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ELECTRON TRANSPORT AND ADENOSINE TRIPHOSPHATASE ACTIVITIES IN TURNIP MICROSOME AND SOLUBLE FRACTIONS

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by

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C O N T E N T S

S U M M A R Y

1. Microsomes were isolated from turnip (*Brassica rapa* L.) root storage tissue. Subfractionation (using discontinuous sucrose gradients in the presence of ions) resulted in (a) the separation of the majority of the ribosomes from the membranous vesicles and (b) the further separation of at least two types of smooth vesicles - one considered to be derived from the endoplasmic reticulum and the other from the plasmamembranes.

2. Specific NADH and NADPH dehydrogenases (in the presence of a variety of acceptors), and cytochrome b_{555}, but not cytochrome P-450, were detected in the microsome fraction. The NADH dehydrogenase activities, NADPH-cytochrome c reductase and cytochrome b_{555} were concentrated in one of the smooth membrane fractions, while the other NADPH dehydrogenase activities were distributed more evenly throughout the subfractions. The structure of the electron transport system was determined. The results indicated that, as in animal microsomes, there were two electron transport chains; one specific for each reduced pyridine nucleotide. Attempts to detect mixed function oxidase activity involving the NADPH electron transport chain were unsuccessful. Oxygen consumption was detected in the presence of NAD(P)H and organic substrate, but was shown to be due to peroxidase.

3. The microsome fraction also contained a salt-stimulated

Mg^{++}-activated ATPase which was concentrated in the smooth membrane fractions. Activity was assayed at high pH (7.8) to eliminate interference from acid phosphatase which was also present in the microsomes. There was a specificity for the anion but not cation in the salt-stimulation of the Mg^{++}-ATPase. This activity was further stimulated by carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol, valinomycin, nigericin and NH_4Cl, with a synergistic effect between CCCP and valinomycin. Activity was insensitive to oligomycin, phlorizin and ouabain. Based on similarity to the chloroplast ATPase, it was proposed that this ATPase was situated on the outside of the vesicle and moved H^+ to the inside, leaving the outside alkaline. It is suggested that this resulted in movement of the anion to maintain electrical neutrality, and exchange of the proton for the cation to maintain pH neutrality.

4. The soluble supernatant fraction contained two reductase activities. Partial purification showed that one was specific for both NADH and FeCN but that the other showed little specificity for either donor or acceptor. It was suggested that the former may be solubilized from the microsomal membranes during homogenizing of the tissue and that the latter may be the equivalent of the animal DT diaphorase.

5. Slicing turnip tissue induced 20-100% loss of microsomal NADH dehydrogenase activities within 10 minutes. Partial recovery of some activities occurred on subsequent aging of the disks, and these

recoveries were sensitive to cycloheximide and, in part, to 6-methylpurine. Aging turnip disks also induced increases in the soluble dehydrogenase activities as well as the development of an NADH-cytochrome c reductase (often not present in fresh tissue). Increases were sensitive to 6-methylpurine, ethionine, puromycin, and cycloheximide. Partial purification of the NADH-cytochrome c reductase showed that it was highly specific for both donor and acceptor and was highly sensitive to pCMB. However, its function was not resolved. Although no correlation could be found between these activity changes and physiological changes in the disks, there did appear to be a correlation between some changes in the microsomal reductases and reported changes in the ultrastructure of the endoplasmic reticulum.

D E C L A R A T I O N

The investigations described in this thesis were carried out in the Botany Department, University of Adelaide, from May 1968 to May 1971. The following papers were written during the period of study:

1. Characterization of Electron Transport in Turnip Microsomes: by J. M. Rungie and J. T. Wiskich. Aust. J. Biol. Sci. (in press).
2. Changes in Microsomal Electron Transport of Plant Storage Tissues Induced by Slicing and Aging: by J. M. Rungie and J. T. Wiskich. Aust. J. Biol. Sci. (in press).
3. Soluble Electron Transport Activities in Fresh and Aged Turnip Tissue: by J. M. Rungie and J. T. Wiskich. Planta (Berl.) (in press).
4. Salt-Stimulated Adenosine Triphosphatase from Smooth Microsomes of Turnip: by J. M. Rungie and J. T. Wiskich. (Submitted to Plant Physiol. October 1971).

To the author's belief and knowledge, this thesis contains no material previously submitted for a degree in any University by the author or by any other person, except where due reference is made in the text.

J. M. Rungie

A C K N O W L E D G E M E N T S

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A B B R E V I A T I O N S

ADP	adenosine diphosphate
AMP	adenosine monophosphate
APAD(H)	3-acetyl pyridine adenine dinucleotide (reduced)
APADP(H)	3-acetyl pyridine adenine dinucleotide phosphate (reduced)
ATP	adenosine triphosphate
BSA	bovine serum albumin
CCCP	carbonyl cyanide m-chloro-phenylhydrazone
cyt.	cytochrome
DCPIP	2,6-dichlorophenol indophenol
DMA	dimethylaniline
DNP	2,4-dinitrophenol
E	extinction
E^{M}	molar extinction coefficient
E'_{o}	oxidation-reduction potential
EDTA	ethylenediaminetetra acetate
ER	endoplasmic reticulum
FeCN	potassium ferricyanide
FP	flavoprotein
gfw	gram fresh weight
G-6-P	glucose-6-phosphate
IAA	indoleacetic acid

K_M	Michaelis constant
MFO	mixed function oxidation
NaBGlP	β sodium glycerophosphate
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
NOQNO	2-nonyl-4-hydroxyquinoline-N-oxide
NT	3,3'-(4,4'-biphenylene)-bis-(2,5-diphenyl-2H-tetrazolium chloride)
pCMB	para-chloromercuribenzoate
P_i	inorganic phosphate
RNA	ribonucleic acid
tes	tris(hydroxymethyl)methylethanesulphonic acid
tris	tris(hydroxymethyl)aminomethane

C H A P T E R I

GENERAL INTRODUCTION

A. INTRODUCTION

The energetics of chloroplasts and both plant and animal mitochondria have been intensively studied (Lehninger, 1964; Pullman and Schatz, 1967; Packer *et al.*, 1970; Schwartz, 1971). The membranes of these organelles contain specifically orientated electron transport chains in which electron flow is geared to phosphorylation of ADP to ATP (by an ATPase) or other energy requiring processes (volume changes and ion movements). It has been proposed that electron flow results in either the formation of high energy chemical intermediates (chemical theory, Slater, 1953), or in the separation of charge across the membrane setting up a proton and electrical gradient (chemiosmotic theory, Mitchell, 1966) although there is increasing support for the latter (Robertson, 1968).

The corresponding properties of the other cell membranes have received little attention, particularly in plants. Membranes of the nucleus, golgi, lysosome, ER and plasmalemma have all been isolated with some degree of purity from animal cells and generally contain phosphatase and electron transport activities (Roodyn, 1965; Reid, 1967; Fleischer *et al.*, 1971). However, while most activities have not been characterized the evidence indicates:

(i) that they perform a variety of minor energetic and synthetic processes dependent on reduced nucleotides and high energy phosphorylated compounds from other sources;

(ii) that the electron transport and phosphatase activities act independently (i.e. are not coupled as in chloroplasts and mitochondria).

In different cells the functions of these membranes (and of the soluble enzymes) must be often quite different. Hence the properties of these fractions are likely to differ between tissue types and in particular between plants and animals. The following discussion considers the properties and functions of the electron transport and phosphatase activities in the post-mitochondrial fractions (i.e. isolated from the supernatant after isolation of the mitochondria) in animals. The corresponding activities are then considered in plants with respect to the animal activities, and the contrasting requirements in plant cells. The changes in these activities in both animals and plants which can be induced, and which occur during the development of the tissue, are also considered.

B. DEFINITION OF POST-MITOCHONDRIAL FRACTIONS

The technique of differential centrifuging as a method of isolating the various fractions according to their sedimentation properties has been well reviewed by Hayashi (1959) and Roodyn (1965). The microsome fraction by definition (Siekevitz, 1965) is the high

speed pellet resulting from the post-mitochondrial supernatant. It will clearly contain a number of different membrane types depending upon the tissue from which it is isolated. In tissues rich in lysosomes this fraction is first separated and the remaining pellet referred to as the microsomes. The microsome fraction appears in electron micrographs as single membrane vesicles (about 50-100 nm diameter) sometimes with attached ribosomes, and is considered to be derived predominantly from the ER (Claude, 1969). However, the total animal microsomal fraction has been subfractionated by:

(i) differential centrifuging (Siekevitz and Palade, 1956);

(ii) centrifuging through linear density gradients (Amar-Costesec *et al.*, 1969);

(iii) centrifuging through discontinuous density gradients in the presence of ions (Dallner, 1963).

This has resulted in the separation of rough (i.e. ER) and smooth membranes and the further separation of smooth membranes into smooth ER and plasmamembranes.

Subfractioning plant microsomes has been limited to:

(i) separation of free and membrane-bound ribosomes in pea roots (Loening, 1961), potato tuber (Sampson and Laties, 1968) and bean seeds (Payne and Boulter, 1969);

(ii) an attempt to isolate plasmamembranes from bean cotyledons (Lai and Thompson, 1971).

This is surprising in view of such major differences as the presence of an extensive tonoplast in mature plant cells which must affect the composition of the microsome fraction.

The soluble fraction is the post-microsomal supernatant. It is derived from:

(i) soluble cytoplasmic contents;

(ii) vacuolar contents;

(iii) material released from the particulate fraction during preparation.

Prolonged centrifuging of the soluble fraction yields very little further sedimentable protein (Palade and Siekevitz, 1956).

Anderson and Green (1967) have reviewed the problems of contamination between fractions, particularly concerning the soluble fraction, i.e. both random absorption of soluble enzymes onto membranes and release of bound enzymes into the soluble fraction. Conclusive evidence for the origin of a particular enzyme is difficult to obtain, the ease with which a bound enzyme is solubilized often being used as a criterion for specificity of binding (Hallaway *et al.*, 1970).

C. ANIMAL MICROSOMAL AND SOLUBLE ACTIVITIES

1. Animal microsomal electron transport

Studies on microsomal electron transport have generally concentrated on the total fraction isolated from a few animal tissues

with specific functions and which are rich in ER (e.g. liver, heart, brain, kidney). Hence, it is assumed that the electron transport activities are associated with the membranes derived from the ER. This has been supported by subfractioning the microsomes.

(a) NADH-specific electron transport

Properties

This system is summarized in Figure 1 and has been reviewed by Strittmatter (1968). It consists of an NADH-specific flavoprotein (NADH-cytochrome b_5 reductase, Strittmatter, 1965) which interacts with cytochrome b_5 probably via at least one unknown intermediate proposed for a variety of reasons (Dallner *et al.*, 1966; Sato *et al.*, 1969). A number of artificial acceptors are reduced in the presence of NADH and these activities are distinguished from the mitochondrial inner-membrance activities by insensitivity to amytal, rotenone and antimycin A (Sottocasa *et al.*, 1967; Ikuma and Bonner, 1967; Takesue and Omura, 1970b). However, activity (with cytochrome c) is characteristic in its sensitivity to sulphydryl group poisons, which could be relieved by prior incubation of the microsomes with NADH. This is due to the binding of NADH to its dehydrogenase through a sulphydryl group such that pCMB (for example) is only bound in the absence of NADH (Strittmatter, 1965). Similar protection has also been reported in animal lactate dehydrogenase (Holbrook and Stinson, 1970), and the mitochondrial outer membrane (rotenone-insensitive) but

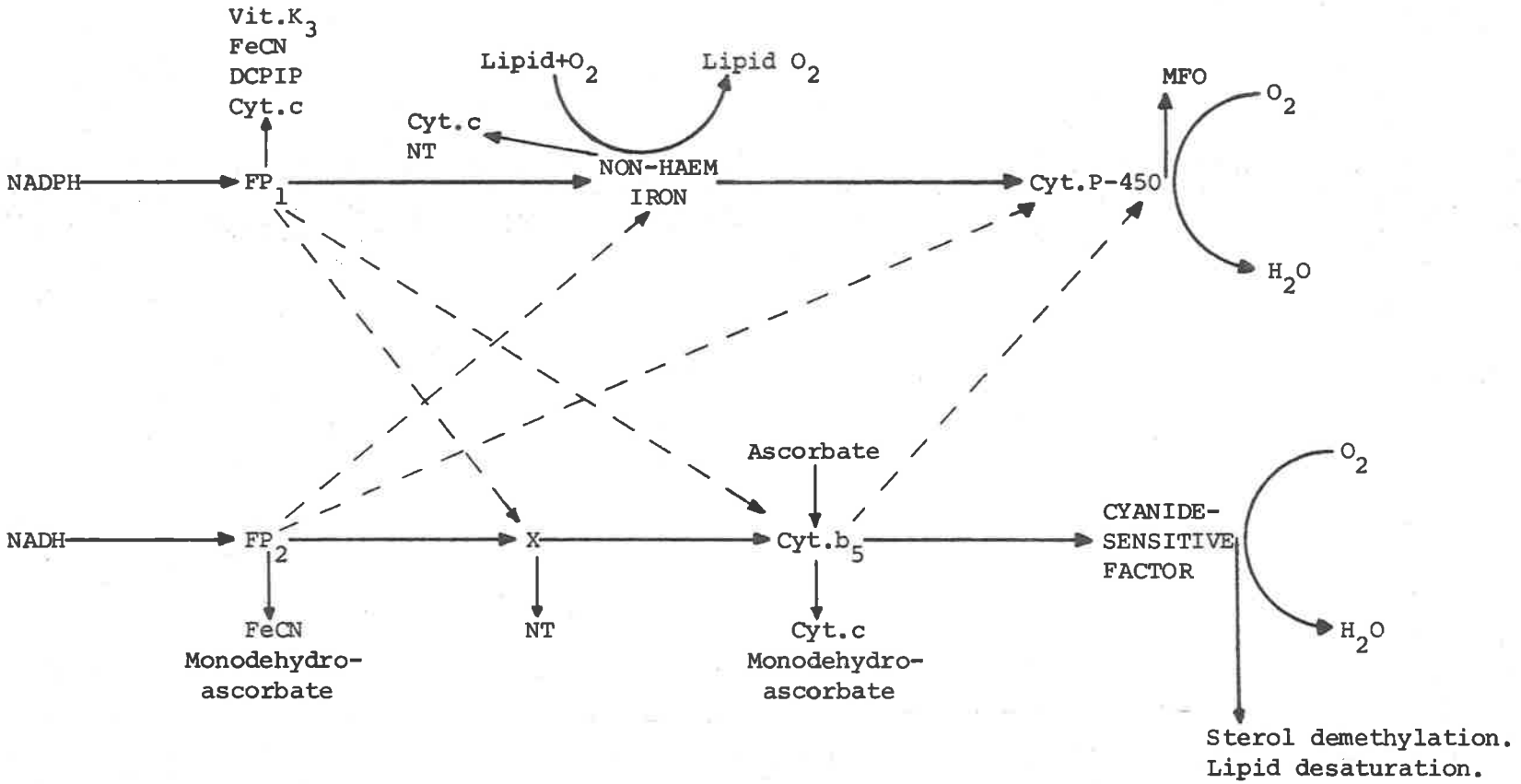


Figure 1. Schematic summary of electron transport in animal microsomes.

not inner membrane NADH-cytochrome c reductase (Ragan and Garland, 1969). Inhibition of the latter actually increases by preincubation with NADH (Tyler *et al.*, 1965).

The NADH-cytochrome b_5 reductase has been solubilized by extraction with hot ethanol (Mahler *et al.*, 1958), incubation with *naja naja* snake venom (Strittmatter, 1967) and with liver lysosomes (Takesue and Omura, 1970a; Sargent *et al.*, 1970). Solubilization did not affect FeCN reduction, increased cytochrome b_5 reduction, largely inhibited DCPIP reduction and completely inhibited cytochrome c reduction (Strittmatter and Velick, 1956; Takesue and Omura, 1970b).

