pHM 26 pH meter.

Figure 3


% of maximum activity
100
80
60
40
20
0
α-NADH-cytochrome c
β-NADH-cytochrome c
50 mM phosphate buffer
50 mM tris buffer
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5

pH range from 6.5 to 8.5; and agrees with that previously reported[55].

Since the reduced nicotinamide adenine nucleotides are unstable in acidic solutions[77], the pH employed in all assays was 7.4; which represents a near isobestic point on the activity curves for the reduction of both cytochrome c and DCPIP by α- and β-NADH, and gives optimal comparison therefore in characterization studies of these enzymes.

Response of the Microsomal α- and β-NADH-DCPIP and cytochrome c Reductases to Sulfhydryl Reagents

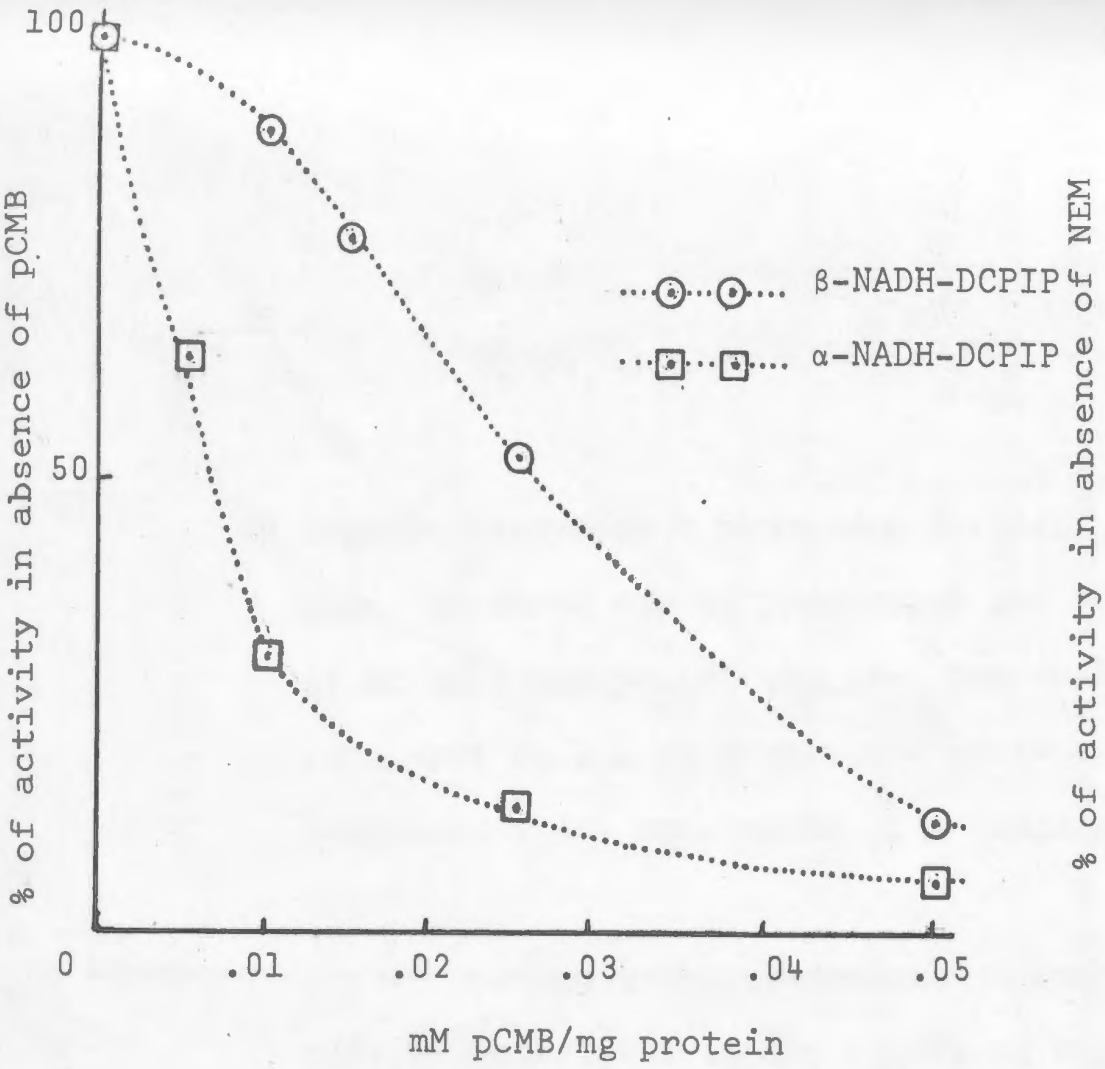
Sulfhydryl groups have long been considered to be involved either directly or indirectly in the activity of many nucleotide-dependent enzymes[57-59]. To demonstrate this for the α- and β-NADH interaction with the microsomal DCPIP and cytochrome c reductases, the sulfhydryl group reagents pCMB and NEM were employed. Since it has been found that mercurial inhibition is dependent upon protein concentration[60], these reagents were titrated against a specific amount of microsomal protein (Figs. 4 and 5). Therefore, the enzymatic inhibitions observed between the various reductases are directly comparable to each other.

It is evident from the titration curves that a differential reactivity exists between the α- and β-NADH-linked reductases in their response to the sulfhydryl group

Fig. 4. Comparison of the effect of pCMB and NEM on the microsomal α- and β-NADH-DCPIP reductase activity.

Sonicated liver microsomes at a protein concentration of 1 mg/ml were pre-incubated at 4°C in a reaction mixture containing 125 mM sucrose, 1 mM EDTA, 25 mM tris-HCl buffer, (pH 7.4) and varying concentrations of either pCMB or NEM. After 10 min of incubation the reaction mixture was assayed for α- and β- NADH-DCPIP reductase activity as described in Methods.

Figure 4


100
50
0
% of activity in absence of pCMB
.01
.02
.03
.04
.05
mM pCMB/mg protein
β-NADH-DCPIP
α-NADH-DCPIP

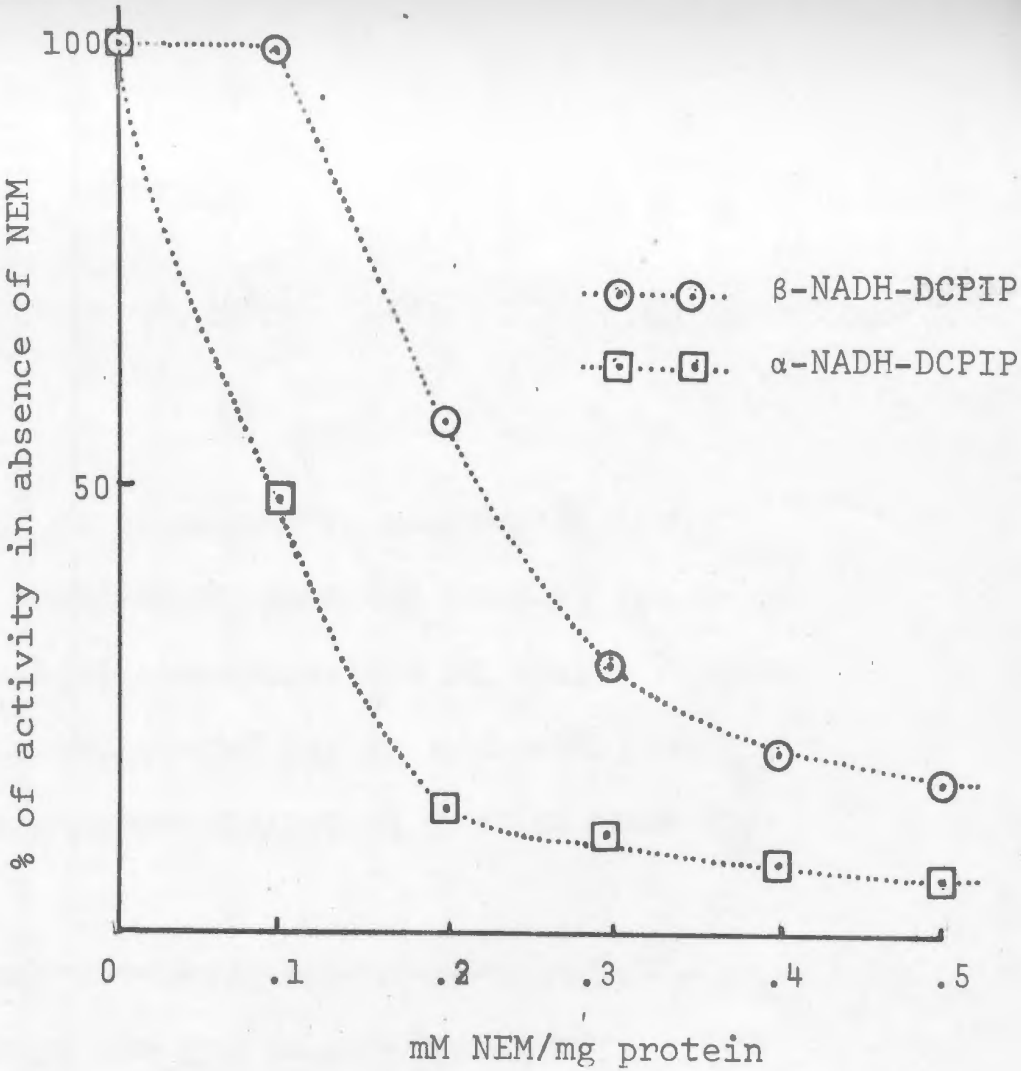
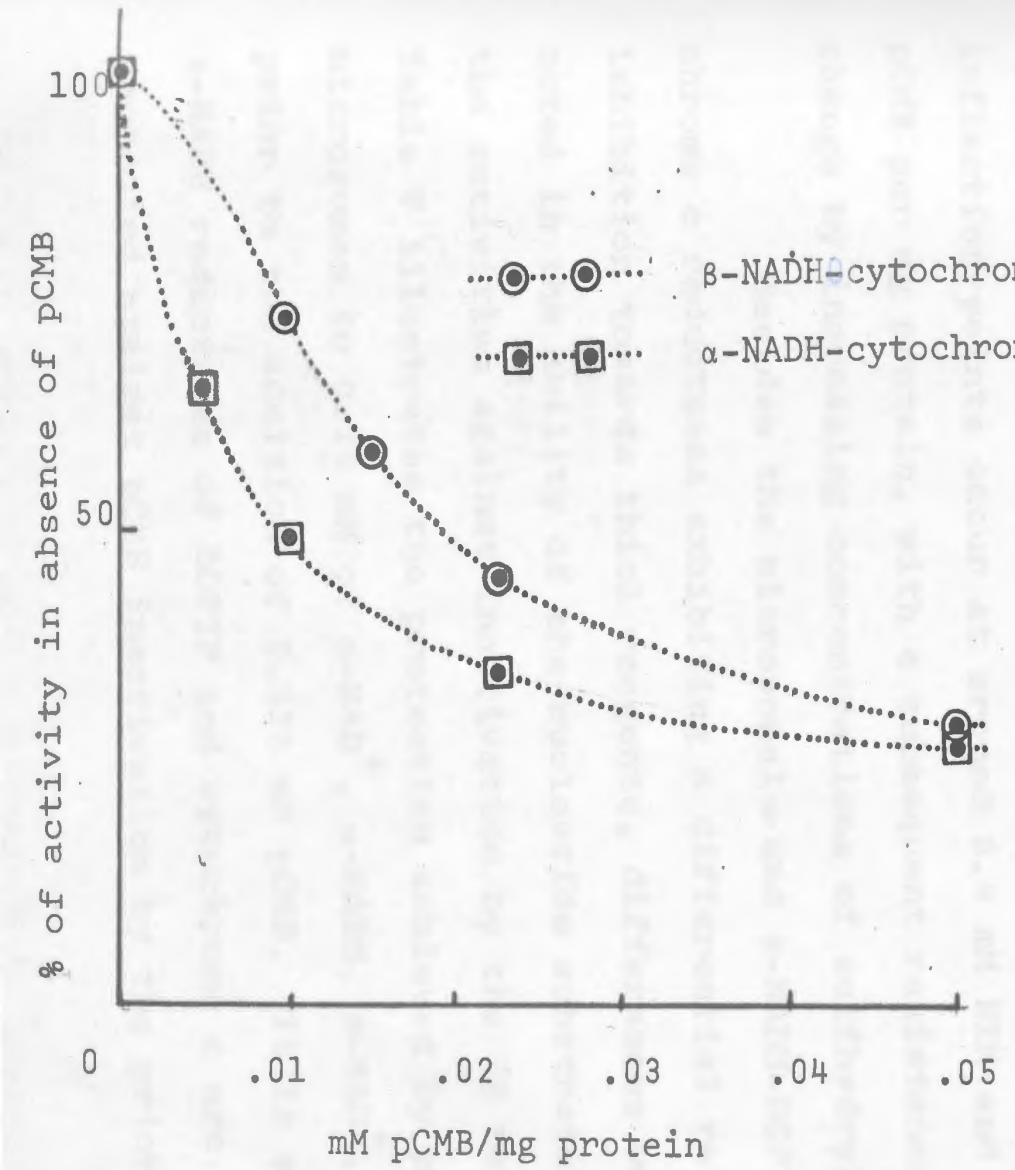
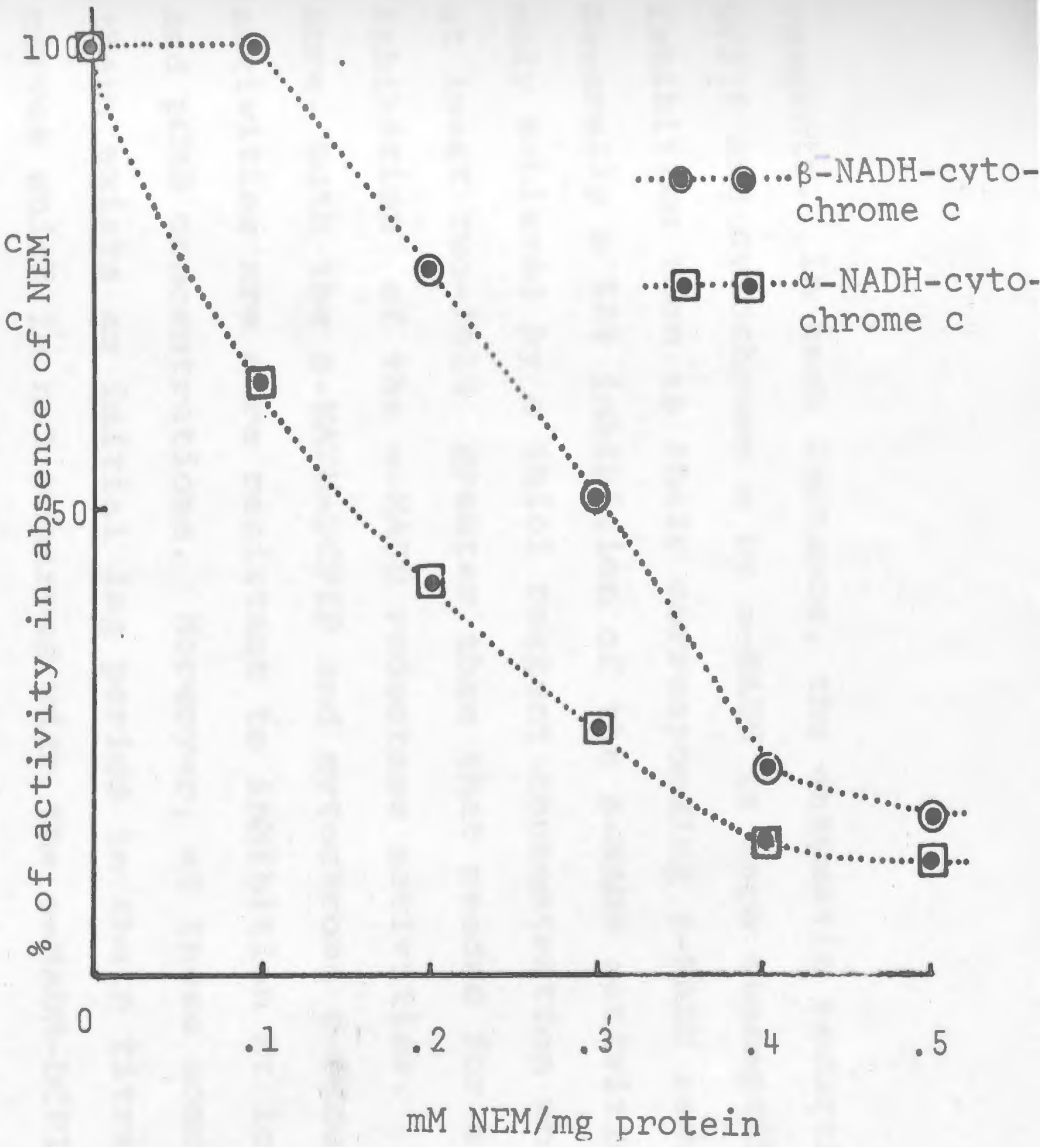

100
50
0
% of activity in absence of NEM
.1
.2
.3
.4
.5
mM NEM/mg protein
β-NADH-DCPIP
α-NADH-DCPIP

Fig. 5. Comparison of the effect of pCMB and NEM on the microsomal α- and β-NADH cytochrome c reductase activity.

Sonicated liver microsomes at a protein concentration of 1 mg/ml were pre-incubated at 4°C in a reaction mixture containing 125 mM sucrose, 1 mM EDTA, 25 mM tris-HCl buffer (pH 7.4) and varying concentrations of either pCMB or NEM. After 10 min of incubation the reaction mixture was assayed for α- and β-NADH-cytochrome c reductase activity as described in Methods.

Figure 5


100
50
0
% of activity in absence of pCMB
β-NADH-cytochrome c
α-NADH-cytochrome c
.01
.02
.03
.04
.05
mM pCMB/mg protein


100
50
0
% of activity in absence of NEM
β-NADH-cyto-chrome c
α-NADH-cyto-chrome c
.1
.2
.3
.4
.5
mM NEM/mg protein

reagents. In each instance, the enzymatic reduction of DCPIP and cytochrome c by α-NADH is more susceptible to inhibition than is their corresponding β-NADH reduction. Generally a 50% inhibition of the β-NADH activities is only achieved by a thiol reagent concentration which is at least two-fold greater than that needed for a similar inhibition of the α-NADH reductase activities. Furthermore, both the β-NADH-DCPIP and cytochrome c reductase activities are more resistant to inhibition at low NEM and pCMB concentrations. Moreover, at these concentrations there exists an initial lag period in their titration curves which is not observed with the α-NADH-DCPIP and cytochrome c reductases. In all cases however, similar inflection points occur at around 0.4 mM NEM and 0.05 mM pCMB per mg protein, with a subsequent resistance to further change by increasing concentrations of sulfhydryl reagent.

Besides the microsomal α- and β-NADH-DCPIP and cytochrome c reductases exhibiting a differential rate of inhibition towards thiol reagents, differences were also noted in the ability of the nucleotide substrates to protect the activities against inactivation by the SH reagents. Table V illustrates the protection achieved by pre-exposing microsomes to 0.25 mM of α-NAD^+, α-NADH, β-NAD^+, or β-NADH, prior to the addition of 0.025 mM pCMB. It is seen that the β-NADH reductases of DCPIP and cytochrome c are completely protected against pCMB inactivation by the prior addition

TABLE V *

α- AND β-NUCLEOTIDE PROTECTION OF THE α- AND β-NADH-DCPIP AND CYTOCHROME c REDUCTASE ACTIVITIES AGAINST pCMB INACTIVATION

Additions	α-NADH-linked reductase		β-NADH-linked reductase	
	Electron acceptor		Electron acceptor	
	DCPIP	cytochrome c	DCPIP	cytochrome c
	% activity	% activity	% activity	% activity
α- and β-NADH [a]	100	100	100	100
0.025 mM pCMB + α- or β-NADH [b]	15	36	53	46
0.25 mM α- or β- nucleotide [c] + 0.025 mM pCMB				
α-NAD^+	24	56	78	66
α-NADH	103	107	-	-
β-NAD^+	103	90	102	108
β-NADH	- [d]	-	102	147

Table V.

* Figures represent the average value of three experiments, the mean deviation being less than 10%.

[a] α- and β-NADH-DCPIP and cytochrome c reductase activities were assayed in the absence of pCMB as described in Methods.

[b] Sonicated liver microsomes (1 mg/ml) were preincubated at 4°C for 10 min in a reaction mixture containing 125 mM sucrose, 1 mM EDTA, 25 mM Tris-HCl buffer (pH 7.4) and 0.25 mM pCMB and assayed for α- and β-NADH-DCPIP and cytochrome c activity as described in Methods.

[c] 0.25 mM α- or β- nucleotides were added to the preincubation mixture prior to the addition of 0.025 mM pCMB. When studying α- or β-NAD^+ the appropriate reduced nucleotide was used to start the reaction.

[d] Inconclusive value in that both the α- and β-NADH are enzymatically active in reducing DCPIP and cytochrome c.

of either β-NAD^+ or β-NADH; indeed the β-NADH-cytochrome c reductase activity is stimulated 47% by pCMB added after β-NADH. On the other hand, the corresponding α-NADH-linked reductases, while being protected by α-NADH are not protected by α-NAD^+, although β-NAD^+ does confer protection. Furthermore, the α-NADH-cytochrome c reductase activity was only slightly stimulated (7%) by pCMB if added after α-NADH.

Response of the microsomal α- and β-NADH-DCPIP and cytochrome c reductases to X-irradiation

A relationship often exists between the reactivity of protein SH groups towards different sulfhydryl reagents and their sensitivity towards X-irradiation[61-63]. In order to substantiate and reaffirm the contrast in vulnerability between the α- and β-NADH-linked reductases, as seen with the sulfhydryl reagents, fresh sonicated microsomes (0.2 mg/ml) were subjected to X-irradiation and assayed for activity. Figs. 6 and 7 represent such a dose-response curve.

