THE PROPERTIES AND FUNCTION OF NADH-SEMIDEHYDROASCORBATE REDUCTASE IN MICROSOMAL AND MITOCHONDRIAL FRACTIONS

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ABSTRACT

The involvement of cytochrome b_5 reductase and cytochrome b_5 in the activity of semidehydroascorbate ($AH^{\cdot}$) reductase has been investigated by comparing the properties of $AH^{\cdot}$ reductase to those of ferricyanide reductase and rotenone insensitive cytochrome c reductase.

The $AH^{\cdot}$ and cytochrome c reductases were localized in the microsomal and outer mitochondrial membrane fractions, a localization which coincides with that of cytochrome b_5 reductase.

The $AH^{\cdot}$ reductase showed a Km value for NADH of 1 μM and a pH optimum of 7.4, which agrees favourably with that of ferricyanide and cytochrome c reductases. The $AH^{\cdot}$ and cytochrome c reductases showed a similar sensitivity to sulphydryl inhibition as ferricyanide reductase and all three enzymes were protected from sulphydryl inhibition by NADH.

Highly purified cytochrome b_5 reductase did not show any $AH^{\cdot}$ or cytochrome c reductase activities. When purified cytochrome b_5 was added to the system, both $AH^{\cdot}$ and cytochrome c reductase activities could however be demonstrated. DOC solubilization of microsomes, followed by DEAE cellulose chromatography, showed that $AH^{\cdot}$ and cytochrome c reductases were resolved together and separated from a major ferricyanide reductase peak.

When cytochrome b_5 was released from intact mitochondria and microsomes by trypsin treatment, the cytochrome c reductase was inactivated more effectively than the AH• reductase.

The AH• and cytochrome c reductase showed similar lipid dependence. Both enzymes were inactivated by acetone extraction and reactivated by lipid micelles containing lecithin and oleic acid. Both enzymes showed similar sensitivity towards detergent inhibition.

It is concluded that cytochrome b_5 reductase and cytochrome b_5 are components of the membrane bound AH• reductase. Certain dissimilarities observed between the AH• and cytochrome c reductases could be explained by an additional component of the AH• reductase not yet identified, which is not shared by the cytochrome c reductase.

Possible functions of the AH• reductase have also been discussed. The enzyme may play a role in maintaining high levels of NAD in the cells of tissues that contain high concentrations of ascorbic acid, such as steroid producing tissues. The AH• reductase may also be an important protector of mitochondrial and microsomal membranes by reducing the semidehydroascorbate radical which is believed to be a powerful catalyst of lipid peroxidation. Thirdly, the enzyme probably has an important function in maintaining the ascorbate of the cell in a reduced state. Ascorbate may function in the cell as an enzyme cofactor.

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CONTENTS

page

ABBREVIATIONS

The following abbreviations have been used in this thesis:

AH_2	ascorbic acid
$AH^{\bullet}$	semidehydroascorbic acid
A	dehydroascorbic acid
ACTH	adrenocorticotropic hormone
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
p-CMB	p-chloromercuribenzoate
DEAE	diethylamino ethyl cellulose
DOC	deoxycholate, sodium salt
EDTA	(ethylenedinitrilo) tetraacetate, disodium salt
FeCN	ferricyanide
GSH	glutathione
GSSG	oxidized glutathione
LH	luteotropic hormone
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
O.D.	optical density
TBA	thiobarbituric acid
TTFA	thenoyltrifluoroacetone

INTRODUCTION

Ascorbic acid occurs as such and in its oxidized form in nearly all plant and animal tissues. The concentrations of ascorbic acid in various animal tissues differ considerably, and with the highest concentrations being found in the adrenal cortex, anterior pituitary, eye lens, and liver (1). Lowry (2) however reported that the average values of ascorbic acid in adrenal, liver and kidney of 40 animal species which synthesize their own ascorbic acid, fall within a reasonably narrow range for each tissue:

	mg ascorbic acid per 100 g tissue		
	adrenal	liver	kidney
40 animal species			
average	140 (12 mM)	23 (2 mM)	12 (1 mM)
range	104-295	18-40	4-25
guinea pig on cabbage diet	135	28	12

Guinea pigs and primates lack two enzymes (D-glucuronolactone reductase and L-gulonolactone oxidase) essential for the synthesis of ascorbic acid (3,4) and rely on dietary sources of the vitamin. Tissue concentrations of ascorbic acid found in guinea pig fed on cabbage diet are close to the average figures reported for other animals, and there is good evidence that human tissues have the same range of ascorbic acid concentrations as those of guinea pig (2).

When dietary intake in guinea pig is lowered below the minimal requirement of the vitamin, tissue levels of ascorbic acid are affected and eventually clinical symptoms of deficiency are observed. In guinea pig minimal signs of scurvy are observed when tissue levels are about one-third of the normal values in adrenal, liver, and kidney, whereas in severe scurvy the ascorbic acid concentration has dropped to less than one-twentieth of the normal values in the same tissues (2).

The guinea pig is highly susceptible to lack of vitamin C and the pathological alterations that appear are known to be similar to those found in human scurvy. The most outstanding symptoms are tendency to hemorrhage and slow wound healing. Poor dentine formation leads to abnormal tooth development in the guinea pig and in man loosening of teeth and spongy gums are observed in scurvy. Painful joints, edema, and anemia are also found in advanced stages of scurvy. All signs and symptoms of scurvy are rapidly reversed by feeding ascorbic acid or its oxidized form, dehydroascorbic acid. A few analogues of ascorbic acid also possess antiscorbutic activity but are less effective (5).

The function of ascorbic acid

1. Ascorbic acid in the adrenal gland

As mentioned earlier, the adrenal gland has the highest ascorbic acid concentration of all tissues in the body, of the order of 20 mM. Bourne (6) and Giroud and Leblond (7) established by histochemical studies that the adrenal cortex

was the main site of ascorbic acid, although the presence of ascorbic acid in the medulla was reported by Harris and Ray (8).

Sayers et al (9) originally reported the observation that adrenal ascorbic acid decreased in response to stress or ACTH stimulation. Slusher and Roberts (10) found that ascorbic acid lost from the adrenal gland on ACTH stimulation of hypophysectomized rats could be quantitatively recovered from the adrenal vein, and that this loss preceded the secretion of corticosteroids by this gland. Harding and Nelson (11) demonstrated that while ascorbic acid decreased 50% under maximal ACTH stimulation of hypophysectomized rats, the concentration of glutathione in the adrenal gland remained unchanged. No detectable amount of either of the oxidized forms, dehydroascorbic acid or oxidized glutathione was observed in adrenals from ACTH stimulated or control animals.

Kitabchi (12, 13) reported an inhibition by ascorbate of steroid C21-hydroxylase in beef adrenal microsomes associated with lipid peroxidation and an inhibition of beef adrenal mitochondrial 11β-hydroxylase activity which was not associated with lipid peroxidation. He advanced the hypothesis that ACTH, by stimulating the discharge of ascorbic acid from the adrenal tissue, might reverse the inhibition of these enzymes and thus allow increased steroid biosynthesis (14). Harding et al (15), contradicting Kitabchi's findings, showed that ascorbic acid could support steroid 11β-hydroxylation in vitro. They demonstrated that ascorbic acid is capable of providing reducing equivalents to the steroid oxygenase system, and conclude that

an ascorbate-supported reversal of electron flow, coupled to steroid hydroxylation, exists in adrenocortical mitochondria. ACTH may then exert its rapid effects on steroid biosynthesis through making ascorbic acid available to the mitochondrial electron transport chain.

Similar studies to those discussed above have been done on the rat ovary. The high concentration of ascorbate in the corpus luteum is decreased in response to LH stimulation and this is followed by an increase in steroidogenesis (16, 17). Sulimovici and Boyd (18) reported an inhibitory effect of ascorbate on the cholesterol sidechain cleavage enzymes in the mitochondria of the rat ovary. Their evidence supports the theory that increased steroid production is facilitated by LH stimulated release of ascorbic acid from the endocrine tissue.

2. Lipid peroxidation initiated by ascorbic acid

Ascorbic acid may be involved in permeability changes or breakdown of intracellular membranes in vivo. Peroxidation of unsaturated lipids occurs rapidly in vitro when tissue particulate fractions are exposed to oxygen, and Barber (19) showed that ascorbic acid is the cofactor for peroxidation in homogenates.

Hunter et al (20) have shown that mitochondrial swelling, which is initiated by ascorbate is associated with peroxidation of mitochondrial membrane lipids. Hoffsten et al (21) using ascorbate, found a close correlation between

rate of production of TBA (thiobarbituric acid) positive substances with the rate of swelling, and concurrent with these changes was the consumption of increased amount of oxygen. It was concluded that the onset of lipid peroxidation in the mitochondrial membranes leads to permeability changes which result in mitochondrial swelling.

Robinson (22) found that a decrease in the turbidity of the vesicles of brain microsomal fractions, in the presence of ascorbate or ascorbate and Fe^{+2}, was accompanied by lipid peroxide formation as measured by the TBA reaction and oxygen uptake.

Wills (23) reported lipid peroxide formation in liver microsomes initiated by ascorbate or NADPH but not NADH. His evidence suggests that the ascorbate induced peroxidation may involve part of the NADPH electron transport chain, which is normally concerned with hydroxylations but can be switched to oxidize unsaturated lipids in the endoplasmic reticulum.

Lipid peroxidation in vitro in vitamin E deficiency as tested by the TBA test has been reported in numerous animal tissues (24, 25, 26). Kitabchi and Williams (27) found that in vitamin E deficiency the extent of adrenal lipid peroxidation in vitro was about 10 times higher than in any other tissue tested. Diminution of adrenal ascorbate in the deficient group by various methods, reduced this in vitro lipid peroxidation. It was suggested that with gradual diminution of tocopherol, the presence of the high ascorbic acid concentration in the adrenal supernatant solution contributed to this high in vitro lipid peroxidation.

3. Oxygenase reactions involving ascorbic acid.

Some of the metabolic disorders observed in scurvy have been directly associated with enzymes that require ascorbate as a cofactor. Proline hydroxylase and dopamine-β-hydroxylase have both been shown to require ascorbate as a cofactor, whereas the ascorbate requirement of p-hydroxy-phenylpyruvic acid oxidase appears to be nonspecific.

Early studies by several workers (28) established that formation and maintenance of normal collagen in the animal required L-ascorbic acid. Recently Kivirikko and Prockop (29) showed that hydroxylations of proline and lysine residues in protocollagen required molecular oxygen, ascorbate, ferrous iron and α-ketoglutarate as cofactors. Hutton et al (30) using peptidylproline as a substrate for the enzyme, showed that ascorbate could be partially replaced by pteridines, whereas no compounds were found that could replace the other cofactors. These findings might explain the observation that small amount of collagen may be synthesized in the absence of demonstrable ascorbic acid, while rapid synthesis is dependent on its presence (31).

Dopamine-β-hydroxylase catalyses the conversion of dopamine to norepinephrine, a key reaction in the formation of the neurohormones, norepinephrine and epinephrine. The enzyme is a copper containing monooxygenase and has been found in adrenal medulla and brain (32). While the enzyme is relatively nonspecific for the substrate, the hydroxylation reaction which uses molecular oxygen, is specific for ascorbate. The

ascorbate was shown to reduce oxidized copper in the enzyme, which then subsequently reacted with oxygen. The enzyme was stimulated markedly by certain dicarboxylic acids such as fumarate and α-ketoclutarate.

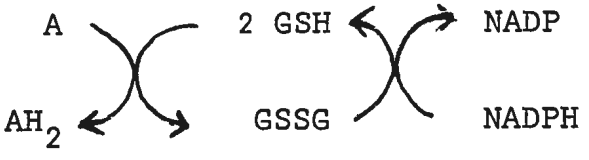
A third enzyme, thymine-7-hydroxylase (33) found in Neurospora, has been reported to require ascorbate, ferrous iron and α-ketoglutarate as cofactors. The significance of the dicarboxylic acid control of these three ascorbate requiring hydroxylating enzymes is not known.

Ascorbic acid has long been shown to be essential for normal tyrosine oxidation. Scorbutic guinea pigs were found to excrete increased amounts of p-hydroxyphenylpyruvic and p-hydroxyphenyllactic acids in response to an administered dose of tyrosine (34), and the excretion of these acids ceased if ascorbic acid was also administered. The enzyme p-hydroxyphenylpyruvic acid oxidase was found to require ascorbic acid for normal activity; however, studies with purified enzyme showed that ascorbate is not an essential factor (35) but probably acts in crude systems as a reducing agent to protect the enzyme from inactivation. Similarly tryptophan oxygenase has been reported to require ascorbic acid for maximal activity (36).

Oxidation - reduction reactions involving ascorbic acid

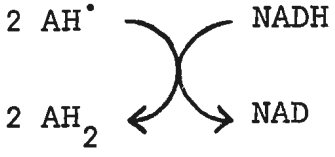
Almost all terminal oxidases in plant and animal tissues are capable of directly or indirectly catalysing the oxidation of ascorbic acid. These enzymes include ascorbic acid oxidase, cytochrome oxidase, phenolase, peroxidase and laccase. In addition, the oxidation of ascorbate is readily induced under aerobic conditions by many metal ions, hemochromogens and quinones. The oxidation product of these reactions is either dehydroascorbic acid or the extremely active radical, semidehydroascorbic acid.

Parallel with these oxidizing systems, enzyme systems capable of reducing both forms of the oxidized vitamin, have been demonstrated in plant tissue. An enzyme, dehydroascorbate reductase, uses glutathione as the reductant,



and oxidized glutathione is subsequently regenerated by the enzyme glutathione reductase at the expense of NADPH.

A second reduction mechanism found in plants, yeast and bacteria (37) involves NADH as the reductant and semidehydroascorbate as the active form of the oxidized ascorbate:



The enzyme, semidehydroascorbate reductase, is a flavoenzyme and has been purified 60-fold from yeast (38). A similar enzyme mechanism was described by Staudinger and coworkers in animal tissues (39, 40, 41). A NADH-semidehydroascorbate oxidoreductase (EC 1.6.5.4) was localized in the microsomal and mitochondrial fractions of adrenal, kidney and liver. The enzyme caused an oxidation of NADH in the presence of ascorbate and oxygen and the reaction was greatly stimulated on addition of ascorbic acid oxidase. Dehydroascorbate could not replace ascorbate in the system, but greatly enhanced the reaction. Electron spin resonance spectroscopy studies later showed that a semidehydroascorbate was the effective electron acceptor in the reaction (42), and was formed from ascorbate by the action of ascorbic acid oxidase or by reaction with dehydroascorbate.

The physiological significance of these oxidation-reduction reactions is not clear at the present moment. In plants ascorbate has been shown to play a role in photo-oxidation. Trebst et al (43) showed that ascorbate as well as cysteine, hydroquinone and other compounds could compete with and override the photo-oxidation of H_2O in intact chloroplasts. Recently Böhme and Trebst (44) reported a stoichiometry of ATP formation, ascorbate oxidation and O_2 uptake of 0.5:1:1 (P/2e= 0.5) in intact chloroplasts. Yamashita and Butler (45) measured the same P/2e = 1 with fresh chloroplasts, so there seems to be some confusion as to where the ascorbate

might enter the electron transport sequence in system II of photo-oxidation.

In animal tissues, Devine and Rivers (46) have demonstrated a possible function for ascorbate and semidehydroascorbate reductase in the intracellular oxidation of NADH. They found a marked shift in the NAD/NADH ratio in adrenal and placenta of scorbutic guinea pigs, although the total NAD and NADH concentrations were unaltered compared to normal animals. The activity of semidehydroascorbate reductase was unaffected in the scorbutic animals, but the decrease in tissue concentration of ascorbic acid was followed by depression of NAD/NADH ratios. The authors suggest that in steroid producing tissues this depressed ratio could lead to inhibition of the conversion of pregnenolone to progesterone, a reaction that requires NAD as a cofactor.

Properties of NADH-semidehydroascorbate reductase

Frunder et al (47) showed that reduced microsomal cytochrome b_5 was oxidized in the presence of ascorbate and ascorbate oxidase, and they postulated an electron transport mechanism in microsomes involving cytochrome b_5 reductase and cytochrome b_5 leading to semidehydroascorbate. Recently Staudinger (48) claimed that an AH$^\cdot$ reductase, an enzyme not involving cytochrome b_5 was responsible for the major part of the AH$^\cdot$ reductase activity of microsomes and only a minor part of the activity was due to cytochrome b_5 and a cytochrome b_5-AH$^\cdot$ reductase (49). Staudinger further suggested that the

$AH^{\bullet}$ reductase activity of cytochrome b_5 reductase demonstrated by Iyanagi and Yamazaki (50) represents only a very minor portion of the $AH^{\bullet}$ reductase in intact microsomes or may in fact be due to impurities in their enzyme preparation.

In the present work the involvement of cytochrome b_5 reductase and cytochrome b_5 in the reduction of semidehydroascorbate has been investigated. A comparative study of $AH^{\bullet}$ reductase and the two enzymes cytochrome b_5 reductase and rotenone insensitive cytochrome c reductase was undertaken, since the latter enzyme is believed to involve cytochrome b_5 reductase and cytochrome b_5 (78).

Possible functions of the $AH^{\bullet}$ reductase in microsomal and mitochondrial fractions have also been discussed.

CHAPTER I

THE PROPERTIES OF SEMIDEHYDROASCORBATE REDUCTASE

MATERIALS AND METHODS

Materials -

Dehydroascorbic acid was obtained from Schwarz/Mann, Orangeburg, New York. ICI America, Inc. donated Lubrol WX. All biochemicals were obtained from Sigma Chemical Co. Other materials used were of Analytical Reagent grade of purity.

Methods -

Preparation of subcellular fractions

Male albino rats (150 - 250 g) of the Sprague-Dawley strain were used for all experiments, except for the tissue distribution of AH• reductase, in which sheep tissues obtained from a local slaughter house were used. The rats were sacrificed by a blow on the head, the livers removed and washed in ice cold sucrose medium (0.3 M sucrose, 1 mM EDTA and pH adjusted to 7.5 with 1 M KOH). Subcellular fractions of liver and other tissues were prepared by the method of Sedgwick and Hübscher (51).

In most studies, freshly prepared subcellular fractions were used. Otherwise the fractions were stored at -20° and used within 2 weeks. The AH• reductase, which was the most labile of the enzymes studied, had lost about 30% of its activity at the end of a 2 week storage.

Enzyme Assays

1. Semidehydroascorbate reductase was assayed by a modification of the method of Nazemi and Staudinger (52). The oxidation of NADH was followed spectrophotometrically at 340 mμ in an assay medium containing 0.05 M potassium phosphate buffer, pH 6.9, 1 mM EDTA, 6 mM ascorbate, 6 mM dehydroascorbate, 0.3 mM KCN and 0.1 mM NADH. The assay was carried out at 37°. (For characterization of enzyme assay, see "Kinetic properties", page 25.)
2. Ferricyanide reductase was assayed at 25° by following spectrophotometrically the reduction of ferricyanide at 420 mμ. The assay medium contained 0.05 M tris-HCl buffer, pH 7.4, 1 mM EDTA, 0.1 mM NADH and 0.5 mM potassium ferricyanide.
3. NADH-cytochrome c reductase was assayed at 25° by following the reduction of cytochrome c at 550 mμ in an assay medium containing 0.5 M Tris-HCl buffer, pH 7.4, 1 mM EDTA, 0.1 mM NADH, 50 μM cytochrome c (Type III, Sigma) and when indicated 1.5 μM rotenone.
4. Malate dehydrogenase was assayed at 25° using the method of Ochoa (53).
5. Cytochrome oxidase was assayed by the method of Schnaitman et al (54) using a Clark electrode at 23°.