Cytochrome b_5 is loosely attached to the microsomal membrane and is easily solubilized resulting in loss of cytochrome c reductase activity. Solubilization occurs during reductase solubilization as well as by incubation of the microsomes with detergents (Ito and Sato, 1968; Sato *et al.*, 1969), trypsin (Sato *et al.*, 1969; Omura and Takesue, 1970) and pancreatic lipase (Garfinkel, 1957; Strittmatter, 1967). Purified cytochrome b_5 (E'_o = + 0.02) has absorption maxima at 413 nm (oxidized) and 423, 526, 556 nm (reduced) (Strittmatter, 1968). However, several apparently distinct varieties of cytochrome b_5 (based on their absorption maxima) have been reported (Ichikawa and Yamano, 1965; Kusel *et al.*, 1969; Okada and Okunuki, 1969). Although predominantly microsomal, the cytochrome has also been observed in the membranes of the mitochondrion (outer), golgi (Davis and Kreil,

1968; Ichikawa and Yamano, 1970), nuclei and plasmalemma (Fleischer *et al.*, 1971).

Much of the information concerning the sites of reduction of the acceptors (Fig. 1) has been obtained by disrupting the membranes and may not apply to the intact membrane.

(i) FeCN may be reduced at two sites in the intact microsomes (flavoprotein and cytochrome b_5). While release of cytochrome b_5 prevents further cytochrome c reduction, it may not affect the rate of FeCN reduction which can still occur at the flavoprotein (Kuylenstierna *et al.*, 1970; Hara and Minakami, 1971a).

(ii) DCPIP may also be reduced at two sites in the intact microsomes (Takesue and Omura, 1970b).

(iii) Cytochrome b_5 is probably reduced at 'X' and not directly by the flavoprotein in intact microsomes. However, the solubilized NADH-dehydrogenase rapidly reduces cytochrome b_5 indicating exposure of a site not normally available.

Hence it is preferable to at least support the proposed scheme (Fig. 1) with data obtained from intact membranes.

Functions

Several functions for this electron transport chain have been suggested.

(i) The only natural acceptor in Figure 1 is cytochrome c (largely located inside the mitochondrion; González-Cadavid and

Campbell, 1967). The close contact often observed between ER and mitochondria in the cell may suggest interaction between the chains on these membranes.

(ii) Oxygen does react very slowly with cytochrome b_5 and may be the terminal acceptor (Sottocasa, 1968).

(iii) NADH dependent reduction of monodehydroascorbate (Heath and Fiddick, 1965; Iyangi and Yamazaki, 1969; Hara and Minakami, 1971b) but not cystine or glutathione (Heath and Fiddick, 1965) has been reported. The advantage of this system is that it keeps cytochrome b_5 fully reduced which favours monodehydroascorbate reduction.

(iv) Cytochrome b_5 may be involved in the reduction of a cyanide-sensitive factor in lipid desaturation (Fig. 1) (Oshino *et al.*, 1971).

(v) The NADH (and NADPH)-induced microsomal shrinkage (Kamino and Inouye, 1970) may involve all or part of this electron transport chain.

(vi) Although the NADH-cytochrome c reductase is stimulated by ions (Sottocasa, 1968), there is no evidence for ion movement coupled to electron flow.

(b) NADPH-specific electron transport

Properties

Also present in animal microsomes is an NADPH specific electron transport chain (Fig. 1). It consists of at least an NADPH-specific flavoprotein, a non-haem iron complex and a CO-binding

pigment (cytochrome P-450) (Omura *et al.*, 1965). A number of acceptors are reduced at about equal rates probably directly from the flavoprotein (Williams and Kamin, 1962; Dallner *et al.*, 1966; Ernster and Orrenius, 1965; Ichikawa and Yamano, 1969). Partial protection of reductase activity from pCMB inhibition by preincubation with NADPH was detected by Williams and Kamin (1962) but not by Strittmatter (1959). The flavoprotein has been solubilized by incubation with lipase (Williams and Kamin, 1962) and trypsin (Omura and Takesue, 1970).

Cytochrome P-450 has a single broad absorption peak at 450 nm when reduced in the presence of CO (Omura *et al.*, 1965) and has a haemoprotein structure characteristic of a cytochrome (Omura and Sato, 1964). The CO-complex is disassociated by light with maximum effect at 450 nm (Omura *et al.*, 1965). The cytochrome is readily solubilized but the treatments usually result in denaturation with a corresponding shift in the absorption peak to 420 nm (cytochrome P-420, Omura and Sato, 1967). Cytochrome P-450 is not present in plasma-membranes but has been detected in nuclei (Fleischer *et al.*, 1971), golgi (Ichikawa and Yamano, 1970) and mitochondria (Whysner and Harding, 1968; Ishidate *et al.*, 1969). However, Brunner and Bygrave (1969) have suggested that its presence in mitochondria was due to contamination as it could be removed by repeated washing.

Functions

It has been proposed (Cohen and Estabrook, 1971; Hilderbrandt and Cohen, 1971) that reduced cytochrome P-450 can "activate" oxygen which is then available for the oxidation of a variety of organic substrates (RH) (Fig. 1). Such oxidations have been termed mixed function oxidations (MFO) by Mason (1965), as one oxygen atom is incorporated into the substrate and the other into water.

$$NADPH + H^+ + RH + 0{:}0 \rightarrow NADP^+ + ROH + H_2O$$

MFO are concentrated in microsomes (Mason, 1965) and are involved in synthetic and detoxifying oxidations of fatty acids, steroids, phospholipids, cholesterol, amino acids and a variety of aromatic hydrocarbons.

Much evidence has now accumulated for the involvement of the NADPH electron transport chain in MFO reactions:

(i) Parallel induction by drugs of the electron transport chain and MFO activities (Ernster and Orrenius, 1965).

(ii) Recombination of the purified components to recover MFO activity (Omura *et al.*, 1965).

(iii) Inhibition of MFO by inhibiting or denaturing the various components of the electron transport chain (Mason, 1965; Omura *et al.*, 1965; Mason *et al.*, 1965; Smuckler *et al.*, 1967; Das *et al.*, 1968).

However, although the NADPH chain is present in most animal tissues (Siekevitz, 1963), MFO activity appears to be limited to

only a few tissues (Mason, 1965). Hence reducing cytochrome P-450 may be only one of many functions of this electron transport chain.

However, some of the NADPH-dependent oxidations are exceptional:

(i) Lipid peroxidation does not involve cytochrome P-450, suggesting that the reactions occur at sites on the NADPH chain before the cytochrome (Fig. 1) (Hochstein and Ernster, 1963; Lumper *et al.*, 1968).

(ii) Sterol demethylation (Gaylor and Mason, 1968) and lipid desaturation (Oshino *et al.*, 1966; 1971; Sato *et al.*, 1969), unlike the other oxidations, require either NADH or NADPH and are cyanide-sensitive. Despite earlier reports (Oshino *et al.*, 1966) evidence now indicates (Oshino *et al.*, 1971) that these oxidations involve cytochrome b_5 which reduces the cyanide-sensitive factor (Fig. 1). This factor has recently been isolated and is thought to be a cytochrome (Gaylor *et al.*, 1970).

(c) Interaction between the two chains

Figure 1 summarizes the electron transport scheme for animal microsomal membranes. (There is now evidence for at least one other NADH- (Schulze *et al.*, 1970) and NADPH- (Kamino and Inouye, 1970) flavoprotein.) Limited interaction occurs between the two chains such that reduced components on one chain may reduce components on the other (Estabrook and Cohen, 1969; Sato *et al.*, 1969). However,

the situation is complex as the rate of cytochrome c reduction by NADPH is much less than by NADH, while sterol demethylation and lipid desaturation occur at equal rates with the two reduced nucleotides. The microsomal volume changes induced by both NADH and NADPH (Kamino and Inouye, 1970) again suggest interaction between the chains. Recently a synergistic effect between NADH and NADPH in MFO has been reported (Cohen and Estabrook, 1971; Hilderbrandt and Estabrook, 1971). It appears that cytochrome P-450 may require two electrons for full reduction, the first from the NADPH chain and the second from the NADH chain. Subfractioning the microsomes usually results in all the electron transport activites sedimenting together, particularly NADH and NADPH-cytochrome c reductase (Imai *et al.*, 1966; Amar-Costesec *et al.*, 1969). This suggests that the two chains are intrinsically part of the same membrane, rather than existing separately to carry out their own specific functions.

2. Animal soluble electron transport

Properties

The NAD(P)H dehydrogenase activities detected in the animal soluble supernatant fraction have been well reviewed (Ernster *et al.*, 1962; de Duve *et al.*, 1962; Roodyn, 1965; Anderson and Green, 1967). The predominant activity is the DT diaphorase (Ernster *et al.*, 1962) characterized by:

(i) oxidation of both NADH and NADPH (slightly favouring the

latter);

(ii) reduction of FeCN and DCPIP (at about equal rates) but not cytochrome c;

(iii) high sensitivity to low concentrations of dicoumarol and pCMB;

(iv) no pH optimum but favouring alkaline conditions.

A variety of other soluble reductases have been reported but with slightly different properties (Williams *et al.*, 1959; Wosilait, 1960; Ernster *et al.*, 1962). According to the above criteria these other reductases probably result from DT diaphorase. The partial dicoumarol-sensitivity of the liver microsomal DCPIP, FeCN and vitamin K_3 but not NT or cytochrome c reduction (Dallner, 1963) indicates some DT diaphorase associated with these membranes. However, it is not known if this is specifically microsomal or due to contamination from the soluble fraction. Bacteria also contain a soluble DT diaphorase (Wosilait and Nason, 1954b; Bragg, 1965), as well as an antimycin A-insensitive NADH-specific cytochrome c reductase (Horio and Kamen, 1960; 1962; Horio *et al.*, 1969). A variety of other NADH-dehydrogenases have been solubilized from the membrane fractions (Chapter V). However, there is no evidence to suggest that these are solubilized under preparation conditions employing only differential centrifuging. Hence, the DT diaphorase may be the only soluble reductase in animal tissues.

Low levels of cytochrome b_5 (Mangum *et al.*, 1970) and cytochrome P-450 (Katagiri *et al.*, 1968) have been reported in the animal soluble fraction. Their origin is not known but they may be released from the microsomal membranes during homogenizing.

Functions

A definite function of the DT diaphorase has not been determined but may include:

(i) oxidation of extra-mitochondrial reduced pyridine nucleotides;

(ii) in reductive synthesis of vitamin K and coenzyme Q;

(iii) linked to mitochondrial electron flow yielding an amytal-insensitive, antimycin A- and dicoumarol-sensitive respiration (Ernster *et al.*, 1962; Conover and Ernster, 1962).

3. Animal microsomal and soluble phosphatases

A number of phosphatase activities have been detected in the animal microsomal and soluble fractions (Roodyn, 1965). The microsomal phosphatases are gradually being assigned to the various constituent membranes as separation becomes more effective.

(a) Acid and alkaline phosphatases

The acid phosphatases are concentrated in the lysosome fraction (Robinson and Willcox, 1969; Takesue and Omura, 1970a). Activity detected in the microsomal and soluble fractions is probably due to contamination from small and broken lysomes (Morton, 1961).

The alkaline phosphatases do not appear to be associated with the lysosomes and are concentrated in the soluble fraction with low activity in the microsomes. The soluble (but not microsomal activity) is activated by divalent cations (Novikoff, 1952; Allard *et al.*, 1954; Dixon and Webb, 1958). The alkaline phosphatases (unlike the acid phosphatases) are probably normally functional in animal cells for general phosphate hydrolysis.

(b) G-6-Pase

G-6-Pase is concentrated in the membranes derived from the ER with little or no activity associated with the other membranes or soluble fraction (Dixon and Webb, 1958; Siekevitz, 1963; Dallner, 1963; Reid, 1967; Stetten and Burnett, 1967; Nordlie and Johns, 1968; Robinson and Willcox, 1969; Fleischer *et al.*, 1971). The low activities reported in mitochondrial and soluble fractions (Roodyn, 1965) have not been shown to be specific for G-6-P (McGilvery, 1961).

G-6-Pase appears to be limited to a few specific animal tissues (liver, intestine, kidney) and is involved in glycogenolysis releasing glucose to the blood. In tissues without G-6-Pase activity (e.g. muscle) G-6-P can be metabolized by the glycolytic pathway (White *et al.*, 1964). G-6-Pase may also be involved in glucose secretion in liver (Siekevitz, 1963) and glucose to fructose conversion which occurs during absorption in the intestine (Ginsburg and Hers, 1960).

(c) Nucleoside phosphatases

A variety of nucleoside mono-, di- and triphosphatase activities have been detected in the microsome fraction. There is an ATPase associated with the ribosomes which is probably involved in protein synthesis (Miall and Walker, 1967), and at least two ATPases associated with the membranes. Both membrane-bound ATPases are activated by Mg^{++} (Benedetti and Emmelot, 1968) and one further stimulated synergistically by $Na^{+} + K^{+}$ (Skou, 1965). They are concentrated (with an AMPase) in the lightest smooth microsome fraction (Glauman and Dallner, 1970) and are now considered to be associated with the plasmamembranes (Emmelot and Bos, 1966; Benedetti and Emmelot, 1968; Avruch and Wallach, 1971; Jorgensen *et al.*, 1971).

While the function of the Mg^{++}-ATPase is unknown, the Na^{+}-K^{+}-stimulated Mg^{++}-ATPase is strongly implicated in the coupled cellular accumulation of K^{+} and extrusion of Na^{+} at the plasmamembrane (Skou, 1965). Activity has been detected in a variety of animal tissues and is inhibited by ouabain, an inhibitor of some energy requiring-ion transport in animal cells (Siekevitz, 1963; Skou, 1965; Csaky, 1965; Bakkeren and Bonting, 1968a; 1968b). Presumably, there must be a "sidedness" to the membrane on which such an ATPase is situated. However, as disruption of the isolated vesicles stimulates the ATPase activity but $Na^{+} + K^{+}$ are still required (Jorgensen and Skou, 1971), a proton gradient is probably not involved.

4. Induced and developmental changes in the animal microsomal and soluble systems

Study of the changes in the microsomal system in animals has provided information about their synthesis, structure and function. Microsomal enzyme levels have been followed most commonly:

(i) in developing liver;

(ii) in normal turnover in adult liver;

(iii) in adult livers of drug (e.g. phenobarbital)-treated animals.

In all cases activities first appear in the rough membranes and then subsequently in the smooth membranes indicating that the attached ribosomes are likely to be involved in synthesizing specific proteins to be incorporated into the membranes (Sargent and Vadlamudi, 1968; Omura and Kuriyama, 1971). The subsequent appearance of activities in the smooth membranes may be due to a rapid incorporation of the enzymes into the nearest smooth membranes (Omura and Kuriyama, 1971) or loss of ribosomes from rough membranes (Ernster and Orrenius, 1965).

In developing rat liver increase in NADPH dehydrogenases precedes increase in the NADH activities and cytochromes b_5 and P-450 (Dallner *et al.*, 1966; Omura *et al.*, 1967; Sargent and Vadlamudi, 1968). Phenobarbital treatment, however, results in a rapid increase in the smooth ER membranes and specifically in NADPH-cytochrome c reductase, cytochrome P-450 and MFO activity, with little or no

increase in the NADH dehydrogenases and cytochrome b_5. The specific increase in the components of the MFO system apparently supplies the mechanism for "detoxifying" the administered drug (Ernster and Orrenius, 1965; Estabrook and Cohen, 1969). Kuriyama *et al.* (1969) have shown that these phenobarbital-induced increases are due to drastic reduction in the rates of enzyme degradation rather than increase in rates of synthesis.

The phosphatase activities have been only briefly considered. G-6-Pase but not ATPase increases in developing rat liver (Dallner *et al.*, 1966) and in the early stages of induced alloxan diabetes (Jakobsson and Dallner, 1968). However, there was no increase in either phosphatase in liver microsomes of phenobarbital-treated rats (Orrenius *et al.*, 1965).

All activity increases are inhibited by inhibitors of animal cytoplasmic RNA (actinomycin D) and protein (puromycin) synthesis (Ernster and Orrenius, 1965; Dallner *et al.*, 1966). This work has concentrated on the microsome fraction as changes could be correlated with changes in the ER (Orrenius *et al.*, 1965; Dallner *et al.*, 1966). Probably for this reason, corresponding studies on the soluble fraction have not been attempted.

D. PLANT MICROSOMAL AND SOLUBLE ACTIVITIES

1. Plant microsomal electron transport

(a) NADH-specific electron transport

The limited information available for the plant microsome fraction is largely derived from general intracellular characterizations of chemical and enzymic properties. Antimycin A-insensitive NADH-cytochrome c, -FeCN and -DCPIP reductase and NADH-oxidase activities have been detected in microsomes isolated from a variety of plant tissues (Goddard and Stafford, 1954; Martin and Morton, 1955; 1956a; 1956b; Hackett, 1957; Crane, 1957; Loening, 1961; Beevers, 1961; Ragland and Hackett, 1961; 1964). Some of the reported reductase activities are presented in Chapter III (Table 2) - the considerable variation probably depends on the tissue used and the method of isolation.