The inactivation of the α- and β-NADH-DCPIP reductases was found to proceed in a parallel manner, the α-NADH reduction of DCPIP being slightly more susceptible to the ionizing radiation than is the β-NADH reduction. In addition, X-irradiation markedly enhanced (by 47%) the activity of the β-NADH-cytochrome c reductase, whereas the α-NADH-cytochrome c reductase activity was readily inactivated. Furthermore, the activity of β-NADH-cytochrome c reductase was nc

Fig. 6. Effect of X-irradiation on the microsomal α- and β-NADH-cytochrome c reductase activity.

Sonicated hepatic microsomes (0.2 mg/ml) were irradiated in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 1 mM EDTA under the conditions given in Methods. After various doses the α- and β-cytochrome c reductase activity was measured as described in Methods and expressed as a percentage of the unirradiated control.

Figure 6

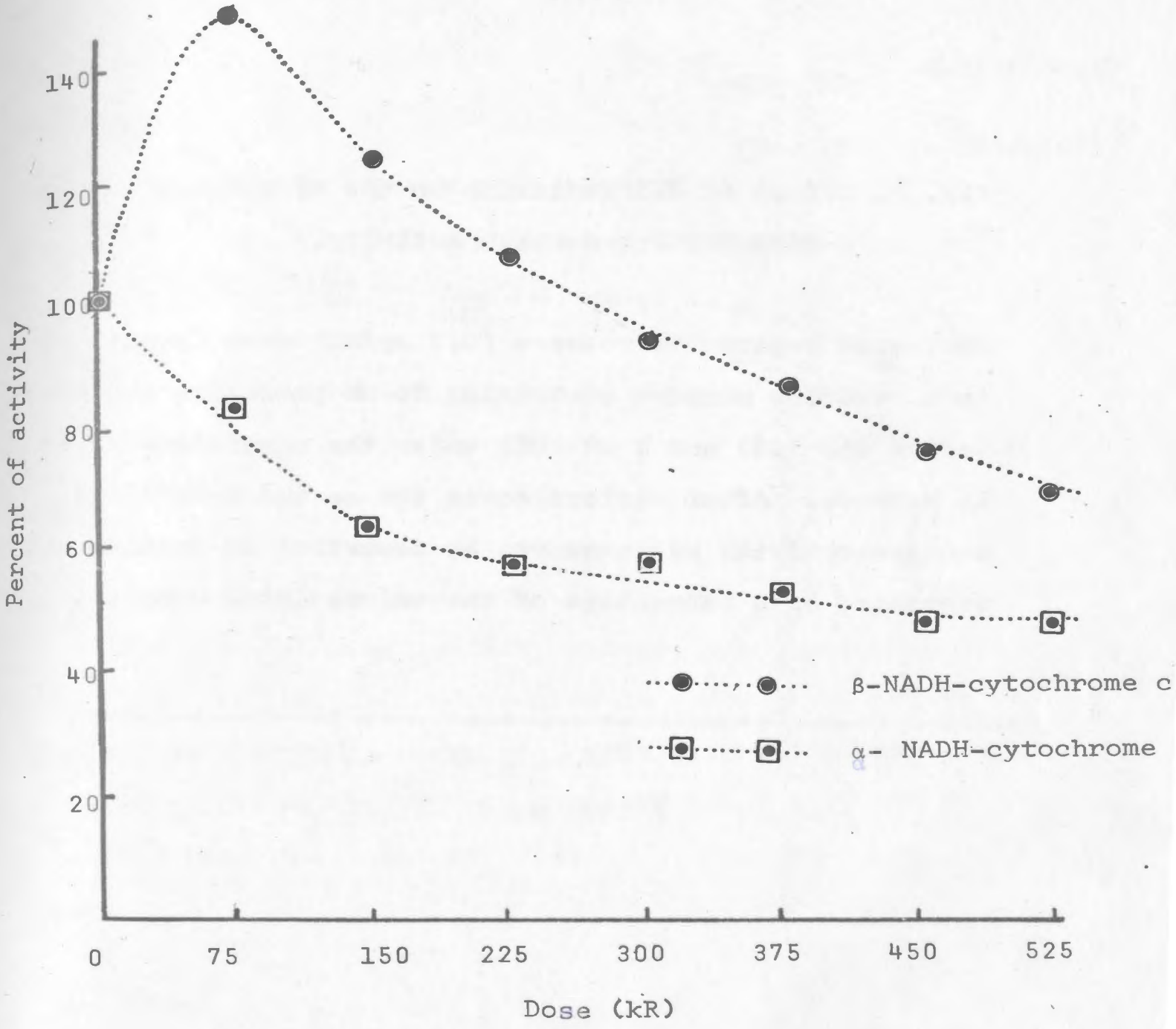
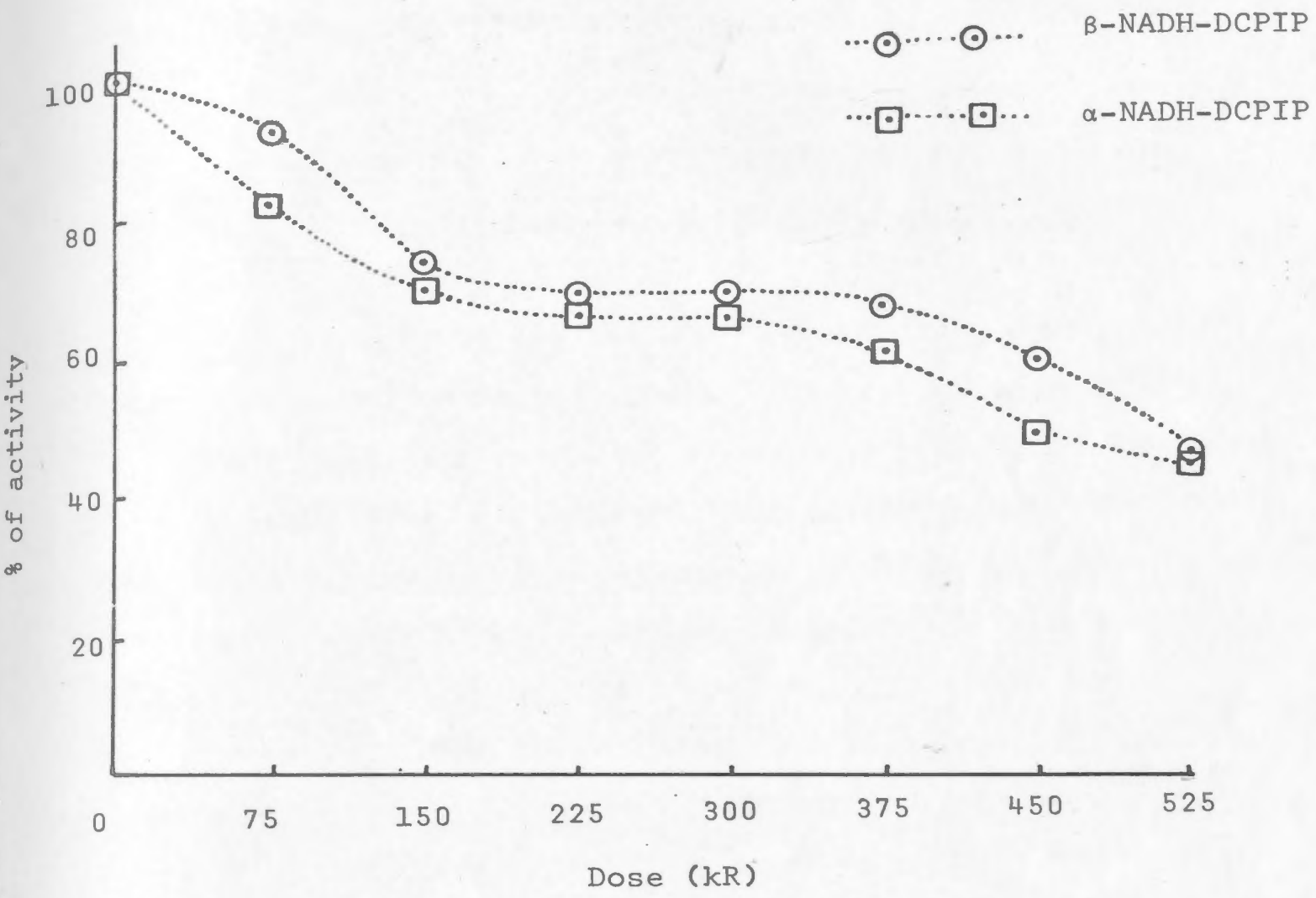

140
120
100
80
60
40
20
Percent of activity
0
75
150
225
300
375
450
525
Dose (kR)
β-NADH-cytochrome c
α- NADH-cytochrome

Fig. 7. Effect of X-irradiation on the microsomal α- and β-NADH-DCPIP reductase activity.

Sonicated hepatic microsomes (0.2 mg/ml) were irradiated in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 1 mM EDTA under the conditions given in Methods. After various doses the α- and β-DCPIP reductase activity was measured as described in Methods and expressed as a percentage of the unirradiated control.

Figure 7


β-NADH-DCPIP
α-NADH-DCPIP
100
80
60
40
20
% of activity
0
75
150
225
300
375
450
525
Dose (kR)

inactivated by 263 kRads, an amount which causes a 50% inactivation of the α-NADH-cytochrome c reductase system. Indeed, a dose of 525 kRads inactivated the β-NADH-cytochrome c reductase by only 32%.

Response of microsomal α- and β-NADH-DCPIP and cytochrome c reductases to trypsin digestion; comparison with β-NADH-$Fe(CN)_6^{-3}$ reductase

Incubating sonicated microsomes (1 mg/ml) with varying amounts of trypsin for 10 min at 4°C results in a differential effect on the α- and β-NADH reductases of DCPIP and cytochrome c. For example, Fig. 8 illustrates that the α-NADH-cytochrome c reductase activity is apparently unaffected under these conditions by trypsin concentrations as high as 500 μg/mg protein, and was even stimulated at concentrations which resulted in a 50% inactivation of the β-NADH-cytochrome c reductase activity. Furthermore, it may be seen that at low trypsin concentrations the α-NADH-cytochrome c reductase activity is stimulated 40% relative to its original level; whereas at the same trypsin concentration with β-NADH-cytochrome c activity, maximal stimulation still occurs but is only 17%.

Fig. 9 illustrates the effects of trypsin on the α- and β-NADH-DCPIP reductase activities. In conjunction with the apparent insensitivity of the α-NADH-cytochrome c reductase to trypsin, both the α- and β-NADH-DCPIP activities appear fairly resistnat to trypsin digestion. However, it may be

Fig. 8. Response of the α- and β-NADH-cytochrome c reductase activity of sonicated microsomes after preincubation with trypsin.

Sonicated microsomes were preincubated with varying amounts of trypsin as described in Methods and assayed for α- and β-NADH-cytochrome activity. The results are expressed as a percentage of the activity in a control, incubated in the presence of trypsin inhibitor, in the presence of trypsin and trypsin inhibitor, and in the absence of trypsin. α- and β-NADH-cytochrome activity was assayed as described in Methods

Figure 8

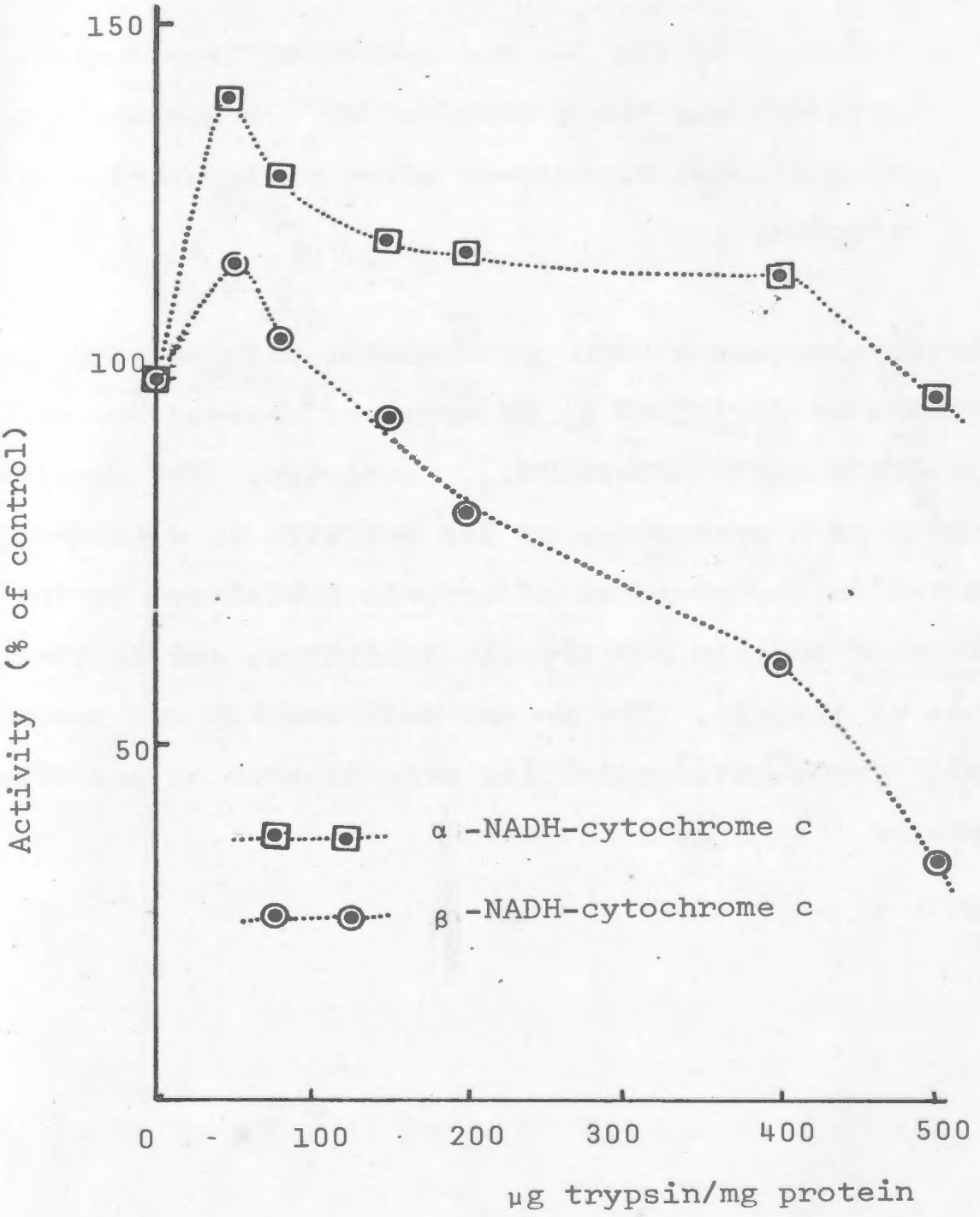

150
100
50
Activity (% of control)
α -NADH-cytochrome c
β -NADH-cytochrome c
0
100
200
300
400
500
µg trypsin/mg protein

Fig. 9. Response of the α- and β-NADH-DCPIP reductase activity and the β-NADH-$Fe(CN)_6^{-3}$ reductase activity of sonicated microsomes after preincubation with trypsin.

Sonicated microsomes were preincubated with varying amounts of trypsin as described in Methods and assayed for α- and β-NADH-DCPIP and β-NADH-$Fe(CN)_6^{-3}$ activity. The results are expressed as a percentage of the activity in a control, incubated in the presence of trypsin inhibitor, in the presence of trypsin and trypsin inhibitor, and in the absence of trypsin. The α- and β-NADH-DCPIP and β-NADH-$Fe(CN)_6^{-3}$ reductase activities were assayed as described in Methods.

Figure 9

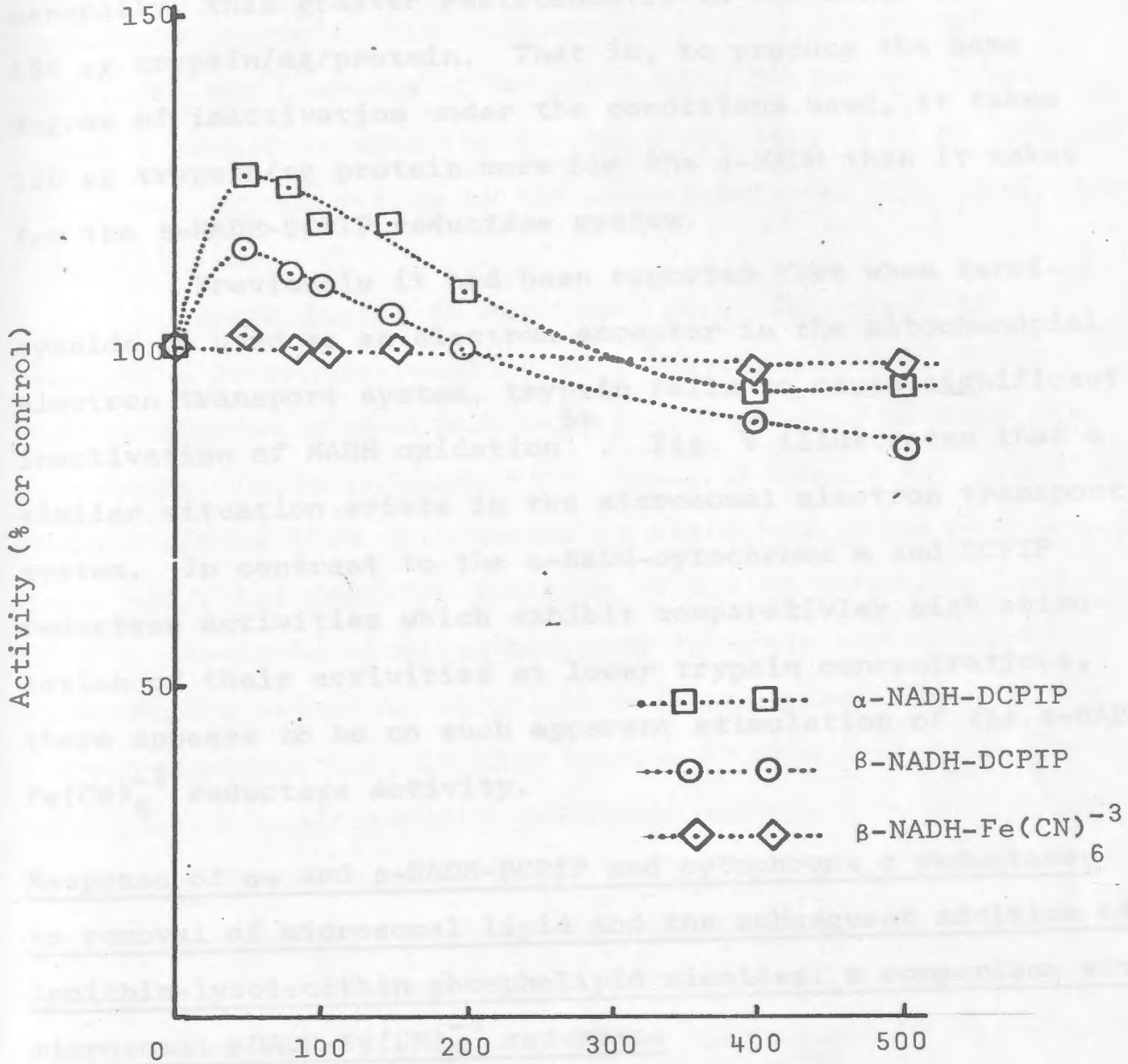
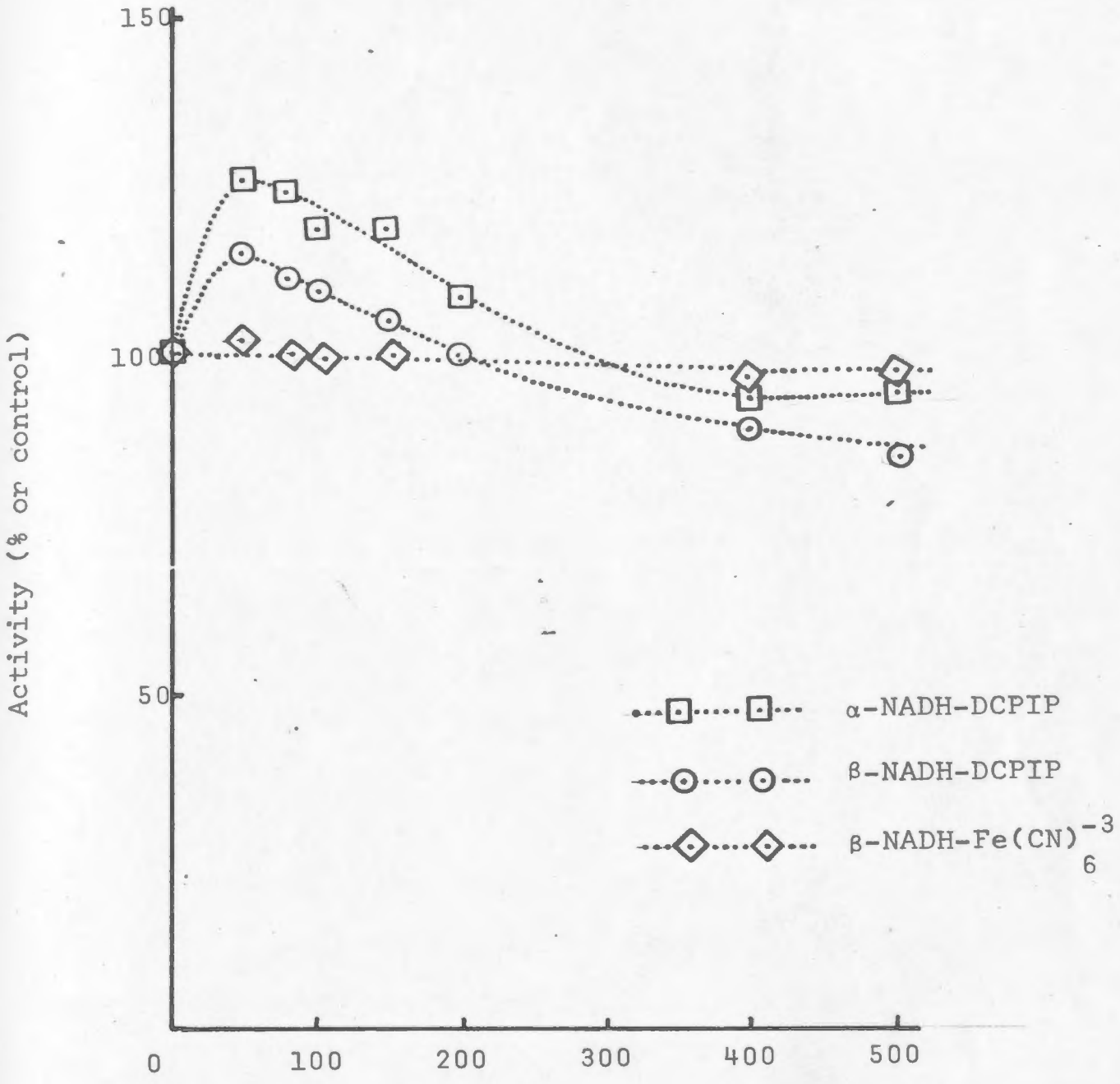

150
100
50
Activity (% or control)
0
100
200
300
400
500
α-NADH-DCPIP
β-NADH-DCPIP
β-NADH-Fe(CN)$_6^{-3}$

Fig. 9. Response of the α- and β-NADH-DCPIP reductase activity and the β-NADH-$Fe(CN)_6^{-3}$ reductase activity of sonicated microsomes after preincubation with trypsin.