Protein was estimated by the method of Lowry et al (55) using bovine serum albumin as a standard.

Inhibitors

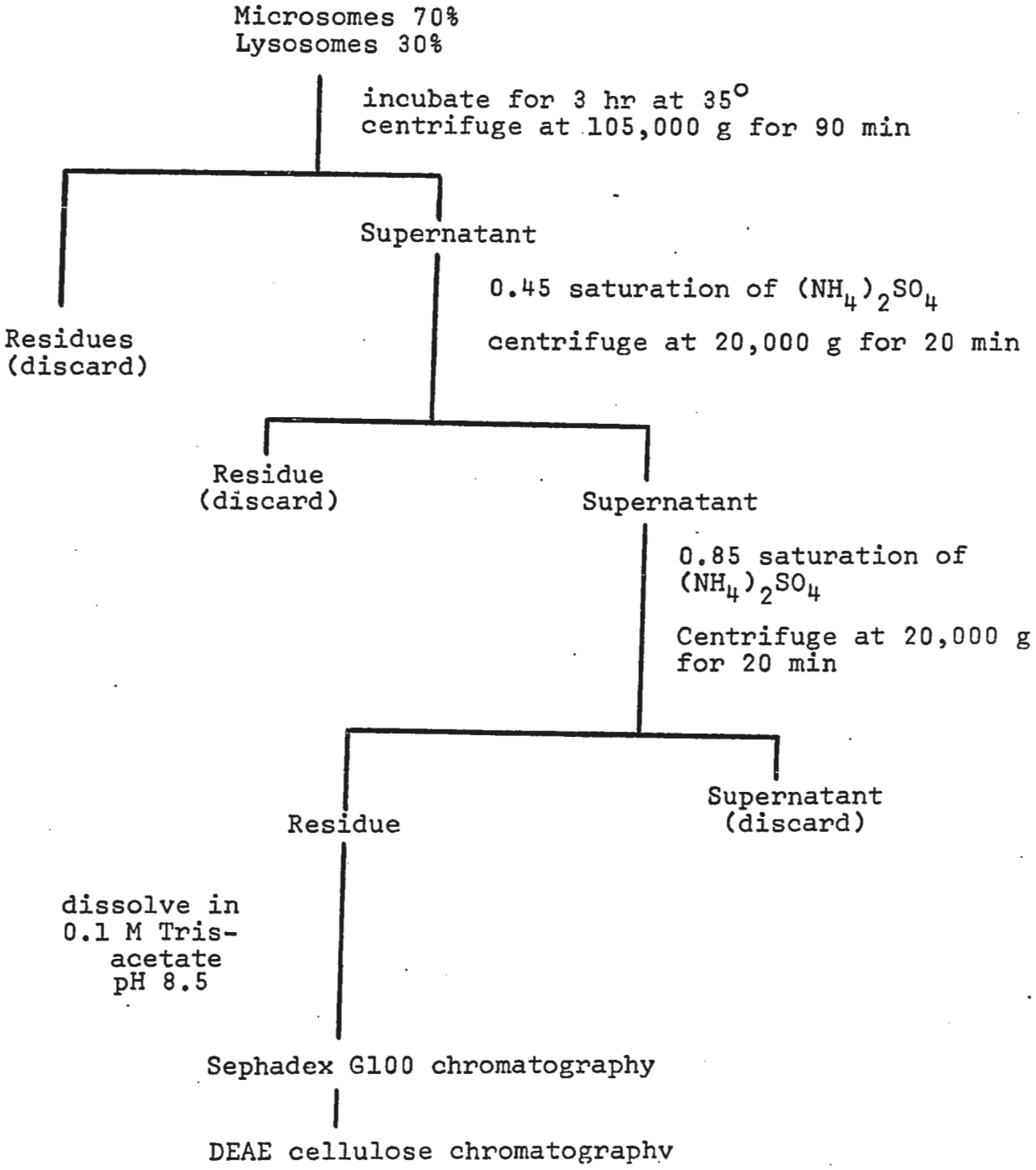
All inhibitors studied were added directly to the assay medium except p-chloromercuribenzoate. Microsomal protein (1 mg/ml) was preincubated in 50 mM Tris-HCl buffer, pH 7.5 with varying quantities of p-CMB for 10 minutes at 0°. 0.1 mM NADH was added prior to p-CMB as indicated. Suitable aliquots were taken from the preincubation mixture for enzyme assays.

Mitochondrial subfractionation

Subfractionation of the mitochondria was carried out essentially by the method of Sottocasa et al (56). After washing the mitochondria twice in 1/2 and 1/4 of the original volume of sucrose medium, they were suspended in 10mM Tris-phosphate buffer, pH 7.5 (50 mg in 7.5 ml buffer). After standing for 5 minutes at 0° the mitochondria were contracted by adding 1/3 volume of 1.8 M sucrose containing 2 mM ATP and 2 mM $MgSO_4$. Following another 5 minutes at 0° the suspension was sonicated in 5 ml aliquots for 20 seconds in a Branson cell disruptor at a power of 50 watts. The inner membrane and unbroken mitochondria were sedimented at 9,500 g for 10 minutes and the supernatant fluid centrifuged at 40,000 g for 10 minutes to bring down inner membrane fragments. The outer membrane preparation was obtained by centrifuging the 40,000 g supernatant at 105,000 g for 60 minutes. The sedimented fractions were resuspended in sucrose medium and sonicated prior to enzyme assay.

Solubilization and purification of microsomal NADH dependent reductases

1. Lysosomal digestion. Microsomal protein was solubilized by the method of Takesue and Omura (57). Microsomes (7 mg protein/ml) were incubated with lysosomes (3 mg protein/ml) in 0.1 M Tris-maleate buffer, pH 5.7, 1 mM EDTA, at 35°. The incubation was carried out anaerobically under nitrogen to minimize lipid peroxidation. At the end of a 3 hour incubation period the digest was centrifuged at 105,000 g for 90 minutes. The solubilized enzymes were purified by ammonium sulfate fractionation and gel chromatography on Sephadex G-100. The gel eluate was purified further by ion exchange chromatography on DEAE cellulose using a linearly increasing gradient of KCl (0 - 0.1 M) in 0.05 M Tris-HCl buffer, pH 8.5. The purification procedure is outlined in Scheme I (page 16).
2. Deoxycholate solubilization. Microsomes were solubilized with deoxycholate by a modification (58) of the method of Lu and Coon (59). Microsomes (25 mg protein/ml) were stirred gently for 20 minutes at 5° in a solubilizing solution containing 0.1 M potassium citrate buffer, pH 7.7, 20% glycerol and 0.5% sodium deoxycholate. The mixture was centrifuged at 105,000 g for 1 hour and the supernatant decanted and diluted with 3 volumes of ice cold deionized

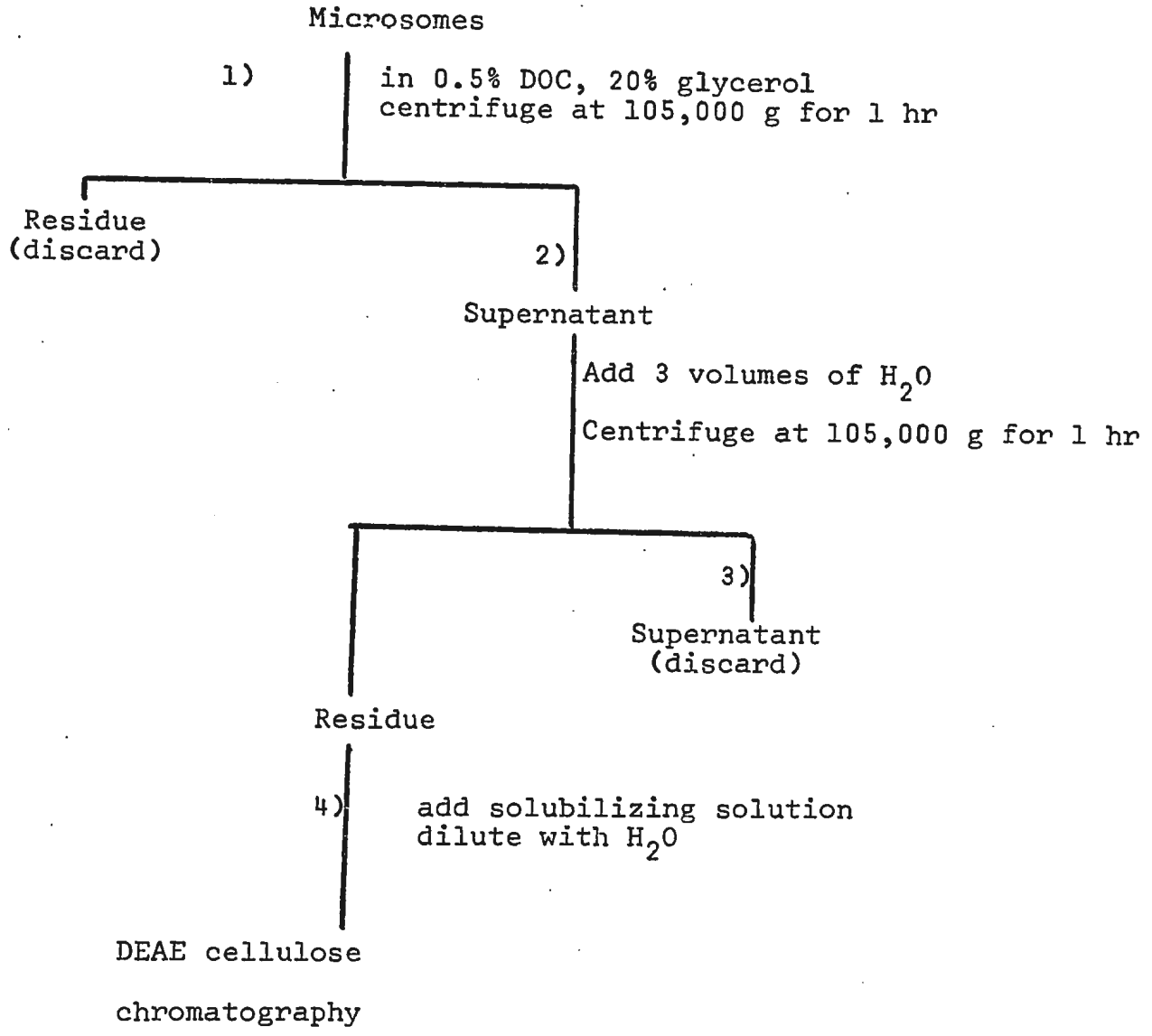


Scheme I. Outline of the purification method for microsomal NADH dependent reductases following lysosomal digestion.

water. After stirring for 20 minutes at 5°, the centrifugation was repeated at 105,000 g for 1 hour and the resulting pellet resuspended in the original solubilizing solution by gentle homogenization. The mixture was allowed to stand for 20 minutes at 0°, and then diluted with 3 volumes of ice cold deionized water and applied to a DEAE cellulose column previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.7, containing 0.05% deoxycholate. The column was eluted with an increasing linear concentration gradient of KCl (0-0.6 M) in the same buffer and the eluate collected in 6 ml fractions. The purification procedure is outlined in Scheme II (page 18).

Cytochrome b_5 was purified from pig liver by the method of Omura and Takesue (60). Cytochrome b_5 was assayed spectrophotometrically by recording the difference spectrum (reduced minus oxidized) after the addition of a few grains of sodium dithionite to the experimental cuvette. A molar extinction coefficient increment of 185 cm^{-1} mM^{-1} between 424 and 409 mμ (61) was assumed in these difference spectra.

Trypsin treatment of mitochondria was done by the method of Kuylenstierna et al (62). Mitochondria (4 mg protein/ml) were preincubated for 10 minutes at 30° in the presence of 5 mM potassium phosphate buffer, pH 7.5, 0.25 M sucrose and with varying quantities of a freshly made up solution of trypsin. At the end of this period soya bean trypsin



Scheme II. Outline of the purification method for microsomal NADH dependent reductases following DOC solubilization.

inhibitor (in a three-fold weight excess) was added and enzyme activities assayed without delay. Microsomes were preincubated with trypsin as described by Orrenius et al (63). Microsomes (10 mg protein/ml) 50 mM Tris-HCl buffer, pH 7.5, and varying quantities of trypsin were incubated for 10 minutes at 30°. The reaction was stopped as described above and enzyme assays immediately started.

Acetone extraction of microsomal lipids was carried out using 90% aqueous acetone at -20° by the method of Jones and Wakil (64). Lipid micelles added to the extracted microsomes contained 5 mg L-α-lecithin (Type III-E, Sigma), 1.2 mg oleic acid in 1 ml 0.02 M Tris-HCl buffer, pH 8.0, and 1 mM EDTA.

Spectrophotometric determinations were carried out with a Beckman DB-G recording spectrophotometer, except when otherwise stated.

RESULTS

Intracellular distribution of AH• reductase

The intracellular distribution of NADH-semidehydroascorbate reductase and rotenone insensitive NADH-cytochrome c reductase was determined in four different tissues of sheep (Table I). The figures presented in Table I are an average of two experiments, but the intracellular distribution of the two enzymes in rat liver and kidney from several experiments showed a similar distribution and specific activities as those of the sheep. The intracellular distribution of the two enzymes in guinea pig liver was also found to be similar to that of rat and sheep liver.

The AH• reductase in the three tissues, liver, adrenal and kidney showed a similar distribution pattern between mitochondria and microsomes, with a mitochondrial total activity about two-fold that of the microsomes. The specific activity of the mitochondrial enzyme was higher than that of the microsomal enzyme in all three tissues, and this makes it unlikely that microsomal contamination could account for the mitochondrial activity.

In contrast to the specific activity of the AH• reductase, the rotenone insensitive cytochrome c reductase showed a two- to five-fold higher specific activity in microsomes over that of mitochondria. The enzyme recovery in the

TABLE I

TISSUE DISTRIBUTION OF AH• REDUCTASE AND ROTENONE INSENSITIVE CYTOCHROME c REDUCTASE [a]

	AH• Reductase		r.i. Cyt. c reductase	
Tissue fraction	units[b]/mg protein	% of total	units[c]/mg protein	% of total
Liver mitochondria	0.088	44.6	0.76	15.0
Liver lysosomes	0.046	15.4	2.09	28.4
Liver microsomes	0.077	26.6	4.08	57.0
Liver supernatant	<0.004		0.06	0.07
Heart microsomes	<0.004		0.15	
Adrenal mitochondria	0.092	43.2	0.76	24.1
Adrenal microsomes	0.054	19.5	1.82	43.8
Kidney mitochondria	0.118	52.5	1.32	21
Kidney microsomes	0.054	27.0	6.83	48

[a] Subcellular fractions of sheep liver, heart, adrenal and kidney were prepared by the method of Sedgwick and Hubscher (51). The fractions were assayed for protein, AH• reductase and rotenone insensitive cytochrome c reductase activities as described in "Materials and Methods".

[b] One unit is equivalent to an absorbance change of 1.0 per 2 minutes.

[c] One unit is equivalent to an absorbance change of 1.0 per minute.

microsomal fraction was therefore about 50% or more of the total enzyme activity in the post nuclear homogenate, which is about twice the recovery of the AH• reductase in the same fraction.

Whilst levels of AH• reductase activity seem to be similar in liver, adrenal and kidney, no activity could be demonstrated in heart muscle. The rotenone insensitive cytochrome c reductase activity in heart muscle was also low. The reason for this low activity could be that cytochrome b_5 which is believed to be a component of the liver rotenone insensitive cytochrome c reductase is absent from heart tissue (35). Furthermore, the heart cytochrome c reductase may even be a different enzyme from that of the liver enzyme (66).

Intramitochondrial localization of AH• reductase

The mitochondria were fractionated by the method of Sottocasa et al (56). The method essentially consists of swelling, shrinking and sonicating the mitochondria and separating the fragments by differential centrifugation. The enzyme activities measured were found to be unaltered by this treatment, whereas digitonin treatment of mitochondria, which is also employed in mitochondrial fractionation (67) resulted in activation of both AH• reductase and rotenone insensitive cytochrome c reductase.

The intramitochondrial distribution of AH$^{\bullet}$ reductase in rat liver mitochondria is presented in Table II. Rotenone insensitive cytochrome c reductase and cytochrome oxidase were used as outer and inner membrane markers respectively, and malate dehydrogenase was used as matrix marker. It is seen that 12% of the AH$^{\bullet}$ reductase was recovered in the 105,000 g pellet with specific activity five-fold that of the 9,500 g pellet.

The rotenone insensitive cytochrome c reductase, which is recognized as an outer membrane enzyme in rat liver mitochondria (68) had a similar distribution. A little over 20% of the rotenone insensitive cytochrome c reductase was recovered in the 105,000 g pellet with a specific activity ten-fold that of the 9,500 g pellet. Microsomal contamination would contribute to the recovery of the r.i. cytochrome c reductase in the 105,000 g pellet to a much greater extent than in the case of the AH$^{\bullet}$ reductase. The reason for this being that the specific activity of the microsomal r.i. cytochrome c reductase is several times higher than that of the mitochondrial enzyme whereas the AH$^{\bullet}$ reductase shows a higher specific activity in the mitochondria than the microsomes (Table I).

Most of the cytochrome oxidase, on the other hand, was recovered in the 9,500 g pellet with enriched specific activity compared to that of the unfractionated preparations. About 78% of the malate dehydrogenase was recovered

TABLE II

INTRAMITOCHONDRIAL DISTRIBUTION OF $AH^{\bullet}$ REDUCTASE [a]

Fraction	Protein		$AH^{\bullet}$ Reductase		Cyt. c oxidase		r.i. cyt. c reductase		Malate dehydrogenase	
	mg	% of total	units/mg protein [b]	% of total	units/mg protein [c]	% of total	units/mg protein [d]	% of total	units/mg protein [d]	% of total
Unfractionated	124	100	0.112	100	0.39	100	0.645	100	6.9	100
9,500 g (P)	96	77.5	0.089	71.5	0.45	91	0.514	61.5	6.95	77.8
40,000 g (P)	6.5	5.2	0.158	11.3	0.34	4.6	0.994	8.1	4.87	3.7
105,000 g (P)	3.5	2.8	0.417	12.4	0.32	2.4	5.05	22.3	0.76	0.3
105,000 g (S)	20.8	16.8	<0.004	-	0.03	1.4	0.262	6.8	8.42	20.4
Recovery		102.3		95.2		99.4		98.7		102.2

[a] Mitochondria were fractionated by the method of Sottocasa et al (56). Cytochrome oxidase and r.i. cytochrome c reductase were used as enzyme markers for inner and outer membranes respectively. Malate dehydrogenase was used as matrix enzyme marker.