"b" cytochromes have been detected in a variety of plant tissues (Hill and Scarisbrick, 1951; Hill and Hartree, 1953; Martin and Morton, 1955; 1957; Smith and Chance, 1958; Beevers, 1961; Shichi and Hackett, 1962a; 1962b; Shichi *et al.*, 1963a; 1963b; Shichi and Hackett, 1966; Moore, 1967). These include cytochromes b_{555}, b_{558}, b_{559} (cytochrome b_3) and b_{561}. Shichi and Hackett (1966) and Moore (1967) have shown that cytochrome b_{555} is microsomal, although both workers also detected it in the soluble fraction. Moore (1967) concluded that cytochrome b_{555} and the animal microsomal cytochrome b_5 are identical. Cytochrome b_3 has also been detected in the microsomes (Morton, 1958) but is present in other fractions

(Lundegardh, 1958). The localization of cytochromes b_{558} and b_{561} is unknown.

No attempt has been made to determine the structure of this electron transport chain but it appears to be similar to that in animal microsomes. While the functions are also unknown, all those suggested for animals may equally well apply for plants. Hence an equivalent electron transport chain might be expected.

(b) NADPH-specific electron transport

NADPH-NADP transhydrogenase and antimycin A-insensitive NADPH-cytochrome c, FeCN and DCPIP reductase activities have also been reported in plant microsomes, and are summarized in Chapter III (Table 2) (Martin and Morton, 1955; 1956a; Beevers, 1961; Ragland and Hackett, 1961; 1964; Frear *et al.*, 1969). As in animal microsomes, the NADPH activities were much lower than the NADH activities (except in beet petiole where high activities may have been due to NADH contamination of the NADPH). Cytochrome P-450 has been detected in the microsomes of pea cotyledons (Moore, 1967), cucumber endosperm (Murphy and West, 1969), *Claviceps* (Ambike *et al.*, 1970), sorghum (Liljegren, 1971), jerusalem artichoke and potato tubers and *Arum* and *Sauromatum* spadices (Bendall, 1971). However, none was detected in the microsomes of cotton leaves (Frear *et al.*, 1969), etiolated mung bean hypocotyls and maize roots (Bendall, 1971). Again no attempt has been made to determine the structure of this chain.

The only two reports of plant microsomal MFO are summarized in Table 1. Only one of the two hydroxylations by cucumber microsomes showed oxygen substitution in accordance with the definition of MFO (oxygen substitution was not examined in cotton microsomes). Both activities required organic substrate, oxygen and reduced nucleotide. However, there were some marked differences to the animal activities, particularly in lack of NADPH-specificity and SKF525A-sensitivity (an inhibitor of animal microsomal MFO). This was more like the animal microsomal lipid desaturating and sterol demethylating system (Fig. 1). The lack of nucleotide specificity may result from a different interaction of the two chains in plants or possibly the involvement of a third (non-specific) dehydrogenase. The low MFO activity also detected in cotton leaf soluble fraction (Frear, 1968) would suggest an activity quite different to the animal membrane-bound complex. Hydroxylation by plant microsomes, requiring NADPH (and tetrahydrofolic acid) has often been reported (Nair and Vining, 1965; Russell and Conn, 1967; Stafford, 1969; Stohs, 1969). However, no attempt has been made to demonstrate cytochrome P-450 involvement.

Hence, as in animal microsomes, an NADPH chain is probably present in microsomes from most plant tissues. However, insufficient information is available to comment on the distribution of the plant MFO. Again, the function of the NADPH chain (in activities other

TABLE 1. Summary of the properties of reported MFO activity in plant microsomes.

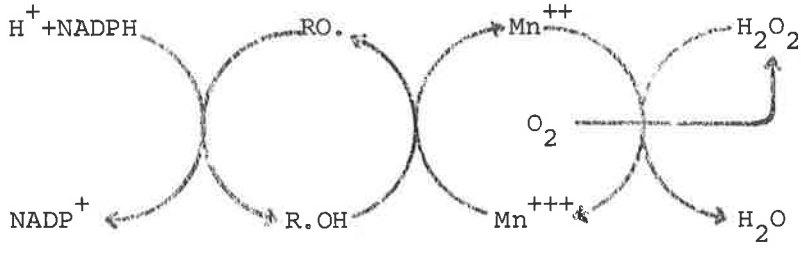
Reference	Frear *et al.* (1969)	Murphy and West (1969)
Tissue	Cotton leaf (+ others)	Cucumber endosperm
Fraction	Microsomal (10% soluble)	Microsomal only
Reaction	N-demethylation of monuron	(a) Hydroxylation of kaurene (b) Hydroxylation of kaurenealdehyde
O_2 required	+	(a) + (b) +
O_2 substitution	?	(a) in -OH (b) not in -OH
Cytochrome P-450	-	+
CO inhibition	35%	100%-reversed by light with max. at 450 nm
NADH/NADPH	50%	(a) 50% (b) 30%
SKF525A inhibition	0	?
pCMB inhibition	100%	?
pH optimum	?	?

than MFO) is unknown. The significance of the different properties of the plant (compared with animal) microsomal MFO is also unknown.

2. Plant microsomal and soluble peroxidases

Peroxidases are widely distributed in plants, the majority being soluble with some microsomal and less frequently mitochondrial activity (Haskins, 1955; Yonetani and Ohnishi, 1966; Kanazawa *et al.*, 1967; Plesnicar *et al.*, 1967). It is not known whether activity in the membrane fractions is specific (Kanazawa *et al.*, 1967) or due to contamination from soluble enzymes (Jansen *et al.*, 1960), or small peroxidase containing bodies (Hogg, 1969).

Peroxidases are capable of catalysing a variety of activities (Nicholls, 1962). They may oxidize NADH or NADPH in the presence of oxygen and an organic compound (RO.) by the following reaction (Akazawa and Conn, 1958; Gamborg *et al.*, 1961; Nicholls, 1962).



In this reaction NADPH supports twice the NADH rate and the NAD(P)H:O ratio is 0.5 (i.e. same as MFO). It is necessary to distinguish this peroxidatic activity in the microsomes from MFO (particularly when assaying oxygen consumption or NAD(P)H oxidation). Although both Mn^{++} and H_2O_2 are implicated, the lack of requirement for either

or both in the crude cell fractions may not be evidence for lack of peroxidase (Yamazaki and Souzu, 1960; Gamborg *et al.*, 1961). Unlike the MFO, this peroxidase reaction does not consume the organic compound as the requirement is catalytic. However, peroxidases may also catalyse hydroxylations under certain conditions, the incorporated oxygen being derived from molecular oxygen (Buhler and Mason, 1961).

Peroxidases are haemoproteins containing a 'b' type haem (Yamazaki *et al.*, 1967; Kanazawa *et al.*, 1967). It is this pigment in bean leaf microsomes which Hill and Scarisbrick (1951) may have identified as cytochrome b_3 (Morita, 1956). The so-called "low-spin" peroxidases bind cyanide (and fluoride) which results in a spectral shift of the oxidized peroxidase absorption peak (Yamazaki *et al.*, 1967). The lack of this property has been used to distinguish cytochrome b_3 from plant peroxidase in the microsome fraction (Lundegardh, 1958).

The functions of the plant peroxidases are largely speculative (Chance and Maehly, 1961):

(i) oxidation of reduced nucleotides under certain conditions in the cell;

(ii) detoxification (equivalent to MFO in animals) (Lieb and Still, 1969);

(iii) synthesis of physical and chemical barriers to fungal infection (Stahmann *et al.*, 1966);

(iv) in cell wall and lignin synthesis (Bonner and Varner, 1965).

3. Plant soluble electron transport

While soluble reductase activities have been reported from a variety of plant tissues, there has been no attempt to characterize them (Beevers, 1961). These activities have been assayed in the crude soluble fraction and may be due to more than one enzyme. The results indicate that there may be two reductases, one favouring NADH oxidation and FeCN and quinone reduction (Wosilait and Nason, 1954a; Ragland and Hackett, 1964), the other oxidising either NADH or NADPH and favouring DCPIP reduction (Martin and Morton, 1956a; Hackett, 1958; Marré *et al.*, 1962; Ragland and Hackett, 1964). NADH-cytochrome c reductase activity has been reported in the soluble fraction of cauliflower buds (Crane, 1957) and potato tuber (Hackett, 1958). However, properties and nucleotide specificity were not determined and the activity from potato was lost on dialysis. In contrast to animals, low MFO activity (8% of microsomal activity) has been reported in the soluble fraction from bean leaves (Frear, 1968). Bacterial MFO is concentrated in the soluble fraction and generally is specific for NADH rather than NADPH (Cardini and Jurtshuk, 1970).

As previously mentioned, the localization of the b cytochromes in plants has not been well characterized. Shichi and Hackett (1966) and Moore (1967) have both reported that the cytochrome b_{555} present in the microsomes was also present in the soluble

fraction. Moore also detected cytochrome b_3 in the soluble fraction in contrast to Morton (1958). Despite the soluble MFO activity, no soluble cytochrome P-450 has been detected in higher plants, although it has been reported in fungi (Shechter and West, 1971) and bacteria (Appleby, 1969; Yu and Gunsalus, 1970; Cardini and Jurtshuk, 1970). The cytochrome P-450 reported by the latter authors was, however, associated with lipids.

Hence, the soluble reductase activities are somewhat different to animals. The NAD(P)H-DCPIP reductase may correspond to the DT diaphorase (with equivalent functions). However, the origins and functions of the other reductase activities are unknown.

4. Plant microsomal and soluble phosphatases

(a) Acid and alkaline phosphatases

High acid but probably not alkaline phosphatase is present in the soluble fraction (Morton, 1961; Thompson, 1969) with small proportions of what appear to be the same activity in the cell wall, mitochondria and microsomes (Gahan and McLean, 1969; Atkinson and Polya, 1967; Sahulka, 1969; McLean and Gahan, 1970). Presumably the majority of activity is contained in the large central vacuoles of mature plant cells and has a similar hydrolytic function to the animal enzyme. Again, it is not clear whether the membrane-bound activities are due to contamination. The study of other specific phosphatases, particularly in the membrane fractions, has been

confused by this acid phosphatase activity. It is not surprising that, with the lack of general alkaline conditions in mature plant cells, alkaline phosphatase activity is not present.

(b) G-6-Pase

G-6-Pase has received little attention in plants. Activity was 10 fold greater in the microsome than mitochondria fraction of bean seedlings (soluble fraction not assayed) (Das and Mukherjee, 1964), but was evenly distributed between microsome and soluble fractions of bean cotyledons (Thompson, 1969). While Das and Mukherjee (1964) made no attempt to eliminate acid phosphatase (assayed at pH 6.5), Thompson (1969) assayed in the presence of 4 mM EDTA and 4 mM KF (at pH 6.5) to inhibit alkaline and acid phosphatase, respectively. The assumption that all activity in the presence of these two inhibitors could be attributed to G-6-Pase is doubtful. Hence, there is no conclusive evidence for specific G-6-Pase activity in plants. This is not surprising as this activity is only present in particular animal tissues where it performs specific functions, probably not relevant to plants.

(c) Nucleoside phosphatases

Studies on the nucleoside phosphatases in plants have concentrated on detecting a microsomal Na^+-K^+-stimulated ATPase, corresponding to that found in animal microsomes, which may be implicated in ion movement. However, early work was again complicated

by the acid phosphatase and the so-called "ATPases" were generally characterized by:

(i) low pH optima;

(ii) lack of substrate specificity;

(iii) presence also in the soluble fraction;

(iv) inhibition by Mg^{++};

(v) stimulation (but not synergistically) by Na^{+} and K^{+};

(vi) insensitivity to ouabain;

(Brown and Altschul, 1964; Bonting and Caravaggio, 1966; Dodds and Ellis, 1966; Greuner and Neumann, 1966; Atkinson and Polya, 1967). The latter authors concluded that activity was entirely due to acid phosphatase.

More recently apparently specific ATPases which require Mg^{++} have been reported in particular plant fractions. Activity was sometimes stimulated by monovalent ions but was insensitive to ouabain. Hansson and Kylin (1969) and Kylin and Gee (1970) reported two microsomal (prepared in the presence of detergent) ATPase activities with acid pH optima, which were stimulated by Na^{+} and K^{+} depending upon the ratio of the ions. This varied between plant species and is the only report in plants of such a synergistic effect. Sexton and Sutcliffe (1969) reported an ATPase in young pea roots with a neutral pH optima but which was not monovalent-ion stimulated.

Specific ATPases showing some salt stimulation and with high pH optima have been detected in the cell wall, mitochondria, microsome and possibly soluble fractions (Reid *et al.*, 1964; Fisher and Hodges, 1969; Fisher *et al.*, 1970; Lai and Thompson, 1971). Only the activity reported by Fisher and Hodges (1969) and Fisher *et al.* (1970) required both Mg^{++} and monovalent ions for maximum stimulation. Corresponding to the location of Na^{+}-K^{+} ATPase in animal plasmamembranes, Lai and Thompson (1971) have recently attempted to isolate plasmamembranes with this activity from bean cotyledons. However, there is no evidence that the isolated fraction is plasmamembranes and, although the activity is salt-stimulated, there is neither a specific requirement for Mg^{++} nor a significant synergistic effect between Na^{+} and K^{+}.

The apparent lack of a Na^{+}-K^{+} ATPase in higher plants is again not surprising as coupled K^{+} uptake and Na^{+} extrusion has not been demonstrated (Robertson, 1968). Plant cells do accumulate salts (i.e. both monovalent cations and anions) by a mechanism which requires energy and it is therefore surprising that workers have only tested for the effects of cations with no regard for the anion.

5. Induced and developmental changes in the plant microsomal and soluble systems

Changes in the ER in plants in response to a variety of conditions have been reported (see Jackman and Van Steveninck, 1967,

for review):

(i) germination and senescence;

(ii) changes in the environment of the mature plant;

(iii) slicing and aging of plant tissues.

The latter is of particular interest as it can be controlled experimentally by adding compounds to the aging medium. Hence, this provided a system equivalent to that in liver, where changes in the microsome fraction and in the ultrastructure of the ER may be correlated. The induced ultrastructural changes have been studied (in an attempt to characterize the well-established induced physiological changes) (Jackman and Van Steveninck, 1967; Fowke and Setterfield, 1967; Van Steveninck, 1970). However, changes in the microsome fraction have received little attention.

(a) Induced physiological changes

The most commonly observed effect of slicing plant storage tissues is the immediate increase in respiration. This is not a result of increased oxygen supply (MacDonald, 1967a) but may be due (Laties, 1967) to release of a protein and RNA synthesis inhibitor, allowing increased synthesis and therefore increased oxygen consumption. The induced respiration appears to be sensitive to the competitive inhibitor malonate, while the basal (fresh) rate remains insensitive (Laties, 1967). This indicated lack of Krebs cycle activity in fresh tissue. However, the stimulation of fresh tissue

respiration to the maximum of aged tissue by DNP (Kahl, 1971) indicated that the Krebs cycle is active and limited by ADP in fresh tissue, but presumably not operating at a sufficient rate for malonate to be effective. This is supported by the recent work of ap Rees and Royston (1971) who demonstrated malonate inhibition of fresh carrot disk respiration, and Krebs cycle activity in both fresh and aged disks.

The development of an antimycin A- (Hackett *et al.*, 1960; Kahl *et al.*, 1969; Floyd and Rains, 1971) and an azide- and cyanide- (Beevers, 1961) insensitive respiration has also been reported. However, as antimycin A-insensitivity developed before cyanide-insensitivity (Kahl *et al.*, 1969) these two effects may not be related.

The increase in antimycin A-insensitive NADH-cytochrome c reductase activity in the mitochondria from 24 hour aged potato disks (Hackett *et al.*, 1960) may result from an increase in an outer membrane reductase similar to that present in animal mitochondria (Sottocasa *et al.*, 1967). The development of such an activity could result in the development of an antimycin A-insensitive, cyanide-sensitive respiration (Chapter VIII, Fig. 5).