Sonicated microsomes were preincubated with varying amounts of trypsin as described in Methods and assayed for α- and β-NADH-DCPIP and β-NADH-$Fe(CN)_6^{-3}$ activity. The results are expressed as a percentage of the activity in a control, incubated in the presence of trypsin inhibitor, in the presence of trypsin and trypsin inhibitor, and in the absence of trypsin. The α- and β-NADH-DCPIP and β-NADH-$Fe(CN)_6^{-3}$ reductase activities were assayed as described in Methods.

Figure 9


150
100
50
0
100
200
300
400
500
Activity (% or control)
α-NADH-DCPIP
β-NADH-DCPIP
β-NADH-Fe(CN)$_6^{-3}$

noted that the α-NADH-DCPIP activity is relatively more resistant to trypsin than is the β-NADH-DCPIP reductase. Generally, this greater resistance is in the order of 100 µg trypsin/mg/protein. That is, to produce the same degree of inactivation under the conditions used, it takes 100 µg trypsin/mg protein more for the α-NADH than it takes for the β-NADH-DCPIP reductase system.

Previously it had been reported that when ferricyanide is used as an electron acceptor in the mitochondrial electron transport system, trypsin fails to cause significant inactivation of NADH oxidation[64]. Fig. 9 illustrates that a similar situation exists in the microsomal electron transport system. In contrast to the α-NADH-cytochrome c and DCPIP reductase activities which exhibit comparativley high stimulation of their activities at lower trypsin concentrations, there appears to be no such apparent stimulation of the β-NADH-$Fe(CN)_6^{-3}$ reductase activity.

Response of α- and β-NADH-DCPIP and cytochrome c reductases to removal of microsomal lipid and the subsequent addition of lecithin-lysolecithin phospholipid micelles: a comparison with microsomal β-NADH-$Fe(CN)_6^{-3}$ reductase

Direct evidence has been reported for a specific phospholipid requirement of lecithin and lysolecithin in the β-NADH-cytochrome c reductase system of hen liver microsomes[48].

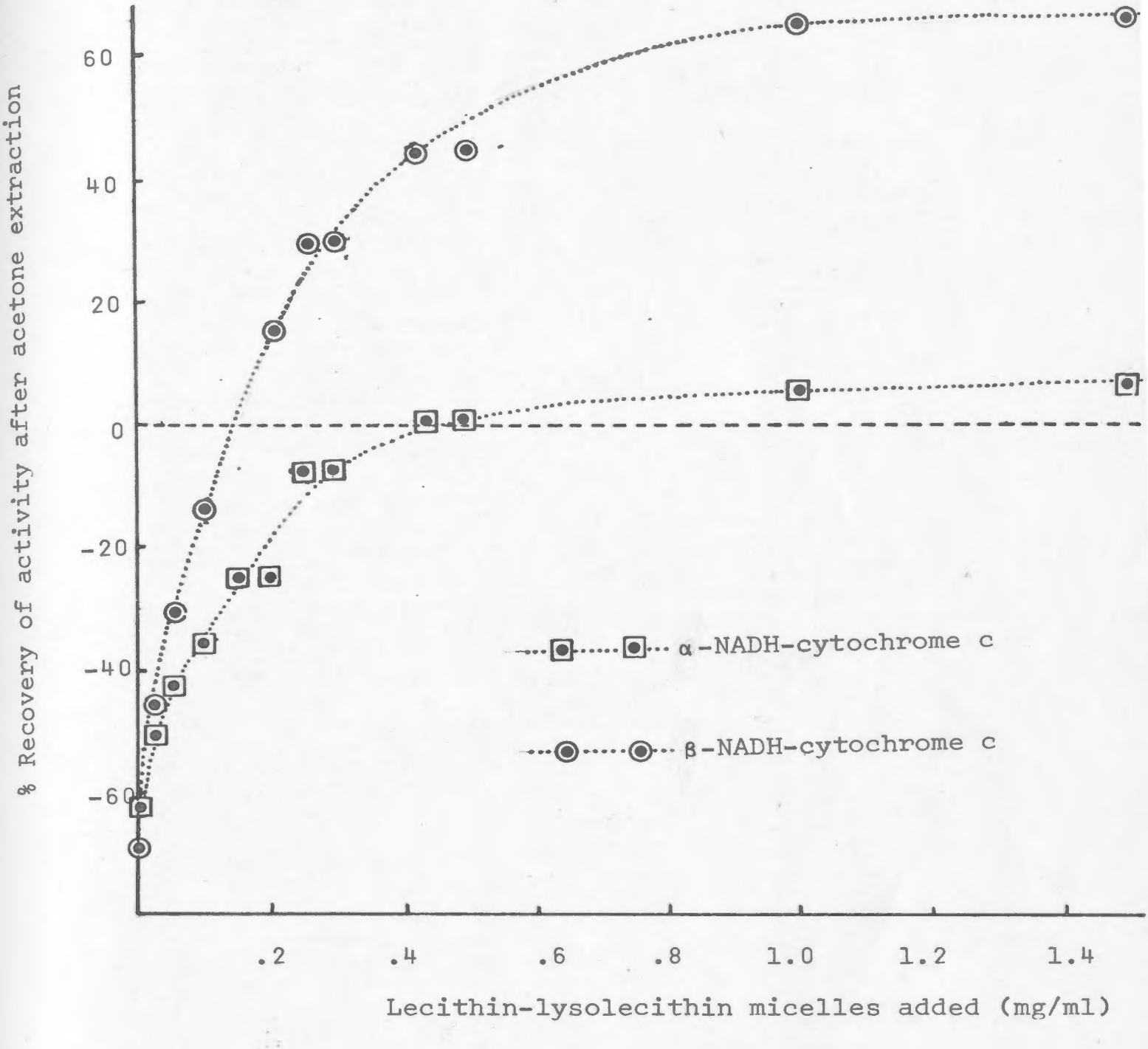
A 10% aqueous acetone extraction was performed on the microsomal fraction of rat liver to demonstrate: (a) the lipid dependence of the hepatic microsomal electron transport chain; (b) the response of α- and α-NADH-DCPIP and cytochrome c reductase activity to lipid removal; (c) to compare the α- and β- reductases to the subsequent addition of phospholipid micelles.

Accordingly, it was found (Fig. 10) that the extraction procedure resulted in a 70% reduction of the β-NADH-cytochrome c reductase activity; likewise, the α-NADH-cytochrome c reductase activity, being decreased 62%, is also greatly affected by the removal of microsomal lipid. On the other hand, although the addition of 0.5 mg/ml of the lecithin-lysolecithin micelles not only restores the β-NADH-cytochrome c reductase activity to its original level of the untreated microsomes, it also causes a 50% stimulation, an effect never realized with α-NADH-cytochrome c reductase. In addition, it may also be seen that 0.1 mg/ml of micelles is needed to completely restore the β-NADH-cytochrome c reductase activity to its original level. In contrast, this concentration of lipid micelles causes little enhancement of the α-NADH-cytochrome c reductase. In fact, the α-NADH-cytochrome c reductase activity is only restored to its original level by a lipid micelle concentration five times higher than that needed for the complete restoration of β-NADH-cytochrome c reductase.

Fig. 10. Effect of lecithin-lysolecithin micelles on the α- and β-NADH-cytochrome c reductase activity of acetone-extracted microsomes.

Lecithin-lysolecithin micelles were solubilized by sonic oscillation and the indicated amounts preincubated with acetone-extracted microsomes (0.5 mg/ml) as described in Methods. The α- and β-NADH-cytochrome c activity was measured as described in Methods and the results expressed as a percentage of the activity in a control, in which the experimental conditions were exactly the same except that intact sonicated microsomes were used instead of acetone-extracted microsomes. The values of 0 represents the reductase activity of intact microsomes prior to acetone extraction and the addition of the micelles.

FIGURE 10


% Recovery of activity after acetone extraction
60
40
20
0
-20
-40
-60
α-NADH-cytochrome c
β-NADH-cytochrome c
.2
.4
.6
.8
1.0
1.2
1.4
Lecithin-lysolecithin micelles added (mg/ml)

In contrast to the α- and β-NADH-cytochrome c reductase, the β-NADH-$Fe(CN)_6^{-3}$ reductase activity of hen liver microsomes is not affected by 10% aqueous acetone extraction[48]. Figure 11 illustrates that a similar situation exists in rat liver microsomes. It may be seen that although the acetone extraction decreases the β-NADH-$Fe(CN)_6^{-3}$ reductase activity by only 4%, the addition of 0.5 mg/ml micelles causes an analogous enhancement of activity to a level approximately corresponding to that seen for the β-NADH-cytochrome c reductase.

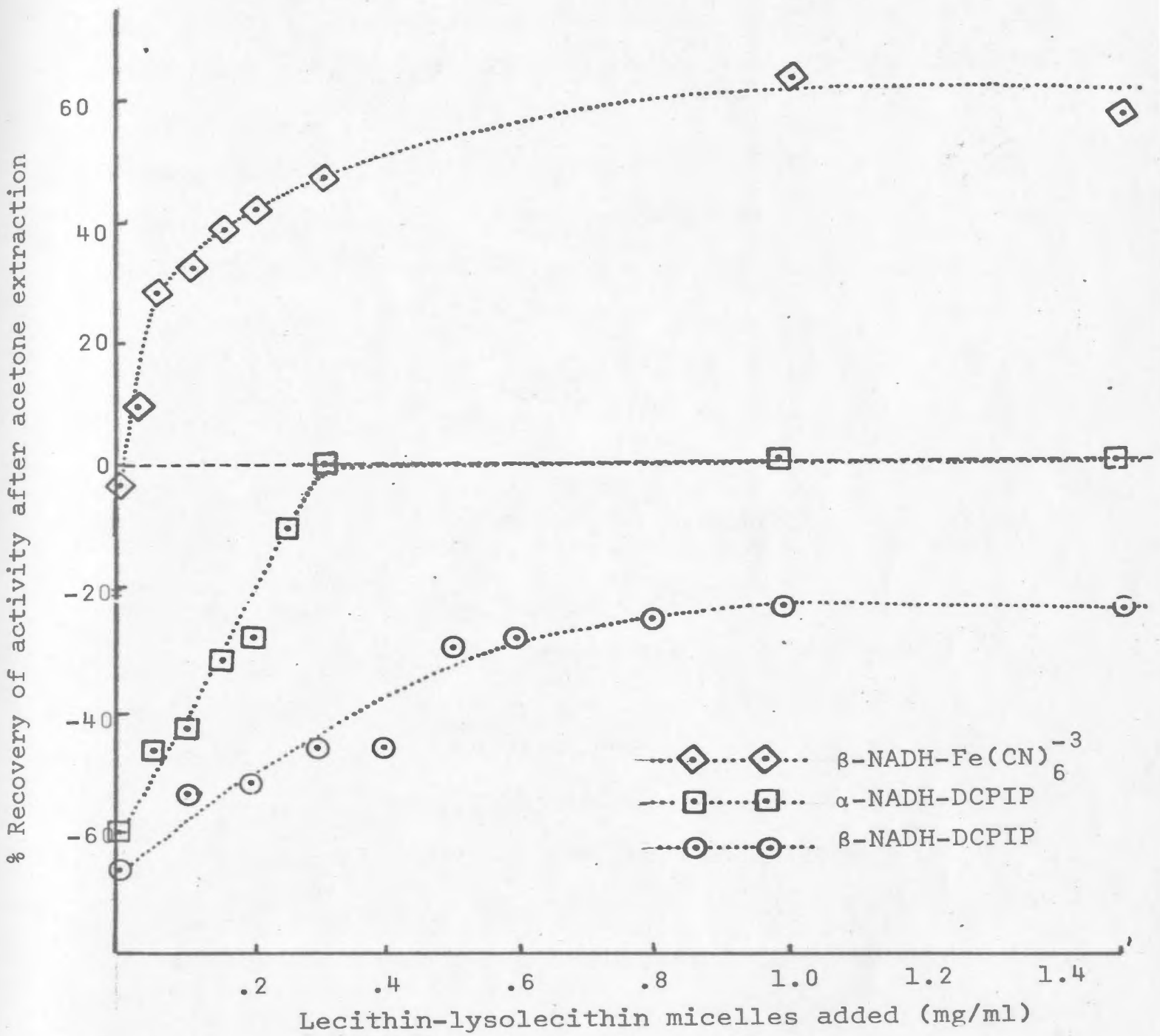
As is the case with the α- and β-NADH cytochrome c reductases, the 10% aqueous acetone extraction results in decreasing both the α- and β-DCPIP reductases to about the same extent (Fig. 11). In contrast to that observed with β-NADH-cytochrome c reductase however, the addition of lipid micelles fails to restore the activity of β-NADH-DCPIP reductase to its original level. Yet the α-NADH-DCPIP reductase activity may be fully restored. Furthermore, this restoration of activity by the lipid micelles is analogous to the restoration observed for the α-NADH-cytochrome c activity. In each case 0.5 mg/ml micelles causes 100% recovery of activity and additional micelles cause little enhancement.

It appears therefore that in the α-NADH-linked reductase system, when using either DCPIP or cytochrome c as the final electron acceptor, a common response to the addition of the phospholipid is observed. Likewise, the β-NADH-linked reductase system, although responding

Fig. 11. Effect of lecithin-lysolecithin micelles on the α- and β-NADH-DCPIP reductase activity and the β-NADH-$Fe(CN)_6^{-3}$ reductase activity of acetone-extracted microsomes.

Lecithin-lysolecithin micelles were solubilized by sonic oscillation and the indicated amounts preincubated with aceton extracted microsomes (0.5 mg/ml) as described in Methods. The α- and β-NADH-DCPIP and β-NADH-$Fe(CN)_6^{-3}$ activity was measured described in Methods and the results expressed as a percentage of the activity in a control, in which the experimental condit ions were exactly the same except that intact sonicated micro-somes were used instead of acetone-extracted microsomes. The value of 0 represents the reductase activity of intact micro-somes prior to acetone extraction and the addition of the micelles.

FIGURE 11


60
40
20
0
-20
-40
-60
% Recovery of activity after acetone extraction
.2
.4
.6
.8
1.0
1.2
1.4
Lecithin-lysolecithin micelles added (mg/ml)
β-NADH-Fe(CN)$_6^{-3}$
α-NADH-DCPIP
β-NADH-DCPIP

differently from the α-NADH-linked system, also exhibits characteristic response to the addition of lipid micelles when either $Fe(CN)_6^{-3}$ or cytochrome c is used as the final electron acceptor. β-NADH-DCPIP reductase does not exhibit a similar response to the added phospholipid, a result however, that is in agreement with that previously reported — that the β-NADH-DCPIP activity, after 10% aqueous acetone extraction, fails to respond to the addition of total microsomal lipid micelles[48]. The loss of this activity, therefore, may be due to the loss of other,as yet unknown, factors which are required for the β-NADH-DCPIP reductase activity.

Response of the microsomal α-NADH-DCPIP and cytochrome c reductase and β-NADH-DCPIP, cytochrome c and $Fe(CN)_6^{-3}$ reductases to thenoyltrifluroacetone

The enzymatic activity of microsomal NADH-semidehydroascorbate reductase has been reported to be susceptible to inhibition by TTFA, while that of β-NADH-cytochrome c reductase is reported to be resistant[65]. The sensitive component of TTFA inhibition has been suggested to be non-heme iron[66-68], and too it has been reported that the microsomal NADH-cytochrome c reductase complex contains non-heme iron[69-71]. It was therefore of interest to see whether the microsomal α-NADH-linked reductases, in particular α-NADH-cytochrome c reductase, were sensitive to TTFA inhibiton.

Fig. 12. Comparison of the effect of TTFA on the microsomal α-NADH and β-NADH-linked activities.

Sonicated liver microsomes were preincubated at 4°C for 5 min in a final protein concentration of 1 mg/ml in the presence of 0.25 M sucrose, 2 M EDTA, and varying concentrations of freshly prepared TTFA. At the end of the incubation period the reaction mixture was tested for the relevant α- and β-NADH-linked reductases activities as described under Methods. The reductase activity of a similar reaction mixture devoid of TTFA served as a control.

Figure 12

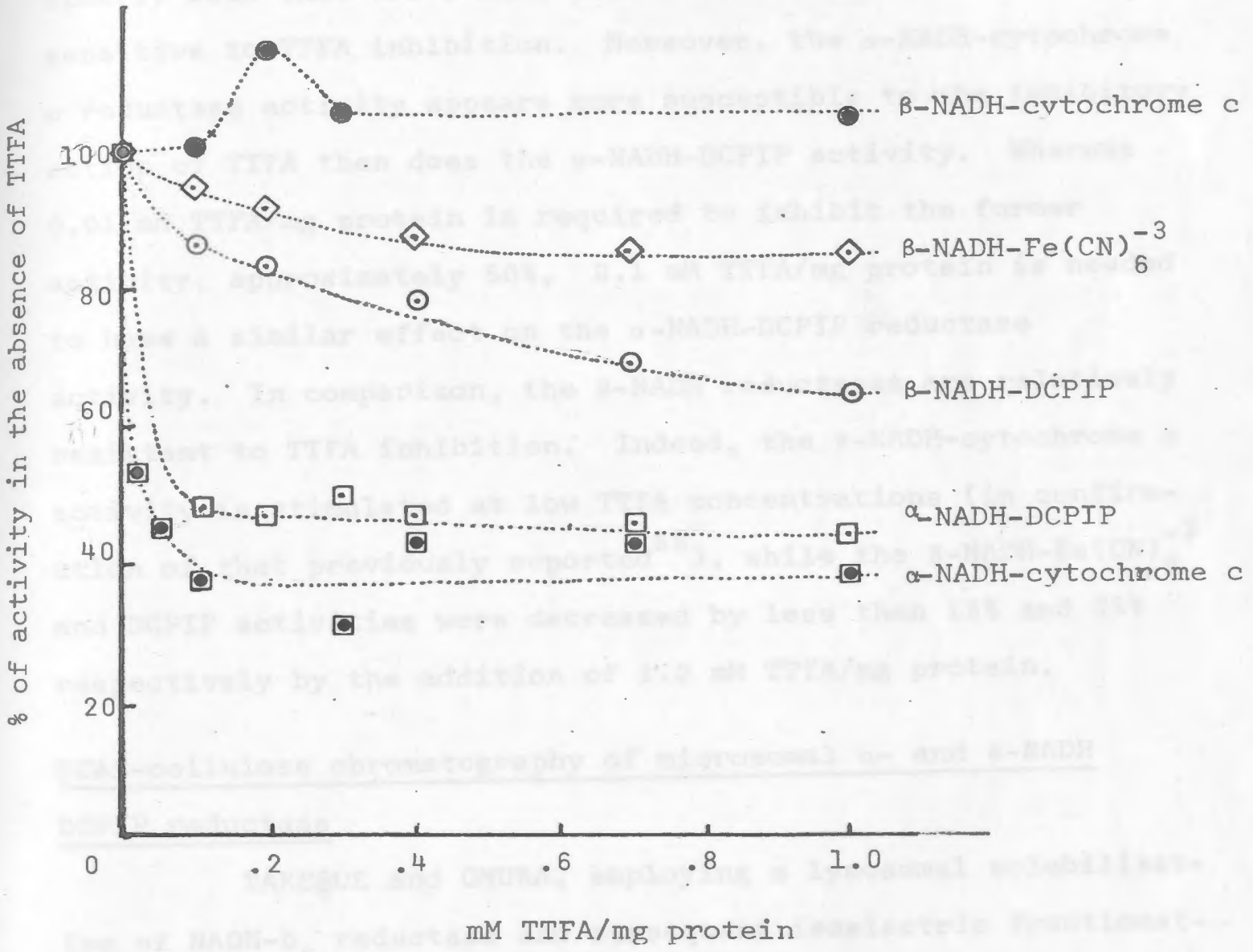

% of activity in the absence of TTFA
100
80
60
40
20
0
.2
.4
.6
.8
1.0
mM TTFA/mg protein
β-NADH-cytochrome c
β-NADH-Fe(CN)$_6^{-3}$
β-NADH-DCPIP
α-NADH-DCPIP
α-NADH-cytochrome c

Fig. 12 represents the effect that increasing concentrations of TTFA have on microsomal α- and β-NADH reductase activities when either DCPIP, cytochrome c, or $Fe(CN)_6^{-3}$ are used as the final electron acceptor. It is clearly seen that the α-NADH-linked reductases are extremely sensitive to TTFA inhibition. Moreover, the α-NADH-cytochrome c reductase activity appears more susceptible to the inhibitory action of TTFA than does the α-NADH-DCPIP activity. Whereas 0.01 mM TTFA/mg protein is required to inhibit the former activity, approximately 50%, 0.1 mM TTFA/mg protein is needed to have a similar effect on the α-NADH-DCPIP reductase activity. In comparison, the β-NADH reductases are relatively resistant to TTFA inhibition. Indeed, the β-NADH-cytochrome c activity is stimulated at low TTFA concentrations (in confirmation of that previously reported[65]), while the β-NADH-$Fe(CN)_6^{-3}$ and DCPIP activities were decreased by less than 15% and 35% respectively by the addition of 1.0 mM TTFA/mg protein.