Enzyme activities were assayed as described in "Materials and Methods". (P) and (S) indicate pellet and supernatant respectively.

[b] One unit is equivalent to an absorbance change of 1.0 per 2 minutes.

[c] One unit is equivalent to one µatom of oxygen consumed per minute.

[d] One unit is equivalent to an absorbance change of 1.0 per minute.

in the 9500 g pellet, which shows that the inner membrane-matrix fraction was not broken to any great extent by the fractionation procedure.

Although the method used for fractionating mitochondria in the present work did not yield very clean membrane fractions, the enzyme distribution between the fractions clearly indicates that AH• reductase is localized in the outer mitochondrial membrane.

Kinetic properties of the AH• reductase

Both the microsomal and mitochondrial AH• reductase showed similar or identical kinetic behaviour and the results reported here are mostly from studies of the microsomal enzyme.

The pH optimum for the AH• reductase was found to be about 7.4 in both Tris-HCl buffer and potassium phosphate buffer (Fig. 1). When assayed in phosphate buffer the enzyme showed a somewhat higher specific activity in the vicinity of the pH optimum than when assayed in Tris-HCl buffer. At pH 7.4 the dehydroascorbate preparation used in the assay was found to be rather unstable; it gradually turned yellow and gave an interference at 340 mμ, the wavelength used for assaying the enzyme. The pH employed in all assays was 6.9, at which the dehydroascorbate gave only about one-fifth the rate of interference at 340 mμ compared to that at pH 7.4.

The enzyme showed a linear response to increased concentration of protein up to a concentration of 2 mg protein/3 ml or up to a rate equivalent to a change in optical density of

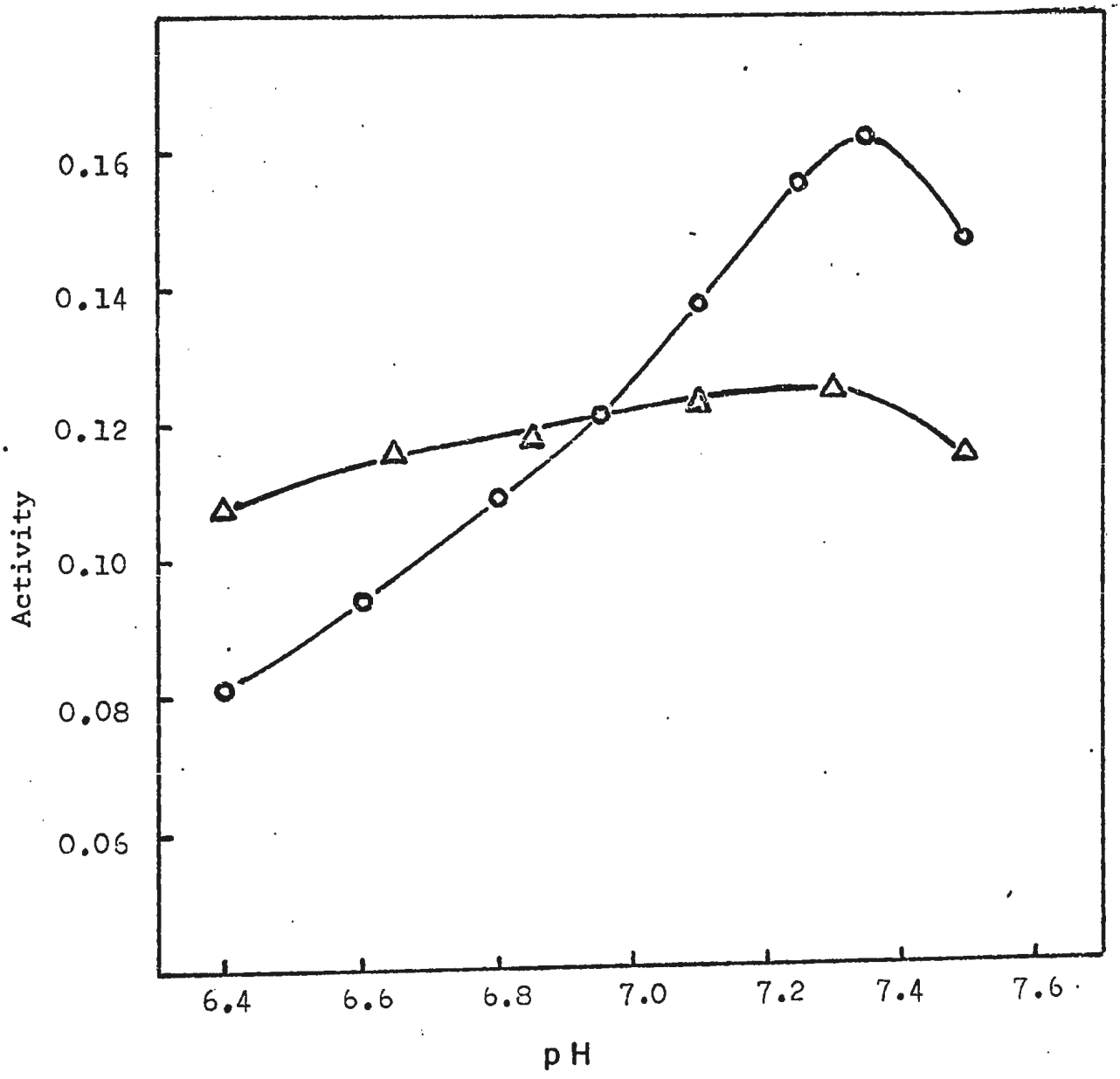


Figure 1. The effect of pH on the activity of AH· reductase. The enzyme activity was measured as described in "Materials and Methods" using 0.05 M potassium phosphate buffer (-o-o-) and 0.05 M Tris-HCl buffer (-Δ-Δ-) in the pH range 6.4-7.5. 1.4 mg microsomal protein in a total volume of 3 ml was used for all assays and the pH was measured at the end of each assay.

0.120 O.D. units per two minutes). The reaction rate was linear until less than 5 μM NADH was left in the reaction medium and this took 5 - 40 minutes depending on the enzyme activity present. Enzyme activity equivalent to a change in O.D. units of 0.015 per 2 minutes was the sensitivity limit of the enzyme assay.

Boiled enzyme was inactive and when ascorbate was omitted from the assay medium, no enzyme activity was detected.

The Km value for NADH of 1 μM (Fig. 2) obtained in the present study was markedly different from the 50 μM Km value reported by Lumper et al (69), and falls within a reasonably close range of the Km values for NADH in the microsomal FeCN and cytochrome c reductases, which are 2.7 μM (70) and 5.8 μM (71) respectively. Lumper et al (69) used a solubilized enzyme preparation for measuring the Km, which might explain the discrepency observed between their data and the presently reported results. Lubrol WX treatment (0.1 mg Lubrol/mg protein) of the subcellular fractions had no measurable effect on the Km, which suggests that the substrates have free access to the enzyme in the untreated membrane.

The enzyme was found to be specific for NADH, with no measurable reaction (less than 0.004 Δ O.D./2 minutes/mg protein) taking place with the same concentration of NADPH.

It was discovered that α-NADH could replace β-NADH in both AH• reductase and cytochrome c reductase, although the reaction rate (Vmax) with α-NADH was only 60% of that with β-NADH. The apparent Km for α-NADH was 2.5 μM in the AH• reductase. This ability to accept both α- and β-NADH as electron donors has interesting implications.

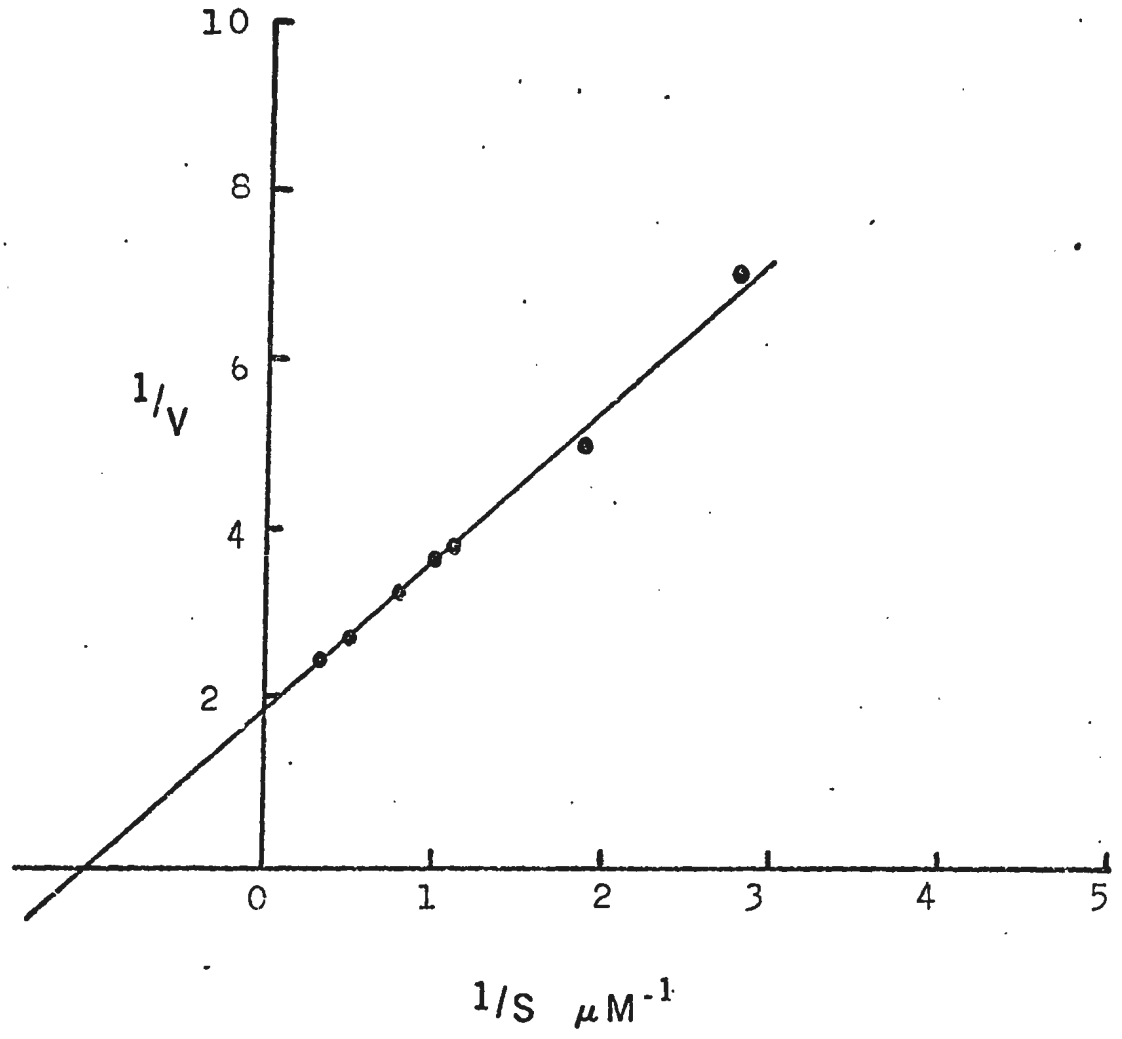


Figure 2. A Lineweaver-Burk plot of the effect of NADH concentration on the reaction rate of AH· reductase. The rate of reaction was followed at NADH concentration of 5 μM and less in a Unicam SP800 spectrophotometer with a 0-0.1A scale expansion accessory and external chart recorder.

Since cytochrome b_5 reductase is the only flavoprotein reported (72) that is able to reduce α-NADH and both AH• and cytochrome c reductases are able to do so it seems possible that these three enzyme activities involve the same flavoprotein. The large steric differences between the two forms of the nucleotide make it difficult however to believe that the flavoprotein has only one acceptor site that accepts both α- and β-NADH. As the FeCN reductase did not accept α-NADH, an existence of a second flavoprotein might possibly explain this behaviour. The flavoprotein that accepts β-NADH and reacts with FeCN, might feed electrons into a second flavoprotein that accepts α-NADH and subsequently the second flavoprotein would react with cytochrome b_5 and then possibly with semidehydroascorbate or cytochrome c.

Effect of inhibitors

Most inhibitors studied affected the three enzymes, the AH•, FeCN and cytochrome c reductases, similarly. 0.4 mM dicumarol inhibited the AH• reductase 50% (Table III) while the FeCN and cytochrome c reductases were inhibited 50% at concentrations of 0.15 mM and 0.45 mM respectively. TTFA was found however to inhibit the AH• reductase effectively (0.24 mM TTFA causing 50% inhibition), whereas it had no inhibitory effect on either the FeCN or cytochrome c reductases.

AMP and ATP had slight inhibitory effect on the AH• reductase whereas ADP stimulated the enzyme activity about 10%.

TABLE III

THE EFFECT OF INHIBITORS ON THE ACTIVITY OF AH· REDUCTASE[a]

Inhibitor	% Activity
None	100
0.4 mM dicumarol	50
0.24 mM TTFA	50
0.40 mM TTFA	20
5 mm AMP	90
5 mM ADP	110
5 mM ATP	88
10 mM EDTA	75
1 mM $MnSO_4$	95
10 μM $FeSO_4$	115

[a] The enzyme activity was assayed as described in "Materials and Methods" in the presence of the listed compounds at the concentrations indicated.

1 mM $MnSO_4$ had no significant effect on the AH• reductase, while 10 μM $FeSO_4$ stimulated the enzyme activity in the presence of 1 mM EDTA. 10 mM EDTA inhibited the enzyme 25%, but had no significant effect at concentrations below 5 mM.

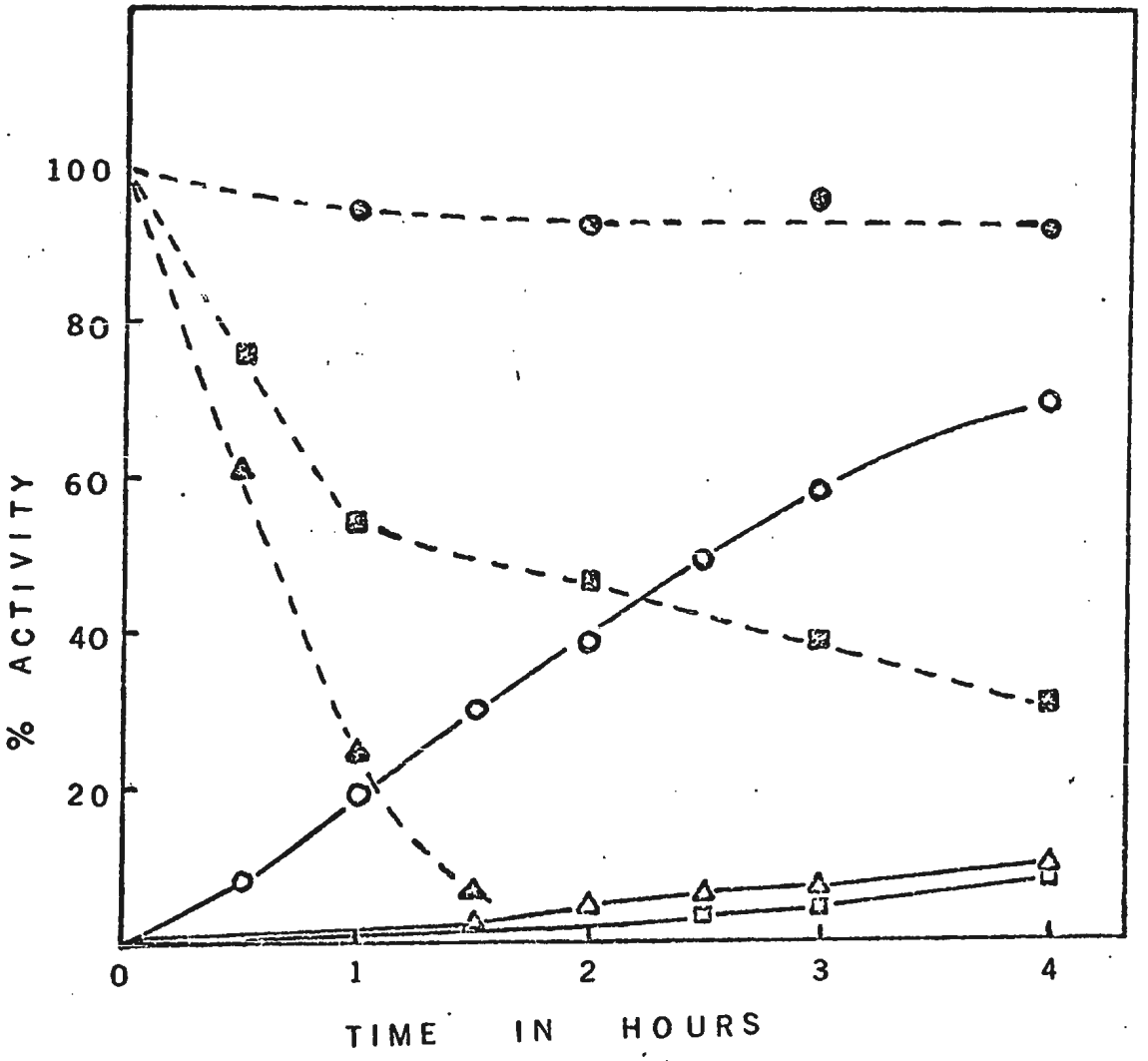
Rotenone, azide, KCN (1 mM), progesterone (0.5 mM), o-phenanthroline (0.1-1 mM), and hydroxyquinoline (0.1-1 mM) had no significant effect on the activity of AH• reductase or the FeCN and cytochrome c reductases.

Solubilization of microsomal AH• reductase

Lysosomal digestion of microsomes selectively releases the NADH-cytochrome b_5 reductase from the membrane (57). When the flavoenzyme was assayed as NADH-FeCN reductase, about 80% of the enzyme activity was released from the microsomes without much inactivation. If, however, the enzyme was assayed, using cytochrome b_5 as electron acceptor, the reductase showed allotopic behaviour, and activity for this acceptor was increased several fold on solubilization from the membrane (73).

The NADH-cytochrome c and NADH-AH• reductases were solubilized less effectively than the FeCN reductase by lysosomal digestion of microsomes at 35°, as seen in Figure 3. During a 2 - 3 hour digestion period the AH• reductase lost 75 - 85% of its initial enzyme activity and the activity of the cytochrome c reductase fell to 40% of its initial value during the same incubation time. Part of this inactivation was found to be due to aging at 35°, as about 40% of the AH• reductase and 20% of the cytochrome c reductase activities were lost during a two hour incubation of microsomes in the absence of lysosomes

Figure 3. Solubilization and inactivation of NADH dependent reductases by incubation of lysosome-microsome suspension at 35°. Lysosome-microsome suspension in 0.1 M Tris-maleate buffer, pH 5.7, 1 mM EDTA was incubated at 35°. At the times indicated, a portion of incubated suspension was taken out, rapidly cooled by ice and centrifuged at 105,000 g



for 90 minutes to give a clear supernatant. Enzyme activities were assayed in the whole digest and the supernatant and expressed as % of the initial enzyme activity. The dotted lines represent enzvme activities in the whole digest, solid lines represent the solubilized enzyme activities.