Mitochondria isolated from some tissues (e.g. skunk cabbage and Arum spadix) exhibit a cyanide-insensitive respiration. This has now been shown to be due to a branch in the mitochondrial electron transport chain terminating in an unidentified cyanide-insensitive

oxidase (Bendall and Bonner, 1971). However, there is no evidence to suggest that this develops in the mitochondria in aging potato tissue, which remain cyanide-sensitive (Hackett *et al.*, 1960). Hence, it would appear that the developed cyanide-resistance in aged tissue may result from changes within the cell such that the mitochondria "see" a less effective cyanide concentration.

The respiratory rise is initially blocked by both RNA and protein synthesis inhibitors. However, by 8 hours after slicing, the rise becomes insensitive indicating that all transcriptional and translational events leading to an induced respiration develop during this period (Click and Hackett, 1963; Laties, 1965; Bryant and ap Rees, 1971).

With aging, tissue slices develop the ability to accumulate ions, the time to develop maximum capacity depends on the tissue, temperature and ion species (Stiles and Dent, 1946; Robertson *et al.*, 1947; Laties, 1967; MacDonald, 1967b). Van Steveninck (1962) has shown that this may be due to a decreased cation efflux rather than an increased influx. As disks become contaminated with bacteria during aging, it has been suggested that this may contribute to ion uptake (MacDonald, 1967b). However, Palmer (1970a) has shown that development of uptake in contaminated and aseptic beet disks is similar although absolute rates are somewhat higher in the contaminated disks. Although inhibitors of RNA and protein synthesis prevent

the development of ion accumulation, it is now generally considered that this is not a direct effect (MacDonald *et al.*, 1966; Polya, 1968; Van Steveninck, 1970).

The immediate source of energy for salt accumulation is a matter of debate. Some workers favour ion movement linked energetically to an oxygen terminated electron transport system (Briggs *et al.*, 1961; Atkinson *et al.*, 1966; Polya and Atkinson, 1969), and this appears to hold for both the low and high salt uptake mechanisms (Osmond and Laties, 1968). However, there is also evidence for a dependence on ATP or similar high energy compounds (MacDonald *et al.*, 1966; Smith, 1970; Lüttge *et al.*, 1971).

(b) Induced RNA and protein synthesis changes

The dependence of development of activities with aging on RNA or protein synthesis has been largely interpreted from the effect of inhibitors of these processes. In plants, ethionine, 6-methylpurine and actinomycin D are usually used to inhibit RNA synthesis, and cycloheximide and puromycin to inhibit protein synthesis (Glasziou, 1969). Within a few hours of slicing, leucine incorporation (Click and Hackett, 1963) and enzyme synthesis (e.g. phenylalanine ammonia lyase; Stone *et al.*, 1969) become insensitive to inhibitors of RNA synthesis but remain sensitive to inhibitors of protein synthesis. This is interpreted as a rapid messenger RNA synthesis (to the extent where it is no longer limiting) but continuous protein synthesis.

However, Bryant and ap Rees (1971) have shown that while net RNA synthesis was complete by 8 hours in carrot disks, synthesis continued for at least 4 days.

The various limitations of experiments involving such inhibitors have been well documented by Glasziou (1969) and Filner *et al.* (1969). In particular the effects may not be specific.

(i) Ethionine may also inhibit protein synthesis (Zucker, 1963; Ariyoshi and Takabatake, 1970) or limit ATP content (Davies, 1964; Atkinson and Polya, 1968; Polya and Atkinson, 1969).

(ii) 6-methylpurine may inhibit other processes as it strongly inhibits respiration (Gayler and Glasziou, 1968).

(iii) Cycloheximide may also limit ATP by uncoupling respiration (MacDonald and Ellis, 1969; Ellis and MacDonald, 1970), although it did not effect respiration in a variety of tissues (Kirk, 1970; Ellis and MacDonald, 1970; ap Rees and Bryant, 1971). However, Chinnadurai (1970) has shown that, while fungus respiration was stimulated by cycloheximide, protein but not RNA synthesis was inhibited.

Slicing induces a rapid increase in polyribosomes in carrot (Leaver and Key, 1967) and a massive synthesis of ribosomes in potato (Laties, 1965; 1967; Sampson and Laties, 1968). It has also been shown that ribosomes bind to the membranes soon after slicing, which accounts for the apparent rapid reduction in free

ribosome numbers. Laties (1967) has suggested that this is a random effect but the electronmicrographs of Fowke and Setterfield (1968) show that in jerusalem artichoke binding is specific for the ER. It is not known what induces this binding; however, the ER in actively growing plant cells is often rough, while in mature plant cells it is often smooth (Lund *et al.*, 1958; Loening, 1961; Payne and Boulter, 1969; Robards and Kidwai, 1969). It is thought that bound and free ribosomes synthesize different proteins and it has been suggested that bound ribosomes in plants may synthesize storage protein (Opik, 1968; Payne and Boulter, 1969). However, in aging jerusalem artichoke disks (Fowke and Setterfield, 1968), germinating bean seeds (Payne and Boulter, 1969), and developing bean cotyledons (Opik, 1968) ribosomes are bound to the ER at the time when these membranes are rapidly increasing. This would suggest that bound ribosomes (as in liver) synthesize the protein part of the ER membranes. As ribosome synthesis is dependent on RNA synthesis (Sampson and Laties, 1968), actinomycin D-sensitive activity increases may reflect limiting ribosomes (number or type) rather than synthesis of new messenger RNA.

(c) Induced changes in enzyme activities

Increases in a variety of enzymes in aging disks have been reported (see Kahl, 1971, for review). In many (but not all) cases inhibitor studies implicate RNA and protein synthesis. The onset of these syntheses (consuming ATP) would result in increased ADP and

therefore increased respiration.

(i) Aging potato disks for 24 hours results in 4-7 fold increases in microsomal NADH dehydrogenases (Hackett *et al.*, 1960; Ben Abdelkader, 1969) which are partially inhibited by cycloheximide (Ben Abdelkader, 1969).

(ii) Aging potato disks also results in an increase in soluble NADPH dehydrogenase which reaches a maximum after about 70 hours (Marré *et al.*, 1962; Kahl, 1971).

(iii) Phosphatase activity changes have been reported in jerusalem artichoke (Edelman and Hall, 1965) and potato disks where G-6-Pase reached a maximum after 48 hours (Kahl, 1971). Phosphatase activities have been investigated more thoroughly in germinating and senescing plant tissues. Thompson (1969) reported increases in both microsomal and soluble G-6-Pases (particularly the latter) during bean germination. Increases in acid phosphatase in excised leaf tissues has often been observed (Parish, 1968; Wyen *et al.*, 1971).

(iv) Aging also induces increases in peroxidase activity in mitochondria, microsomes and soluble fraction (Kanazawa *et al.*, 1967; Gayler and Glasziou, 1968; Bastin, 1968; Kahl, 1971), which were inhibited by both RNA and protein synthesis inhibitors (Gayler and Glasziou, 1968; Asahi and Majima, 1969). Development has also been reported in germinating and developing seedlings (Pilet and Galston, 1955; Haskins, 1955), senescing leaves (Parish, 1968; Weston, 1969)

and infected plant tissues (Kanazawa *et al.*, 1965).

(d) Induced ultrastructural changes

As previously mentioned, slicing and aging plant storage tissues induces changes in the ER. These have been observed in beetroot (Jackman and Van Steveninck, 1967), jerusalem artichoke (Fowke and Setterfield, 1968) and swede but not carrot (Van Steveninck, 1970). The ER of beetroot vesiculated two hours after slicing and of swede after about 40 hours. With further aging there was a subsequent increase in the ER of beetroot and jerusalem artichoke. These increases may correspond to the reported increases in microsomal phospholipids (Tang and Castelfranco, 1968) and reductases (Hackett *et al.*, 1960; Ben Abdelkader, 1969) in aging potato slices. In aged beetroot (Van Steveninck, 1970) and jerusalem artichoke (Fowke and Setterfield, 1968) ribosomes became attached to the ER membranes. Fowke and Setterfield also noted a granulation of the nucleolus possibly signifying ribosome synthesis.

Jackman and Van Steveninck (1970) reported no other significant ultrastructural changes in beetroot, despite the apparent disintegration of mitochondria extracted from 12 hour aged tissue (Van Steveninck and Jackman, 1967). Increases in numbers of mitochondria have, however, been reported in aging disks of potato (Lee and Chasson, 1966; Kahl, 1971) and sweet potato (Asahi and Majima, 1969). "Stabilization" of the plasmalemma in carrot and of the tonoplast in

swede has also been observed.

The lack of similarity both between the effects on the same membranes in different tissues and different membranes in the same tissue suggests that the changes in the ER are not due to general osmotic damage or a change in the conditions for fixation within the cell. The reported vesiculation of the ER with little effect on the other organelles in senescing bean cotyledons (Opik, 1968) further suggests that this is a specific effect.

E. THE PRESENT STUDY

This work characterizes the electron transport and phosphatase properties of the post-mitochondrial fractions in one particular plant tissue, and the changes induced by slicing and aging. Although it was advantageous to restrict the study to one tissue, others were studied from time to time to test whether the results were part of general phenomena in plants. Turnip tissue was chosen because of its availability and suitability for both spectrophotometric work (i.e. lack of pigments) and aging (i.e. uniform tissue without active polyphenol oxidase). The study was carried out with the following general aims in mind:

1. General characterization

The plant mitochondrial fraction has been well characterized, but relatively little is known about the post-mitochondrial fractions.

2. Comparison with corresponding animal fractions

As plant and animal cells are quite different, the functions and therefore the properties of the post-mitochondrial fractions are likely to be quite different.

3. Role of the fractions in the overall energetics of the cell

In the intact cell the various cytoplasmic membrane-bound and soluble enzymes may interact connecting various pools of ATP, reduced and oxidized nucleotides, glutathione, cysteine, ascorbate etc. Further elucidation of such functions can be made by attempting to correlate slicing and aging induced biochemical and physiological changes.

4. General membrane concept

All membranes may have a basically similar enzymic principle which manifests itself according to the actual physiological function of the membrane. Hence the energetic properties of the post-mitochondrial membranes are considered in light of the general energetic principles of the mitochondria and chloroplasts.

C H A P T E R I I

METHODS

A. PLANT MATERIAL

Fresh white turnip (*Brassica rapa* L.), swede (*Brassica napobrassica* Mill.), beetroot (*Beta vulgaris* L.), radish (*Raphanus sativus* L.), parsnip (*Pastinaca sativa* L.), potato (*Solanum tuberosum* L.) and carrot (*Daucus carota* L.) were obtained commercially. Peas (*Pisum sativum* L.) were grown in moist vermiculite and the roots harvested when about 5-10 mm long. Wheat (*Triticum* L. spp.), oats (*Avena sativa* L.), sunflower (*Helianthus annuus* L.), soybean (*Glycine max* Merr.) and maize (*Zea mays* Tourn.) were grown on moist gauze over aerated water and the roots also harvested when about 5-10 mm long.

B. SLICING AND AGING ROOT STORAGE TISSUE

Disks (1 mm thick) were cut using a hand microtome from cylinders (1 cm diameter) of tissue. The disks were aged in 10^{-4} M $CaSO_4$ aerated with a stream of filtered air at about $17^{o}C$ (unless otherwise stated). The aging solution was changed several times in the first hour and then about four times per day. $CaSO_4$ was used in the aging solution as it prevented loss of turgidity of the disks caused by some chemicals (e.g. cycloheximide, chloramphenicol). Disks were rinsed three times in distilled water and chilled before homogenizing.

C. TISSUE PHYSIOLOGY

1. Manometry

Respiratory rates of turnip disks were measured at 25°C using standard manometric techniques with air as the gas phase (Umbreit *et al.*, 1964). The disks were blotted dry, weighed to the nearest mg and transferred to 2.5 ml 12 mM phosphate buffer (pH 7.2) in a Warburg vessel. The number of disks and weight of tissue were kept as constant as possible (i.e. 7 disks weighing approximately 700 mg). The centre well contained 0.3 ml 4N KOH and filter paper wick to absorb all CO_2. Manometers were shaken in a Paton bath at 25°C at 150 oscillations per minute. Respiration rates were measured over 2 hours and are expressed as the average of 2 estimations (± 5 µl O_2/hour/gfw).

Attempts to measure respiration with a Rank oxygen electrode were unsuccessful due to biphasic rates of O_2 uptake. This was thought to be due to O_2 trapped in the intercellular spaces as the first (slow) phase could be considerably (but not completely) diminished by infiltration of the disks with water.

2. Conductivity

Salt (KCl) accumulation by turnip disks was measured by the changes in the conductivity of the external solution using a Pye Conductance Bridge (11700) (Robertson, 1941). 5g disks were shaken in 15 ml 40 mM KCl at 25°C and the conductivity of the solution measured at 30 minute intervals.

D. PREPARATION OF MICROSOMES

Root storage tissues (400 g) were homogenized in a Braun juice extractor into 30 ml 0.3 M sucrose containing 8 mM EDTA and 0.5 g BSA. The pH was kept at 7.4 during the homogenization by the drop-wise addition of 1 M tris. The mixture was strained through muslin and centrifuged at 27,000 g (maximum) for 15 minutes to remove cell debris, mitochondria and the larger mitochondrial fragments. The supernatant was then centrifuged at 150,000 g (average) for 45 minutes to sediment the microsomes. The microsome pellet was washed by re-suspending in 0.4 M sucrose, with the aid of a Potter-Elvehjem homogenizer, and re-centrifuging at 150,000 g (average) for 45 minutes. The final pellet was re-suspended in approximately 10 ml 0.4 M sucrose. All apparatus was pre-chilled and all operations were performed at $4^{o}C$.

The isolation procedure is known to yield active mitochondria showing respiratory control (Millard *et al.*, 1965). It was assumed that this minimised mitochondrial damage lessening any contamination of the microsomal pellet.

Young growing roots (40 g) were homogenized with a mortar and pestle in 30 ml 0.3 M sucrose containing 8 mM EDTA and 0.25 g BSA. The brei was strained through muslin and the pH immediately adjusted to 7.4 with 1 M tris. The preparation then followed the above procedure.

Disks were usually homogenized in the Braun juice extractor

as for whole tissue, except that the resulting solid phase was collected and rehomogenized (twice). The liquid phase from the three homogenizations was pooled and treated as above. When only 25 g were used, disks were homogenized using a mortar and pestle as for young roots.

E. SUBFRACTIONATION OF MICROSOMES

Microsomes were subfractioned once by differential centrifuging (Chapter III, Table 10). However, the method normally used (Chapters III, IV, VI, VII) was adapted from the method of Dallner (1963) for subfractioning liver microsomes.

1. Method for Chapters III, IV and VI

Unwashed microsomes from 600 gm turnip tissue were re-suspended in 90 ml 0.25 M sucrose containing 15 mM CsCl and 7.0 ml layered over 4.5 ml 1.3 M sucrose containing 15 mM CsCl. Centrifuging at 50,000 rpm (Type 50 Ti rotor) for 90 minutes resulted in a pellet (fraction 1) and 2 inseparable layers (fraction 2) at the sucrose boundary. The total layers were re-suspended in 90 ml 0.25 M sucrose containing 10 mM $MgCl_2$, and 7.5 ml layered over 4.0 ml 1.15 M sucrose containing 10 mM $MgCl_2$. Centrifuging at 50,000 rpm for 90 minutes resulted in 3 layers at or near the boundary (fractions 2 top, middle, bottom). The four fractions were washed and each re-suspended in 10 ml 0.25 M sucrose.

2. Method for Chapter VII

It was later shown that lowering the sucrose concentration of the bottom layer in the presence of $MgCl_2$ from 1.15 M to 0.90 M did not affect fraction 2 (middle) but resulted in pelleting of fraction 2 (top) and (bottom). Hence, in Chapter VII the microsomes were subfractioned as above except that the bottom sucrose layer in the presence of $MgCl_2$ was lowered to 0.90 M. This resulted in a much cleaner separation of fraction 2 (middle).

3. Variations

As this method partly depends on whether the membranes have bound ribosomes, the initial tissue homogenization was done in the absence of EDTA, which often causes a disassociation of the bound ribosomes (Palade and Siekevitz, 1956; Lund *et al.*, 1958; González-Cadavid and Campbell, 1967). The presence or absence of EDTA in the isolating medium did not affect the microsomal reductase activities.

The method of Dallner (1963) originally employed a rotor with a fixed tube angle of 20°. However, Bergstrand and Dallner (1969) reported that this gave poor separation of rough and smooth microsomes and that the tube angle should be at least 34° to avoid mixing. The Ti50 rotor used in this work has a fixed tube angle of 26°. Hence some mixing between fractions may be expected.