DEAE-cellulose chromatography of microsomal α- and β-NADH DCPIP reductase

TAKESUE and OMURA, employing a lysosomal solubilization of NADH-b_5 reductase and subsequent isoelectric fractionation have reported the resolution of two protein components with identical enzymatic activities[52]. In that study NADH-DCPIP-reductase activity of microsomes was inactivated 50% by the lysosomal digestion. However, the residual activity was solubilized by this treatment and purified in parallel

Fig. 13. DEAE-cellulose chromatography of lysosomal solubilized hepatic microsomes.

As described in Methods, lysosomal solubilized microsomal extract was eluted by a continuous linear concentration gradient of KCl in 50 mM tris buffer (pH 8.5) from a DEAE-cellulose column (2.5 x 45 cm). The eluate was collected in 5 ml fractions at a rate of 0.6 ml/min and analyzed for α- and β-NADH-DCPIP, cytochrome c, and β-NADH-$Fe(CN)_6^{-3}$ reductase activity as described in Methods. Enzyme activities are expressed as n moles of substrate reduced/min/ml of eluate.

Figure 13

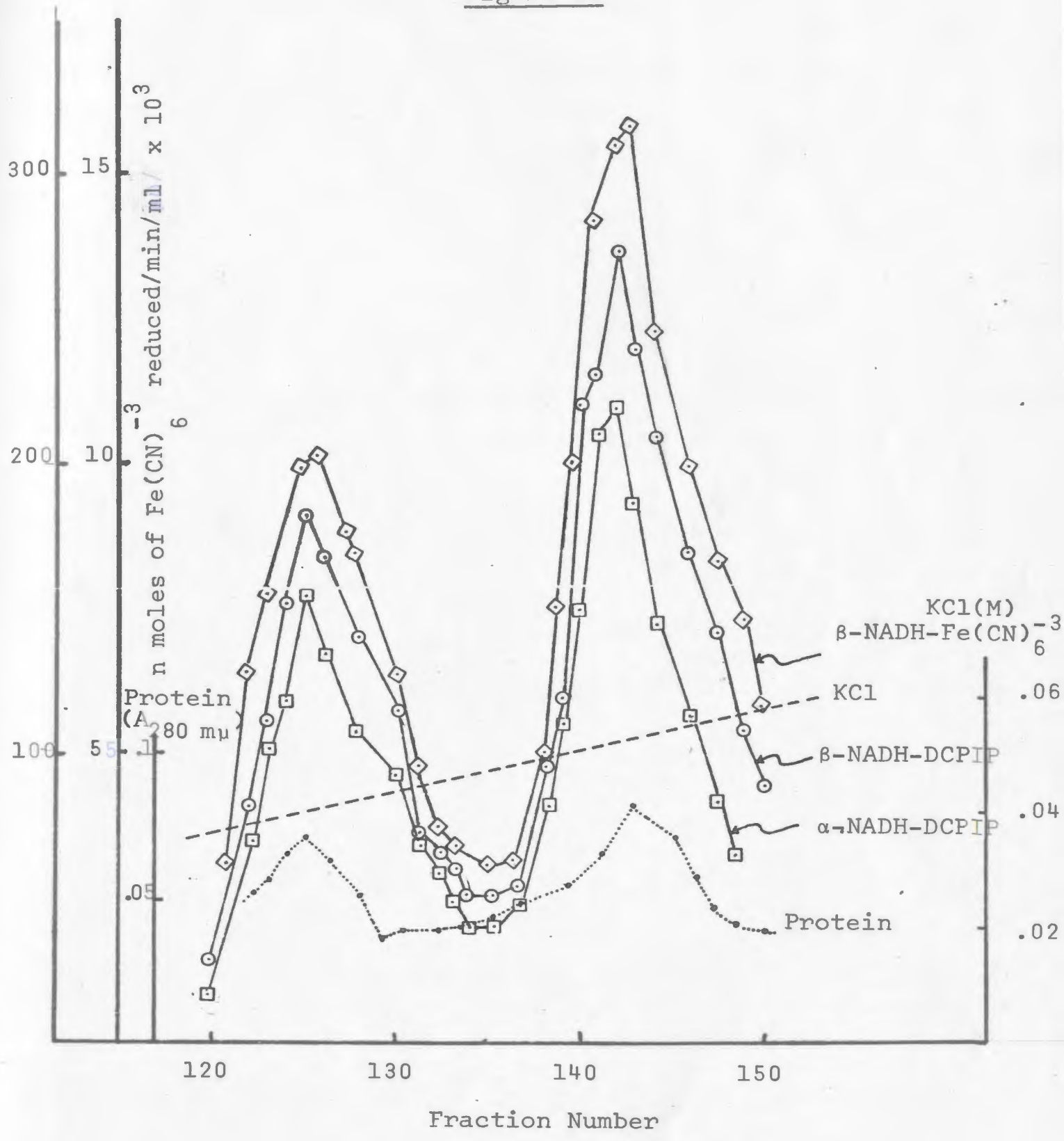

n moles of DCPIP reduced/min/ml
300
200
100
15
10
5
n moles of Fe(CN)6-3 reduced/min/ml x 10^3
Protein (A280 mμ)
.1
.05
KCl(M)
β-NADH-Fe(CN)6-3
KCl
β-NADH-DCPIP
α-NADH-DCPIP
Protein
.06
.04
.02
120
130
140
150
Fraction Number

Fig. 14. Isoelectric Fractionation of NADH-cytochrome b_5 reductase.

The two components from the "lysosomal solubilized DEAE-purified NADH-cytochrome b_5 reductase" (Fig. 13) were pooled and about 0.5 mg was subjected to isoelectric fractionation as described in Methods. After electrophoresis, the contents of the column were eluted from the bottom, and collected in 1 ml fractions. The pH and α- and β-NADH DCPIP, cytochrome c and β-NADH-$Fe(CN)_6^{-3}$ reductase activity of the fractions was measured as described in Methods. Enzyme activities are expressed as n moles substrate reduced/min/ml of eluate.

Figure 14

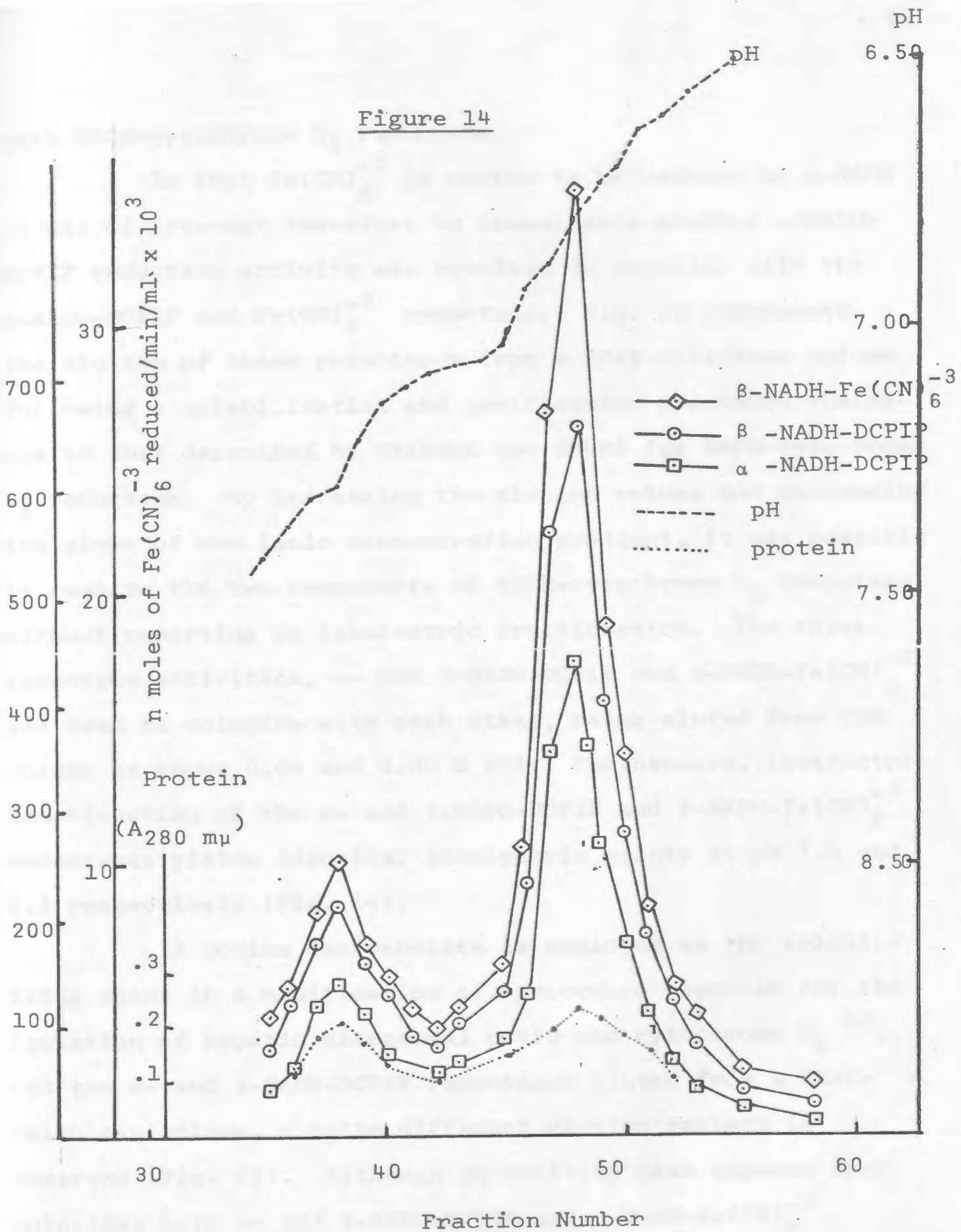
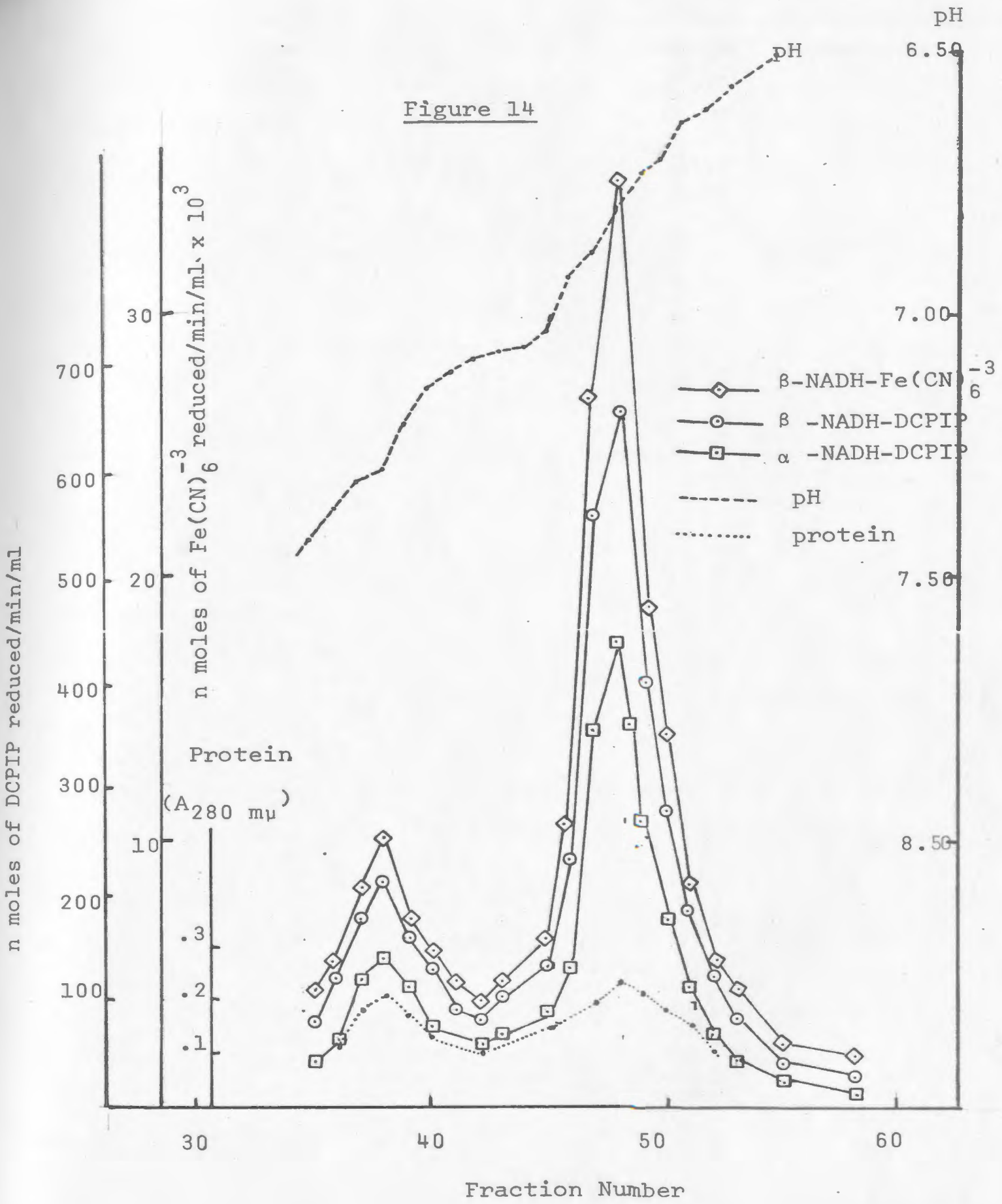

pH
6.50
7.00
7.50
8.50
β-NADH-Fe(CN)$_6^{-3}$
β -NADH-DCPIP
α -NADH-DCPIP
pH
protein
n moles of DCPIP reduced/min/ml
n moles of Fe(CN)$_6^{-3}$ reduced/min/ml x 10^3
Protein
($A_{280\ m\mu}$)
Fraction Number

Fig. 14. Isoelectric Fractionation of NADH-cytochrome b_5 reductase.

The two components from the "lysosomal solubilized DEAE-purified NADH-cytochrome b_5 reductase" (Fig. 13) were pooled and about 0.5 mg was subjected to isoelectric fractionation as described in Methods. After electrophoresis, the contents of the column were eluted from the bottom, and collected in 1 ml fractions. The pH and α- and β-NADH DCPIP, cytochrome c and β-NADH-$Fe(CN)_6^{-3}$ reductase activity of the fractions was measured as described in Methods. Enzyme activities are expressed as n moles substrate reduced/min/ml of eluate.

Figure 14


pH
6.50
7.00
7.50
8.50
n moles of DCPIP reduced/min/ml
n moles of $Fe(CN)_6^{-3}$ reduced/min/ml x 10^3
Protein ($A_{280\ m\mu}$)
β-NADH-$Fe(CN)_6^{-3}$
β -NADH-DCPIP
α -NADH-DCPIP
pH
protein
Fraction Number

with NADH-cytochrome b_5 reductase.

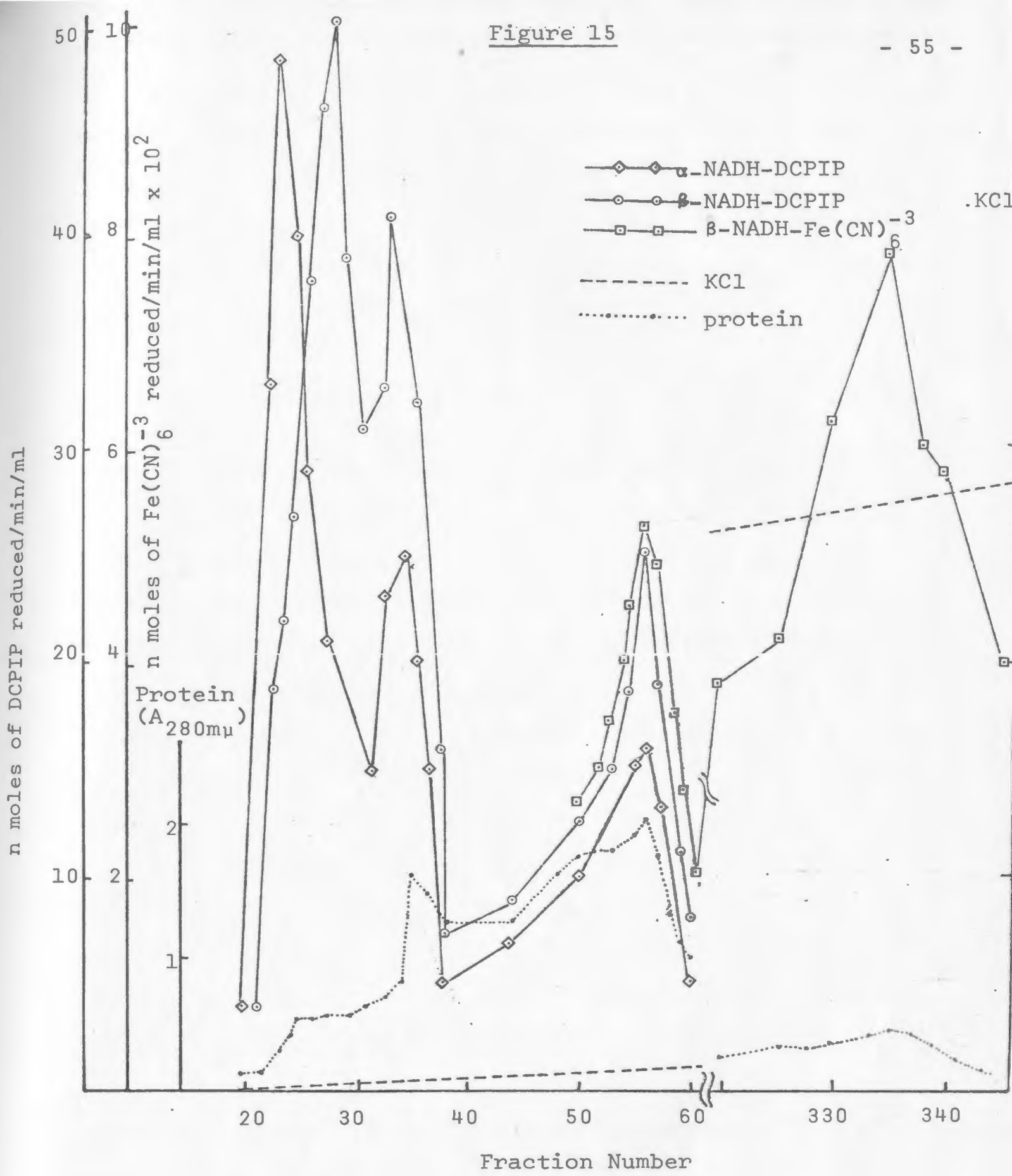
In that $Fe(CN)_6^{-3}$ is unable to be reduced by α-NADH it was of interest therefore to investigate whether α-NADH-DCPIP reductase activity was resolved in parallel with the β-NADH-DCPIP and $Fe(CN)_6^{-3}$ reductase. Fig. 13 represents the elution of these reductases from a DEAE-cellulose column following a solubilization and purification procedure analogous to that described by TAKESUE and OMURA for NADH-cytochrome b_5 reductase. By increasing the elution volume and decreasing the slope of the ionic concentration gradient, it was possible to resolve the two components of NADH-cytochrome b_5 reductase without resorting to isoelectric fractionation. The three reductase activities, α- and β-NADH-DCPIP and β-NADH-$Fe(CN)_6^{-3}$ are seen to coincide with each other, being eluted from the column at about 0.04 and 0.05 M KCl. Furthermore, isoelectric fractionation of the α- and β-NADH-DCPIP and β-NADH-$Fe(CN)_6^{-3}$ reductases yields identical isoelectric points at pH 7.3 and 6.8 respectively (Fig. 14).

If sodium deoxycholate is employed as the solubilizing agent in a modification of a procedure reported for the isolation of hepatic microsomal p-450 and cytochrome b_5 [53], and the α- and β-NADH-DCPIP reductases eluted from a DEAE-cellulose column, a quite different elution pattern is observed (Fig. 15). Although an activity peak appears that coincides both α- and β-NADH-DCPIP and β-NADH-$Fe(CN)_6^{-3}$

Fig. 15. DEAE-cellulose chromatography of DOC solubilized hepatic microsomes.