Δ-Δ-Δ- AH$_2$ reductase activity

□-□-□- Cytochrome c reductase activity

o-o-o- FeCN reductase activity

under the same conditions.

In order to test whether this inactivation might be partly due to lysosomal lipases acting on lipid components of the membrane, lipid micelles, known to activate the two enzymes in acetone extracted microsomes, were added to the digest at the end of a 3 hour digestion. The cytochrome c reductase activity was restored from 40% to 70% of its original activity but no activation was observed with the AH$^{\bullet}$ reductase, which suggests that lipid hydrolysis is not a primary factor in the inactivation of the AH$^{\bullet}$ reductase although it could be for cytochrome c reductase.

After a 3 hour digestion at 35° the solubilized microsomes were subjected to $(NH_4)_2SO_4$ fractionation and gel chromatography on Sephadex G100. The elution pattern in Figure 4 shows that the solubilized AH$^{\bullet}$ reductase was eluted in the same fractions as the FeCN reductase, whereas the cytochrome c reductase activity was displaced by one fraction from the peak of the FeCN reductase and can be accounted for by cytochrome b_5 contamination in the active fractions. Further purification by ion-exchange chromatography on DEAE cellulose resulted in loss of the AH$^{\bullet}$ and cytochrome c reductase activities, whereas 70% of the FeCN reductase was recovered from the column. Table IV shows a summary of the purification. As can be seen the specific activities of the FeCN reductase and the AH$^{\bullet}$ reductase increased in parallel, from the digested supernatant until the latter was lost on the DEAE cellulose column.

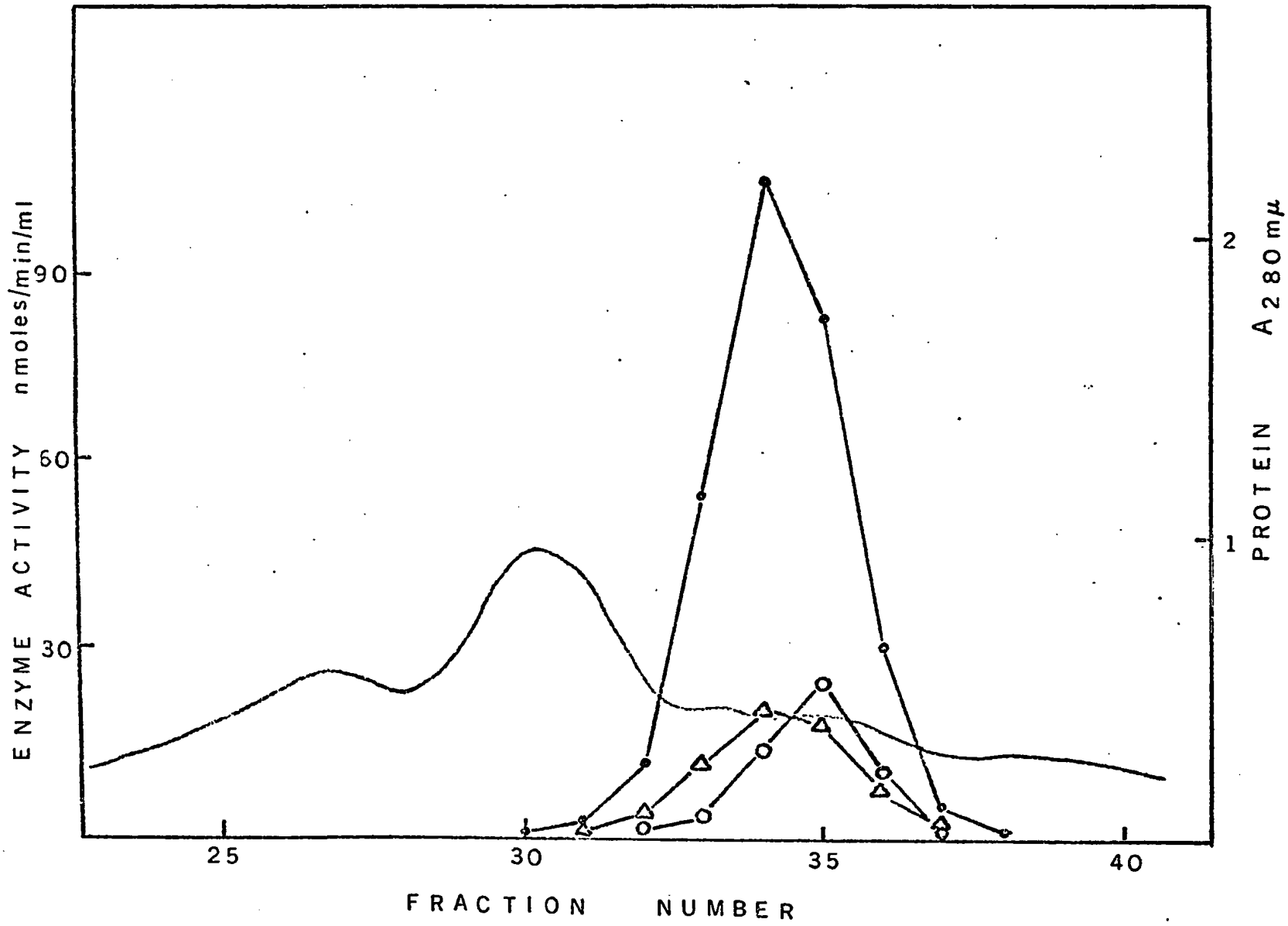

ENZYME ACTIVITY nmoles/min/ml
90
60
30
PROTEIN A 280 mμ
2
1
25
30
35
40
FRACTION NUMBER

Figure 4.

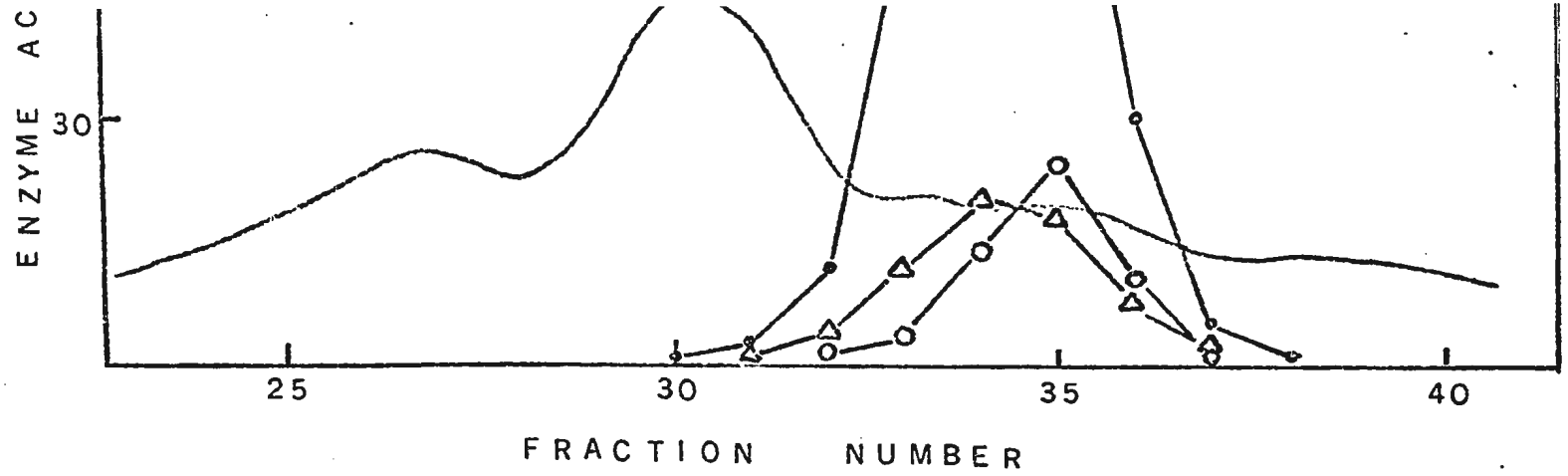


Figure 4.

Figure 4. Gel filtration of enzyme extract on Sephadex G100. The column (2.5 x 90 cm) was equilibrated with 20 mM Tris-HCl buffer, pH 8.5 and the column was eluted with the same buffer at a flow rate of about 40 ml per hour. The eluate was collected in 9 ml fractions, and the enzymes assayed as described in "Materials and Methods". Enzyme activities are expressed as nmoles substrate reduced per minute per ml of eluate.

. -·-·- FeCN reductase activity

-o-o- Cytochrome c reductase activity x 100

-Δ-Δ- AH· reductase activity x 800

——— Protein

TABLE IV

PURIFICATION OF MICROSOMAL FeCN, $AH^{\cdot}$ AND CYTOCHROME c REDUCTASES BY LYSOSOMAL DIGESTION [a]

Fraction [d]	Total protein (mg)	FeCN Reductase units/mg protein [b]	FeCN Reductase % of total	$AH^{\cdot}$ Reductase units/mg protein [c]	$AH^{\cdot}$ Reductase % of total	Cyt. c Reductase units/mg protein [b]	Cyt. c Reductase % of total
Before incubation	1660	0.94	100	0.113	100	1.41	100
After incubation	1460	0.93	87	0.014	11	0.595	37
Supernatant	317	2.12	43	0.020	3.3	0.170	2.3
$(NH_4)_2SO_4$ cut	91	6.7	39	0.065	3.1	0.465	1.8
pooled fr. 30-39	36	17.6	40	0.125	2.4	0.55	1.4
DEAE cellulose pooled fr.	7	44.3	28	<0.004	-	<0.004	

[a] The fractions were assayed for protein, FeCN, $AH^{\cdot}$ and cytochrome c reductase activities as described in "Materials and Methods".

[b] One unit is equivalent to an absorbance change of 1.0 per minute.

[c] One unit is equivalent to an absorbance change of 1.0 per 2 minutes.

[d] The fractions refer to Scheme I, page 16.

The specific activity of the cytochrome c reductase did not show much increase on the Sephadex column.

When the active fractions from the Sephadex column were assayed in presence of 0.2% Triton X-100 no change in the three enzyme activities was observed. The same concentration of Triton X-100 was shown to completely abolish the AH$^{\bullet}$ and cytochrome c reductase activities in intact microsomes. This suggests that the AH$^{\bullet}$ and cytochrome c reductase activities released from the membrane are not the true membrane bound enzymes, but may be artifacts produced by the solubilization. The cytochrome c reductase activity is probably due to the presence of cytochrome b_5, but the concentration of cytochrome b_5 in the eluted fractions was too low (< 1 μM) to contribute much to the AH$^{\bullet}$ reductase activity.

The cytochrome b_5 reductase after purification on DEAE cellulose showed no AH$^{\bullet}$ or cytochrome c reductase activities (Table V). By addition of cytochrome b_5 to the reaction mixture, both cytochrome c and AH$^{\bullet}$ reductase activities could be demonstrated as seen in Table V. The reaction rates of the reconstituted system were quite different however from the reaction rates of the membrane bound enzymes. The cytochrome c reductase showed about one-third of the reaction rate of the membrane bound enzyme when compared to that of the FeCN reductase, while the AH$^{\bullet}$ reductase activity of the reconstituted system showed about one and a half the reaction rate of the membrane bound enzyme by the same comparison.

TABLE V

RELATIVE ENZYME ACTIVITIES OF PURIFIED CYTOCHROME b_5 REDUCTASE

Electron Acceptor	Units/mg protein		
	Microsomes	Cyt. b_5 red.	Cyt. b_5 red. + cyt. b_5 [c]
FeCN [a]	0.85	48.6	46.1
Cytochrome c [a]	1.65	-	34.2
AH• [b]	0.093	-	7.8

Enzyme activities of intact microsomes and purified cytochrome b_5 reductase were assayed as described in "Materials and Methods".

[a] One unit is equivalent to an absorbance change of 1.0 per minute.

[b] One unit is equivalent to an absorbance change of 1.0 per 2 minutes.

[c] The concentration of cytochrome b_5 used in the enzyme assays was 7 μM for FeCN reductase and 25 μM in the assays for cytochrome c and AH• reductase.

Ascorbic acid is known to reduce cytochrome b_5 (74) whereas semidehydroascorbate has been shown to oxidize cytochrome b_5 non-enzymatically (74, 75). The AH$^\cdot$ reductase activity of the reconstituted system does suggest that cytochrome b_5 might be involved in the membrane bound enzyme as well as cytochrome b_5 reductase.

Lu and Coon (59) recently used deoxycholate solubilization of microsomes and subsequent chromatography on DEAE cellulose to resolve microsomal cytochromes and constitutents of microsomal electron transport complexes. Following a similar procedure (outlined in Scheme II, page 18) the three NADH reductases, FeCN, AH$^\cdot$, and cytochrome c reductase, were successfully solubilized when microsomes were treated with 0.5% deoxycholate in 20% glycerol and citrate buffer, pH 7.7. No inhibition or inactivation of enzyme activities was observed; on the contrary some activation occurred in the case of AH$^\cdot$ and cytochrome c reductases, as seen in Table VI.

The DOC treated microsomes were subjected to ion exchange chromatography on DEAE cellulose and eluted with linear KCl gradient in 0.05 M Tris-HCl buffer, pH 7.7, containing 0.05 % deoxycholate. Figure 5 shows the elution pattern of the three reductases. The AH$^\cdot$ reductase and cytochrome c reductase activities were resolved together and were separated from the major FeCN reductase peak. Cytochrome b_5 was identified in the cytochrome c reductase peak, and is probably an integral part of a cytochrome c reductase particle that is being eluted in these fractions.

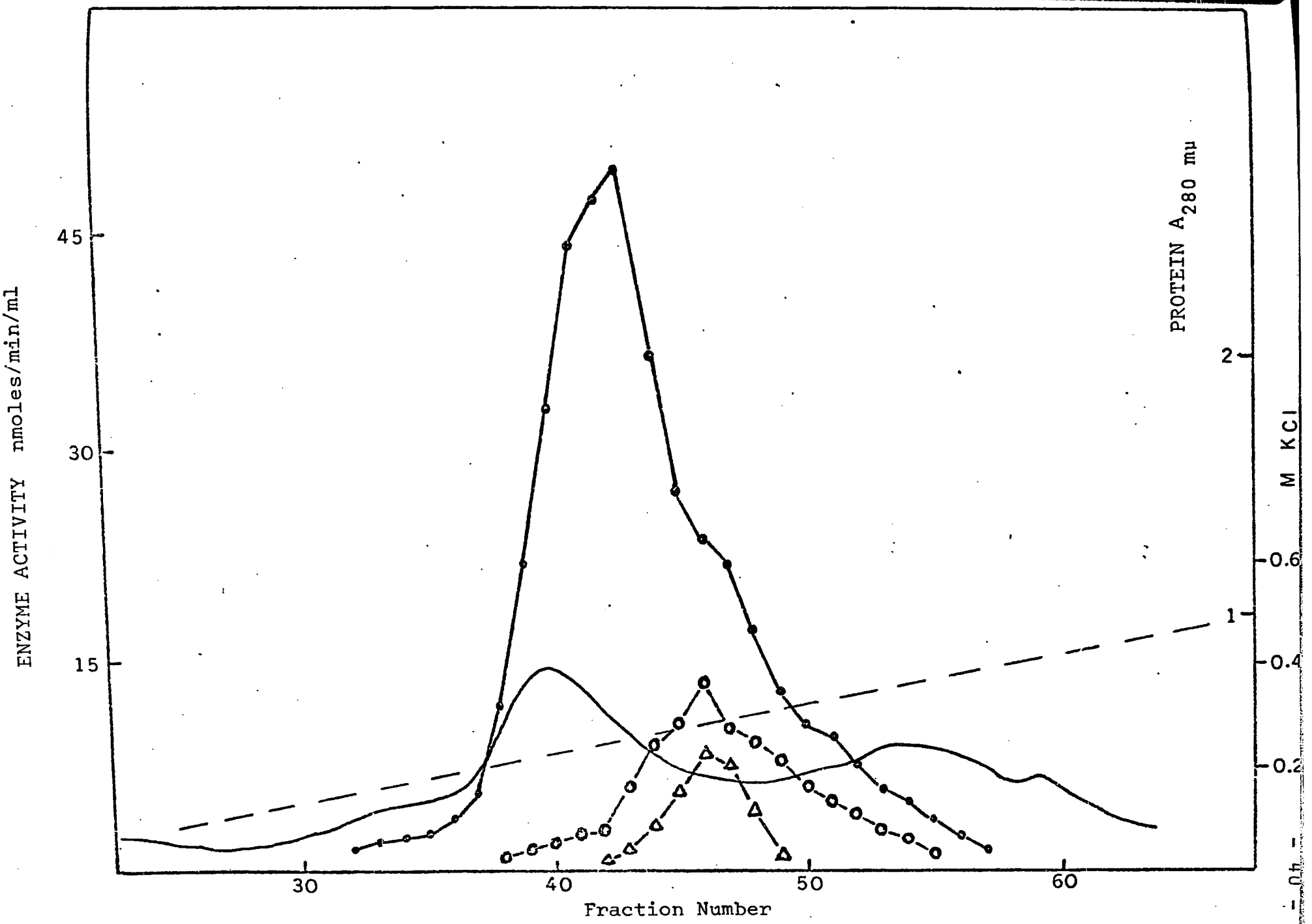
TABLE VI

PURIFICATION OF MICROSOMAL FeCN, AH• AND CYTOCHROME c REDUCTASES BY DOC TREATMENT [a]

Fraction	total protein (mg)	FeCN Reductase units/mg protein	FeCN Reductase % of total	AH• Reductase units/mg protein	AH• Reductase % of total	Cyt. c Reductase units/mg protein	Cyt. c Reductase % of total
1	600	0.67	100	0.109	100	1.65	100
2	515	0.70	89.5	0.114	89	1.81	94
3	268	0.23	15.4	-	-	0.42	11.3
4	251	1.20	75	0.180 [b] (0.02)	69 (7.7)[b]	3.24	82.5
pooled fr. 38-53	59.5	4.5	72.5	0.066	6.5	20.7	134

[a] The fraction numbers refer to the purification steps outlined in Scheme II. Enzyme activities were assayed as described in "Materials and Methods" and enzyme units are the same as described in Table IV.

[b] The AH• reductase activity in fraction 4 was reassayed at the same time as the fractions collected from the DEAE cellulose column, and the units per mg protein were found to have dropped to 0.02.