F. PREPARATION OF SOLUBLE FRACTION

Homogenizing of tissues for the preparation of the soluble

fraction was the same as for preparation of the microsomes except that 2 mg BSA/gfw was used. The soluble fraction was the supernatant fraction after sedimenting the microsomes. It was dialysed against 0.3 M sucrose containing 5 mM phosphate buffer (pH 7.2) and 2 mM EDTA for 24 hours at 4°C with numerous changes, or eluted through Sephadex G-25 (25 cm x 1 cm diameter). Any volume changes during dialysis were corrected for.

G. SOLUBLE ENZYME PURIFICATION

1. $(NH_4)_2 SO_4$ fractionation

Standard fractionation technique was followed (Strittmatter, 1967). 44 g $(NH_4)_2 SO_4$ was added with constant stirring over 15 minutes to 250 ml dialysed soluble fraction (i.e. 30% $(NH_4)_2 SO_4$). Concentrated NH_4OH was added dropwise to keep the pH at 7.2. This solution was stirred for a further 15 minutes and then centrifuged at 27,000 for 15 minutes. The pellet and supernatant were separated and the procedure repeated with the supernatant to 55% (i.e. add 44 g), 75% (i.e. add 35 g), and 100% (i.e. add 120 g). The pellets were re-suspended in 10 ml 5 mM phosphate buffer (pH 7.2) and dialysed against large volumes of the same solution for approximately 12 hours. After dialysis the volumes of these solutions were readjusted to 10 ml if necessary. All operations were performed at 4°C.

2. Sephadex elution

Standard Sephadex elution technique was used (Andrews, 1964).

Sephadex G-25 (medium) and G-200 columns were equilibrated at 4°C with 5 mM phosphate buffer (pH 7.2) for at least 24 hours before use. Columns were approximately calibrated with BSA and NaCl. 10.0 ml samples (soluble fraction) were loaded at the top of Sephadex G-25 columns (25 cm x 1 cm diameter), eluted with phosphate buffer, and 5.0 ml fractions collected. Sephadex G-200 columns (100 cm and 120 cm x 1.1 cm diameter) were loaded at the bottom (10.0 ml) and 5.0 ml fractions collected off the top. All operations were performed at 4°C.

H. ENZYME ASSAYS

1. Oxidation-reduction activities

All oxidation-reduction activities (except NT reductase) were assayed spectrophotometrically using a Beckman spectrophotometer, model DB, monitored directly on a Beckman, linear-log, 5" strip chart recorder. The assay medium contained 0.25 M sucrose; 5 mM (for soluble fraction) or 20 mM (for microsome fraction) phosphate buffer (pH 7.2) and 0.1 - 0.2 ml microsomes or soluble fraction in a total volume of 2.5 ml at 25°C. Substrates were used at, at least, saturating concentrations. The reaction was started by adding the last-named reactant, and the initial rate was measured.

(i) NAD(P)H-cytochrome c reductases.

0.5 mM cytochrome c; 0.3 mM NAD(P)H. Cytochrome c $E^{M}_{550} = 19.8 \times 10^{3}$ (Morton, 1958).

(ii) NAD(P)H-DCPIP reductases.

0.04 mM DCPIP; 0.3 mM NAD(P)H. DCPIP $E^{M}_{600} = 15.5 \times 10^{3}$

(Strittmatter and Velick, 1956).

(iii) NAD(P)H-FeCN reductases.

0.8 mM FeCN; 0.3 mM NAD(P)H. FeCN $E^{M}_{420} = 1.05 \times 10^{3}$

(Morton and Sturtevant, 1964).

(iv) Transhydrogenases.

0.3 mM NAD(P)H; 0.3 mM APAD(P). APAD(P) $E^{M}_{375} = 5.2 \times 10^{3}$

(Ragland and Hackett, 1964).

(v) NAD(P)H-cystine reductases.

0.8 mM cystine; 0.3 mM NAD(P)H. NAD(P)H $E^{M}_{340} = 6.2 \times 10^{3}$

(Pabst Laboratories, 1961).

(vi) NAD(P)H-glutathione reductases.

0.3 mM glutathione; 0.3 mM NAD(P)H. NAD(P)H $E^{M}_{340} = 6.2 \times 10^{3}$.

(vii) $Na_2 SO_3$-cytochrome c reductase.

0.5 mM cytochrome c; 10 mM $Na_2 SO_3$. Cytochrome c

$E^{M}_{550} = 19.8 \times 10^{3}$.

(viii) NAD(P)H oxidases.

0.3 mM NAD(P)H. NAD(P)H $E^{M}_{340} = 6.2 \times 10^{3}$.

(ix) G-6-P dehydrogenase.

10 mM G-6-P; 0.3 mM NADP. NADPH $E^{M}_{340} = 6.2 \times 10^{3}$.

(x) NAD(P)H-NT reductases.

The incubation medium was as previously described but containing 0.5 ml microsomes; 1.2 mM NAD(P)H; 0.75 mM NT in a total volume of 5.0 ml. The reaction was started by adding NT, and 1.0 ml samples were withdrawn at 0, 5, 10 minutes and blown into 1.5 ml of formalin-triton mixture (Lester and Smith, 1961) at room temperature. These solutions were centrifuged at 2,500 g for 10 minutes and read at 505 nm. Activity expressed as ΔE_{505}/min/mg protein (Dallner *et al.*, 1966).

2. Mixed function oxidases

The assay system contained 20 mM phosphate buffer (pH 7.2); 0.25 M sucrose; 0.6 mM NAD(P)H (or 0.6 mM NADP + 10 mM G-6-P + 0.02 ml G-6-P dehydrogenase {Sigma type XI}); 2 mM organic substrate; 0.5 ml microsomes in a total volume of 2.5 - 3.0 ml. This system was used for assaying (i) NAD(P)H oxidation; (ii) O_2 consumption; (iii) product formation.

(i) NAD(P)H oxidation was assayed spectrophotometrically, NAD(P)H $E^{M}_{340} = 6.2 \times 10^3$.

(ii) O_2 consumption was assayed manometrically, or polarographically in a sealed perspex vessel with a Clark oxygen electrode (Yellow Springs Instrument Co., Ohio, U.S.A.) connected to a 10" strip chart recorder (Honeywell-Brown, Middlesex, U.K.).

(iii) DMA oxidation: DMA-N-oxide was estimated by the method of Ziegler and Pettit (1964). As no DMA-N-oxide was available for a

standard curve, approximate estimations were made using $E^{M}_{420} = 8.2 \times 10^{3}$ (Ziegler and Pettit, 1964). Formaldehyde was estimated by the method of Nash (1953).

Aniline oxidation: Aniline was estimated by the method of Greenberg and Lester (1946) and p- aminophenol by the method of Kato and Gillette (1965).

Phenol oxidation: The method of Posner *et al.* (1961) was used to simultaneously estimate consumption of phenol and production of hydroquinone, catechol and resourcinol.

3. Peroxidase

Peroxidase was assayed by the method of Gregory (1966) in which the rate of ascorbate oxidation in the presence of H_2O_2 is measured. The assay medium contained 1.0 ml ascorbic acid (1-5 mM); 1.0 ml 0.2 M H_2O_2; 1.0 ml 0.4% benzidine in 0.1 M sodium citrate buffer (pH 5.3); 0.2 ml microsomes or 0.05 ml soluble fraction. The reaction was started with the enzyme and the time taken for a blue colour to suddenly develop (indicating complete oxidation of ascorbic acid). Activity expressed as µmoles ascorbic acid oxidized/min/ml fraction.

4. Absorption spectra

Absorption spectra (of microsomes and soluble fraction) were measured with a Beckman DB spectrophotometer using cuvettes with a 1 cm light path. Samples were reduced with sodium dithionite (and

read against oxidized samples for difference spectra). Cytochrome b_{555} was approximately estimated using cytochrome b_5 $E^{M}_{425-405} = 160 \times 10^3$ (Von Der Decken, 1967) on the assumption that the value for cytochrome b_{555} would be similar (Moore, 1967).

Cytochrome P-450 was assayed by the method of Murphy and West (1969) in which CO was bubbled into the sample cuvette after reduction with dithionite and read against a reference containing dithionite reduced microsomes.

5. Phosphatases

Phosphatases were usually assayed by determining the release of Pi from the substrate.

(i) G-6-P and NaBGlP: The assay medium contained 0.25 M sucrose; 0.25 ml microsomes or 0.05 ml soluble fraction; 20 mM G-6-P or 10 mM NaBGlP; 20 mM NaHmaleate-NaOH buffer (pH 5.4 or 6.5) or tris-HCl buffer (pH 8.0) in a total volume of 1.0 ml.

(ii) ATP: The assay medium contained 0.25 M sucrose; 0.1 ml microsomes; 2 mM ATP; 20 mM tes-tris buffer (pH 7.8); 2 mM $MgCl_2$; 50 mM KCl in a total volume of 1.0 ml.

Reactions were started by the addition of enzyme, shaken for 30 minutes at 25°C then terminated by the addition of 1.0 ml 5.5% perchloric acid and diluted to 5.0 ml with water. Inorganic phosphate was estimated on the total sample by the method of Marsh (1959). This method was used in preference to the other methods for Pi analysis

as it eliminates molybdate catalysed acid hydrolysis of ATP. Standards were run using K_2HPO_4.

Acid phosphatase was also assayed by the method of Linhardt and Walter (1963) by estimating p-nitrophenol formed from p-nitrophenylphosphate hydrolysis. The assay medium contained 5 mM p-nitrophenylphosphate; 50 mM sodium citrate buffer (pH 4.8) and 0.2 ml microsomes or 0.02 ml soluble fraction in a total volume of 1.0 ml. After 30 minutes incubation at 25°C, 4.0 ml 0.1 N NaOH was added and E read at 405 nm against the control. Standards were run using p-nitrophenol.

I. CHEMICAL ASSAYS

(i) pCMB was routinely assayed by the method of Boyer (1954).

(ii) Protein was assayed by the method of Lowry *et al.* (1951) with the $CuSO_4$ solution in 1% citrate (rather than tartrate). BSA was used as a standard.

(iii) RNA was estimated by the method of Loening (1961) assuming that 31 µg of the hydrolysed RNA/ml gives an extinction of 1.0.

J. ELECTRON MICROSCOPY

The turnip microsomal subfraction pellets were fixed for 2 hours in 4% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) and then rinsed for 24 hours in sodium cacodylate. The pellets were post-fixed for 5 hours in 1% osmic acid in 0.01 M sodium veronal-acetate (pH 7.2) and then left overnight in veronal-acetate. The pellets were

dehydrated in a graded acetone series and embedded in Araldite. Sections were mounted on filmed, carbon stabilized 200 mesh copper grids and stained for 30 minutes in uranyl acetate, rinsed in water, then post-stained for 3 minutes with lead and rinsed in 70% alcohol. Observations were made with a Seimens Elmiskop 1 Electron Microscope with an objective aperture of 50 μ, operating at 80 Kv.

K. CHEMICALS

All reagents were of the highest possible grade available. Biochemicals were obtained from Sigma Chemical Co. CCCP, valinomycin, hooded cobra (*naja naja*) venom were obtained from Calbiochem. Inc. APAD(P) was obtained from P. L. Biochemicals; trypsin (powder) from Difco Labs; NT from Aldrich Chemical Co., amytal from Eli Lilly and Co. Ltd; phlorizin from Fluka; chloramphenicol from Parke, Davis and Co., and Sephadex from Pharmacia. Nigericin was a gift from Dr K. R. West.

C H A P T E R I I I

MICROSOMAL ELECTRON TRANSPORT

INTRODUCTION

The lack of information on electron transport in the microsome fraction of plants relative to animals is described in Chapter I. In this Chapter oxidation-reduction activities in the microsome fractions isolated from a variety of plant tissues are summarized. The structure of the electron transport system in turnip is determined using several acceptors; inhibitor sensitivities; membrane disruption; and membrane fractionation.

RESULTS

A. GENERAL CHARACTERIZATION

The oxidation-reduction activities in the microsome fractions isolated from a variety of plants are summarized in Table 1. Similar activities were detected in oats, sunflower, soybean and maize roots, and carrot, parsnip, swede and potato storage tissues. Cytochrome c, DCPIP and FeCN were reduced in the presence of NADH and NADPH (usually less than 10% NADH rate). These reductase activities were characteristic of all microsome fractions tested. However, using the same isolation procedure, there was considerable variation in magnitude of activities both between tissues and within tissues of different ages (e.g. pea roots; similar results were obtained with wheat roots).

TABLE 1. Plant microsomal oxidation-reduction activities. Preparation of microsomes and assay conditions are as described in Chapter II. NT reduction is expressed in ΔE_{505} ($\times 10^3$)/min/gfw. All other activities are in μmoles NAD(P)H oxidized/min/gfw.

	Turnip	Beetroot	Radish	4-day old Pea Roots	8-day old Pea Roots	12-day old Pea Roots	Etiolated Pea Hypocotyl	Wheat Root Tips	Etiolated Wheat Coleoptile Tips
NADH-cyt.c reductase	2.7	15.7	8.7	111	139	101	52.8	37.5	79.4
NADPH-cyt.c reductase	0.2	0.2	0.5	8.0	2.0	6.1	2.0	4.0	4.2
NADH-FeCN reductase	9.0	48.5	24.8	546	753	500	358	430	718
NADPH-FeCN reductase	1.3	8.2	3.5	72.4	53.8	55.6	71.8	286	162
NADH-DCPIP reductase	3.9	15.2	7.4	133	141	68.0	112	137	225
NADPH-DCPIP reductase	0.3	1.1	2.1	0	0	5.0	14.2	78.0	53.8
NAD(P)H-janus green B reductase	0	-	-	-	-	-	-	-	-
NAD(P)H-methylviologen reductase	0	-	-	-	-	-	-	-	-
NADH-NT reductase	0.14	2.1	-	-	0.3	-	-	-	-
NADPH-NT reductase	0.05	0.12	-	-	0.5	-	-	-	-
Na_2SO_3-cyt.c reductase	0	-	-	-	-	-	-	-	-
NAD(P)H-cystine reductase	0	0	-	-	0	-	0	-	-
NAD(P)H-glutathione reductase	0	0	-	-	0	-	-	-	-
NADH-APAD reductase	0.7	-	-	-	0	-	-	0	-
NADH-APADP reductase	0	-	-	-	0	-	-	0	-
NADPH-APAD(P) reductase	0	-	-	-	0	-	-	0	-
NAD(P)H oxidation	0	0	-	-	0	-	-	-	0
G-6-P dehydrogenase	0	-	-	-	0	-	-	0	0
cytochrome b_{555}	+	0	-	-	+	-	-	+	-
cytochrome P-450	0	-	-	-	0	-	-	-	-

Although the activities (per gfw) from storage tissues were lower than from young growing tissues, the activities were much the same when expressed on a protein basis (Table 2). These rates were comparable with activities previously reported for plant microsomes, with the exception of the high NADPH dehydrogenase in beet petiole (Martin and Morton, 1955; 1956a) and the low NADH-cytochrome c reductase in some tissues (Martin and Morton, 1955; Crane, 1957; Ragland and Hackett, 1961) (Table 2).

Unlike FeCN, DCPIP and cytochrome c (Fig. 4), NT reduction could not be monitored directly as the coloured product must first be solubilized (Dallner, 1963). The reduction of NT over one hour is shown in Figure 1. After an initial rapid reduction, there was a loss of colour presumably due to the breakdown of the product (Dallner, 1963). Hence, activities were calculated from Δ E for the first 10 minutes. The exceptionally high NADPH:NADH ratio for NT reduction in pea and turnip microsomes (Table 2) has also been noted in animal microsomes (Williams and Kamin, 1962; Dallner *et al.*, 1966). However, from the results with beet microsomes, this would appear to be due to a disproportionate loss of NADH activity which has also been reported for liver microsomes (Dallner, 1963).

Plant microsomes from several tissues lacked transhydrogenase activity {in contrast to Ragland and Hackett (1964) and Murphy and West (1969)} and did not reduce cystine or glutathione. While NADH

TABLE 2. Comparison of microsomal reductase activities with reported activities from plants. Preparation of microsomes and assay conditions are as described in Chapter II. Activities are expressed in μmoles NAD(P)H oxidized/min/mg protein (NT reduction ΔE_{505}/min/mg protein).