As described in Methods, hepatic microsomes were solubilized by DOC and the extract eluted by a continuous linear concentration gradient of KCl in 50 mM tris-HCl buffer (pH 8.5) from a DEAE-cellulose column (2.5 x 45 cm). The eluate was collected in 5 ml fractions at a rate of 0.6 ml/min and analyzed for α- and β-NADH-DCPIP, cytochrome c, and β-NADH-$Fe(CN)_6^{-3}$ reductase activity as described in Methods. Enzyme activities are expressed as n moles of substrate reduced/min/ml of eluate.

Figure 15


α-NADH-DCPIP
β-NADH-DCPIP
β-NADH-Fe(CN)6-3
KCl
protein
n moles of DCPIP reduced/min/ml
n moles of Fe(CN)6-3 reduced/min/ml x 10-2
Protein (A280mμ)
Fraction Number
50
40
30
20
10
10
8
6
4
2
2
1
20
30
40
50
60
330
340

reductase activity (tubes 50-60), β-NADH-$Fe(CN)_6^{-3}$ reductase is resolved in tubes 335-355 devoid of α- or β-NADH-DCPIP activity. Conversely, α- and β-NADH-DCPIP reductase activity is isolated together (tubes 32-38) devoid of β-NADH-$Fe(CN)_6^{-3}$ reductase activity. On the other hand, both α- and β-NADH-DCPIP reductase activity, although overlapping are resolved from each other in tubes 22 and 29 respectively.

Electron donor and acceptor specificity on the isolated reductases from lysosomal and deoxycholate DEAE-cellulose chromatography

In an attempt to partially characterize the isolated reductases obtained from the deoxycholate and lysosomal DEAE-cellulose chromatography, various electron acceptors were tested for their efficiency in oxidizing either α- or β-NADH. Table 6 represents such a comparison using the peak tubes from each isolated fraction as the source of the reductase.

It may be seen that the electron acceptors, $Fe(CN)_6^{-3}$, p-benzoquinone, and the napthoquinones, have no detectable activity with either the α- or β-NADH-DCPIP reductase fractions (i.e. Columns 1 and 2). Moreover, these same acceptors are unable to be reduced by α-NADH or have a low α/β ratio in the intact microsomal membrane (see Table I). The acceptors which are active in the α-NADH-DCPIP reductase fraction (i.e. Column I), however, are able to oxidize α-NADH at a higher rate than β-NADH; indeed the α/β ratios have all increased up to 45-fold from their

TABLE VI *

ELECTRON ACCEPTOR SPECIFICITY ON THE ENZYMATICALLY ACTIVE FRACTIONS ISOLATED FROM DEAE-CELLULOSE CHROMATOGRAPHY

I [a]			II [b]			III [c]			IV [d]		
Units [e]		α/β [h]	Units		α/β	Units		α/β	Units		
α-NADH [f]	β-NADH [g]		α-NADH	β-NADH		α-NADH	β-NADH		α-NADH	β-NADH	
48	22	2.2	21	50	0.42	< 0.079	2	0	22	49	0.44
< 0.166 [i]	< 0.166	0	< 0.166	< 0.166	0	< 0.166	750	0	< 0.166	3050	0
13	8	1.6	< 0.079 [l]	< 0.079	0	< 0.079	< 0.079	0	5	7	0.71
< 0.315 [j]	< 0.274 [k]	0	< 0.315	< 0.274	0	< 0.315	< 0.274	0	< 0.315	< 0.274	0
< 0.315	< 0.274	0	< 0.315	< 0.274	0	< 0.315	< 0.274	0	35	98	0.35
< 0.315	< 0.274	0	< 0.315	< 0.274	0	< 0.315	20	0	< 0.315	38	0
< 0.315	< 0.274	0	< 0.315	< 0.274	0	< 0.315	144	0	< 0.315	679	0
238	14	2	< 0.315	4	0	6	134	0.04	40	651	0.06
93	16	5.8	< 0.315	44	0	4	69	0.05	95	556	0.17
48	30	1.6	< 0.315	51	0	< 0.315	< 0.274	0	< 0.315	< 0.274	0
< 0.315	< 0.274	0	< 0.315	< 0.274	0	< 0.315	< 0.274	0	< 0.315	< 0.274	0
< 0.315	17	0	< 0.315	15	0	< 0.315	30	0	< 0.315	38	0

Table VII.

The various enzymatically active fractions following either DOC or lysosomal solubilization and DEAE-cellulose chromatography were tested for reductase activity with the indicated electron acceptors and either α- or β-NADH as primary reductant. The various enzymatic activities were measured as described in Methods and are expressed as n moles of substrate oxidized or reduced per min per ml of eluate.

* Figures represent the average of three values; the mean deviations being 10% or less.

[a] Fraction number 27 following DOC solubilization and DEAE-cellulose chromatography.

[b] Fraction number 29 following DOC solubilization and DEAE-cellulose chromatography.

[c] Fraction number 345 following DOC solubilization and DEAE-cellulose chromatography.

[d] Purified NADH-cytochrome b_5 reductase; after lysosomal solubilization the two components (i.e. Fig. 13) of the flavoprotein were pooled and assayed for activity.

[e] n moles of substrate oxidized or reduced/min/ml of eluate.

[f] Enzymatic activity with α-NADH as primary reductant.

[g] Enzymatic activity with β-NADH as primary reductant.

[h] Ratio of α-NADH reductase activity to β-NADH reductase activity.

[i, j, k, l] lower limit of detection under the experimental conditions.

microsomal value. On the other hand, all the electron acceptors tested (with the exception of DCPIP) are incapable of oxidizing α-NADH in the β-NADH-DCPIP fraction (Column II); furthermore, the active electron acceptors in this fraction are identical to those in the α-NADH-DCPIP fraction (with the exception of cytochrome c.) Therefore, it appears that these two fractions are nearly identical in respect to their electron acceptors; but differ only in their electron donor specificity. The $Fe(CN)_6^{-3}$ peak (i.e. Column III), while having no apparent α- or β-NADH-DCPIP reductase activity, also exhibits negligible α-NADH oxidation. In addition, its highest reductase activity is with those electron acceptors which are incapable of being reduced by α-NADH — namely $Fe(CN)_6^{-3}$ and p-benzoquinone. Likewise, this same situation is seen to exist for the isolated cytochrome b_5 reductase.

Reconstitution of the microsomal NADH-cytochrome c reductase

To obtain direct evidence on the involvement of cytochrome b_5 in the α- and β-NADH mediated reduction of cytochrome c and DCPIP, the microsomal NADH-cytochrome c reductase system was reconstituted. Microsomal NADH-b_5 reductase was prepared by lysosomal digestion of fresh rat liver microsomes as described in Methods. The flavoprotein was purified 400-fold which resulted in a 273 mu to 460 mu O.D. ratio of 5.9, a value somewhat lower than that reported by previous workers[57]. Cytochrome b_5 was prepared from hog liver microsomes in a procedure identical to that described by OMURA

and TAKESUE, for the preparation of cytochrome b_5 from rat liver[50]. The spectral properties of the purified cytochrome b_5 were identical with those previously described for the cytochrome from other animal sources[40,50]. Furthermore, the cytochrome was found to be fully reducible by either α- or β-NADH and the purified NADH-cytochrome b_5 reductase prepared from rat liver microsomes.

The cytochrome b_5 mediated reduction of DCPIP and cytochrome c by either α- or β-NADH and the flavoprotein, NADH-b_5 reductase, is illustrated in Figs. 16 and 17. As is evident (Fig. 16) the α-NADH reduction of DCPIP is solely contingent upon the presence of the flavoprotein under the experimental conditions and is evidently unaffected by the addition of purified cytochrome b_5. Reducing equivalents apparently pass directly to DCPIP via the reduced flavin-nucleotide complex of the flavoprotein instead of going through cytochrome b_5. In contrast, if β-NADH is used as the source of reducing equivalents, cytochrome b_5 will mediate the transfer of electrons from the reduced reductase to DCPIP. It may be seen that the flavoprotein and cytochrome b_5 contribute approximately 55% and 45% respectively to the total β-NADH-DCPIP reductase activity.

Similarly, although microsomal NADH-cytochrome c reductase possesses an absolute requirement for cytochrome b_5 [55,56] it is seen that cytochrome b_5 will more efficiently reduce cytochrome c with β-NADH than with the corresponding α-NADH

Fig. 16. The addition of purified cytochrome b_5 to purified NADH-cytochrome b_5 reductase: The effect on the α- and β-NADH-DCPIP activity.

Cytochrome b_5 and the flavoprotein, NADH-cytochrome b_5 reductase, were prepared and purified as described in Methods. The flavoprotein was purified 400-fold and exhibited a 273 to 460 OD ratio of 5.9. The cytochrome b_5 was found to be fully reducible by either α- or β-NADH and the purified NADH-cytochrome b_5 reductase. The α- and β-NADH-DCPIP reductase activity was assayed as described in Methods. The α- and β-NADH-DCPIP reductase activity of the purified flavoprotein was measured before and after the addition of varying amounts of purified cytochrome b_5. The cytochrome was added directly to the assay medium and the reaction started immediately by the addition of either α- or β-NADH. The α- and β- NADH-DCPIP reductase activity is expressed as n moles of DCPIP reduced/min/ml of purified NADH-cytochrome b_5 reductase.

Figure 16

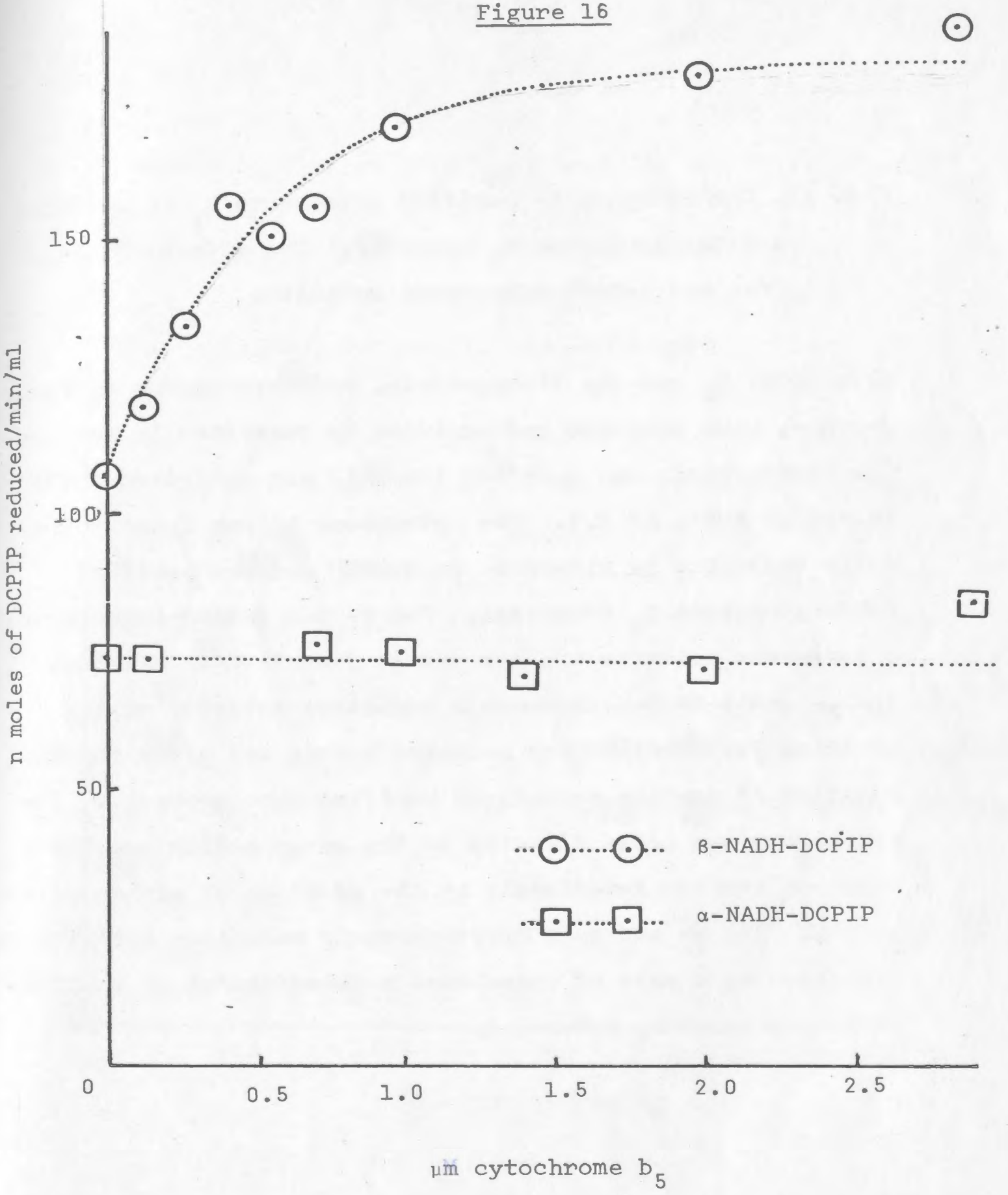
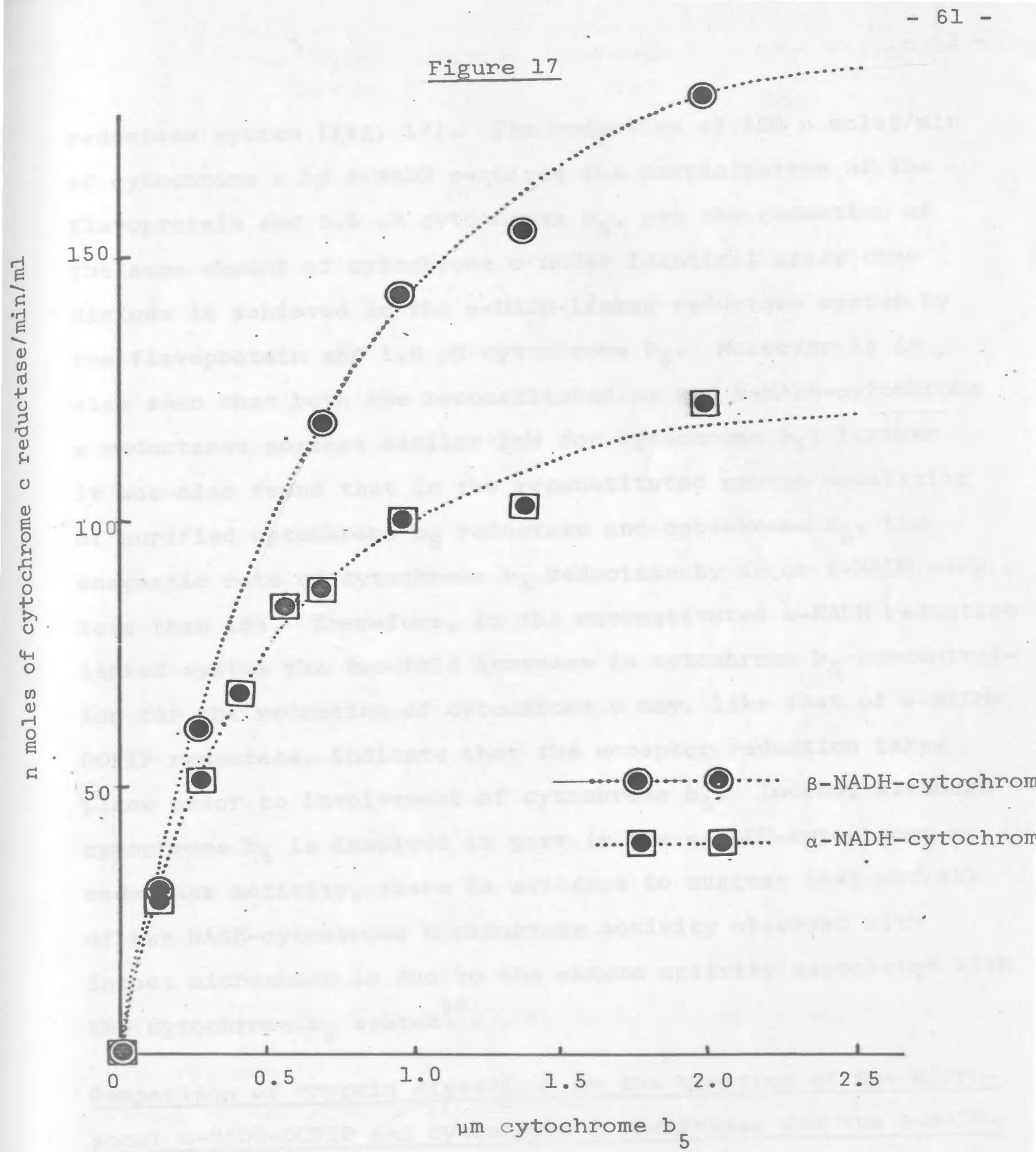

150
100
50
0
0.5
1.0
1.5
2.0
2.5
n moles of DCPIP reduced/min/ml
µm cytochrome b_5
β-NADH-DCPIP
α-NADH-DCPIP

Fig. 17. The addition of purified cytochrome b_5 to purified NADH-cytochrome b_5 reductase: The effect on the α- and β-NADH-cytochrome activity.

Cytochrome b_5 and the flavoprotein, NADH-cytochrome b_5 reductase, were prepared and purified as described in Methods. The flavoprotein was purified 400-fold and exhibited a 273 to 460 OD ratio of 5.9. The cytochrome b_5 was found to be fully reducible by either α- or β-NADH and the purified NADH-cytochrome b_5 reductase. The α- and β-NADH-cytochrome c reductase activity was assayed as described in Methods. The α- and β-NADH-cytochrome c reductase activity of the purified flavoprotein was measured before and after the addition of varying amounts of purified cytochrome b_5. The cytochrome was added directly to the assay medium and the reaction started immediately by the addition of either α- or β-NADH. The α- and β-NADH-cytochrome c reductase activity is expressed as n mole of cytochrome reduced/min/ml of purified NADH-cytochrome b_5 reductase.

Figure 17


n moles of cytochrome c reductase/min/ml
150
100
50
0
0.5
1.0
1.5
2.0
2.5
µm cytochrome b5
β-NADH-cytochrom
α-NADH-cytochrom

reductase system (Fig. 17). The reduction of 100 n moles/min of cytochrome c by β-NADH requires the participation of the flavoprotein and 0.5 μM cytochrome b_5, yet the reduction of the same amount of cytochrome c under identical assay conditions is achieved in the α-NADH-linked reductase system by the flavoprotein and 1.0 μM cytochrome b_5. Moreover it is also seen that both the reconstituted α- and β-NADH-cytochrome c reductases possess similar Km's for cytochrome b_5; further it was also found that in the reconstituted system consisting of purified cytochrome b_5 reductase and cytochrome b_5, the enzymatic rate of cytochrome b_5 reductase by α- or β-NADH vary less than 10%. Therefore, in the reconstituted α-NADH reductase linked system the two-fold increase in cytochrome b_5 concentration for the reduction of cytochrome c may, like that of α-NADH-DCPIP reductase, indicate that the acceptor reduction takes place prior to involvement of cytochrome b_5. Indeed, although cytochrome b_5 is involved in part in the α-NADH-cytochrome c reductase activity, there is evidence to suggest that not all of the NADH-cytochrome c reductase activity observed with intact microsomes is due to the enzyme activity associated with the cytochrome b_5 system[56].

Comparison of trypsin digestion on the kinetics of the microsomal α-NADH-DCPIP and cytochrome c reductases and the β-NADH-DCPIP, cytochrome c, and $Fe(CN)_6^{-3}$ reductases

The mitochondrial rotenone-insensitive NADH-cytochrome c reductase is very sensitive to inactivation by trypsin,

yet the Michaelis constants for β-NADH and cytochrome c remain unaltered during the inactivation process. If, however, $Fe(CN)_6^{-3}$ is used as the electron acceptor, NADH oxidation becomes resistant to trypsin inactivation while the Km for $Fe(CN)_6^{-3}$ increases significantly[64]. It was of interest therefore to see if such kinetic specificity existed in the microsomal electron transport system, and if so, to what extent trypsin modified the kinetics of the α-NADH-linked reductases.

Both the α- and β-NADH-DCPIP reductases have been found to be relatively unaffected by trypsin digestion (Fig. 9). Moreover, the apparent Michaelis constants for β-NADH and DCPIP as substrates of the β-NADH-DCPIP reductase system were found to be 0.40 μM and 1.00 μM respectively; and these constants remain unaltered when the system is exposed to trypsin (i.e. see Fig. 18). Similarly, the Michaelis constants for the microsomal β-NADH-cytochrome c reductase remain unaltered (Table VII) and even at a trypsin concentration sufficient to cause a 70% dimination in maximal rate (Fig. 8).

In contrast, the Michaelis constants for the α-NADH-linked reductases (together with $Fe(CN)_6^{-3}$) all greatly increase upon exposure to trypsin. Fig. 19 illustrates that the Km for α-NADH, at infinite DCPIP concentration, increases from 0.67 to 10.00 μM with increasing trypsin concentration. Indeed, at a trypsin concentration which has little detectable effect upon

Fig. 18. β-NADH-DCPIP reductase activity of sonicated hepatic microsomes after preincubation with trypsin: The effect on the Km for β-NADH.