ENZYME ACTIVITY nmoles/min/ml
45
30
15
PROTEIN A_{280} mμ
2
1
M KCl
0.6
0.4
0.2
30
40
50
60
Fraction Number

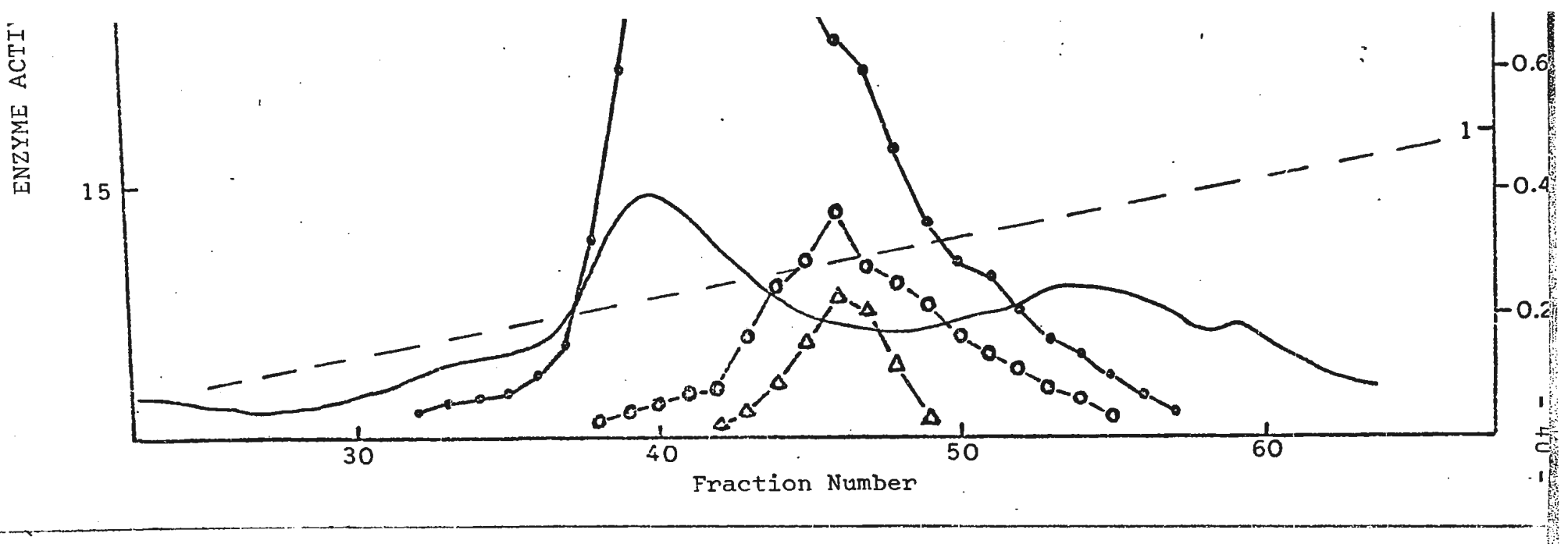


Figure 5. Chromatography of microsomal extract on DEAE cellulose. The column (1.2 x 28 cm) was eluted with a linear KCl gradient in 0.05 M Tris-HCl buffer, pH 7.7, containing 0.05% DOC. 6 ml fractions were collected and enzymes assayed as described in "Materials and Methods". Enzyme activities are expressed as nmoles substrate reduced per minute per ml of eluate.

-Δ-Δ- AH• reductase activity x 80

-o-o- Cytochrome c reductase activity

-·-·- FeCN reductase activity

——— Protein

- - - KCl molarity

The instability of the AH• reductase had already resulted in extensive loss of enzyme activity when the enzyme was eluted from the DEAE cellulose column and further purification was therefore not attempted.

The attempted solubilization of AH• reductase by lysosomal digestion showed that the enzyme is probably not a property of the cytochrome b_5 reductase. The AH• reductase activity that was solubilized and purified along with the cytochrome b_5 reductase could be an artifact of the solubilization or it could be due to contamination of AH• reductase. The AH• reductase activity of the cytochrome b_5 reductase-cytochrome b_5 system however suggests that these two proteins might be involved in the membrane bound AH• reductase.

When the microsomes were solubilized by DOC treatment the AH• reductase activity followed the cytochrome c reductase on purification and not the FeCN reductase. This further suggests that the true membrane AH• reductase is associated with the cytochrome c reductase particle and not with the cytochrome b_5 reductase itself.

A purification of cytochrome c reductase particles was attempted using the method of Mackler (76). Rat liver microsomes (20 mg protein/ml) were suspended in 0.5% sodium deoxycholate in Tris-HCl buffer, pH 8.0 and the mixture centrifuged at 105,000 g for 1 hour. The supernatant fluid was dialysed against 30 volumes of 0.02 M phosphate buffer for

18 hours, and the dialysed fraction centrifuged at 105,000 g for 1 hour. The AH·, cytochrome c and FeCN reductases were recovered in the loosely packed residue from the dialysed fraction with the cytochrome c reductase showing a five-fold increase in specific activity compared to microsomes. The rapid inactivation of the AH· reductase, however, resulted in poor yields of the enzyme activity and further work on purified preparations was not considered feasible.

Involvement of cytochrome b_5 in AH· reductase

The proteolytic action of trypsin releases membrane-bound cytochrome b_5 from both microsomes and mitochondria. This release is followed closely by inactivation of cytochrome c reductase (62, 63). As seen in Figures 6 and 7, pre-treatment of microsomes and mitochondria with trypsin leads to loss of enzyme activity of the three reductases studied. The cytochrome c reductase was effectively inactivated at trypsin concentrations that left the FeCN and AH· reductases almost unaltered. The mitochondrial cytochrome c and AH· reductases appeared to be more sensitive to trypsin than the microsomal cytochrome c and AH· reductases whilst the FeCN reductase showed similar sensitivity in both fractions. The AH· reductase was slightly activated at low trypsin concentrations but at high concentrations it showed greater sensitivity to the protease action than the FeCN reductase.

Table VII shows the effect of adding purified cytochrome b_5 to trypsin treated microsomes. Added cytochrome b_5

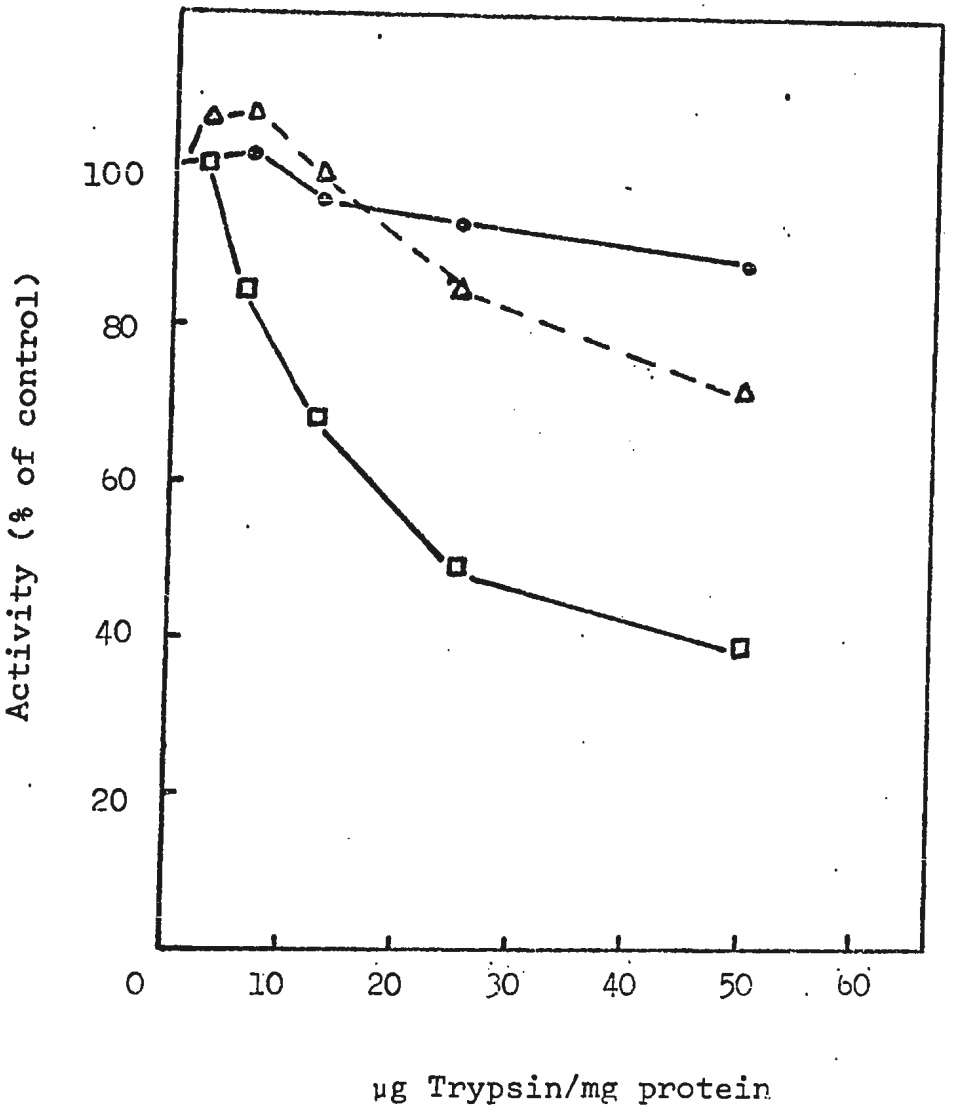


Figure 6. NADH dependent reductases in intact mitochondria after preincubation with trypsin. Mitochondria (4 mg protein/ml) were preincubated with varying amounts of trypsin and assayed for enzyme activities. The results are expressed as a percentage of the activity in a control, incubated in absence of trypsin with trypsin inhibitor and trypsin added at the end of a 10 minute incubation. Cytochrome c and FeCN reductases were assayed in presence of 0.25 M sucrose and 1.5 µM rotenone. AH· reductase was assayed as described in "Materials and Methods".

-Δ-Δ- AH· reductase activity

-□-□- Cytochrome c reductase activity

-●-●- FeCN reductase activity

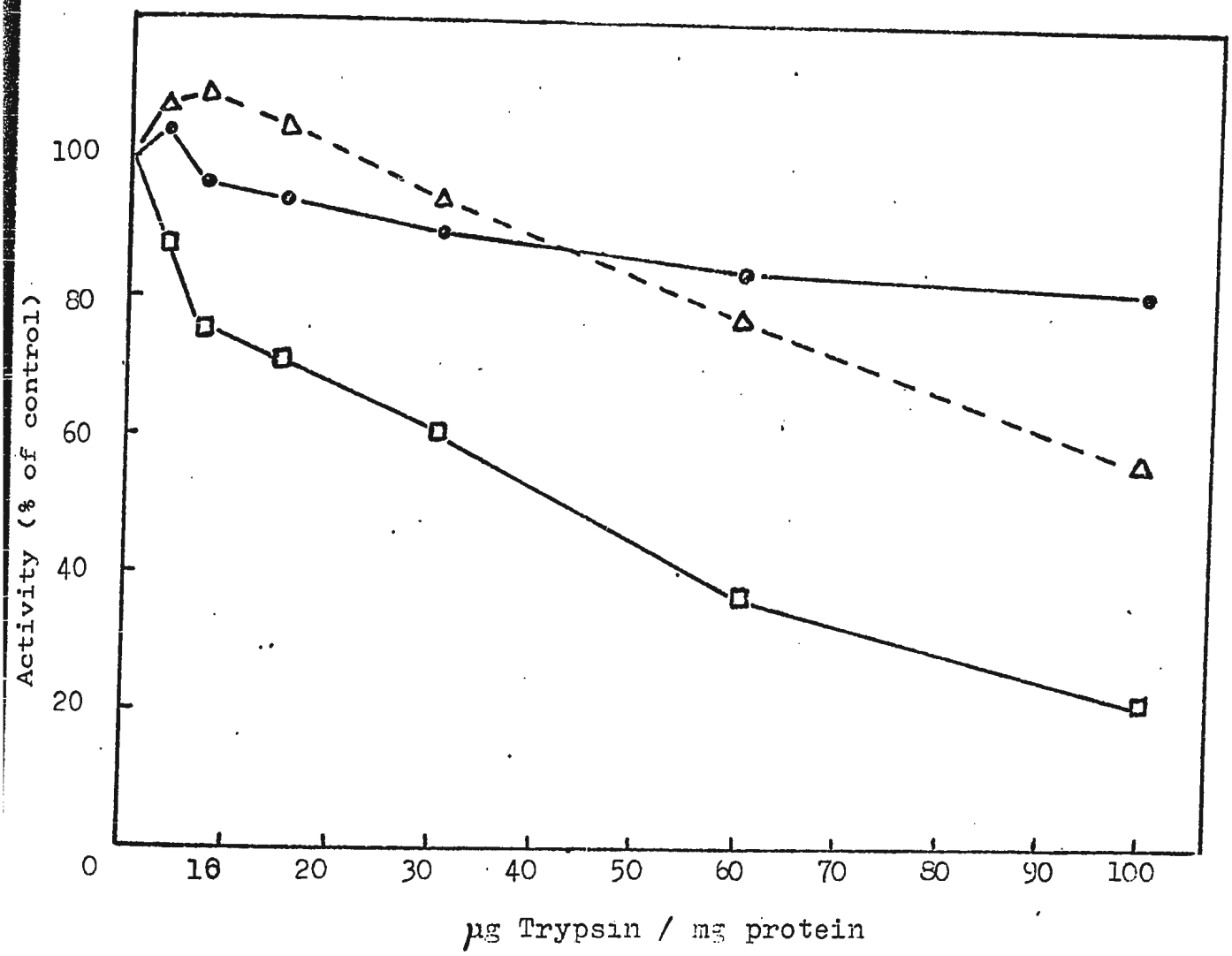


Figure 7. NADH dependent reductases in microsomes after pre-incubation with trypsin. Microsomes (10 mg protein/ml) were incubated with varying amounts of trypsin and assayed for enzyme activities as described in "Materials and Methods". The results are expressed as a percentage of the activity in a control, incubated in absence of trypsin with trypsin inhibitor and trypsin added at the end of a 10 minute incubation.

-Δ-Δ- AH• reductase activity

-□-□- Cytochrome c reductase activity

-●-●- FeCN reductase activity

TABLE VII

CYTOCHROME b_5 REACTIVATION OF AH• AND CYTOCHROME c REDUCTASES IN TRYPSIN TREATED MICROSOMES [a]

µg trypsin/mg protein	% Activity				
	FeCN reductase	AH• reductase [b]		Cyt. c reductase [b]	
		-cyt. b_5	+cyt. b_5	-cyt. b_5	+cyt. b_5
none	100	100	130	100	120
10	85	123	137	70	110
100	70	63	90	40	83
200	58	11	23	8	30

[a] Microsomes were preincubated with trypsin as described in "Materials and Methods". AH•, cytochrome c and FeCN reductases were assayed immediately after trypsin reaction had been stopped. Cytochrome b_5 was added to give maximal stimulation.

[b] The concentration of cytochrome b_5 used in the enzyme assays varied from 7 - 25 µM, as it was found that the concentration of cytochrome b_5 required for maximum activation in the trypsin treated microsomes was higher than in the control.

stimulated both AH• and cytochrome c reductase activities in untreated microsomes. Whilst this stimulatory effect was somewhat reduced in the case of AH• reductase on trypsin treatment, it was enhanced in the case of cytochrome c reductase. The cytochrome c reductase activity could not be restored to control levels in the trypsin treated microsomes, and the stimulation observed in all samples might be explained by the action of cytochrome b_5 reductase, the activity of which is being enhanced by the proteolytic action of trypsin.

If the inactivation of AH• reductase is due to removal of cytochrome b_5 from the membrane, the presence of the cytochrome seems to be less critical for the AH• reductase than for the cytochrome c reductase. Trypsin concentrations that inactivate the cytochrome c reductase 50% inactivate only 20% of the AH• reductase and indicates that cytochrome b_5 is not a rate limiting step in the reduction of AH• by NADH.

Lipid dependence of microsomal AH• reductase

Table VIII shows that acetone extraction of microsomes markedly decreased the activities of AH• and cytochrome c reductase, whereas the FeCN reductase was left unaffected. Both activities were restored on addition of lipid. Jones and Wakil (64) demonstrated a high lipid specificity in restoring the cytochrome c reductase activity in acetone extracted microsomes. Lipid micelles made of lecithin and oleic acid or lecithin and lysolecithin restored the enzyme activity as well as extracted microsomal lipid. In the present investigation AH• reductase

TABLE VIII

LIPID DEPENDENCE OF MICROSOMAL NADH DEPENDENT REDUCTASES [a]

	% Activity		
	Before extraction	After extraction	Lipid added [b]
FeCN reductase	100	95	103
$AH^{\bullet}$ reductase	100	< 10	115
Cyt. c reductase	100	35	127

[a] Microsomal lipids were extracted with 90% aqueous acetone at -20° (64). Enzyme activities were assayed before and after extraction as described in "Materials and Methods".

[b] Lipid micelles were prepared by dispersing 5 mg lecithin and 1.2 mg oleic acid in 1 ml 0.02 M Tris-HCl buffer, pH 8.0, 1 mM EDTA. After centrifugation the clear lower layer was used and a suitable amount added to the extracted microsomes to give a maximum stimulation of enzyme activities.

was found to show similar lipid dependence as the cytochrome c reductase, and the figures shown represent restoration using lecithin and oleic acid in Tris-HCl buffer, pH 7.5. The AH$^{\bullet}$ reductase was found at all times to be more sensitive than the cytochrome c reductase to the extraction procedure; reactivation of both activities was, however, most successful if the cytochrome c activity was not extracted below 30% of residual activity.

Further evidence of lipid dependence is the sensitivity of the AH$^{\bullet}$ and cytochrome c reductases to various detergents. When assayed in presence of deoxycholate, oleic acid or nonionic detergents, such as Triton X-100, rapid loss in activity was observed when a certain concentration was reached. Of the three detergents mentioned oleic acid was the most effective inhibitor. Concentrations as low as 0.1 mM (0.003%) oleic acid inhibited the AH$^{\bullet}$ reductase 50% and 0.06 mM oleic acid caused the same degree of inhibition of the cytochrome c reductase. At these concentrations slight activation of FeCN reductase was obtained. Figure 8 shows the inhibitory effect of deoxycholate. 50% inhibition of the AH$^{\bullet}$ and cytochrome c reductases was obtained at DOC concentrations of 0.08% and 0.07% respectively. FeCN reductase again was slightly activated at these concentrations. Triton X-100 and DOC were almost equally effective as inhibitors.