Tissue	REDUCTASE ACTIVITIES								Reference
	Cytochrome c		DCPIP		FeCN		NT		
	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADPH	
Turnip root	0.27	0.02	0.39	0.03	0.90	0.13	0.016	0.005	This thesis
Beetroot	1.46	0.01	0.78	0.09	4.01	0.69	0.196	0.012	
Pea root	0.67	0.01	0.62	0.04	2.82	0.28	0.003	0.005	
Beet petiole	0.08	0.11	-	-	-	-	-	-	Martin and Morton (1955)
Beet petiole	0.33	0.21	0.82	0.21	-	-	-	-	Martin and Morton (1956a)
Wheat root	1.25	-	-	-	-	-	-	-	Martin and Morton (1956b)
Cauliflower bud	0.03	-	0.05	-	-	-	-	-	Crane (1957)
Etiolated pea stem	0.04	0.01	-	-	-	-	-	-	Ragland and Hackett (1961)
Etiolated pea stem	-	-	0.69	0.08	0.42	0.04	-	-	Ragland and Hackett (1964)
Cotton leaf	0.11	0.03	0.22	0.07	-	-	-	-	Frear *et al.* (1969)

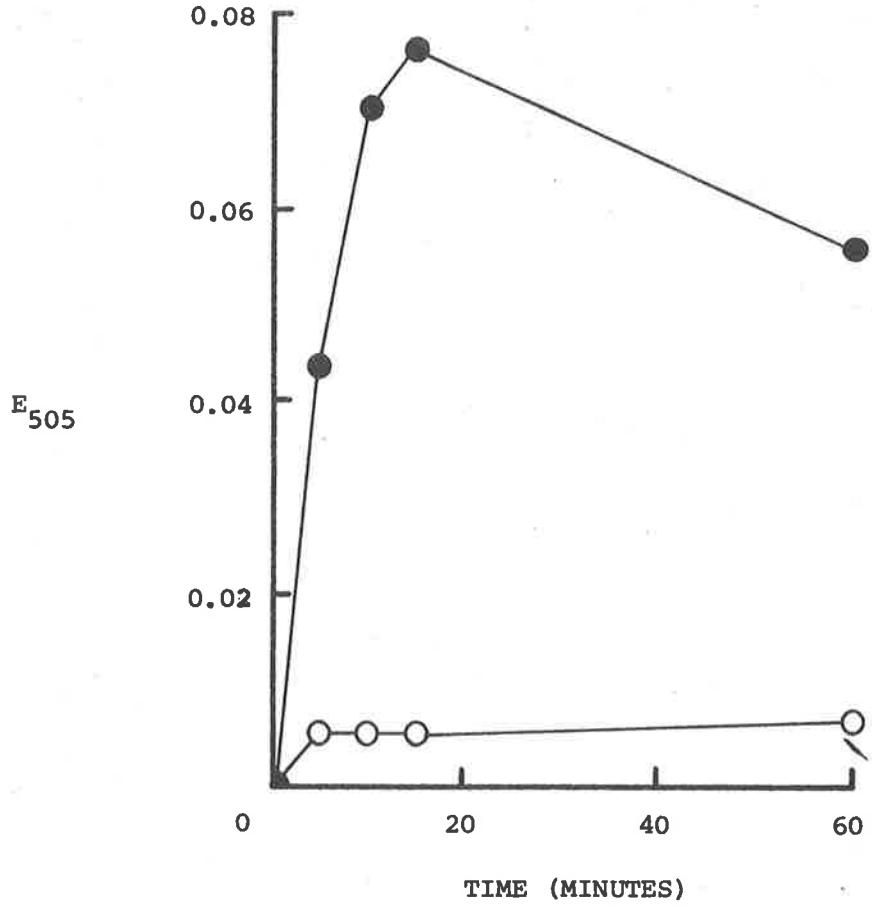


Figure 1. Beetroot microsomal NADH-(●) and NADPH-(○) NT reduction. Assay conditions are as described in Chapter II using 0.05 mg protein/ml.

and NADPH were not oxidized at pH 7.2, there was a low rate of oxidation at lower pH (5.8) probably due to peroxidase activity (Chapter VI). There was also no detectable Na_2SO_3-cytochrome c reductase in the presence or absence of up to 0.1% Triton X-100. This is a predominantly microsomal activity in liver and is considerably stimulated by detergents (Joshi *et al.*, 1969). The lack of G-6-P dehydrogenase activity was not due to hydrolysis of G-6-P as activity with this substrate at pH 7.2 was very low (Chapter VII).

A cytochrome corresponding to plant microsomal cytochrome b_{555} (Moore, 1967) (but not cytochrome P-450) was detected. Absorption maxima of the reduced cytochrome were at 423-425; 524-525; 554-556 nm. This cytochrome could be distinguished from peroxidase by the lack of effect of cyanide and pCMB on the oxidized γ peak (Chapter VI).

B. PROPERTIES OF REDUCTASE ACTIVITIES

1. pH optima

The pH curves of the reductases were broad, usually lacking sharp optima (Fig. 2). This appeared to be a consistent property of both plant (Crane, 1957) and animal (Strittmatter and Velick, 1956) microsomal reductases. The pH optima of the NADH activities were about 7 but higher for the NADPH activities. The peak at pH 6.6 for NADPH-DCPIP reductase was probably due to contaminating activity from the soluble fraction (Chapter V).

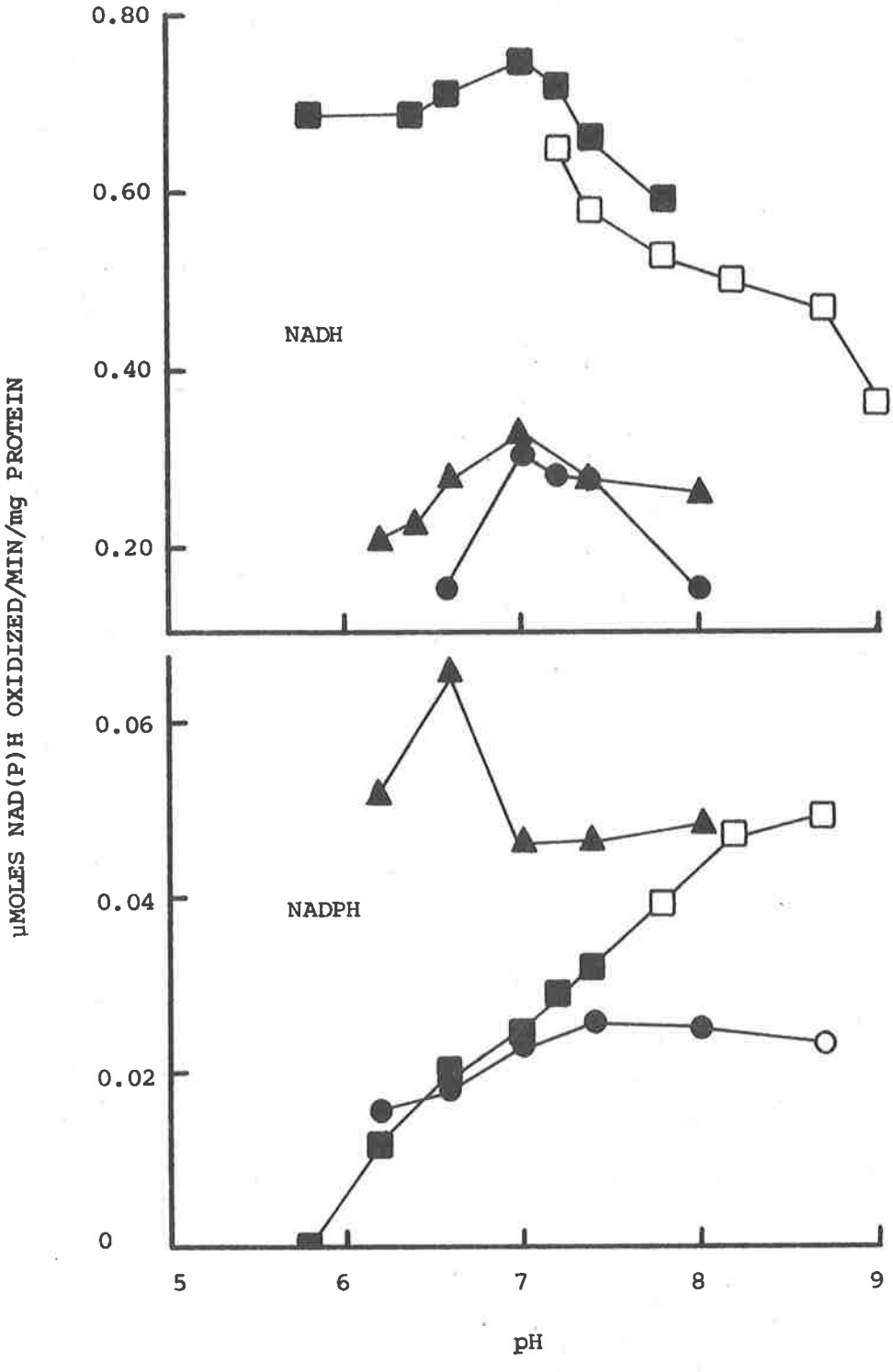


Figure 2. Dependence of the turnip microsomal reductase activities on pH. Assay conditions are as described in Chapter II, using 20 mM phosphate (closed symbols) and tris-HCl (open symbols) buffers. (○ - cytochrome c; △ - DCPIP; □ - FeCN.)

2. Inhibitor sensitivities

The NADH dehydrogenases were characteristic in their insensitivity to inhibitors of lipoyl dehydrogenase (arsenite; Massey, 1963; Basu and Burma, 1960), mitochondrial reductase (amytal, rotenone, antimycin A; Ikuma and Bonner, 1967) and DT diaphorase (dicoumarol; Dallner, 1963) (Table 3). Lack of succinate-cytochrome c reductase activity further supported lack of mitochondrial contamination. The NADH-cytochrome c reductase showed a moderate sensitivity to ionic concentration (Fig. 3). In contrast, similar ionic concentrations stimulated the liver microsomal activity up to 100% probably due to structural changes (Sottocasa, 1968; Kuylenstierna *et al.*, 1970).

There was, however, a characteristic inhibition of the NADH dehydrogenases by sulphydryl group poisons (pCMB and cystine). Figure 4 shows that preincubation of the microsomes with NADH largely protected cytochrome c reductase activity from this inhibition, at least initially. Similar results were obtained with FeCN and DCPIP (Table 4). This demonstrates the presence of an essential sulphydryl group (Strittmatter, 1965) involved in the binding of NADH to its dehydrogenase. The binding of NADH prevented the binding of pCMB. Similar results were obtained with microsomes from wheat, maize and pea roots. NADH-NT reductase was completely inhibited by 10^{-5} M pCMB but protection by NADH was not studied.

Increasing the concentration of NADH added to microsomes

TABLE 3. Inhibitor sensitivities of the turnip microsomal reductase activities. Assay conditions are as described in Chapter II.

Inhibitor	% Inhibition		
	NADH-cyt.c Reductase	NADH-DCPIP Reductase	NADH-FeCN Reductase
$3x10^{-3}$M Arsenite	0	9	0
10^{-6}M Dicoumarol	7	8	0
1mg/ml Antimycin A	0	-	-
$3x10^{-6}$M Rotenone	0	-	-
$5x10^{-4}$M KCN	0	0	0
$3x10^{-3}$M Amytal	29	8	12
$8x10^{-3}$M EDTA	60	33	25

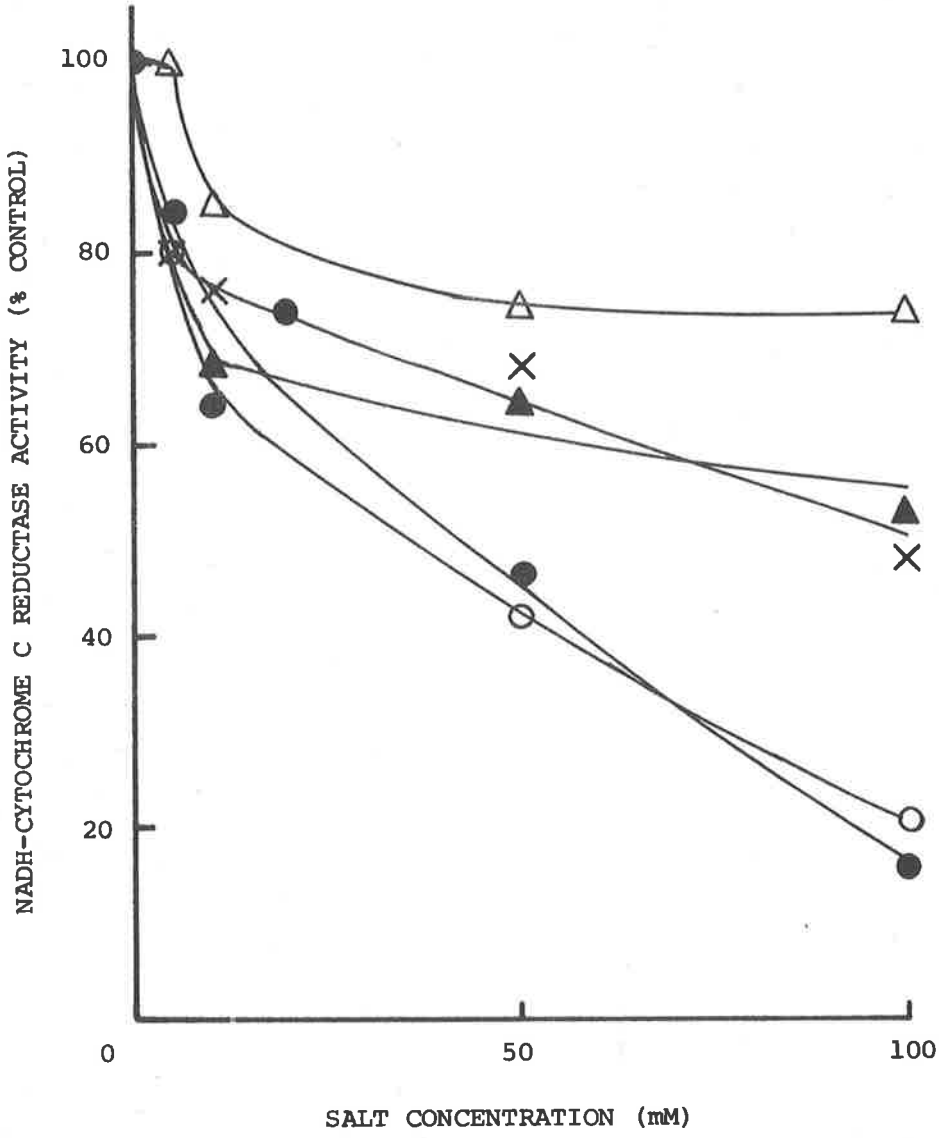


Figure 3. Effect of salts on turnip microsomal NADH-cytochrome c reductase activity. Assay conditions are as described in Chapter II. { ● - $CaCl_2$; ○ - $MgCl_2$; △ - NaCl; × - $(NH_4)_2SO_4$; ▲ - tris-HCl (pH 7.2) }

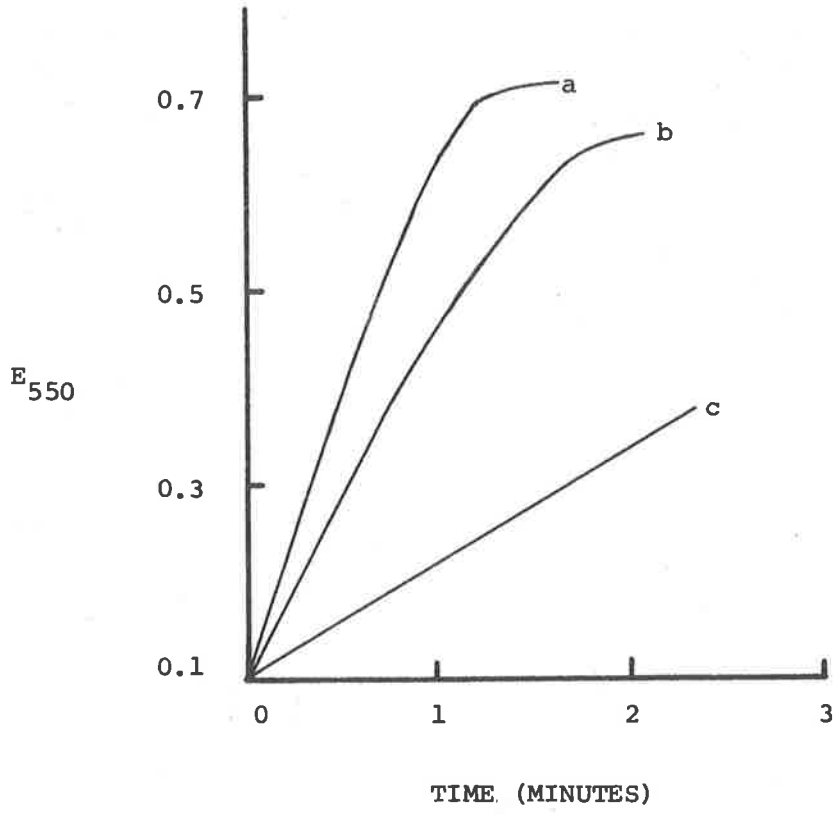


Figure 4. The effect of pCMB (2×10^{-5}M) on turnip microsomal NADH-cytochrome c reduction. Microsomes were preincubated for 2 minutes in the presence of (a) NADH; (b) NADH then after 2 minutes pCMB added; (c) pCMB then after 2 minutes NADH added. Reductase activity was then assayed immediately (as described in Chapter II) starting the reaction with cytochrome c.