Lineweaver-Burk plots illustrating the effect of β-NADH on the reaction rate of DCPIP reduction by sonicated hepatic microsomes which had been preincubated with varying amounts of trypsin (as described in Methods). The rate of reaction was followed at a β-NADH concentration of 5 μM or less, by employing a Coleman 124 spectrophotometer and a 0 - 0.1 A expanded scale on a Coleman 165 external recorder.

a = 500 μg trypsin/mg microsomal protein

b = 200 μg trypsin/mg microsomal protein

c = no trypsin

d = 40 μg trypsin/mg microsomal protein

Figure 18

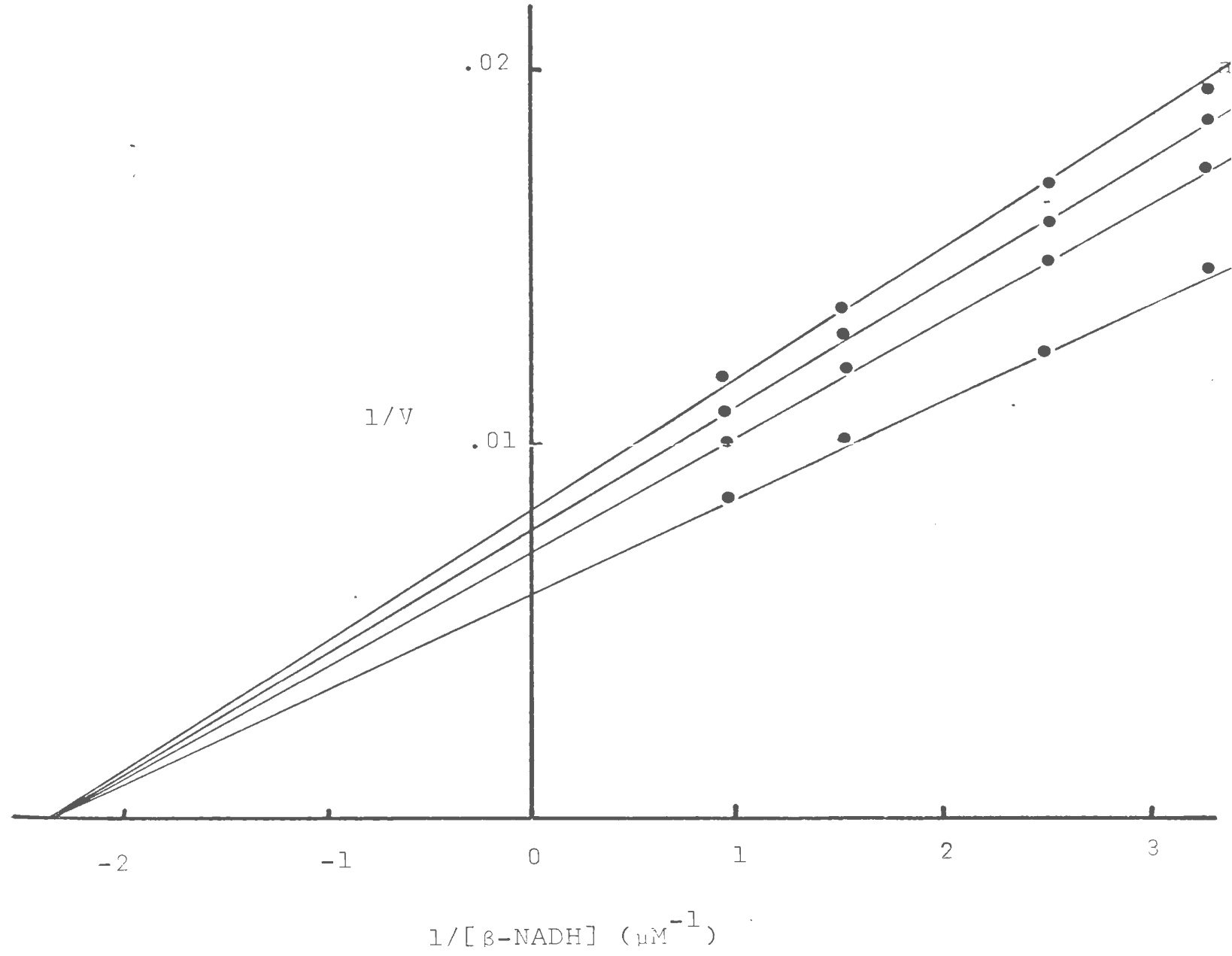


Fig. 19. α-NADH-DCPIP reductase activity of sonicated hepatic microsomes after preincubation with trypsin: The effect on the Km for α-NADH.

Lineweaver-Burk plots illustrating the effect of α-NADH on the reaction rate of DCPIP reduction by sonicated hepatic microsomes which had been preincubated with varying amounts of trypsin (as described in Methods). The rate of reaction was followed at a α-NADH concentration of 5 μM or less, by employing a Coleman 124 spectrophotometer and a 0 - 0.1 A expanded scale on a Coleman 165 external recorder.

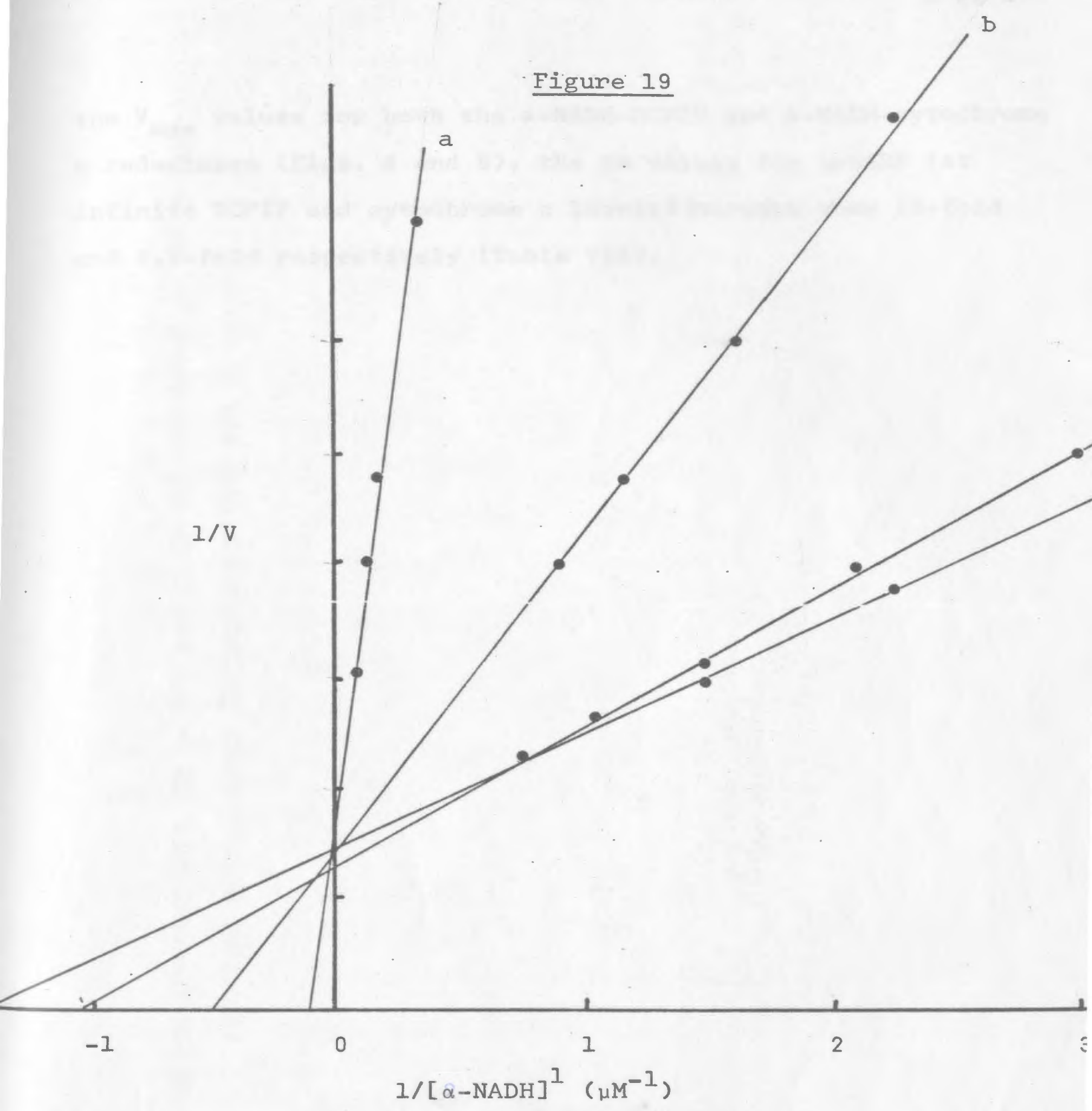
a = 500 μg trypsin/mg microsomal protein

b = 200 μg trypsin/mg microsomal protein

c = no trypsin

d = 40 μg trypsin/mg microsomal protein

Figure 19



the V_{max} values for both the α-NADH-DCPIP and α-NADH-cytochrome c reductases (Figs. 8 and 9), the Km values for α-NADH (at infinite DCPIP and cytochrome c levels) increase some 15-fold and 3.5-fold respectively (Table VII).

TABLE VII *

EFFECT ON THE APPARENT KM'S FOR α- AND β-NADH IN THE MICROSOMAL α- AND β-NADH-LINKED REDUCTASE SYSTEMS AFTER PREINCUBATION WITH 500 µg TRYPSIN

Substrate at infinite concentration	Untreated microsomes Km		Trypsin treated microsomes 500 µg trypsin/mg protein Km		Percent Km 500 µg trypsin/mg protein / untreated x Km	
	α-NADH	β-NADH	α-NADH	β-NADH	α-NADH	β-NADH
DCPIP	0.67×10^{-6}	0.40×10^{-6}	10.0×10^{-6}	0.40×10^{-6}	1492%	100%
Cytochrome c	13.0×10^{-6}	1.5×10^{-6}	57.7×10^{-6}	1.5×10^{-6}	350%	100%
$Fe(CN)_6^{-3}$	- [a]	5.0×10^{-6}	-	50.0×10^{-6}	-	1000%

Table VII.

Sonicated hepatic microsomes were preincubated with 500 g trypsin/mg microsomal protein as described in Methods and the Km's for α- and β-NADH in the reduction of DCPIP, cytochrome c and $Fe(CN)_6^{-3}$ were determined from Lineweaver-Burk plots. The effect of α- or β-NADH on the rate of reduction of DCPIP, cytochrome c or $Fe(CN)_6^{-3}$ was followed at a α- or β-NADH concentration of 5 µM or less, by employing a Coleman 124 spectrophotometer and a 0 - 0.1 A expanded scale on a Coleman 165 external recorder.

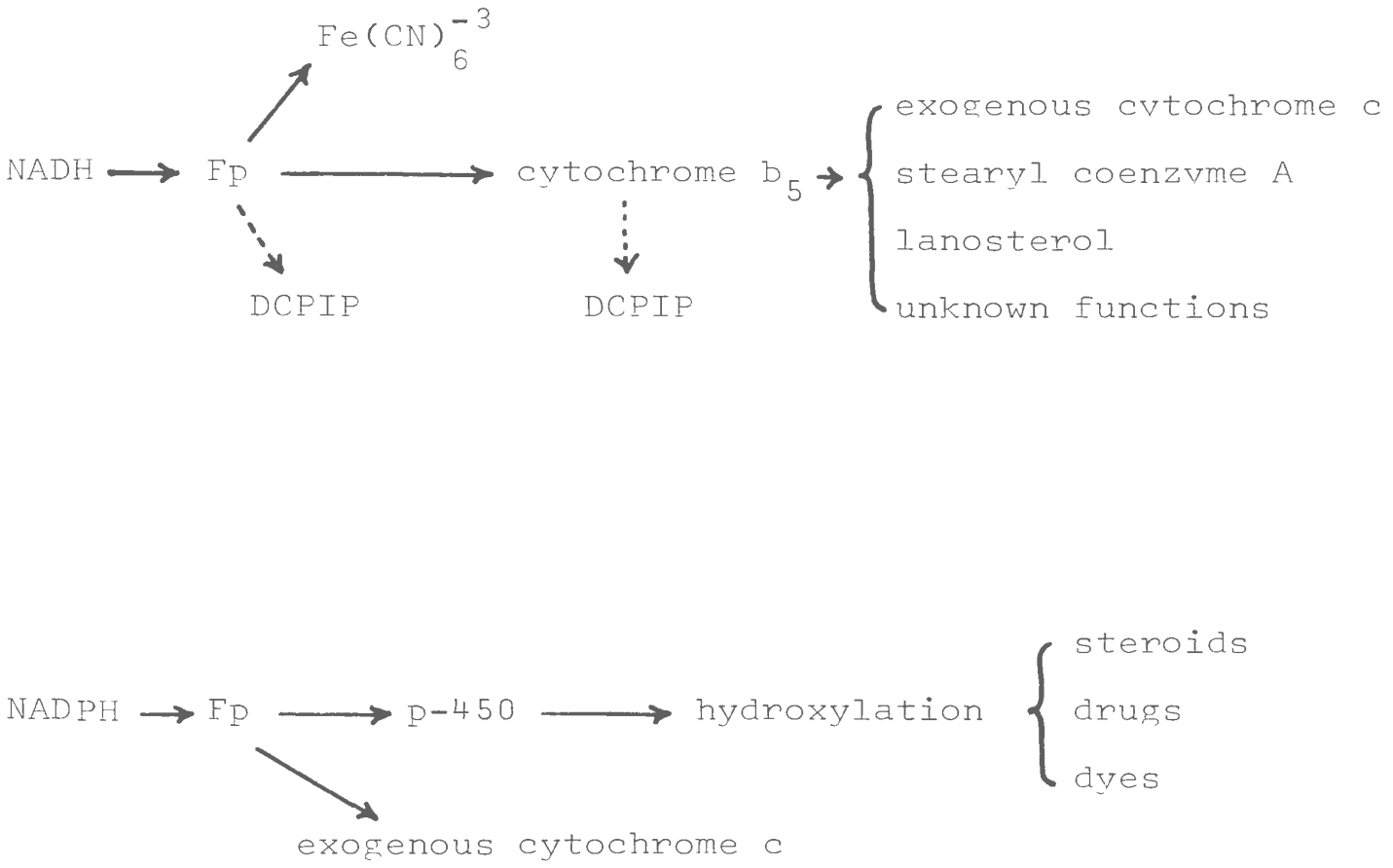
[a] Infinite value in that α-NADH is enzymatically inactive in reducing $Fe(CN)_6^{-3}$.

DISCUSSION

While the existence of the α-anomer of nicotinamide adenine dinucleotide in living cells has been recognized, its biological significance still remains obscure. Although the reduced form of the isomer cannot be substituted for β-NADH in many of the β-NADH-linked enzymes, the evidence presented in this report indicates the existence of certain enzyme systems which catalyze the oxidation of α-NADH.

α-NADH was enzymatically oxidized in the presence of various subcellular fractions of hog adrenal and rat liver, kidney, and heart tissues using either DCPIP or cytochrome c as final electron acceptor (Tables II and III). Since α-NADH cytochrome c reductase was found to be localized in the hepatic microsomal fraction (Table IV), together with a relatively high α-NADH-DCPIP reductase activity, it was possible to investigate these two α-NADH-linked reductase activities within a system in which the biochemical properties of the β-NADH linked microsomal reductases are relatively well established.

Hepatic microsomes are known to contain two electron transport systems (i.e., Scheme A). One consists of the flavoprotein NADPH-cytochrome c reductase and cytochrome P-450[81], and catalyzes the hydroxylation of a number of lipid soluble substances including steroids, drugs, and various dyes[82]. The other electron transport system consists of the flavoprotein



Scheme A. Schematic representation of the microsomal electron transport system

NADH-cytochrome b_5 reductase[83] and cytochrome b_5 and participates in the desaturation of stearyl coenzyme A[84] and lanosterol demethylation[72]. The natural terminal oxidant of the NADH specific system has yet to be discovered[86]. Although liver microsomes are devoid of cytochrome c[86], it is usually used as the *in vitro* electron acceptor[85,55]. Furthermore, it has been shown that the NADH-cytochrome reductase of liver microsomes requires cooperation of NADH-cytochrome b_5 reductase and cytochrome b_5, the NADH-cytochrome b_5 reductase alone being incapable of reducing cytochrome c[55,57]. On the other hand, TAKESUE and OMURA[36,52] have found that the ratio of the NADH-cytochrome b_5 and NADH-$Fe(CN)_6^{-3}$ reductase activities are constant during all steps in their lysosomal solubilization and purification of NADH-cytochrome b_5 reductase, and have therefore concluded that the NADH-$Fe(CN)_6^{-3}$ reductase activity of liver microsomes can be accounted for by the NADH-b_5 reductase alone. Further, by following the NADH-DCPIP reductase activity during the lysosomal solubilization of NADH-cytochrome b_5 reductase purification, it was suggested that about half of the DCPIP reductase activity of intact microsomes is dependent upon the flavoprotein and half dependent on the interaction of the flavoprotein with

another microsomal component, possibly cytochrome b_5 [36,52].

Although the biochemical data is not complete, most of the microsomal components involved in the enzymatic reduction of cytochrome c, $Fe(CN)_6^{-3}$, and to a lesser degree DCPIP, by β-NADH are fairly well established. These same electron acceptors have therefore been used in the present study in investigating the factors responsible for the enzymatic oxidation of α-NADH.

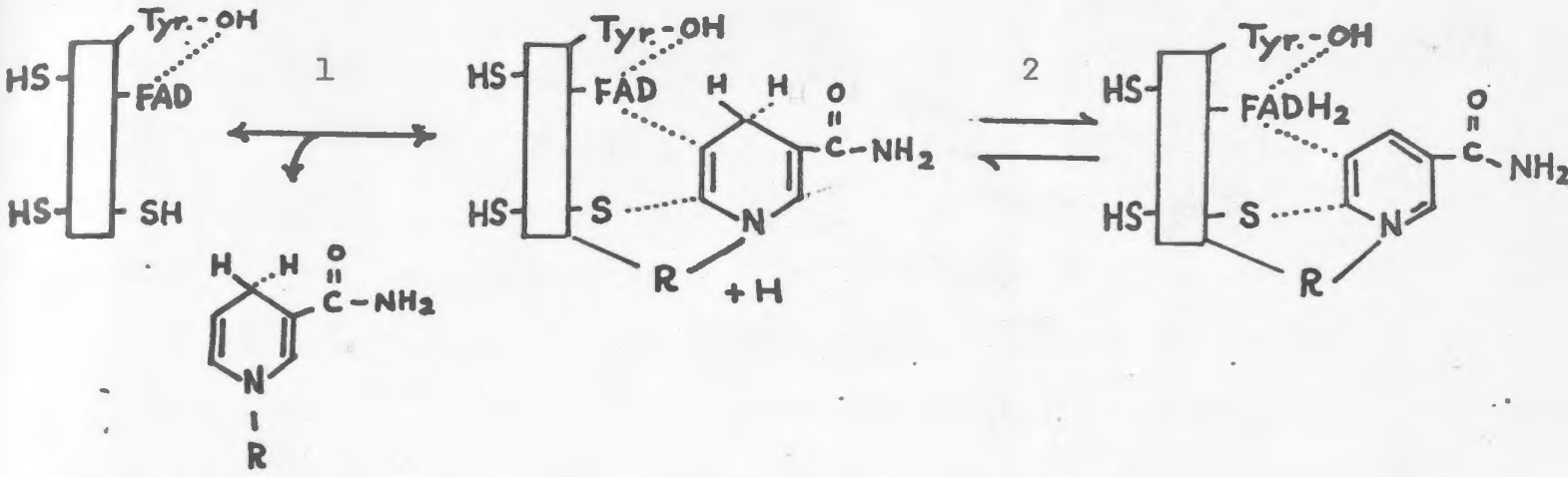
I. Enzymatic Oxidation of α-NADH

The microsomal catalyzed oxidation of the α-NADH may be either explained: (a) by being representative of a very broad specificity of the β-NADH-linked microsomal reductases; or (b) a manifestation of the presence of some hitherto unknown isoenzyme(s) associated with the microsomal electron transport system.

While the present report doesn't completely eliminate the former possibility, it presents strong evidence to support the latter.

Hypothesis A. α-NADH as a donor for microsomal β-NADH-linked reductases -

STRITTMATTER[59,111] has demonstrated that the initial reaction in the enzymatic oxidation of NADH by microsomal cytochrome b_5 reductase involves the participation of only one of the three reactive SH groups and the interaction of the nucleotide with at least two protein sites (i.e. reaction 1 of Scheme B).



Scheme B. The bar represents NADH-cytochrome b_5 reductase depicting the three reactive SH groups and the tyrosyl residue involved in the flavin binding; the hydrogen to the left of position 4 of the dihydropyridine ring represents the hydrogen in position A.

Reaction 2, which is the rate-limiting step in the over-all reaction, initiates the oxidative sequence and involves the direct and stereospecific hydrogen transfer from the 4A position of the nicotinamide ring to the flavoprotein.

It was found that α-NADH may serve as a competitive inhibitor of the β-isomer in the β-NADH-DCPIP and cytochrome c reductase systems (i.e. Fig. 1), resulting in the β-NADH-linked reductase activities not being additive with their corresponding α-NADH-linked activities. Furthermore, β-NAD^+ was found to protect both the α- and β-NADH-DCPIP and cytochrome c reductase activities against pCMB inhibition (Table V). Moreover,

lysosomal solubilization of the microsomal reductases followed by DEAE-cellulose chromatography (Fig. 13) and isoelectric focusing (Fig. 14) revealed that the three reductase activities, α- and β-NADH-DCPIP and β-NADH-$Fe(CN)_6^{-3}$ coincide with each other,yielding identical isoelectric points.

It appears possible therefore that the α-NADH-linked reductase activities may be representative of a broad specificity of the β-NADH-linked reductase system, with either the β-NADH nucleotide binding site accommodating the structural and conformational differences of α- and β-NADH (i.e. see Introduction), or α-NADH interacting at a distinct site, perhaps involving one of the other remaining reactive sulfhydryl groups of the flavoprotein. However, such a conclusion must be tempered by the fact that α-NADH is enzymatically inactive in reducing $Fe(CN)_6^{-3}$ in both the intact microsomal membrane and in the purified NADH-cytochrome b_5 reductase (i.e. Tables I and VI).