The inhibitory effect of DOC was found to be greatly protein dependent and the degree of inhibition decreased in a nonlinear fashion with increased protein concentrations. Triton

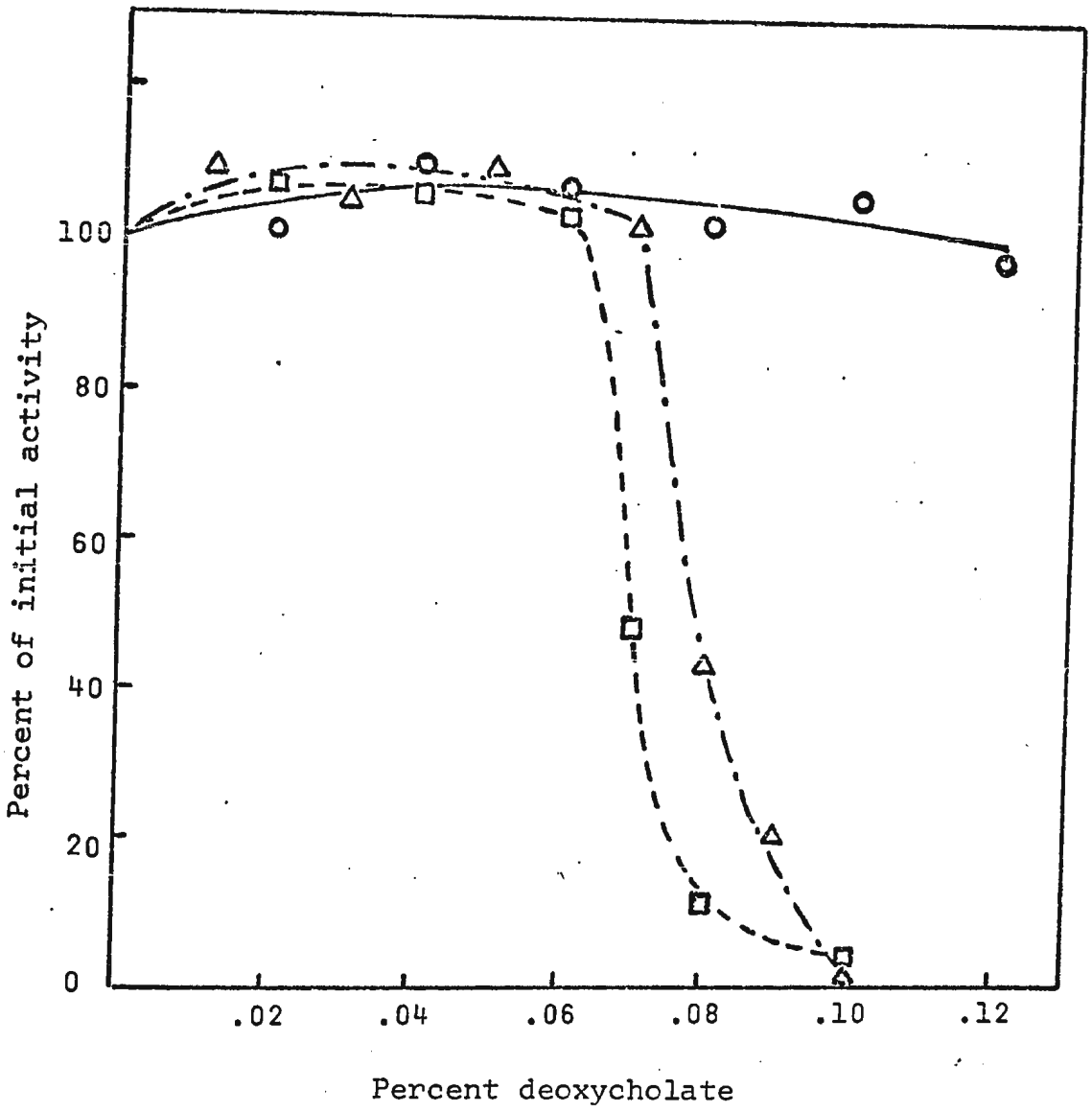


Figure 8. The effect of deoxycholate on NADH dependent microsomal reductases. The enzyme activities were assayed as described in "Materials and Methods" and an equivalent of 1 mg microsomal protein in a total volume of 3 ml was used for all assays. The FeCN and cytochrome c reductases were assayed in a cell of a 2 mm light path.

-Δ-Δ- $AH^{\cdot}$ reductase

-□-□- Cytochrome c reductase

-o-o- FeCN reductase

X-100 and oleic acid were much less protein dependent.

As the FeCN reductase was not significantly altered under the conditions used, the detergents are probably not inhibiting by effecting the protein conformation but rather by interacting with essential lipid components in the membrane.

Sulphydryl dependence of AH• reductase

Microsomal FeCN reductase contains three thiol groups, one of which is associated with the NADH binding site (77). p-Chloromercuribenzoate, N-ethylmaleimide and other thiol reagents are known to inhibit the enzyme effectively and this inhibition can be prevented if excess NADH is added prior to the inhibitor. The sensitivity of AH• reductase and cytochrome c reductase to the thiol reagents was compared to that of FeCN reductase by preincubating microsomes with varying concentrations of p-CMB and taking aliquots for all three enzyme assays from the same mixture (Table IX). It was found that AH• reductase and cytochrome c reductase were as sensitive to p-CMB as FeCN reductase with 10^{-5} M p-CMB causing 50% inhibition, and that this inhibition could be prevented when NADH was added prior to the inhibitor in the incubation mixture. When p-CMB was added after NADH the cytochrome c reductase was markedly stimulated; this observation suggests that p-CMB is binding to a second site in the cytochrome c reductase complex and facilitating a faster flow of electrons by steric alterations at that particular site.

TABLE IX

SULPHYDRYL DEPENDENCE OF MICROSOMAL NADH DEPENDENT REDUCTASES [a]

p-CMB x 10^{-6}M	% Activity - FeCN Reductase	AH• Reductase	Cyt. c Reductase
None	100	100	100
5	81	73	74
10	50	60	47
20	16	< 10	10
20 (NADH present) [b]	86	94	148

[a] p-chloromercuribenzoate in varying amounts was preincubated with microsomes (1 mg protein/ml) in 0.05 M Tris-HCl, pH 7.5, at 0° for 10 minutes. Aliquots were taken from the preincubation mixture and enzyme activities assayed as described in "Materials and Methods".

[b] 0.1 mM NADH was added to the preincubation mixture prior to p-CMB; 0.1 mM NADH was present in the assay medium when the preincubated microsomes were added and the reaction was started by adding the various substrates.

No activation was observed with the AH· reductase, indicating a distinct difference between these two enzyme activities.

The peak fractions from the DEAE cellulose column of DOC treated microsomes were pooled and p-CMB sensitivity of the cytochrome c and FeCN reductases compared. The reductases showed similar sensitivity to p-CMB inhibition, with 10^{-6}M p-CMB causing 50% inhibition. NADH also protected the reductases from p-CMB inactivation. However, the cytochrome c reductase was not stimulated when p-CMB was added in presence of NADH (Table X). Possibly the cytochrome c reductase had already been stimulated to its maximum activity by the DOC treatment, and therefore further stimulation was not obtained through the action of p-CMB. The activity of AH· reductase in the pooled fractions was too low to allow a significant measure of p-CMB inactivation.

Copper sulphate was found to inhibit the AH· reductase as shown in Table XI. Cyanide,which is known to chelate copper ions, reduced the inhibition and NADH was also found to reduce the inhibition when added prior to the copper sulphate. EDTA also protected the enzyme from inactivation by copper. The copper is probably complexing functional sulphydryl groups in the enzyme at or near the NADH binding site. The FeCN and cytochrome c reductases were not inhibited by copper sulphate, suggesting that the functional sulphydryl group of the cytochrome b_5 reductase was not being affected.

TABLE X

SULPHYDRYL DEPENDENCE OF DOC SOLUBILIZED AND PURIFIED NADH DEPENDENT REDUCTASES [a]

p-CMB x 10^{-6}M	% Activity	
	FeCN reductase	Cyt. c reductase
None	100	100
0.2	75	70
1	45	52
2	28	33
1 (NADH present) [b]	102	98
2 (NADH present)	103	89

[a] Fractions 43-49, from the DEAE cellulose column (Figure 5) were pooled and preincubated with varying amounts of p-chloromercuribenzoate, at 0° for 10 minutes. Aliquots were taken from the preincubation mixture and enzyme activities assayed as described in "Materials and Methods".

[b] 0.1 mM NADH was added to the preincubation mixture prior to p-CMB and the enzyme assays were started by adding aliquots of the preincubated protein.

TABLE XI

THE EFFECT OF COPPER SULPHATE ON AH• REDUCTASE [a]

$CuSO_4 \times 10^{-5}$ M	% Activity
None	100
1	103
2	52
3	11
2 (+ 1 mM KCN)[b]	78
2 (+ 0.1 mM NADH)[c]	83
2 (+ 1 mM EDTA)[d]	74

[a] The AH• reductase was assayed as described in "Materials and Methods" except cyanide and EDTA were excluded from the assay medium.

[b] 1 mM KCN was added to the assay medium before the copper sulphate.

[c] NADH was added to the assay medium before copper sulphate and the reaction was started by adding a mixture of ascorbate and dehydroascorbate.

[d] 1 mM EDTA was added to the assay medium before the copper sulphate.

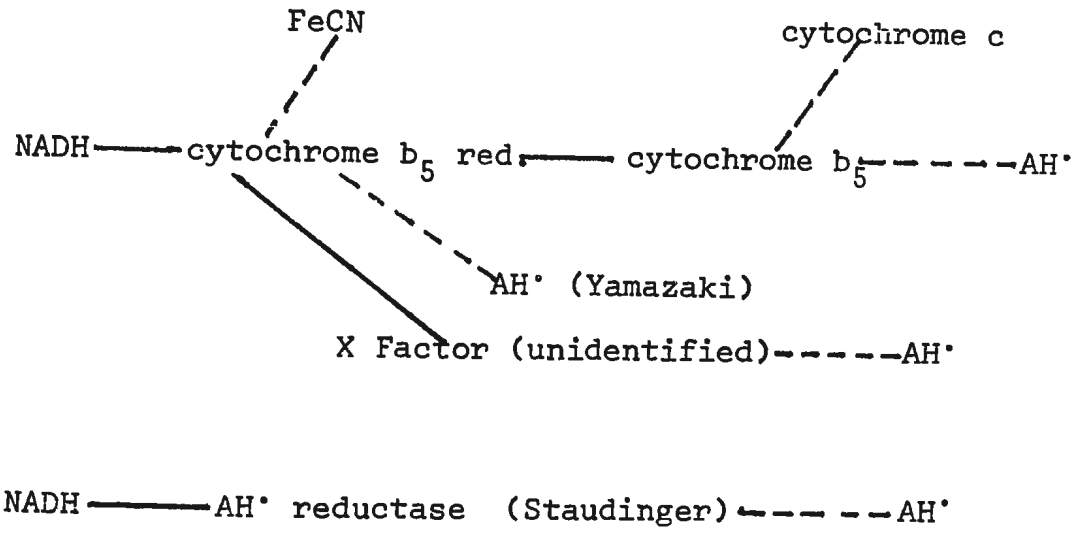
DISCUSSION

Microsomes are known to contain two electron transport chains: one is NADPH specific and involves cytochrome P450, and the other is NADH specific and involves cytochrome b_5. Of the two chains, the NADH specific chain has been suggested to participate in the reduction of cytochrome c (78, 79) by microsomes and the desaturation of stearyl coenzyme A (80).

Two protein components of the microsomal NADH electron transport chain, the NADH-cytochrome b_5 reductase and cytochrome b_5 have been purified and studied by Strittmatter and Velick (81, 70). It was observed that the two proteins combined could function as an NADH-cytochrome c reductase, in which reduced cytochrome b_5 reduces cytochrome c, and Strittmatter (78) suggested this was the mechanism whereby exogenous cytochrome c is reduced by intact microsomes.

An electron transport chain similar to the microsomal NADH-cytochrome b_5 electron transport chain has been described in the outer mitochondrial membrane by Sottocasa et al (56).

Iyanagi and Yamazaki (50) reported that purified NADH-cytochrome b_5 reductase was capable of reducing semidehydroascorbic acid and they suggested that the microsomal NADH-semidehydroascorbate reductase was a property of the flavoenzyme. Staudinger and coworkers (48) however suggested recently that the NADH-semidehydroascorbate reductase is a separate flavoenzyme and that neither cytochrome b_5 reductase nor cytochrome b_5 contribute much to the reduction of semidehydroascorbate by microsomes.



Proposed scheme for the reduction of semidehydroascorbate (AH·) by NADH.

In the present study the tissue distribution and intracellular localization of NADH-semidehydroascorbate reductase showed similarities to that of NADH-rotenone insensitive cytochrome c reductase, which suggests that these two reductases might be linked to the NADH electron transport chains described in microsomes and in the outer mitochondrial membrane.

Further, the AH• reductase showed a Km value of 1 μM for NADH and a pH optimum of 7.4, which agrees favourably with the Km and pH optimum values reported (70, 71) for cytochrome b_5 and cytochrome c reductases, supporting the suggestion that the same flavoprotein is involved in the activity of the three reductases.

Strittmatter (77) demonstrated that only one of the three accessible sulphydryl groups of the cytochrome b_5 reductase was involved in the NADH interaction with the enzyme, and the nucleotide was shown to protect this sulphydryl group from inhibition by sulphydryl reagents. The microsomal AH• and cytochrome c reductases were shown to be protected from p-CMB inhibition by NADH, as was the FeCN reductase, suggesting that the NADH-cytochrome b_5 reductase is involved in the reduction of semidehydroascorbate and cytochrome c. However, under the experimental conditions used, the cytochrome c reductase showed a considerable stimulation when p-CMB was allowed to react with intact microsomes in the presence of NADH. Possibly the p-CMB is binding to a second site in the cytochrome c complex and causing steric alterations that allow

a faster flow of electrons through the complex to cytochrome c although the AH$^{\cdot}$ reduction is not affected.

Cupric ions were found to inhibit the AH$^{\cdot}$ reductase, presumably by complexing functionally active sulphydryl groups. As FeCN and cytochrome c reductases were not inhibited, the sulphydryl groups of the cytochrome b_5 reductase were probably not being affected.

Further evidence for the involvement of the flavo-enzyme NADH-cytochrome b_5 reductase in the activity of AH$^{\cdot}$ reductase is the release and parallel purification of the two microsomal enzyme activities by the method of Takasue and Omura (57). Although much of the AH$^{\cdot}$ reductase activity was destroyed during a three hour digestion of the microsome-lysosome preparation, a little under 5% of the initial enzyme activity was released along with about 45% of the FeCN reductase activity (Table IV). This AH$^{\cdot}$ reductase activity showed an increase in specific activity and an elution pattern from a Sephadex G100 column that parallelled that of the cytochrome b_5 reductase. The solubilized cytochrome c reductase showed a step-wise loss in both specific and total activities under the same conditions, probably because the cytochrome b_5 was being separated from the cytochrome b_5 reductase.

As the solubilized AH$^{\cdot}$ reductase activity was unaffected by detergent concentration that completely inhibited the microsomal enzyme, it appeared as if the cytochrome b_5 reductase did possess an ability to reduce semidehydroascorbate. However, when the enzyme preparation was stored at 0° the AH$^{\cdot}$

reductase activity was lost in 3-5 days when 90% of the FeCN reductase activity still remained, and secondly, the AH• reductase activity disappeared on further purification of the cytochrome b_5 reductase.

In conclusion, the cytochrome b_5 reductase might possess a labile active site capable of reducing semidehydroascorbate, or the flavoenzyme together with an unknown factor is responsible for the solubilized AH• reductase activity. This unknown factor would be of a similar molecular size as cytochrome b_5 reductase and probably a component of the membrane found AH• reductase.

A second method employed to solubilize the microsomal reductases was a deoxycholate treatment of microsomes that successfully solubilized the three enzymes without any loss in activity. Although the lability of the AH• reductase resulted in considerable loss in enzyme activity during the purification procedure (Table VI), the similar elution pattern of the AH• and cytochrome c reductases from the DEAE cellulose column suggests that the two enzyme activities are a property of the same lipoprotein particle that is being eluted from the column under the conditions used. This further suggests that the AH• reductase is probably not a property of the cytochrome b_5 reductase, although the flavoenzyme is possibly involved in a lipoprotein complex responsible for the AH• reductase activity.

Recently Hara and Minakami (75) showed that cytochrome b_5 in intact microsomes as well as soluble cytochrome b_5 was

oxidized by semidehydroascorbic acid, and the latter result was confirmed in the present study. Hara and Minakami further showed that when the reduction of cytochrome b_5 by NADH in intact microsomes was inhibited by detergent treatment, the NADH-AH• reductase activity was reduced in a parallel manner; They concluded from these and other studies that cytochrome b_5 was also involved in the reduction of semidehydroascorbate by microsomes. The same authors (82) also showed that cytochrome c reductase activity of intact microsomes was inhibited by detergent treatment parallel to that of cytochrome b_5 reduction, thus adding further support to the generally accepted hypothesis that cytochrome b_5 is involved in the microsomal reduction of cytochrome c (78, 79).

Ernster and coworkers (62, 63) showed that the inactivation of rotenone insensitive cytochrome c reductase by trypsin treatment parallelled closely the removal of cytochrome b_5 from the membrane, but whether the latter was a cause or an effect of the inactivation could not be decided.

In the present study, the AH• reductase in both mitochondria and microsomes showed a much greater resistance to the proteolytic action than the cytochrome c reductase, although it was more susceptible than the FeCN reductase. Low concentrations of trypsin stimulated the AH• reductase, but after a maximum stimulation was obtained, the reductase lost activity at a comparable rate to that of cytochrome c reductase, probably due to a modification and removal of cytochrome b_5.

When cytochrome b_5 was added to intact microsomes the cytochrome c reductase activity was stimulated 20%. In the cytochrome b_5 deficient microsomes produced by trypsin incubation, the stimulation was enhanced, showing an activation of 30 - 40%. The enhancement is partly due to an increased activity of cytochrome b_5 reductase. Since the cytochrome c reductase activity was not restored to control levels it seems likely that the trypsin is causing a modification at the cytochrome b_5 site in the membrane, resulting in a disruption of electron flow from the cytochrome b_5 reductase to the membrane bound cytochrome b_5 followed by a release of the cytochrome.

The AH$^\bullet$ reductase activity was similarly stimulated 30% when cytochrome b_5 was added to intact microsomes, but in contrast the stimulation was reduced in the cytochrome b_5 deficient microsomes, showing only 10 - 20% increase in activity. The removal of cytochrome b_5 from the membrane seems to be less critical for the activity of AH$^\bullet$ reductase than for the cytochrome c reductase, although the stimulation of the AH$^\bullet$ reductase by trypsin treatment might be masking the inhibitory effect due to loss of cytochrome b_5.

In conclusion a modification of the membrane bound cytochrome b_5 by trypsin is probably the cause of inactivation of both the cytochrome c and AH$^\bullet$ reductases. However, the stimulation of the AH$^\bullet$ reductase suggests that a component of the AH$^\bullet$ reductase that is not shared by the cytochrome c reductase is being altered, resulting in increased enzyme activity that counteracts the inhibition due to cytochrome b_5 modification.

Several microsomal enzymes have been shown to require lipids for their activity (83, 64, 84, 85). Jones and Wakil (64) suggested that lipid specificity shown by cytochrome c reductase in acetone extracted microsomes was dependent on the geometrical dimensions of the micelles rather than on the actual lipid components in the micelles, since synthetic lipids that form the right shape of micelle are just as effective in restoring enzyme activity as micelles of natural lipids.

The AH$^{\bullet}$ reductase was shown to be somwehat more sensitive to acetone extraction than the cytochrome c reductase, a phenomenon also observed with stearyl CoA desaturase (85). Both enzymes however showed similar lipid dependency when activities were restored by adding lipid micelles to the extracted microsomes.

Green and Perdue (86) suggested that intracellular membranes were solubilized by detergents into small lipoprotein subunits, whereas other evidence suggests that complete separation into lipids and proteins occurs (87, 88). When the AH$^{\bullet}$ and cytochrome c reductases were assayed in the presence of detergents, a slight increase in enzyme activity was obtained at low detergent concentrations that solubilized the membrane, as a clearing of the turbid membrane suspension was observed. As the detergent concentration was increased both enzymes showed a similar degree of inactivation, possibly because the

lipoprotein complexes were being disrupted. The FeCN reductase was inhibited about 20% at 10-fold concentrations needed for complete inhibition of AH$^{\bullet}$ and cytochrome c reductase.