TABLE 4. pCMB-sensitivity of the turnip microsomal reductase. Microsomes were preincubated with either NAD(P)H or pCMB (2×10^{-5} M) for 2 minutes, then pCMB or NAD(P)H added (respectively) and the activity assayed immediately. Assays are as described in Chapter II, the reactions being started with the acceptor.

Reductase activity	% Inhibition of initial rate	
	Preincubate with pCMB	Preincubate with reduced nucleotide
NADH-cytochrome c	83	12
NADPH-cytochrome c	-	-
NADH-DCPIP	95	19 (91*)
NADPH-DCPIP	27	36
NADH-FeCN	83	17
NADPH-FeCN	36	12

* preincubated with NADPH

preincubated with pCMB did not result in complete reversal of inhibition (Fig. 5) indicating that inhibition was not strictly competitive as might be expected. This was probably due:

(i) to a very much higher affinity of the sulphydryl group for pCMB than NADH;

(ii) to the involvement of other sulphydryl groups non competitively inhibited by pCMB and not protected by NADH (Strittmatter, 1959).

Hence, calculations of a value for Ki were not attempted.

The NADPH dehydrogenases were considerably less sensitive to pCMB (Table 4). Preincubation with NADPH partially protected NADPH-FeCN but not DCPIP reductase, again suggesting some contamination from the soluble fraction (Chapter V). Also, preincubation with NADPH did not protect the NADH-DCPIP reductase. These results indicate that the NADH activities are all due to the same dehydrogenase while the NADPH activities are due to a second different enzyme.

3. Concurrent reduction of two acceptors

Reduced cytochrome c ($E_o' = + 0.26$) and DCPIP ($E_o' = + 0.22$) reduced oxidized FeCN ($E_o' = + 0.43$) non-enzymically; and reduced cytochrome c reduced DCPIP. The rates of FeCN reduction in the presence of DCPIP and cytochrome c, and of cytochrome c in the presence of DCPIP, were measured to determine if a rate limiting step was present. For example, in the scheme below, if the rate of

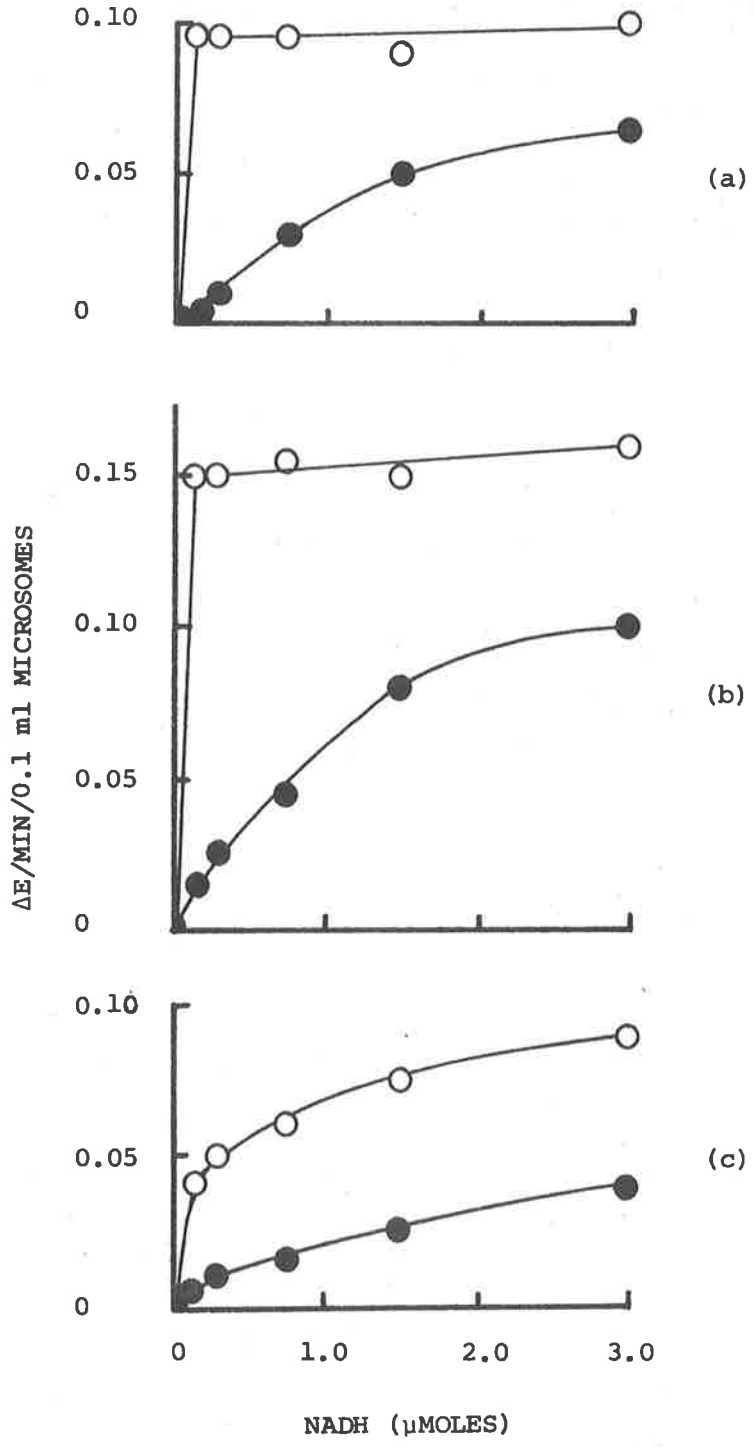
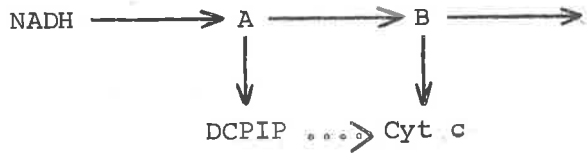


Figure 5. Effect of pCMB on turnip microsomal cytochrome c (a), DCPIP (b), and FeCN (c) reductase activities at varying NADH concentrations. Assay conditions are as described in Chapter II. Microsomes were pre-incubated for 2 minutes in the presence (●) and absence (○) of pCMB (2×10^{-5}M) and the reactions started with NADH.

cytochrome c (but not DCPIP) reduction is limited by the component B, then the addition of DCPIP (reducing cytochrome c non-enzymically) will overcome this.



(a) FeCN and DCPIP

Oxidation-reduction changes of FeCN and DCPIP could be measured spectrophotometrically in a mixture as there was no interference at either absorption peak (420 nm and 600 nm). As reduced DCPIP reduced FeCN non-enzymically there was no net reduction of DCPIP until all the FeCN had been reduced. The initial rate of FeCN reduction is unaffected by the presence of DCPIP. However, the subsequent reduction of DCPIP was twice the normal rate. This effect was also noted for cytochrome c reduction in the presence of FeCN and may be due to prior reduction of intermediates in the electron transport system. There was, however, no evidence of a lag in Figure 4.

(b) FeCN and cytochrome c

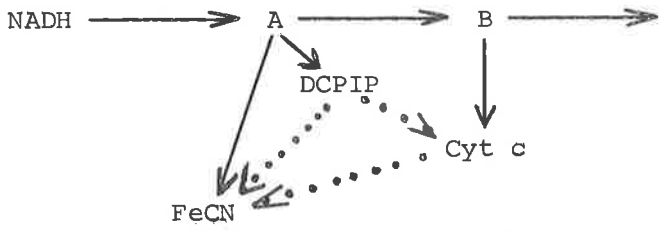
Oxidation-reduction changes of cytochrome c could be measured spectrophotometrically in the presence of FeCN as there was no interference at 550 nm. However, reduced cytochrome c strongly absorbs at 420 nm (γ peak at 415 nm). Hence in a mixture of cytochrome c and FeCN, the overall Δ E at 420 nm was given by the sum of

the decrease due to FeCN reduction and the increase due to cytochrome c reduction. The initial rate of FeCN reduction was also unaffected by the presence of cytochrome c.

(c) Cytochrome c and DCPIP

Oxidation-reduction changes of DCPIP could be measured spectrophotometrically in the presence of cytochrome c as there was no interference at 600 nm. However, DCPIP has a broad absorption peak and absorbs almost as strongly at 550 nm as at 600 nm. Hence in a mixture, ΔE_{550} is given by the sum of the increase due to cytochrome c reduction and the decrease due to DCPIP reduction. Figure 6 shows that the initial rate of cytochrome c reduction (at 550 nm) was greater in the presence of DCPIP (despite any drop in E due to DCPIP reduction). This was accompanied by a lag in DCPIP reduction (at 600 nm). This effect has been noted for several plant tissues and was greatest when the rate of cytochrome c reduction was least (Table 5).

These results are best explained by one electron transport chain with a rate limiting step between the site(s) of FeCN and DCPIP reduction and the site of cytochrome c reduction as in the scheme below. (Evidence in the next section indicates that this rate limiting step is at cytochrome b_{555}).



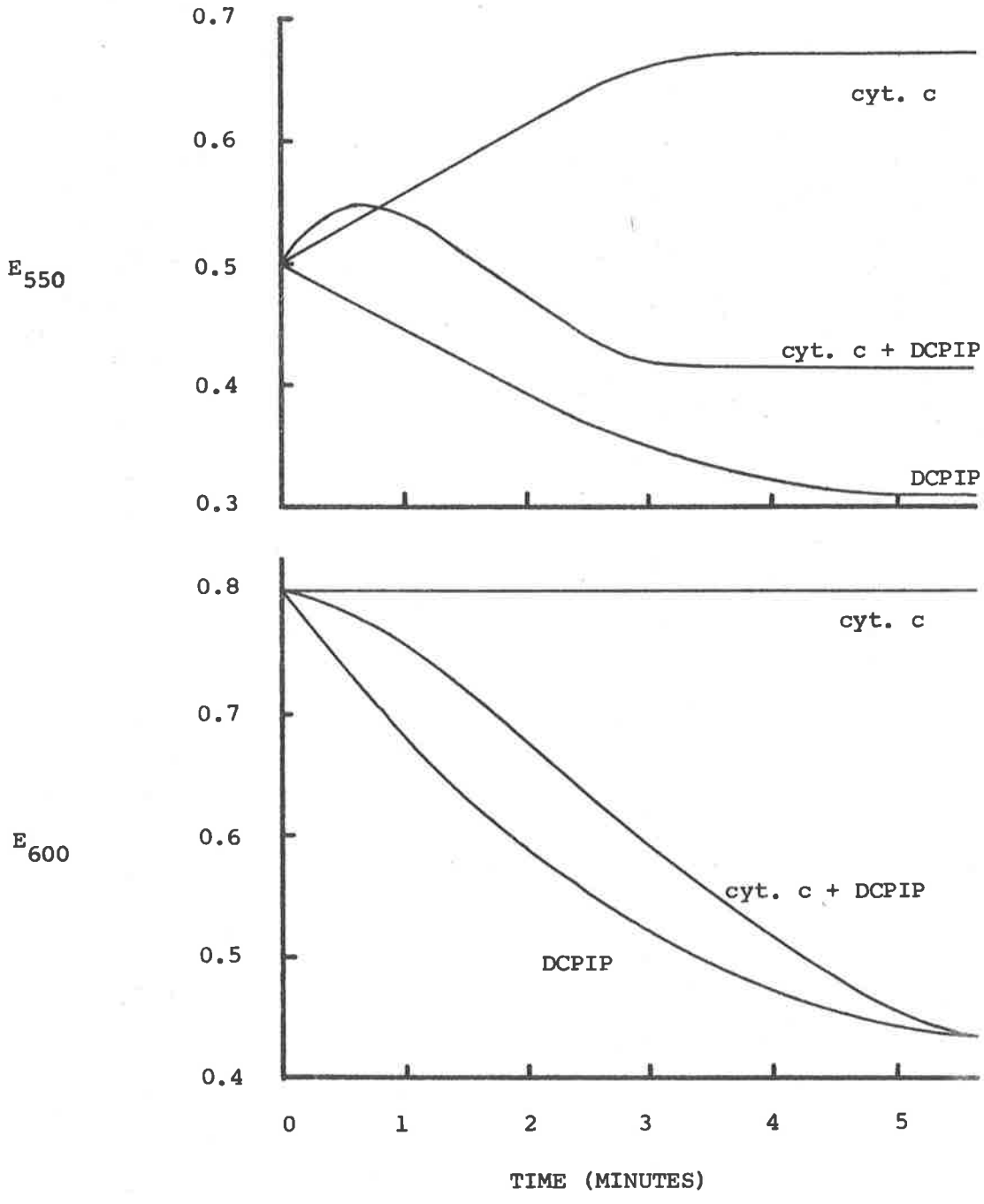


Figure 6. Effect of DCPIP on turnip microsomal cytochrome c reduction at 550 nm, and of cytochrome c on DCPIP reduction at 600 nm. The assay conditions are as described in Chapter II starting the reaction with NADH.

TABLE 5. Effect of DCPIP on the rate of NADH-cytochrome c reduction in plant microsomes. Assay conditions are as decribed in Chapter II measuring the initial rates as shown in Figure 6.