Hypothesis B. α-NADH-linked reductases as a separate enzyme from β-NADH-linked reductases

Since the discovery by SHAW[87] and HUNTER et al[88] of multiple molecular forms of the same enzyme which differ slightly in their electrophoretic mobility, a number of various enzymatically active isoenzymes have been discovered "which catalyze the same main reaction but differ in one or more characteristics, such as kinetic constants, response to stimulators and inhibitors, or even to their action on secondary

substrates"[89]. Using this as a working definition for the existence of isoenzymes, the comparative study of the microsomal α- and β-NADH-linked reductases strongly indicate the existence of isoenzyme reductases which catalyze the oxidation of α-NADH.

Of the data which support this hypothesis, the following are paramount —

1. Substrate specificity -

Substrate specificity studies (Table I) reveal that the most active electron acceptors for the β-NADH-linked reductase (i.e. $Fe(CN)_6^{-3}$ and para-benzoquinone) are completely inactive as substrates in the α-NADH-linked reductase system. However, modification of the electron acceptors greatly affected its reactivity with the α- or β-NADH. Increasing the methylation of the acceptor para-benzoquinone results in a decrease of its β-NADH activity with a concomitant increase in α-NADH-linked reductase activity. Similarly, modification of the napthoquinones by a sulfonic acid or a methyl group dramatically affected either the α- or β-NADH oxidation respectively.

2. pH optimum -

The pH activity curve (Figs. 2 and 3) for the α- and β-NADH reductases with DCPIP or cytochrome c as the final electron acceptor indicate that α-NADH oxidation is optimal at pH 5.7 while β-NADH oxidation is optimal at approximately 6.5 and 8.5 respectively with either DCPIP or cytochrome c. It appears

therefore that the α-NADH-linked reductase activity is more active at a comparatively more acid pH than is the corresponding β-NADH activity.

3. Michaelis constants -

The apparent Km values of α-NADH (Table VII) of 0.67 μM and 13.0 μM respectively, at infinite concentrations of DCPIP and cytochrome c, appear somewhat higher than the corresponding β-NADH values of 0.40 μM and 1.5 μM. The 1.5 μM for β-NADH at infinite concentration of cytochrome c is lower than that reported by HARA et al[56] (i.e. 4.3 μM), for the same substrate in whole microsomes. However, in the present study the microsomes were sonicated and centrifuged and the higher value may well be a reflection of a slower rate of diffusion of β-NADH into the intact microsomal vesicles.

4. Sulfhydryl group sensitivity -

a) Sulfhydryl group reagents and X-irradiation

Both α-NADH cytochrome c and DCPIP reductases are at least 50% more susceptible to inhibition of their enzymatic activities by sulfhydryl group reagents, than are the corresponding β-NADH reductases (Figs. 4 and 5). Moreover, the greater thiol sensitivity of the α-NADH-linked reductase system may also be suggested by the finding that this reductase system is relatively more susceptible to ionizing radiation than is the β-NADH-DCPIP and cytochrome c reductases (Figs. 6 and 7).

It would appear therefore that the two systems possess different functional SH groups. This possibility is supported

by the fact that both the β-NADH-DCPIP and cytochrome c reductase activities are relatively resistant to inhibition at low NEM and pCMB concentration; their titration curves exhibiting an initial resistant phase to the inhibitory action of pCMB and NEM. This initial lag phase may be interpreted to mean that there are proteins present with more reactive thiol groups than β-NADH-linked reductases, or that there are non-functional SH groups in the β-NADH-linked reductase system of higher affinity for the thiol group reagents that must be saturated before the active site is affected, or that the reaction inhibition occurring during this phase is not rate limiting until a finite number of sites are inhibited. This latter possibility has been considered when interpreting a similar pattern of inhibition by antimycin A[90] in the mitochondrial electron transport chain.

Although from these observations alone one cannot determine the molecular basis for the initial resistant phase observed in the β-NADH-DCPIP and cytochrome c reductase activities, one may conclude however that the absence of such a similar phenomena in the α-NADH-linked reductase system further indicates that in this system the SH groups are more susceptible to sulfhydryl group reagents.

b) Nucleotide protection -

It is well established that both β-NADH and β-NAD^+ have the same nucleotide binding site in NADH-b_5 reductase[91,92] and several other enzyme systems[45], the reduced nucleotide

β-NADH binding stronger than the oxidized β-NAD^+ [45,85,92]. Furthermore, STRITTMATTER[58] has shown that β-NADH but not β-NAD^+ is effective in protecting NADH-cytochrome b_5 reductase against p-CMB inhibition.

PHILLIPS and LANGDEN[93], however, have illustrated that the prior addition of the oxidized nucleotide $NADP^+$ not only fully protects NADPH-cytochrome c reductase against p-CMB inactivation but, indeed, causes significant enhancement of its activity. Likewise, it has been found (Table V) that the oxidized nucleotide, β-NAD^+, protects against p-CMB inhibition in both the microsomal β-NADH-DCPIP and cytochrome c reductase system in addition to their corresponding α-NADH-linked reductases. In that β-NAD^+ protects the α-NADH-reductase activities, it would superficially appear that the α- and β-nucleotides share a common site of interaction. However, the molecular situation may well be more complicated. The present data, for example, does not exclude the possibility that the binding of β-NADH or β-NAD^+ at a specific site causes conformational changes which prevent the interaction of p-CMB at a yet distinct α-nucleotide binding site. (Such conformational changes between separate α- and β-NADH interaction sites could also account for the α- and β-NADH-linked reductase activities being non-additive and the apparent competitive inhibition observed between these nucleotides.) Similarly, in that α-NAD^+ offers virtually no protection for either the α- or β-NADH-linked reductases, it may superficially indicate that α-NAD^+

unlike α-NADH, is incapable of forming a stable nucleotide-enzyme complex or, on the other hand, it may mean that it does and α-NADH unsuccessfully competes for the nucleotide binding site, therfore resulting in little apparent enzymatic activity.

Although these phenomena require further investigation one point seems to be significant however — namely, the β-NADH-cytochrome c reductase activity appears "latent". For example, it was observed that in the presence of β-NADH and p-CMB, the β-NADH-cytochrome c reductase activity is stimulated 47%. This phenomena is less apparent with either the α-NADH cytochrome c or α- and β-NADH DCPIP activities. A 50% stimulation can also be achieved with NADPH and p-CMB in the microsomal NADPH-cytochrome c reductase system[93]. Although it is possible to explain these results on strictly a conformational and steric basis, it may well be that cytochrome c reduction via β-NADH or NADPH is dependent on a component not shared by the other reductases studied. This hypothesis is reinforced by the biphasic dose response curve for β-NADH-cytochrome c reductase in which its enzymatic activity is also stimulated 47% by low doses of ionizing radiation (Fig. 6). Moreover, since β-NAD^+ does not protect in the pure NADH-b_5 reductase system, purified according to the method of STPITTMATTER[58], the β-NAD^+ protection observed with microsomal suspensions may well indicate that another species of NADH-cytochrome b_5 reductase is being protected and/or activated, thereby resulting in an apparent fully protected system.

5. Lipid Dependency

It is well known that a number of microsomal enzymes require the presence of lipid for the expression of their activity[105-108]. Indeed, the NADH-cytochrome c reductase system has been isolated in a protein-lipid complex which contained the reductase, cytochrome b_5, non-heme iron, and phospholipids[69-70]. Accordingly the NADH-cytochrome c reductase activity seems to be partially lipid dependent[109-110].

Recently JONES and WAKIL[48] have presented evidence which indicates that phospholipids may be required for the transfer of electrons in the region beyond the flavoprotein of the microsomal NADH-cytochrome c reductase system. It was demonstrated that the microsomal NADH-cytochrome c reductase, but not the NADH-$Fe(CN)_6^{-3}$ reductase activity is inactivated by 10% aqueous acetone extraction and that the former activity can be reactivated by the addition of lecithin-lysolecithin phospholipid micelles. It was not reported however what effect, if any, the addition of the phospholipid micelles had on the NADH-$Fe(CN)_6^{-3}$ reductase activity of the acetone-extracted microsomes.

In accordance with the results of JONES and WAKIL[48] the oxidation of β-NADH by cytochrome c was found to be 70% inactivated by the 10% aqueous acetone extraction, while that observed with $Fe(CN)_6^{-3}$ as the final electron acceptor was found to be inactivated only 4% (Figs. 10 and 11). In addition, however, it was found that after acetone extraction and the

addition of 1mg/ml of the lecithin-lysolecithin phospholipid micelles, both the β-NADH-cytochrome c and $Fe(CN)_6^{-3}$ reductase activities are completely restored and indeed enhanced 60% over their original pre-extracted levels, thereby indicative that these two β-NADH-linked reductases share a common response to the phospholipids.

The α-NADH-linked reductase system, on the other hand, responds differently to the lecithin-lysolecithin micelles. While the addition of 0.5 mg/ml of micelles to the acetone-extracted microsomes restores completely both the α-NADH-cytochrome c and DCPIP reductase activities, additional micelles cause little enhancement.

Although the phospholipid micelles are able to restore completely the α-NADH-DCPIP reductase activity to its original pre-extracted level, only 75% of the original β-NADH-DCPIP activity may be recovered by the addition of the micelles (Fig. 11), a result, however, that is in agreement with that previously reported[48]. The reason why the β-NADH-DCPIP reductase activity is inactivated by acetone extraction and the reason why lipid micelles are able to restore the β-NADH-cytochrome c and $Fe(CN)_6^{-3}$ reductase activities beyond their original level are not understood at the moment. The loss of the β-NADH-DCPIP reductase activity may, however, be due to the loss of other, as yet unknown, factors which are required for this activity.

8. TTFA Inhibition

The heterogeneity between the α- and β-NADH-linked microsomal reductases was further observed in their response to thenoyltrifluroacetone. It was clearly shown (Fig. 12) that the α-NADH-DCPIP and cytochrome c reductases are extremely sensitive to TTFA inhibition, whereas β-NADH-DCPIP, cytochrome c and $Fe(CN)_6^{-3}$ are relatively resistant. In that the enzymatic reduction of the latter two electron acceptors are unaffected by TTFA is in accord with that reported by STAUDINGER et al[65] who also found that the microsomal NADH-semidehydroascorbate reductase activity is sensitive to inhibition by TTFA. Since TAPPEL[67] and WHITTAKER et al[68] have suggested that the sensitive component of TTFA inhibition to be non-heme iron, it may be therefore that this component may be involved in the microsomal electron transport system utilizing α-NADH as the electron donor even though it may not be involved in β-NADH electron transport.

7. Effects of Trypsin

It is well substantiated that the treatment of microsomes with trypsin solubilizes cytochrome b_5 and NADPH-cytochrome c reductase, leaving the major portion of NADH-cytochrome b_5 reductase and cytochrome p-450 in the membrane bound state[93,96]. Moreover, the release of cytochrome b_5 closely parallels the inactivation of microsomal NADH-cytochrome c reductase[94]; however, whether the former is a cuase of or an effect of the inactivation has yet to be determined.

The data presented in this report illustrates that the microsomal α- and β-NADH-linked reductases exhibit a differential sensitivity towards trypsin digestion: the β-NADH-cytochrome c and DCPIP reductases being more highly trypsin-sensitive than the corresponding α-NADH reductases (Fig. 8 and 9). Indeed, at low trypsin concentrations the α-NADH-cytochrome c and DCPIP reductase activities are stimulated 40% and 27% respectively.

On the other hand, as has been found in the mitochondrial electron transport system[64], trypsin fails to cause significant effect on the β-NADH-$Fe(CN)_6^{-3}$ reductase activity. The non-involvement of cytochrome b_5 in the β-NADH-$Fe(CN)_6^{-3}$ activity could explain this trypsin resistance.

The stimulation of the α-NADH-DCPIP or cytochrome c reductase activity by trypsin points to the interesting possibility that the reactivity may represent an emerging activity arising upon modification of the enzyme by trypsin similar to the modification of the mitochondrial NADH dehydrogenase with a subsequent emergence of an NADH-cytochrome c reductase activity[101]. Alternatively, the increased activity may be the reflection of an increased accessibility of the microsomal membrane to α-NADH and/or cytochrome c and DCPIP. Relevant to this possibility is the response of glucose-6-phosphatase and nucleoside diphosphatase activities to trypsin treatment which was similar to those occurring upon treatment of liver microsomes with detergents[102,103].

The molecular basis for trypsin inactivation is yet

obscure. The experiments of SOTTOCASA *et al*[104] suggest that the release of cytochrome b_5 may not necessarily be the rate-limiting reaction in the inactivation of microsomal NADH-cytochrome c reductase; consequently, trypsin inactivation may not simply be caused by the release of cytochrome b_5. The release may rather be a consequence of a modification of cytochrome b_5 by trypsin, as found by ITO *et al*[95], and resulting in an inactivation of the cytochrome towards the NADH-cytochrome b_5 reductase flavoprotein. On the other hand, trypsin digestion may also modify the flavoprotein, as is indicated in Fig. 19 and Table VII. It appears that although their enzymatic rates are little affected (Figs. 8 and 9) the Michaelis constants for both the α-NADH-DCPIP and cytochrome c reductases and β-NADH-$Fe(CN)_6^{-3}$ reductase all greatly increase upon exposure to trypsin. In contrast the Km's for β-NADH-cytochrome c and DCPIP (Fig. 18) remain unaltered while their activities are inhibited by trypsin digestion.

These observations are consistent with and add further support to the hypothesis (p. 59) that the β-NADH reduction of DCPIP and cytochrome c involves the participation of both cytochrome b_5 and cytochrome b_5 reductase, while the reduction of $Fe(CN)_6^{-3}$ and α-NADH reduction of DCPIP (and to a lesser degree, cytochrome c) are dependent upon the flavoprotein (i.e. Figs. 16 and 17). Interestingly, while this assumption appears to be less true for the α-NADH-cytochrome c reductase activity, it is also noticed that a Km increase of 3.5-fold for this activi

significantly less than that of either the α-NADH-DCPIP or β-NADH-$Fe(CN)_6^{-3}$ reductase activities.

8. Solubilization and Purification of the Microsomal α- and β-NADH-linked Reductase

GREEN and PERDUE[112] have suggested that detergents solubilize intracellular membranes into heterogeneous units of small lipoprotein subunits; on the other hand, other evidence suggests the complete separation of membranes into lipids and proteins[73,74]. The DOC solubilization of microsomal membranes followed by DEAE-cellulose chromatography (Fig. 15), results in the elution of several enzymatically active peaks, possibly representing heterogeneous lipoprotein complexes. However, both the α- and β-NADH-DCPIP reductase, although overlapping, can be resolved from each other. This difference in electrophoretic mobility, together with the electron donor and acceptor specificity studies (Table VI) on the isolated reductases would further support the hypothesis of isoenzyme reductases associated with the microsomal electron transport system which can catalyze the oxidation of α-NADH.

II. Hypothetical Model Systems of Microsomal Electron Transport

A. The purification of "NADH-cytochrome b_5 reductase" -

It is generally assumed that in the NADH specific microsomal electron transport chain the natural flow of electrons proceeds from NADH through the flavoprotein, NADH-cytochrome b_5 reductase, to cytochrome b_5[55]. Furthermore, OKAMOTO *et al*[32]

have observed that addition of α-NADH to a suspension of rat liver microsomes gives essentially the same spectral change of cytochrome b_5 to reduced form, indicating therefore the presence of a cytochrome b_5-reducing system in rat liver microsomes that may transfer electrons from α-NADH to cytochrome b_5. However, the immediate mediator of reducing equivalents from α-NADH to cytochrome b_5 may not necessarily be the flavoprotein, NADH-cytochrome b_5 reductase, originally described by STRITTMATTER[5]

This possibility arises from the following observations: Although the properties of NADH-cytochrome b_5 reductase, purified according to the lysosomal digestion method of TAKESUE and OMURA[52], were reported to be nearly identical to those of NADH-cytochrome b_5 reductase, purified according to the "snake venom-solubilization" procedure of STRITTMATTER and VELICK[57], it was observed that the latter possesses (a) a molecular weight of 38,400 [57], (b) is specific for NADH[55], and (c) exhibits a 273 mu/460 mu absorption ratio of 6.9 - 7.3 [57,59]. In contrast the NADH-cytochrome b_5 reductase solubilized by lysosomal digestion displayed[52] (a) a molecular weight of 27,000, (b) an inherent NADPH-linked activity which catalyzed a slow reduction of cytochrome b_5, and (c) an absorption ratio at 273 mu to that of 460 mu of 5.9.

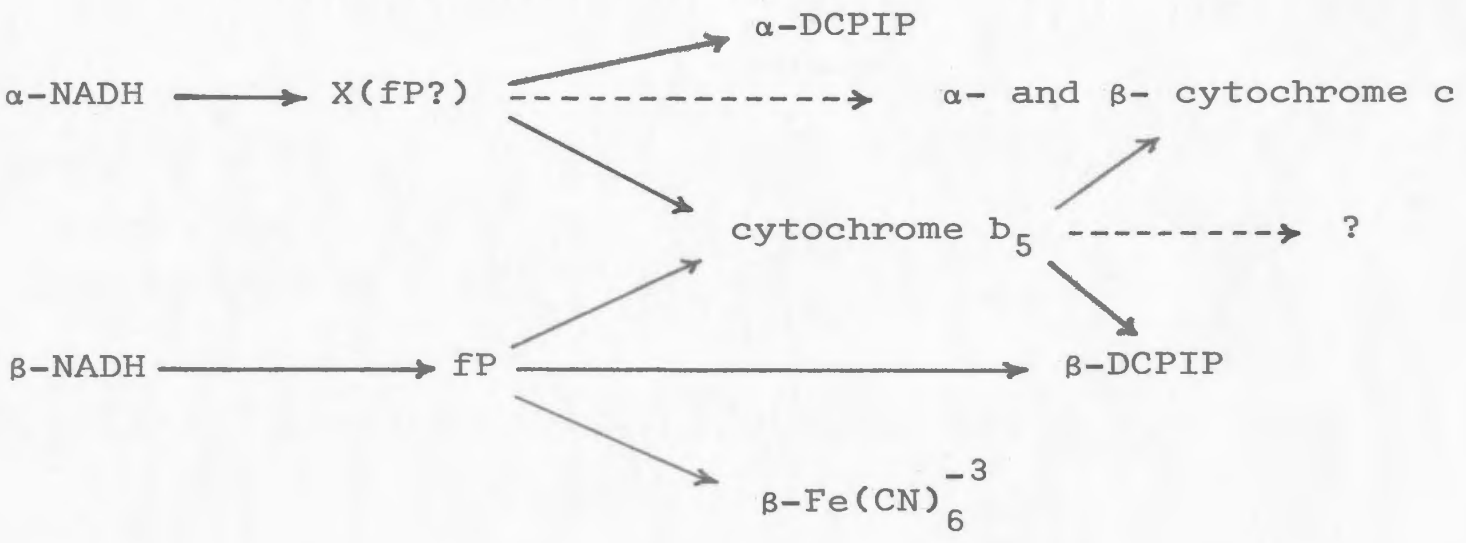
TAKESUE and OMURA[36,52] concluded that the NADH-$Fe(CN)_6^{-3}$ reductase activity of their purified NADH-cytochrome b_5 reductase, solubilized from microsomal membranes by lysosomal digestion, represents the total NADH-$Fe(CN)_6^{-3}$ reductase activity of intact

liver microsomes. Yet, although the flavoprotein has an inherent α-NADH-linked reductase activity (Table VI) which was found to be inseparable from the corresponding β-NADH-linked reductase activity on isoelectric focusing (Fig. 14), it was nevertheless found that α-NADH is enzymatically inactive in reducing $Fe(CN)_6^{-3}$ with either the lysosomal purified NADH-cytochrome b_5 reductase (Table VI) or in the intact microsomal membrane (Table I).

In agreement with the suggestion made by TAKESUE and OMURA[52] that about half of the NADH-DCPIP reductase activity may be dependent on the interaction between NADH-cytochrome b_5 reductase with cytochrome b_5, Fig. 16 shows that indeed cytochrome b_5 will mediate the transfer of electrons from the reduced flavoprotein to DCPIP, the flavoprotein and cytochrome b_5 contributing 55% and 45% respectively to the total β-NADH-DCPIP reductase activity. On the other hand, α-NADH-DCPIP activity seems to be a different enzyme system since it was found that the addition of cytochrome b_5 has no effect on the α-NADH-DCPIP activity. This may well indicate, together with the differences noted between the NADH-cytochrome b_5 reductase purified by snake venom-solubilization and the NADH-cytochrome b_5 reductase purified by lysosomal solubilization, that there exists two forms of cytochrome b_5 reductase within the microsomal membrane.