Dicumarol and TTFA were found to inhibit the AH$^{\bullet}$ reductase, the former presumably by interaction with the flavoproteins (89), whereas the latter inhibitor has been shown to interact with iron sulphur proteins in the succinate dehydrogenase complex in mitochondria (90, 91) and might possibly be behaving similarly in the microsomes. As ferrous ions were found to stimulate the AH$^{\bullet}$ reductase in the presence of EDTA (1 mM) it is possible that non-heme iron is involved in the enzyme activity. High concentrations (> 10 mM) of chelating agents such as EDTA, citrate and pyrophosphate have been reported to inhibit cytochrome b_5 and cytochrome c reductases. AH$^{\bullet}$ reductase was similarly found to be inhibited by 10 mM EDTA, whereas low concentrations of EDTA somewhat stimulated the enzyme. These chelating agents might possibly be affecting a non-heme moiety involved in the cytochrome c complex, as has been suggested by Strittmatter and Velick (92) and Mahler and Elowe (93).

The evidence that supports the suggestion that cytochrome b_5 reductase is involved in the activity of AH$^{\bullet}$ reductase as well as the cytochrome c reductase activity includes: 1) the localization of the three enzymes in the microsomal and outer mitochondrial membrane fractions; 2) similarities in Km values and pH optima; 3) protection by NADH of sulphydryl inhibition;

4) $AH^{\cdot}$ and cytochrome c reductase activities of a reconstituted system of cytochrome b_5 reductase and cytochrome b_5, and apparent involvement of cytochrome b_5 in the membrane bound enzymes. Further, the AH· and cytochrome c reductases showed: 5) similar elution pattern from DEAE cellulose chromatography of DOC solubilized microsomes; and 6) similar lipid dependence and sensitivity to detergent inhibition.

Some dissimilarities between the AH· and cytochrome c reductases were also observed: 1) the specific activity of the cytochrome c reductase was about 5 times higher in microsomes than in mitochondria, whereas the specific activity of AH· reductase was higher in mitochondria than in microsomes; 2) the AH· reductase was inhibited by $CuSO_4$ and TTFA but cytochrome c reductase was not; 3) the two enzymes showed difference in sensitivity to trypsin treatment and cytochrome b_5 was less effective in restoring activity of the AH· reductase in cytochrome b_5 deficient microsomes than the cytochrome c reductase.

The present evidence does favour the suggestion that cytochrome b_5 reductase and cytochrome b_5 are components of the membrane bound AH· reductase. An additional component of the AH· reductase not yet identified could explain the differences observed between the cytochrome c and AH· reductases. The AH· reductase activity of cytochrome b_5 reductase reported by Iyanagi and Yamazaki (50) is either an artificial property caused by lysosomal solubilization or is due to contamination of cytochrome b_5 or other components of the AH· reductase.

However, the possibility that two different flavoproteins are responsible for the activities of the AH$^{\bullet}$ and cytochrome c reductases as suggested by Staudinger and coworkers (48) cannot be ruled out. A final conclusion will have to await further resolution, identification and reconstitution of both of these membrane bound enzyme complexes.

CHAPTER II

THE FUNCTION OF SEMIDEHYDROASCORBATE REDUCTASE

METHODS

Rat liver mitochondria were prepared as described in Chapter I, except the livers were homogenized in a sucrose medium containing 0.33 M sucrose, 0.025 M Tris-HCl buffer, pH 7.4.

Hog adrenals were obtained from a local slaughter house. The adrenals were decapsulated and subcellular fractions were prepared by the method of Sedgwick and Hübscher (51) using a sucrose medium containing 0.3 M sucrose, 1 mM EDTA with pH adjusted to 7.5 with 1 M KOH.

Semidehydroascorbate reductase was assayed as described in Chapter I, except 1.5 µM rotenone was used instead of KCN to inhibit NADH oxidation.

RESULTS

AH• reductase maintaining intracellular NAD levels

Although the ascorbic acid levels in the adrenal cortex are at least five times higher than those found in liver, the AH• reductase activity in both tissues is about the same (Table I).

When the AH• reductase was assayed using ascorbic acid instead of semidehydroascorbic acid as the electron acceptor, the adrenal microsomes were shown to exhibit the same activity as when semidehydroascorbate was used. However, liver microsomes only showed one-fifth or less of their maximum enzyme activity when ascorbate alone was used. It has been demonstrated (94) that the semidehydroascorbate radical is the effective stimulator of NADH oxidation, that is observed in the presence of ascorbate. The adrenal microsomes therefore apparently contain a powerful ascorbic acid oxidase capable of oxidizing ascorbate to semidehydroascorbate radical which is much less effective in liver microsomes. This apparent ascorbic oxidase was stimulated by EDTA and is therefore probably not due to lipid peroxidation. It was inhibited by 0.1 mM o-phenanthroline if 1 mM KCN was present; this inhibition was partly reversed by Fe^{+2} but Cu^{+} had no effect. KCN by itself had no effect, whereas 0.1 mM o-phenanthroline stimulated the activity.

It is suggested that the oxidation of ascorbate by the ascorbate oxidase of the adrenal microsomes would supply the AH• reductase with saturation level of semidehydroascorbate and thus allow the AH• reductase to act at its maximum efficiency in oxidizing NADH in the cell.

Protective function of the AH• reductase in the mitochondria

Ascorbate-induced mitochondrial swelling is possibly due to the semidehydroascorbate radical initiating lipid peroxidation since agents that accelerate the oxidation of ascorbate to semidehydroascorbate (such as Cu^{+2}, Fe^{+3} or cumene hydroperoxide), markedly enhance the rate of mitochondrial swelling.

Ascorbate-induced swelling showed similar characteristics over a wide range of ascorbate concentrations (0.3 - 10 mM). At concentrations of 20 mM the lag period was increased and rate of swelling was considerably slower. When copper or cumene hydroperoxide were added to the swelling medium, the lag period was greatly reduced and rapid swelling occurred (Figure 9). In the absence of ascorbate, copper (5 µM) or cumene hydroperoxide (0.1 mM) had little or no effect. NADH however lengthened the lag period of the swelling induced by ascorbate in the presence of cumene hydroperoxide, but had no effect on the ascorbate-induced swelling in the presence of copper.

When intact mitochondria were assayed in the swelling medium for AH• reductase, in the presence of 1.5 µM rotenone, the enzyme showed the same activity as with sonicated or Lubrol WX treated mitochondria. As the inner membrane is impermeable to NADH (95) this supports the conclusion that the enzyme is located in the outer membrane (Table II) and suggests that the enzyme is functioning in the intact organelles.

Thus the effect of NADH on the cumene hydroperoxide

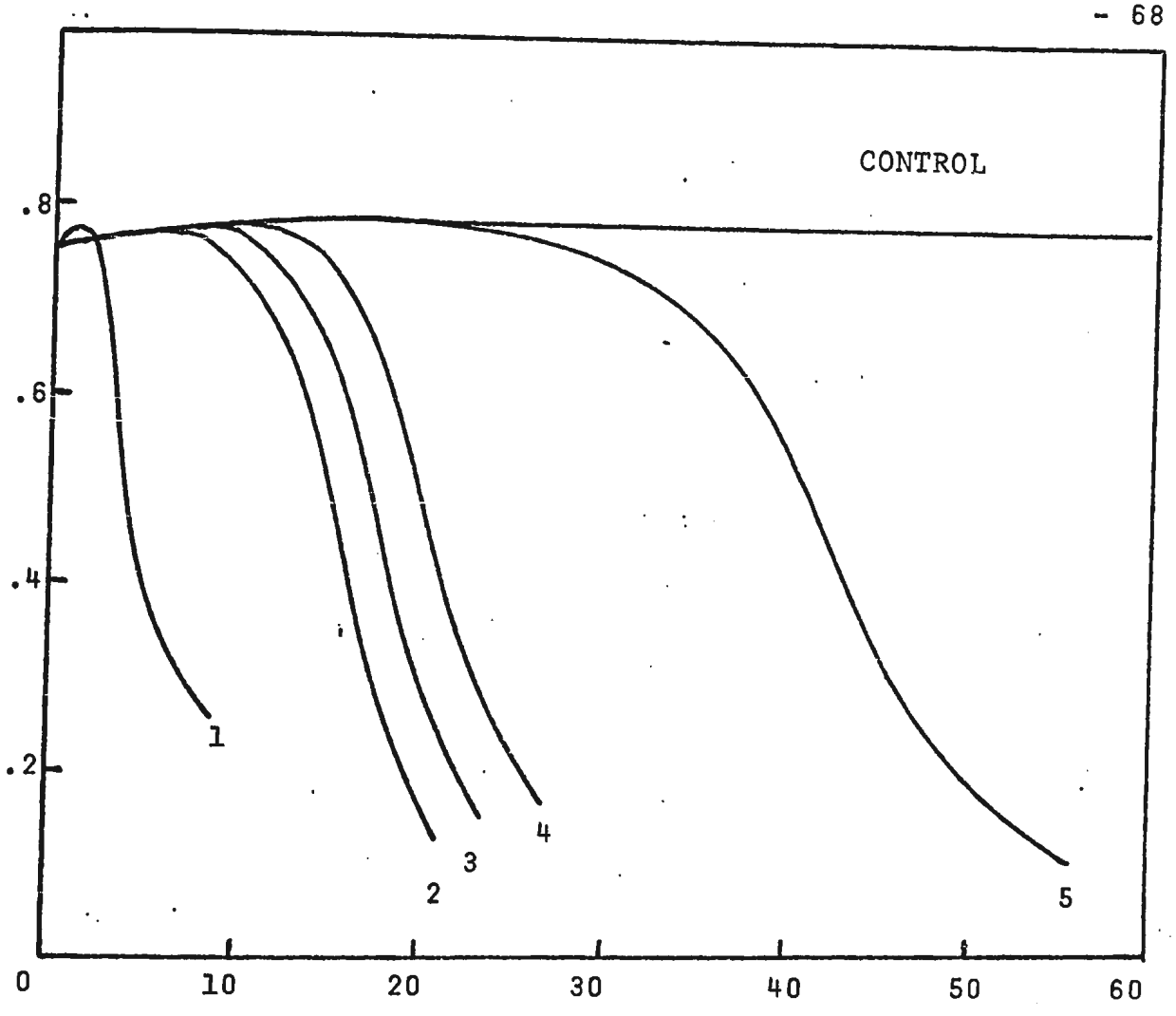


Figure 9. The effect of NADH on cumene hydroxyperoxide stimulation of ascorbate - induced mitochondrial swelling. Mitochondrial swelling was followed by changes in turbidity (D_{520}) in a medium containing 0.33 M sucrose, 25 mM Tris-HCl, pH 7.4, and additives as indicated.

1. 5 mM ascorbate, 5 x 10^{-6} M $CuSO_4$
2. 5 mM ascorbate, 0.1 mM cumene hydroperoxide
3. same as 2; 0.1 mM NADH[a]
4. same as 2: 0.2 mM NADH[a]
5. 0.3 - 10 mM ascorbate

[a] The rate of NADH oxidation in the assay cuvette was 6 nmoles per minute.

stimulation of ascorbate-induced swelling may be due to the action of AH• reductase in the outer membrane. The enzyme could delay the onset of lipid peroxidation by reducing the semidehydroascorbate (produced by the cumene hydroperoxide oxidation of ascorbate) back to ascorbate.

The AH• reductase may therefore play an important role in protecting the mitochondrial and possibly the microsomal membranes from the damaging effect of lipid peroxidation initiated by semidehydroascorbic acid.

AH• reductase maintaining ascorbate in the reduced form

Low concentrations of ascorbate were shown to induce mitochondrial swelling, whereas high concentrations (20 mM) induced only a slow swelling following a long lag period. As can be seen in Figure 10 concentrations as low as 1 μM ascorbate induce mitochondrial swelling although the lag is considerably longer and the rate of swelling slower than observed with ascorbate concentrations in the range of 10 - 30 μM. When NADH was added along with ascorbate, a shortening of the lag period and a marked stimulation of swelling was observed at low concentrations of ascorbate (Figure 11). NADH by itself caused no swelling at the concentrations used.

The swelling by these low concentrations of ascorbate was markedly enhanced by agents oxidizing ascorbate to semidehydroascorbate (e.g. $CuSO_4$, cumene hydroperoxide or dehydroascorbate).

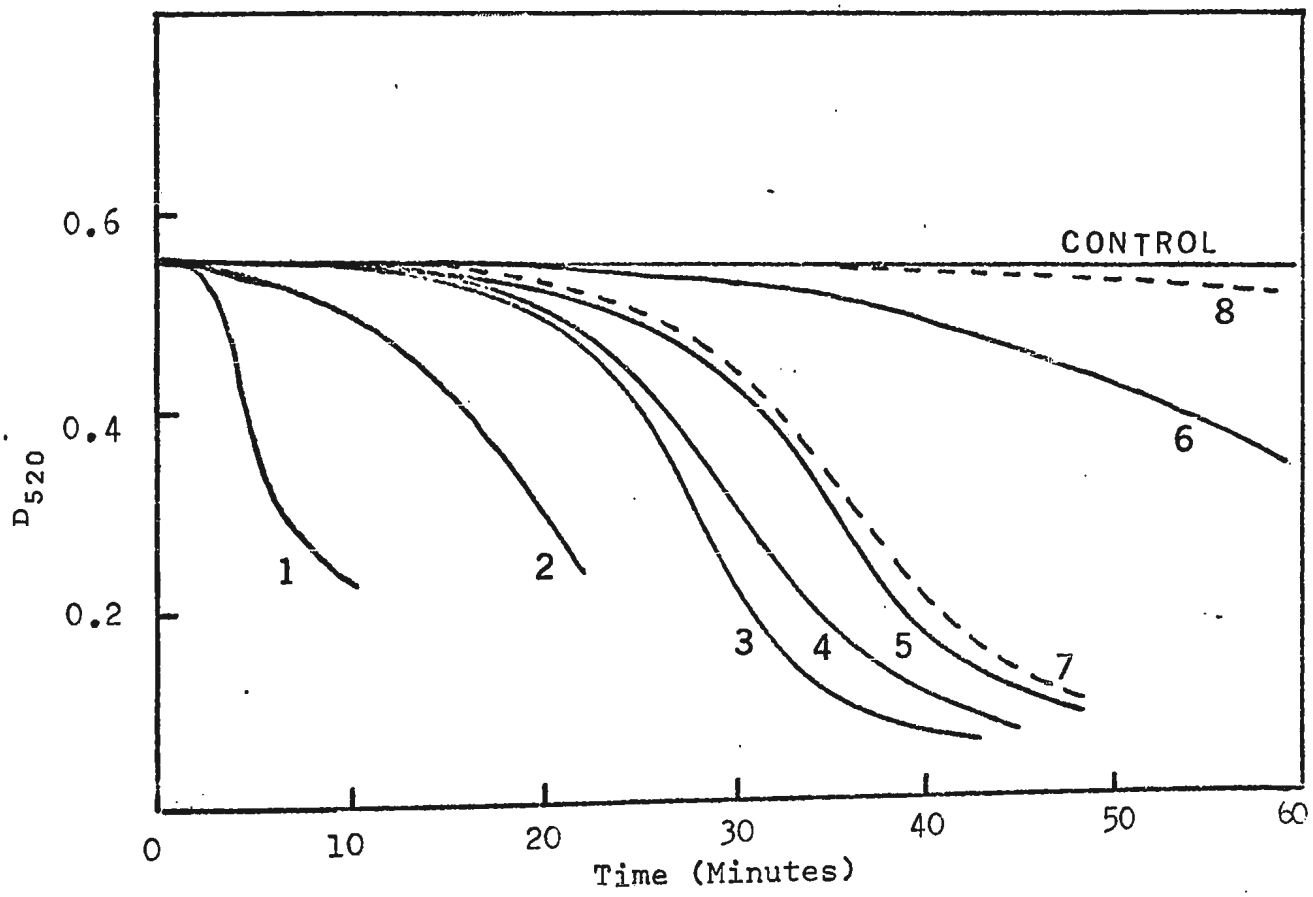


Figure 10 Ascorbate-induced mitochondrial swelling. The effect of different concentrations of ascorbate and ascorbate+NADH on mitochondrial swelling. Mitochondrial swelling was followed by changes in turbidity (D_{520}) in a medium containing 0.33 M sucrose, 25 mM Tris-HCl, pH 7.4, and ascorbate and NADH as indicated.

1. 10 µM ascorbate, 10^{-6} M $CuSO_4$
2. 10 µM ascorbate, 10^{-6} M $FeCl_3$
3. 100 µM ascorbate
4. 30 µM ascorbate
5. 10 µM ascorbate
6. 1 µM ascorbate
7. 1 µM ascorbate, 0.1 mM NADH
8. 0.1 mM NADH

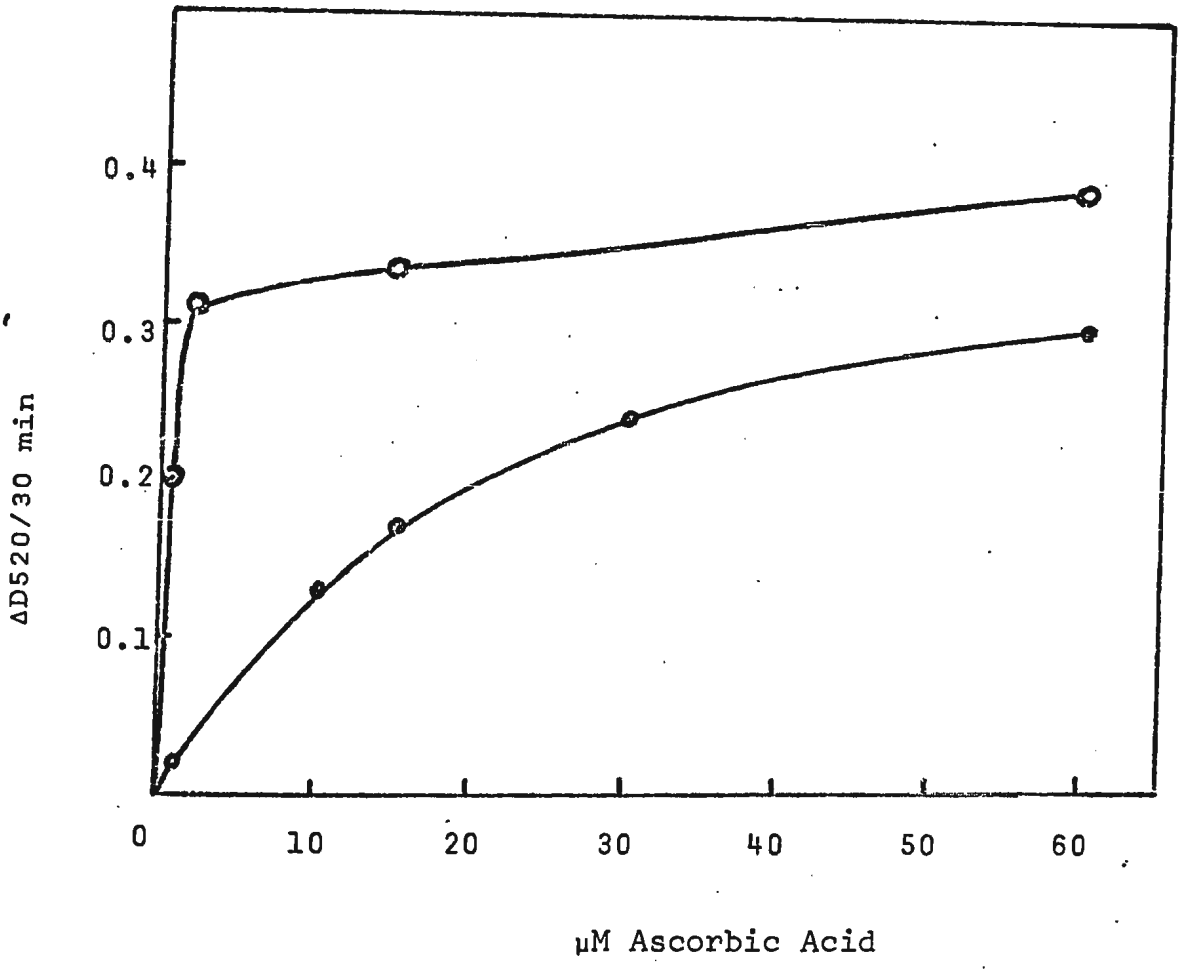


Figure 11. NADH stimulation of ascorbate-induced mitochondrial swelling. The extent of swelling is expressed as the change in turbidity observed 30 minutes after addition of ascorbate.

-●-●- ascorbate

-o-o- ascorbate, 0.1 mM NADH

Agents oxidizing ascorbate further to dehydroascorbate (e.g. excess ascorbate oxidase, or cytochrome c, or Wurster's blue) inhibited mitochondrial swelling. An ascorbate oxidase extract (from cucumber stem) that completely abolished the ascorbate-induced swelling had no effect on the NADH plus ascorbate-induced swelling. Rotenone (1.5 μM) and azide (10 mM) had no significant effect on either the ascorbate or on the NADH plus ascorbate-induced swelling. The NADH enhancement is therefore unlikely to be due to increased electron transport in the inner membrane.

Cyanide (30 μM) prolonged the lag period and slowed down the rate of both ascorbate and NADH plus ascorbate-induced swelling.

The NADH stimulation of ascorbate-induced swelling at low ascorbate concentrations is probably due to the activity of the AH$^{\bullet}$ reductase which in the presence of NADH would maintain the ascorbate in the reduced state. Oxidation of the ascorbate to the semidehydroascorbate radical could result in initiation of lipid peroxidation which is thought to be responsible for the swelling of the mitochondria (21). In the absence of NADH much of the ascorbate at these low concentrations would be aerobically oxidized to dehydroascorbate and would not be available to induce lipid peroxidation.

DISCUSSION

It has been implied (75, 52) that the function of $AH^{\cdot}$ reductase is to maintain the ascorbic acid of the cell in the reduced form. The slow non-enzymatic reduction of dehydroascorbate by glutathione has also been suggested (96) as an important mechanism for keeping ascorbate in the reduced state. However, the latter mechanism may not be very effective in tissues with high ascorbate concentrations where glutathione may exist in concentrations up to 10% of that of ascorbate, such as in the adrenal and the anterior pituitary, or even in liver where these two reductants are found in almost equal concentrations (97).

The high concentrations of ascorbic acid in steroid producing tissues and alterations in ascorbate concentrations preceding active hormone synthesis has lead to the suggestion that ascorbate might be involved in the control of steroidogenesis (98). Devine and Rivers (46) suggested that ascorbic acid was involved in the conversion of pregnenolone to progesterone, which is an important step in the biosynthesis of adrenal corticosteroids and ovarian and testicular steroids. The conversion that occurs in the microsomes requires NAD as a cofactor (99). As the NAD/NADH ratio in the adrenal gland was shown to be lowered from 1.2 in normal guinea pigs to 0.7 in scorbutic animals, when the ascorbic acid levels dropped from 102 to 15 mg % (46), it was suggested that ascorbic acid and the

microsomal $AH^{\cdot}$ reductase might play an important role in maintaining high levels of NAD for this reaction. The ascorbate oxidase that was demonstrated (page 66) in the adrenal microsomes may, by oxidizing ascorbate to semidehydroascorbate, also be of great importance by supplying the enzyme with high substrate concentrations.

The diminution of ascorbic acid in the adrenal cortex and ovary in response to stimulating hormones may be related to other functional properties of ascorbic acid. High concentrations of ascorbic acid have been shown to directly inhibit the mitochondrial steroid hydroxylating enzymes (14, 18) although ascorbic acid has also been shown to stimulate these same enzymes, possibly by supplying electrons to the mitochondrial electron transport chain (15). The location of the $AH^{\cdot}$ reductase in the outer mitochondrial membrane is probably of significance if the above observations are correct. In the resting endocrine tissue it would be of importance to keep the ascorbate in a reduced state, to enable ascorbate to control the activity of the above mentioned enzymes. This could be especially important during active steroid synthesis, when the ascorbate concentration of the cell is decreased.

Lipid peroxidation is a well-recognized phenomenon associated with cellular damage in certain pathological states, such as in vitamin E deficiency (100). Peroxidation of lipids has also been suggested as an important factor in

the regulation of cytoplasmic structure and function (101). Ascorbic acid is known to initiate lipid peroxidation in vitro (23) and Haase and Dunkley (102) have shown that semidehydroascorbate radical is a far more powerful inducer of lipid peroxidation than is ascorbic acid. Hunter et al (20) have also suggested that the semidehydroascorbate radical is responsible for the ascorbate-induced mitochondrial swelling.

It was shown that in ascorbate-induced mitochondrial swelling the lag period was markedly reduced when copper or dehydroascorbate or cumene hydroperoxide were added to the swelling medium. At physiological concentrations of ascorbic acid (2- 10 mM) this enhanced rate of swelling was reduced in the presence of NADH (0.1 - 0.2 mM). Copper (103) or dehydroascorbate (104) are known to greatly increase the concentration of semidehydroascorbate radical, when added to a solution of ascorbic acid. Thus the $AH^{\cdot}$ reductase is probably delaying the onset of lipid peroxidation by reducing the semidehydroascorbic acid back to ascorbate in the presence of NADH. $AH^{\cdot}$ reductase may therefore act as an important protector of both microsomal and outer mitochondrial membranes by reducing the semidehydroascorbate radical, preventing it from initiating extensive lipid peroxidation damage to these intracellular membranes.

At concentrations of ascorbic acid much lower than physiological concentrations (1-20 μM), NADH was shown to stimulate mitochondrial swelling. This again can be explained

by the action of $AH^{\bullet}$ reductase. In the presence of NADH and $AH^{\bullet}$ reductase the autoxidation of ascorbate to dehydroascorbate (105) is prevented. Thus, the low levels of ascorbate are maintained in the reduced state and the ascorbate then probably generates the semidehydroascorbate required for a normal ascorbate-induced mitochondrial swelling to occur.

REFERENCES

1. Meiklejohn, A.P., Vitamins and Hormones 11 (1953) 61.
2. Lowry, O.H., Physiol. Rev. 32 (1952) 431.
3. Chatterjee, I.B., Kar, N.C., Ghosh, N.C. and Guha, B.C., Nature 192 (1961) 163.
4. Chatterjee, I.B., Kar, N.C., Ghosh, N.C. and Guha, B.C., Ann. N.Y. Acad. Sci. 92 (1961) 36.
5. Smith, F., Vitamins 1 (1954) 199.
6. Bourne, G.H., Nature 131 (1933) 874.
7. Giroud, A. and Leblond, C.P., Compt. Rend. Soc. Biol. 115 (1934) 705.
8. Harris, L.J. and Ray, S.N., Biochem. J. 27 (1933) 2006.
9. Sayers, G., Sayers, M.A., Lewis, H.L. and Long, C.N.H., Proc. Soc. Exp. Biol. Med. 55 (1944) 238.
10. Slusher, M. and Roberts, S., Endocrinology 61 (1957) 98.
11. Harding, B.W. and Nelson, D.H., Endocrinology 73 (1962) 97.
12. Kitabchi, A.E., Fed. Proc. 25 (1966) 494.
13. Kitabchi, A.E., Fed. Proc. 26 (1967) 484.
14. Kitabchi, A.E., Steroids 10 (1967) 567.
15. Harding, B.W., Bell, J.J., Oldham, S.B., Wilson, L.D., in Function of the Adrenal Cortex (McKerns, K., ed.)Appleton-Century-Crofts, New York, 1967.
16. Claesson, L., Hillarp, N.A., Hogberg, B. and Hokfelt, B. Acta Endocrin. 2 (1949) 249.
17. Hokfelt, B., Acta Physiol. Scand. 20 (1950) 172.

18. Sulimovici, S. and Boyd, G.S., Steroids 12 (1968) 127.

19. Barber, A., Lipids 1 (1966) 146.

20. Hunter, F.E. Jr., Scott, A., Hoffsten, P.E., Guerra, E., Weinstein, J., Scheider, A., Schutz, B., Fink, J., Ford, L., and Smith E., J. Biol. Chem. 239 (1964) 604.

21. Hoffsten, P.E., Hunter, F.E. Jr., Gebicki, J.M. and Weinstein, J., Biochem. Biophys. Res. Commun. 7 (1962) 276.

22. Robinson, J.D., Arch. Biochem. Biophys. 112 (1965) 170.

23. Wills, E.D., Biochem. J. 113 (1969) 315.

24. Carpenter, M.P., Kitabchi, A.E., McCay, P.B. and Caputto, R., J. Biol. Chem. 234 (1959) 2814.

25. Bieri, J.G. and Anderson, A.A., Arch. Biochem. Biophys. 90 (1960) 105.

26. Pritchard, S.T. and Singh, H., Biochem. Biophys. Res. Commun. 2 (1960) 184.

27. Kitabchi, A.E. and Williams, R.H., J. Biol. Chem. 243 (1968) 3248.

28. Bourne, G.H. in Biochemistry and Physiology of Nutrition (Bourne, G.H. and Kidder, C.W., eds.) Vol. 2, Academic Press, New York, 1953.

29. Kivirikko, K.I. and Prockop, D.J., Proc. U.S. Nat. Sci. 57 (1967) 782.

30. Hutton, J.J., Tappel, A.L., and Udenfriend, S., Arch. Biochem. Biophys. 118 (1967) 231.

31. Robertson, W.,Ann. N.Y. Acad. Sci. 92 (1961) 159.

32. Kaufman, S., Bridgers, W.F. and Baron, J. in Oxidation of Organic Compounds - III, 172 (American Chemical Society, 1968).

33. Abbot, M.T., Schandl, E.K., Lee, R.F., Parker, T.S. and Midgett, R.J.,Biochim. Biophys. Acta 132 (1967) 525.

34. Zannoni, V.G. and LaDu, B.N., J. Biol. Chem. 235 (1960) 2667.

35. LaDu, B.N. and Zannoni, V.G., Nature 177 (1956) 574.

36. Knox, W.E., Piras, M.M. and Tokuyama, K., J. Biol. Chem. 241 (1966) 297.

37. Nason, D., Wossilait, W. and Terell, A.L., Arch. Biochem. Biophys. 48 (1954) 233.

38. Kern, M. and Racker, E., Arch. Biochem. Biophys. 48 (1954) 235.

39. Kersten, W., Schmidt, H. and Staudinger, Hj., Biochem. Z., 326 (1955) 469.

40. Kersten, H., Kersten, W. and Staudinger, Hj., Biochem. Z., 328, (1956) 24.

41. Kersten, H., Kersten, W. and Staudinger, Hj., Biochim. Biophys. Acta 27 (1958) 598.

42. Yamazaki, I., J. Biol. Chem. 237 (1962) 224.

43. Trebst, A., Eck, H., Wagner, S. in Photosynthetic Mechanism of Green Plants, 174 (Kok, B., Jagendorf, A.T., eds., Nat. Acad. Sci. - Nat. Res. Coun. Publ. 1145, 1963).

44. Böhme, H. and Trebst, A., Biochim. Biophys. Acta 180 (1969) 137.

45. Yamashita, T. and Butler, W.L., Plant Physiol. 43 (1968) 1978.

46. Devine, M.M. and Rivers, J.M., J. Nutrition 97 (1969) 295.

47. Frunder, H., Blume, E. and Kluge, H., Biochim. Biophys. Acta, 65 (1962) 146.

48. Schulze, H.-U., Gallenkamp, H. and Staudinger, Hj., Hoppe-Seyler's Z. Physiol. Chem. 351 (1970) 809.

49. Everling, F.B., Weis, W. and Staudinger, Hj., Hoppe-Seyler's Z. Physiol. Chem. 350 (1969) 1485.

50. Iyanagi, T. and Yamazaki, I., Biochim. Biophys. Acta, 172 (1969) 370.

51. Sedgwick, B. and Hübscher, G., Biochim. Biophys. Acta 106 (1965) 63.

52. Nazemi, M. and Staudinger, Hj., Hoppe-Seyler's Z. Physiol. Chem. 349 (1968) 345.

53. Ochoa, S., in Methods in Enzymology, Vol. 1 (Colowick, S.P. and Kaplan, N.O., eds.) Academic Press, New York, 1955, p. 735.

54. Schnaitman, C., Erwin, V.G. and Greenawalt, J.W., J. Cell. Biol. 32 (1967) 719.

55. Lowry, O., Rosebrough, N., Farr, A., and Randall, R., J. Biol. Chem. 193 (1951) 265.

56. Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A., J. Cell. Biol. 32 (1967) 415.

57. Takesue, S. and Omura, T., Biochem. Biophys. Res. Commun.

30 (1968) 723.

58. Holloway, P.W. and Wakil, S.J., J. Biol. Chem. 245 (1970) 1862.

59. Lu, A.Y.H., and Coon, M.J., J. Biol. Chem. 243 (1968) 1331.

60. Omura, T. and Takesue, S., J. Biochem. 67 (1970) 249.

61. Omura, T. and Sato, R., J. Biol. Chem. 239 (1964) 2370.

62. Kuylenstierna, B., Nicholls, D.G., Hovmöller, S.and Ernster, L., Europ. J. Biochem. 12 (1970) 419.

63. Orrenius, S., Berg, A. and Ernster, L., Europ. J. Biochem. 11 (1969) 193.

64. Jones, P.D., and Wakil, S.J., J. Biol. Chem. 242 (1967) 5267.

65. Ichikawa, Y. and Yamano, T., Arch. Biochem. Biophys. 121 (1967) 742.

66. Katsuyuki, I., Omura, T. and Sato, R., J. Biochem. 60 (1966) 274.

67. Schnaitman, C. and Greenawalt, J.W., J. Cell. Biol. 38 (1968) 158.

68. Davis, K.A. and Kreil, G., Biochim. Biophys. Acta, 162 (1968) 627.

69. Lumper, L., Schneider, W. and Staudinger, Hj., Hoppe-Seyler's Z. Physiol. Chem. 348 (1967) 323.

70. Strittmatter, P. and Velick, S.F., J. Biol. Chem. 228 (1957) 785.

71. Penn, N. and Mackler, B., Biochim. Biophys. Acta, 27 (1958) 539.

72. Okamoto, H., Ichiyama, A. and Hayaishi, O., Arch. Biochem. Biophys. 118 (1967) 110.

73. Sargent, J.R., St. Louis, P.J. and Blair, P.A., Biochim. Biophys. Acta 223 (1970) 339.

74. Everling, F.B., Weis, W. and Staudinger, Hj., Hoppe-Seyler's Z. Physiol. Chem. 350 (1969) 886.

75. Hara, T. and Minakami, S., J. Biochem. 69 (1971) 325.

76. Mackler, B. in Methods in Enzymology, Vol. X, (Estabrook, R.W. and Pullman, M.E. eds.) Academic Press, New York, 1967, p.551.

77. Strittmatter, P., J. Biol. Chem. 234 (1959) 2661.

78. Strittmatter, P., in The Enzymes, Vol. 8 (Boyer, P.D., Lardy, H. and Myrbäck, K. eds.) Academic Press, New York, 1963 p.113.

79. MacLennan, D.H., Tzagoloff, A. and McConnell, D.G., Biochim. Biophys. Acta 131 (1967) 59.

80. Holloway, P.W., Biochemistry 10 (1971) 1556.

81. Strittmatter, P. and Velick, S.F., J. Biol. Chem. 221 (1956) 253.

82. Hara, T. and Minakami, S., J. Biochem. 69 (1971) 317.

83. Duttera, S.M., Byrne, W.L. and Ganoza, M.C., J. Biol. Chem. 243 (1968) 2216.

84. Schulze, H.-U., and Staudinger, Hj., Hoppe-Seyler's Z. Physiol. Chem. 352 (1971) 309.

85. Jones, P.D., Holloway, P.W., Peluffo, R.O. and Wakil, S.J., J. Biol. Chem. 244 (1969) 744.

86. Green, D.E. and Perdue, J.F., Proc. Nat. Acad. Sci., U.S. 55 (1966) 1295.

87. Engelman, D.M., and Morowitz, H.J., Biochim. Biophys. Acta 150 (1968) 385.

88. Bont, W.S., Emmelot, P. and VazDias, H., Biochim. Biophys. Acta. 173 (1969) 389.

89. Mahler, H.R., Raw, I., Molinari, R. and doAmaral, D.F., J. Biol. Chem. 233 (1958) 230.

90. Whittaker, P.A. and Redfearn, E.R., Biochem. J. 88 (1963) 15P.

91. Tappel, A.L., Biochem. Pharmacol. 3 (1960) 289.

92. Strittmatter, P. and Velick, S.F., J. Biol. Chem. 221 (1956) 277.

93. Mahler, H.R. and Elowe, D.G., J. Biol. Chem. 210 (1954) 165.

94. Staudinger, Hj., Abraham, R. and Schneider, W., Biochim. Biophys. Acta, 74 (1963) 547.

95. Purvis, J.L. and Lowenstein, J.M., J. Biol. Chem. 236 (1961) 2794.

96. Harding, B.W. Rutherford, E.R. and Nelson, D.H., Program of the 43rd Meeting of the Endocrine Society, New York, 1961 (abstract # 84).

97. Lazarow, A., in Glutathione (Colowick, S., et al, eds.) Academic Press, New York, 1954, p.231.

98. Kitabchi, A.E., Nature 215 (1967) 1385.

99. Koritz, S.B., Arch. Biochem. Biophys. 100 (1963) 349.

100. Tappel, A.L., Vitamins and Hormones 20 (1962) 493.

101. Hochstein, P. and Ernster, L., Ciba Foundation Symposium on Cellular Injury, (1964) p.123.

102. Haase, G. and Dunkley, W.L., J. Lipid Res. 10 (1969) 568.

103. Schubert, J., Copper and Peroxides in Radiobiology and Medicine, p.22, Charles C. Thomas, publisher (1964).

104. Weis, W., Zubrzycki, Z. and Staudinger, Hj., Hoppe-Seyler's Z. Physiol. Chem. 349 (1968) 279.

105. Levandoski, N.G., Baker, E.M., and Canham, J.E., Biochemistry 10 (1964) 1465.