(i) Model System I

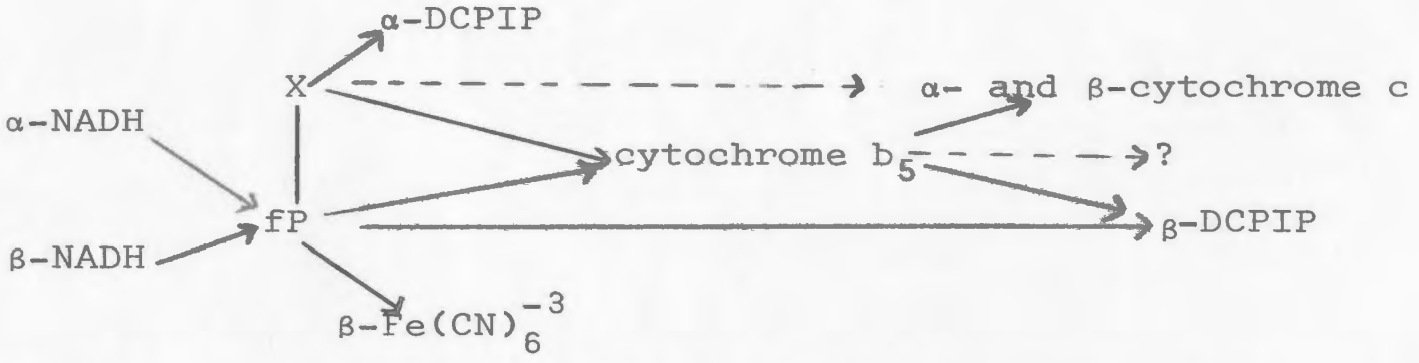
The most plausible interpretation of the information gathered with the purified lysosomal NADH-cytochrome b_5 reductase, as well as that obtained with the reconstituted system (Figs. 16 and 17), may well imply the existence of a microsomal component, perhaps a flavoprotein, accepting electrons from α-NADH and reducing microsomal cytochrome b_5. This concept is diagrammatically illustrated in Scheme C.



Scheme C. Schematic representation of microsomal electron transport illustrating the hypothetical relationship of α-NADH interaction (Model System I).

(ii) Model System II

In that the purified NADH-cytochrome b_5 reductase solubilized by snake venom was originally prepared from calf liver microsomes and that the purified NADH-cytochrome b_5 reductase prepared by lysosomal digestion was from rat liver microsomes, the possibility exists that the differences noted may reflect differences inherent in the enzyme source, or in the method of solubilization rather than differences of the two enzymes within the same tissue, a single flavoprotein accommodating the conformational differences of α- and β-NADH. However, the total microsomal NADH-$Fe(CN)_6^{-3}$ reductase activity may be accounted for by NADH-cytochrome b_5 reductase, purified according to the method of TAKESUE and OMURA; the purified flavoprotein having an inherent α-NADH-linked reductase activity but no detectable α-NADH-$Fe(CN)_6^{-3}$ activity. It is therefore still necessary to postulate a microsomal component (X) which accounts for the α-NADH-linked reductase activity. This concept is represented in Scheme D.

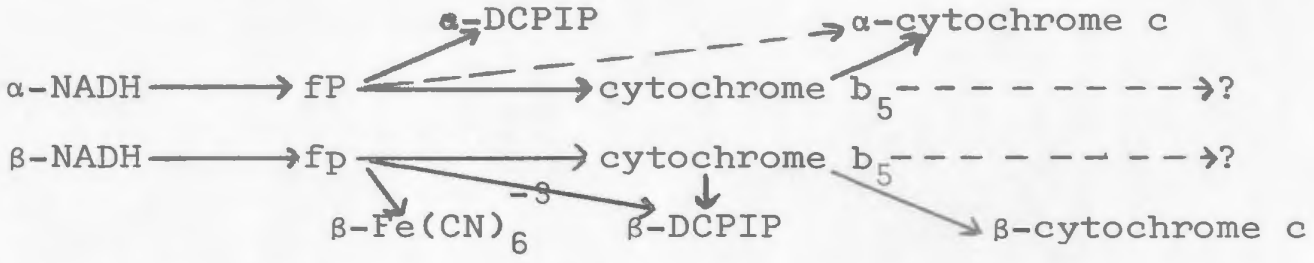


Scheme D. Schematic representation of microsomal electron transport illustrating the hypothetical relationship of α-NADH interaction.

B. Purification of Cytochrome b_5 -

(i) Model System III

Interestingly, in support of and consistent with the hypothesis of the existence of two forms of NADH-cytochrome b_5 reductase, a number of workers have isolated two separable cytochrome b_5 species in their purified preparations of the cytochrome[97-100]. Equally important, the two cytochrome b_5 species were found to have identical spectra[97] but differ in molecular weight[100], in length of their peptide chains[97], and in their crystalline structure[98]. The isolation of the two forms of cytochrome b_5 implies either that multiple forms normally exist in the endoplasmic reticulum or that they are formed as a result of peptide bond cleavage during the isolation procedure. Although neither hypothesis has yet been accepted, the possibility that multiple forms of cytochrome b_5 exist would complement the evidence presented here for the heterogeneity of the microsomal electron transport system. It may well be for example, that α- and β-NADH donate reducing



Scheme E. Schematic representation of microsomal electron transport illustrating the hypothetical relationship of α-NADH interaction to the possible existence of two forms of cytochrome b_5 (Model System III).

equivalents to separate electron transport chains as has been diagrammatically illustrated in Model System III, shown in Scheme E.

III. Occurrence and Biological Significance of the Enzymatic Oxidation of α-NADH

While the enzymatic oxidation of α-NADH has been characterized for hepatic microsomes, several other enzyme systems of mammalian origin catalyze the transfer of electrons from α-NADH to appropriate electron acceptors. Indeed, the enzymatic oxidation of α-NADH by DCPIP and cytochrome c appeared highest in pig adrenal glands and in lesser degrees in rat liver, heart, and kidney tissue (Tables II and III).

IMAI et al[75], in accordance with the data reported by STRITTMATTER[76], have found that the typical heart microsomal fraction contains high concentrations of flavins but only very low concentrations of hemoproteins. Likewise, it seems characteristic of heart microsomes to exhibit only nominal α-NADH reductase activity (Tables III and IV). OKAMOTO et al[32] have purified from the heart tissue cytosol a dehydrogenase activity which was found to accept either α- or β-NADH or NADPH and because of its inhibition by 10^{-9} M dicumerol, is likely to be "DT diaphorase". Whilst OKAMOTO[32] observed that the mitochondrial NADH-dehydrogenase was found to be specific for β-NADH, the presence of a rat liver mitochondrial α-NADH oxidase system which is inhibited by cyanide or azide but

insensitive to inhibition by amytal, rotenone, and antimycin A was reported. This electron transfer system, however, appears to be independent of the well known system coupled to phosphorylation since the latter system is sensitive to the above inhibitors.

In contrast to the low α-NADH-linked reductase activity of heart microsomes, adrenal cytosol exhibited the unique feature of not only possessing the most active α-NADH DCPIP reductase activity of the tissues and subcellular fractions studied, but unexpectedly contained the highest α-NADH-cytochrome c reductase activity and exemplified the only instance of the α-NADH mediated reduction of $Fe(CN)_6^{-3}$. In that the cytosol of other tissues studied exemplified only slight α-NADH-cytochrome c reductase activity, and in view of the fact that 95% of the "DT diaphorase" of the cell is found in the cytosol[80] with cytochrome c being inactive as an electron acceptor[78,79], it was surprising to find the localization of the α-NADH-cytochrome c reductase in this subcellular fraction. It is not known whether the oxidation of α-NADH in the adrenal cytosol is dependent upon the "diaphorase" or is a manifestation of some unknown enzyme activity. The former possibility seems unlikely however in view of its electron acceptor specificity. The

possibility exists, however, that the high adrenal α-NADH reductase activity may be reflective of yet unknown enzyme(s) associated in the steroidogenesis of this tissue. While KAPLAN et al[1] originally found α-NAD^+ to comprise 10 - 15% of purified commercial NAD^+ preparations which were prepared from either liver or yeast, NAKANISHI et al have reported the existence of α-$NADP^+$ in the isthmus of hen oviducts in a molar ratio of nearly 1:1 to that of β-$NADP^+$. Although the existence of the α-anomer of pyridine nucleotides has therefore been reported, its biological significance in living cells still remains obscure. Neither has the enzymatic reduction of α-$NAD(P)^+$ nor the enzymatic oxidation of α-NADPH been yet observed. Nevertheless, by investigating the factors responsible for the microsomal enzymatic oxidation of α-NADH, this present report has strongly implicated that the reductase ability of α-NADH is more than a manifestation of a broad electron donor specificity of the microsomal β-NADH-linked reductase system. Rather, evidence has been presented which suggests that the enzymatic oxidation of α-NADH is a manifestation of hitherto unknown isoenzyme reductases associated with the microsomal electron transport system and may provide the first clue in understanding the biological significance of α-NADH.

FOOTNOTES

(A) Throughout this thesis NADH and NAD^+ are often used without any reference to either the α- or ß-isomer. This is usually done when either direct or indirect reference has been made to the literature and conclusions cited or discussed which are based on reports that have not stated which isomer has been used.

(B) In an attempt to eliminate substrate diffusion barriers and contamination by "DT-diaphorase", the intact microsomal vesicles were sonicated as described in Methods. Relevant to the latter is the fact that the microsomal enzymatic oxidation was found to be unaffected by 10^{-9} M dicumerol.

REFERENCES

1. N.O. KAPLAN, M.M. CIOTTI, F.E. STOLZENBACH and N.R. BACHUR, J. Am. Chem. Soc., 77 (1955) 815.
2. N.O. KAPLAN, S.P. COLOWICK and A. NASON, J. Biol. Chem., 191 (1951) 473.
3. S.P. COLOWICK, N.O. KAPLAN and M.M. CIOTTI, J. Biol. Chem., 191 (1951) 447.
4. R.H. SARMA, V. ROSS and N.O. KAPLAN, Biochem., 7 (1968) 3052.
5. N.O. KAPLAN, Enzymes, 3 (1960) 105.
6. D.W. MILES, D.W. URRY and H. EYRING, Biochem., 7 (1968) 2333.
7. L.J. HAYNES, N.A. HUGHES, G.W. KENNEY and A. TODD, J. Chem. Soc. (1957) 3727.
8. S. SUZUKI, K. SUZUKI, T. IMAI, N. SUZUKI and S. OKUDA, J.Biol. Chem., 240 (1965) PC554.
9. K. SUZUKI, H. NAKANO and S. SUZUKI, J. Biol. Chem., 242 (1967) 3319.
10. J. PREISS and P. HANDLER, J. Biol. Chem., 233 (1958) 493.
11. J. IMSANDE, J. Biol. Chem., 236 (1961) 1494.
12. Y. NAKANISHI, S. SHIMIZU, N. TAKAHASHI, M. SUGIYAMA and S. SUZUKI, J. Biol. Chem., 242 (1967) 967.
13. C. RICCI, U. PALLINI and P. MARTELLI, Biochem. Biophys. Res. Commun., 19 (1965) 296.
14. J. PREISS and P. HANDLER, J. Am. Chem. Soc., 79 (1957) 1514.
15. R.U. LEMIEUX and J.W. LOWN, Can. J. Chem., 41 (1963) 889.

16. G. WEBER, Nature, 180 (1957) 1409.

17. S. SHIFRIN and N.O. KAPLAN, Nature, 183 (1959) 1529.

18. W.L. MEYER, F.R. MAHLER and R.H. BAKER, Biochim. Biophys. Acta, 64 (1962) 353.

19. O. JARDETZKY and N.G. WADE-JARDETZKY, J. Biol. Chem., 241 (1966) 85.

20. D.W. MILES and D.W. URRY, J. Biol. Chem., 243 (1968) 4181.

21. S.F. VELICK, J. Biol. Chem., 233 (1958) 1455.

22. S.F. VELICK in W.D. McELROY and H.B. GLASS, Light and Life, John Hopkins, Baltimore, 1961 p. 108.

23. N.O. KAPLAN in F.E.W. WOLSTENHOLME and C.M. O'CONNOR, Steric Course of Microbiological Reactions. Churchill, London, 1959, p. 37.

24. R.H. SARMA and N.O. KAPLAN, Biochem., 9 (1970) 539.

25. W.A. CATTERALL, D.P. HOLLIS and C.F. WALTERS, Biochem., 8 (1969) 4032.

26. G. CZERLINSKI and F. HOMMES, Biochim. Biophys. Acta, 79 (1964) 46.

27. J.M. SIEGEL, G.A. MONTGOMERY and R.M. BOCK, Arch. Biochem. Biophys., 82 (1959) 288.

28. S. SHIFRIN and N.O. KAPLAN, Advan. Enzymol. 22 (1960) 337.

29. S. SHIFRIN and N.O. KAPLAN in W.D. McELROY and H.B. GLASS, Light and Life, John Hopkins, Baltimore, 1961 p. 144.

30. W. GINGRICH and F. SCHLENK, J. Bact. 47 (1944) 535.

31. W.M. CLARK, N.O. KAPLAN and M.D. KAMEN, Bacteriol. Rev., 19 (1955) 245.
32. H. OKAMOTO, A. ICHIYAMA and O, KAYAISHI, Arch. Biochem. Biophys., 118 (1967) 110.
33. C.F. STRITTMATTER and E.G. BALL, Proc. Natl. Acad. Sci. U.S., 38 (1952) 19.
34. O.H. LOWRY, N.J.ROSEBROUGH, A.L. FARR and R.J. RANDALL, J. Biol. Chem., 193 (1951) 265.
35. H.R. MAHLER, Methods Enzymol., 2 (1955) 688.
36. S. TAKESUE and T. OMURA, J. Biochem., 67 (1970) 259.
37. B.F. VAN GELDER and E.C. SLATER, Biochim. Biophys. Acta, 58 (1962) 593.
38. E.P. STEYN-PARUE and H. BEINERT, J. Biol. Chem., 233 (1958) 843.
39. K.A. SCHELLENBERG and L. HELLERMAN, J. Biol. Chem., 231 (1958) 547.
40. P. STRITTMATTER and S.F. VELICK, J. Biol. Chem., 221 (1956) 253.
41. A.K. KOLI, C. YEARBY, W. SCOTT and K.O. DONALDSON, J. Biol. Chem., 244 (1969) 621.
42. A. KORNBERG, Methods Enzymol., 1 (1955) 441.
43. T. OMURA and R. SATO, J. Biol. Chem., 239 (1964) 2370.
44. B. SEDGWICK and G. HÜBSCHER, Biochim. Biophys. Acta, 106 (1965) 63.
45. S.P. COLOWICK, J. van EYS and J.H. PARK, Comp. Biochem., 14 (1966)1.

46. N.A. HUGHES, G.W. KENNER and A.R. TODD, J. Chem. Soc., (1957) 3733.

47. R.L. LESTER and S. FLEISCHER, Biochim. Biophys. Acta, 47 (1961) 358.

48. P.D. JONES and S.L. WAKIL, J. Biol. Chem., 342 (1967) 5267.

49. C. WOENCKHAUS and P. ZUMPE, Biochem. Z., 343 (1965) 326.

50. T. OMURA and S. TAKESUE, J. Biochem., 67 (1970) 249.

51. S. TAKESUE and T. OMURA, Biochem. Biophys. Res. Commun., 30 (1968) 723.

52. S. TAKESUE and T. OMURA, J. Biochem., 67 (1970) 267.

53. A.Y.H. LU and M.L. COON, J. Biol. Chem., 243 (1968) 1331.

54. O. VERSTERBERG and H. SEVESSON, Acta Chem. Scand., 20 (1966) 820.

55. P. STRITTMATTER and S.F. VELICK, J. Biol. Chem. 221 (1956) 277.

56. T. HARA and S. MINAKAMI, J. Biochem., 69 (1971) 317.

57. T. STRITTMATTER and S.F. VELICK, J. Biol. Chem., 228 (1957) 785.

58. P. STRITTMATTER, J. Biol. Chem., 233 (1958) 748.

59. P. STRITTMATTER, J. Biol. Chem., 234 (1959) 2661.

60. M.R. FRANKLIN and R.W. ESTABROOK, Arch. Biochem. Biophys., 143 (1971) 318.

61. C. LITTLE, T. SANNER and A. PIHL, Europ. J. Biochem., 8 (1969) 229.

62. C. LITTLE, T. SANNER and A. PIHL, Biochim. Biophys. Acta, 178 (1969) 83.

63. T. SANNER and A. PIHL, Scand. J. Clin. Lab. Invest. Suppl. 106, 22 (1968) 53.

64. B. KUYLENSTIERNA, P.G. NICHOLLS, S. HOUMÖLLER and L. ERNSTER, Europ. J. Biochem.,12 (1970) 419.

65. H.-U. SCHULZE, H. GALLENKAMP, and Hj. STAUDINGER, Hoppe-Seyler's Z., Physiol. Chem. 351 (1970) 809.

66. E.R. REDFEARN, P.A. WHITTAKER and J. BURGOS, in T.E. KING, H.S. MASON and M. MORRISON, Oxidases and Related Redox Systems, Wiley, New York, 1965, p. 943.

67. A.L. TAPPEL, Biochem. Pharmacol., 3 (1960) 289.

68. P.A. WHITTAKER and E.R. REDFEARN, Biochem. J., 88 (1963) 15 p.

69. N. PENN and B. MACKLER, Biochim. Biophys. Acta, 27 (1958) 539.

70. D.H. MACLENNAN, A. TZAGOLOFF and D.G. MCCONNELL, Biochim. Biophys. Acta, 131 (1967) 59.

71. B. MACKLER, Methods Enzymol., 10 (1967) 551.

72. J.L. GAYLOR and H.S. MASON, J. Biol. Chem., 243 (1968) 4966.

73. D.M. ENGELMAN and H.J. MOROWITZ, Biochim. Biophys. Acta, 150 (1968) 385.

74. W.S. BONT, P. EMMELOT and H. VAZDIAA, Biochim. Biophys. Acta, 173 (1969) 389.

75. K. IMAI, T. OMURA and R. SATO, J. Biochem., 60 (1966) 274.

76. C.F. STRITTMATTER, Arch. Biochem. Biophys., 102 (1963) 293.

77. O.H. LOWRY, J.V. PASSONNEAU and M.K. ROCK, J. Biol. Chem., 236 (1961) 2756.

78. L. ERNSTER, L. DANIELSON and M. JUNGGREN, Biochim. Biophys. Acta, 58 (1962) 171.

79. C. MARTIUS, Enzymes, 7 (1963) 517.

80. L. ERNSTER, Methods Enzymol., 10 (1967) 309.
81. L. ERNSTER and S. ORRENIUS, Federation Proc., 24 (1965) 1190.
82. A.H. CONNEY, Pharmacol. Rev., 19 (1967) 317.
83. P. STRITTMATTER, Federation Proc., 24 (1965) 1156.
84. P.W. HOLLOWAY, Biochem., 10 (1971) 1556.
85. P. STRITTMATTER, Enzymes, 8 (1963) 113.
86. P. SIEKEVITZ, Federation Proc., 24 (1965) 1153.
87. C.R. SHAW, Science, 149 (1965) 936.
88. R.L. HUNTER and C.L. MARKERT, Science, 125 (1957) 1294.
89. F. SHAPIRA, J.C. DREYFUS and G. SCHAPIRA in D. SHUGAR, Enzymes and Isoenzymes, Academic Press, London, 1970, p305.
90. R.W. ESTABROOK, Biochim. Biophys. Acta, 60 (1962) 236.
91. P. STRITTMATTER, J. Biol. Chem., 234 (1959) 2665.
92. P. STRITTMATTER, J. Biol. Chem., 236 (1961) 2336.
93. A.H. PHILLIPS and R.G. LANGDEN, J. Biol. Chem., 237 (1962) 2652.
94. S. ORRENIUS, A. BERG and L. ERNSTER, Europ. J. Biochem., 11 (1969) 193.
95. A. ITO and R. SATO, J. Cell Biol., 40 (1969) 179.
96. T. OMURA, P. SIEKEVITZ and G.E. PALADE, J. Biol. Chem., 242 (1967) 2389.
97. P. STRITTMATTER and J. OZOLS, J. Biol. Chem., 241 (1966) 4787.
98. T. KAJIHARA and E. HAGIHARA, J. Biochem., 63 (1968) 453.

99. J.R. SARGENT and B.P. VADLAMUNDI, Biochem. J., 107 (1968) 839.

100. A. ITO and R. SATO, J. Biol. Chem., 243 (1968) 4922.

101. T.P. SINGER, Comp. Biochem., 14 (1966) 127.

102. L. ERNSTER, P. SIEKEVITZ and G.E. PALADE, J. Cell Biol., 15 (1962) 541.

103. L. ERNSTER and L. JONES, J. Cell Biol., 15 (1962) 563.

104. G.L. SOTTOCASA, B. KUYLENSTIERNA, L. ERNSTER and A. BERGSTRAND, J. Cell Biol., 32 (1967) 415.

105. S.M. DUTTERA, W.L. BYRNE and M.C. GANOZA, J. Biol. Chem., 243 (1968) 2216.

106. H.-U. SCHULZE and Hj. STAUDINGER, Hoppe-Seyler's Z. Physiol. Chem., 352 (1971) 309.

107. P.D. JONES, P.W. HOLLOWAY, R.O. PELUFFO and S.J. WAKIL, J. Biol. Chem., 244 (1969) 744.

108. M.C. GANOZA and W.L. BYRNE, Federation Proc., 22 (1963) 535.

109. G. DALLNER, P. SIEKEVITZ and G.E. PALADE, Biochem. Biophys. Res. Commun., 20 (1965) 142.

110. G. DALLNER, P. SIEKEVITZ and G.E. PALADE, J. Cell Biol., 30 (1966) 97.

111. P. STRITTMATTER, J. Biol. Chem., 240 (1965) 4481.

112. D.E. GREEN and J.F. PERDUE, Proc. Nat. Acad. Sci. U.S., 55 (1966) 1295.

PUBLICATIONS ARISING FROM THIS WORK -

1. Evidence for a separate α-NADH flavoenzyme in the endoplasmic reticulum. P.J. O'Brien and P. Macaluso, 8th International Congress of Biochemistry, Symposium 8 (1970).

2. Phospholipid dependence of microsomal quinone reductases. P.J. O'Brien and P. Macaluso. Proceedings of the Canadian Federation of Biological Societies, 13 (1970).