Tissue	Rate (μmoles NADH oxidized/min/gfw)		
	NADH-cytochrome c reduction	NADH-cytochrome c reduction (+ DCPIP)	NADH-DCPIP reduction
Wheat root	1.0	12.3	38.3
Oat root	13.8	23.7	86.8
Sunflower root	20.2	31.6	70.6
Pea root	43.1	55.0	163
Turnip	2.2	4.5	10.4
Turnip (microsomes stored at -15°C for 3 days	0.5	3.8	11.0

C. MICROSOMAL DISRUPTION

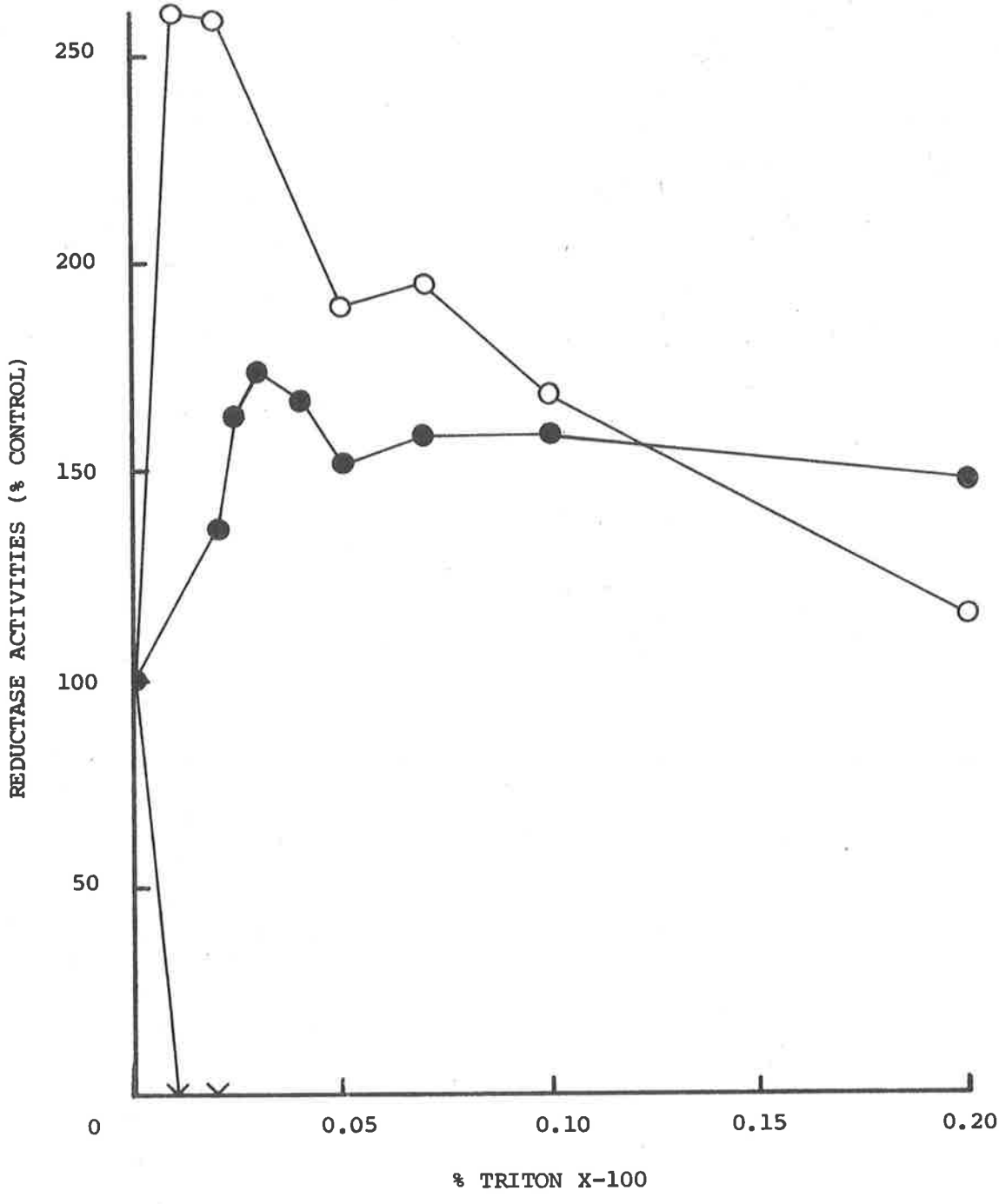
The turnip microsome fraction was subjected to both physical disruption (Triton X-100, sonication) and enzymic digestion (trypsin, *naja naja* venom) in an attempt to elucidate the electron transport systems.

1. Physical

Figure 7 shows that low concentrations of Triton X-100 inhibited NADH-cytochrome c reductase but stimulated NADH - DCPIP and -FeCN reductases. Higher concentrations had no effect on the stimulated NADH - FeCN but inhibited the stimulated NADH - DCPIP reduction rate. This was similar to the effects of deoxycholate on liver microsomes (Ernster, 1958). Sonication, however, resulted in inhibition of all three reductase activities which was greatest for cytochrome c and least for FeCN (Fig. 8). It appeared that, while Triton and sonication inhibited NADPH - cytochrome c reductase activity in a similar manner to the NADH-cytochrome c reductase activity, both NADPH - DCPIP and - FeCN reductase activities were stimulated by high Triton concentrations and by sonication for 30-60 seconds (Table 6).

No attempt was made to test for the solubilization of components after Triton treatment as prolonged contact with detergents (during separation of the fractions) resulted in inactivation (Ernster, 1958; Moulé *et al.*, 1960; Hess and Lagg, 1963; Dallner and Ernster, 1968). Sonication, however, did solubilize 5-10% of the

Figure 7. Effect of incubation of turnip microsomes with Triton X-100 on the NADH dehydrogenase activities. Microsomes were incubated with Triton for 2 minutes at 25°C immediately prior to assaying. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the activities in untreated microsomes (given below in μmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.179); NADH-DCPIP (O) (0.152); NADH-FeCN (●) (0.280).


250
200
150
100
50
0
0.05
0.10
0.15
0.20
REDUCTASE ACTIVITIES (% CONTROL)
% TRITON X-100

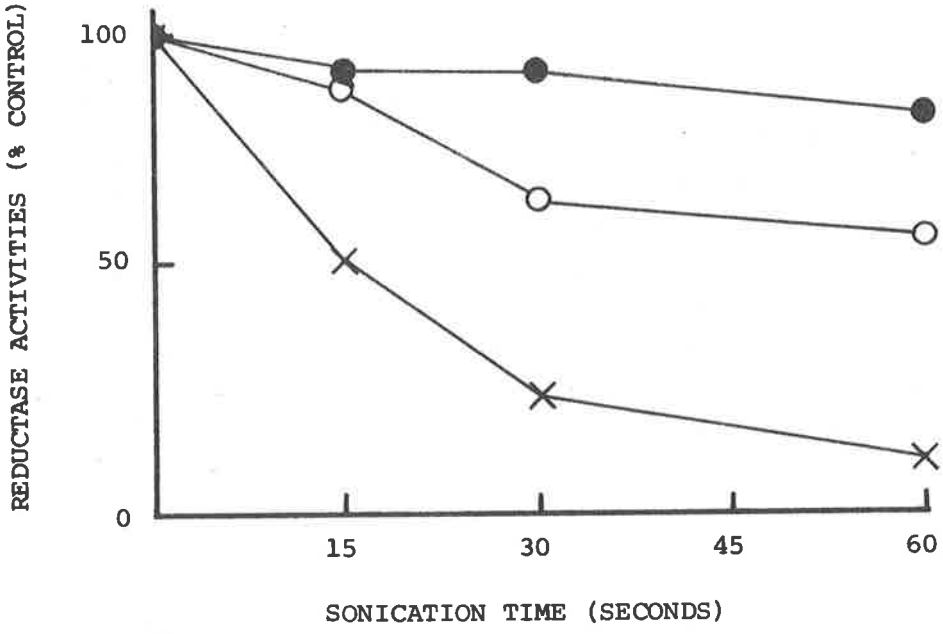


Figure 8. Effect of sonication of turnip microsomes on the NADH dehydrogenase activities. Microsomes were chilled in a salt-ice bath and sonicated with an MSE 100 Watt Ultrasonic Disintegrator with a pre-chilled probe for the times shown. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the activities in untreated microsomes (given below in μmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.663); NADH-DCPIP (○) (0.618); NADH-FeCN (●) (2.81).

TABLE 6. Effect of Triton X-100 and sonication on NADPH oxidations by turnip microsomes. Details of methods are described in Figures 7 and 8 and of assays in Chapter II.

Reductase activities (μmoles NADPH oxidized/min/mg protein)	% Triton X-100							Sonication time (secs)			
	0	0.01	0.02	0.05	0.07	0.10	0.20	0	15	30	60
NADPH-cytochrome c	0.002	0.002	0	0	-	-	-	0.010	0.005	0.010	0.002
NADPH-DCPIP	0.014	0.030	0.030	0.039	0.039	0.028	0.025	0.037	0.037	0.049	0.049
NADPH-FeCN	0.086	-	0.087	0.088	0.117	0.119	0.092	0.282	0.236	0.328	0.282

NADH-FeCN reductase and about 80% of cytochrome b_{555} but failed to solubilize the other reductases (Table 7). The solubilized cytochrome b_{555} could be partially purified by $(NH_4)_2SO_4$ (it precipitated in the 55-75% fraction) and by Sephadex G-200 (it eluted directly after the void volume). Similar results were obtained with wheat and pea root microsomes.

NT reduction was not studied in detail. However, treatment of beetroot microsomes with 0.025% Triton X-100 immediately before assaying inhibited the NADH activity 96% but the NADPH activity only 33%. Liver microsomal NADH-NT reductase was also more sensitive to detergent treatment (deoxycholate) than the NADPH activity (Dallner, 1963). Sonication of beetroot microsomes for 30 seconds caused 89% inhibition of the NADH activity and 100% inhibition of the NADPH activity.

Storage of the turnip microsomal suspension resulted in loss of all NADH dehydrogenases (greatest for NADH-cytochrome c and least for NADH-FeCN) (Table 8). In contrast the NADPH dehydrogenases were stimulated by storage at -15°C but inhibited by storage at higher temperatures. Losses of NADH-cytochrome c reductase on storage of both animal and plant microsomes have been reported (Palade and Siekevitz, 1956; Crane, 1957) although the latter author noted a partial stabilization with BSA.

The hot ethanol extraction method used by Mahler *et al.*

TABLE 7. Effect of sonication of microsomes on the electron transport activities. Turnip microsomes were sonicated for 120 seconds (60 seconds x 2) as described in Figure 8. The sonicated microsomes were assayed and then centrifuged at 50,000 rpm for 2 hours, and the resulting pellets resuspended in an equal volume of 0.4 M sucrose. Assay conditions are as described in Chapter II.

Reductase activities µmoles NAD(P)H oxidized/min/ 0.2ml sample	Untreated microsomes	Sonicated		
		Microsomes	Pellet	Supernatant
NADH-cyt.c	0.011	0.007	0.006	0
NADPH-cyt.c	0.001	-	0.001	0
NADH-DCPIP	0.055	-	0.027	0
NADH-FeCN	0.216	-	0.119	0.032
cyt.b_{555} (µmoles/0.2 ml sample)	0.044	-	0.031	0.106

TABLE 8. Effect of storing turnip microsomes for 70 hours on the reductase activities. The microsome suspension was divided into 5 aliquots, one assayed immediately and the others stored at the stated temperatures. Assay conditions are as described in Chapter II. Reductase activities are expressed in nmoles NAD(P)H oxidized/min/0.2 ml microsomes.

Reductase activities	Fresh	Stored			
		-15°C	4°C	9°C	16°C
NADH-cyt.c	6.9	3.6	2.4	2.1	0
NADH-DCPIP	2	68	40	26	12
NADH-FeCN	162	162	97	65	38
NADPH-cyt.c	0.6	1.2	0.9	0.5	0
NADPH-DCPIP	8.4	9.1	6.8	5.3	3.8
NADPH-FeCN	11	32	11	11	11

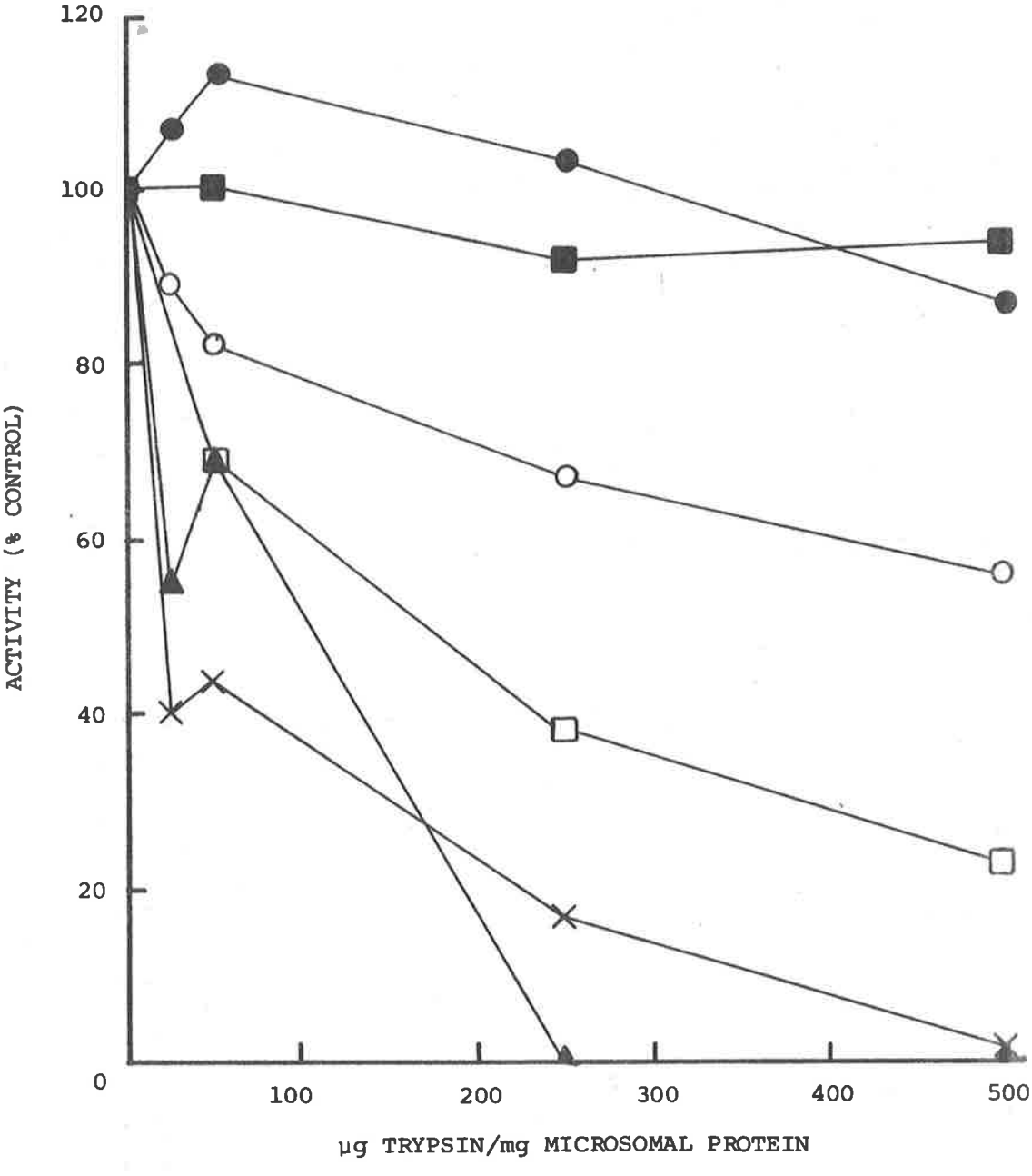
(1958) to solubilize a particulate dehydrogenase and cytochrome failed to solubilize any protein from fresh turnip microsomes.

2. Enzymic

The effect of incubating the microsomes with increasing concentrations of trypsin is shown in Figure 9. Other than a small stimulation of NADH - FeCN reductase at low trypsin concentration, all activities were inhibited. Corresponding to this, there was 80% loss of bound cytochrome b_{555} but only an 8% loss of total protein. The effect of incubating microsomes at a much lower trypsin concentration (too low to stimulate NADH-FeCN reductase) still failed to stimulate the rate of DCPIP or cytochrome c reduction (Fig. 10). Orrenius *et al.* (1969) reported stimulation of liver microsomal NADH-cytochrome c reductase but Kuylenstierna *et al.* (1970) reported inhibition. The effect of trypsin on the other reductases has only been considered by the latter authors who reported no effect on rates of either NADH-FeCN or -DCPIP reductase activity.

Figure 11 shows the facilitating effect of 70 mM KCl on the effect of trypsin on the reductase activities (maximum effect was produced by 30 mM KCl with higher concentrations up to 150 mM having no further effect). As KCl facilitation was quantitatively different for the three activities and also stimulated the activities in the absence of trypsin, this indicated that it may cause a structural change in the membrane rather than an activity change of the trypsin.

Figure 9. Effect of trypsin incubation on the microsomal dehydrogenase activities, protein and cytochrome b_{555}. Microsomes (suspended in 0.4 M sucrose) were equilibrated at 25°C for 1 minute, then varying quantities of freshly made up solution of trypsin added. (Optimum time of incubation with 50 µg trypsin/mg microsomal protein shown to be 1 minute.) After 1 minute, 3 x weight of soybean trypsin inhibitor was added, the whole chilled and the reductases assayed immediately (Kuylenstierna *et al.*, 1970). Activity losses in the control were corrected for. The pellet was then separated from the supernatant by centrifuging at 54,000 g (average) for 45 minutes and protein and cytochrome b_{555} in the pellet assayed. All assay conditions are as described in Chapter II and activities are expressed as a percentage of activities in untreated microsomes (given below in µmoles NAD(P)H oxidized/min/mg protein). NADH-cytochrome c (×) (0.140); NADH-DCPIP (○) (0.243); NADH-FeCN (●) (1.19); NADPH-cytochrome c (▲) (0.010); cytochrome b_{555} (□) (0.29 n moles/mg protein); protein (■) (0.72 mg/ml).


120
100
80
60
40
20
0
ACTIVITY (% CONTROL)
100
200
300
400
500
µg TRYPSIN/mg MICROSOMAL PROTEIN

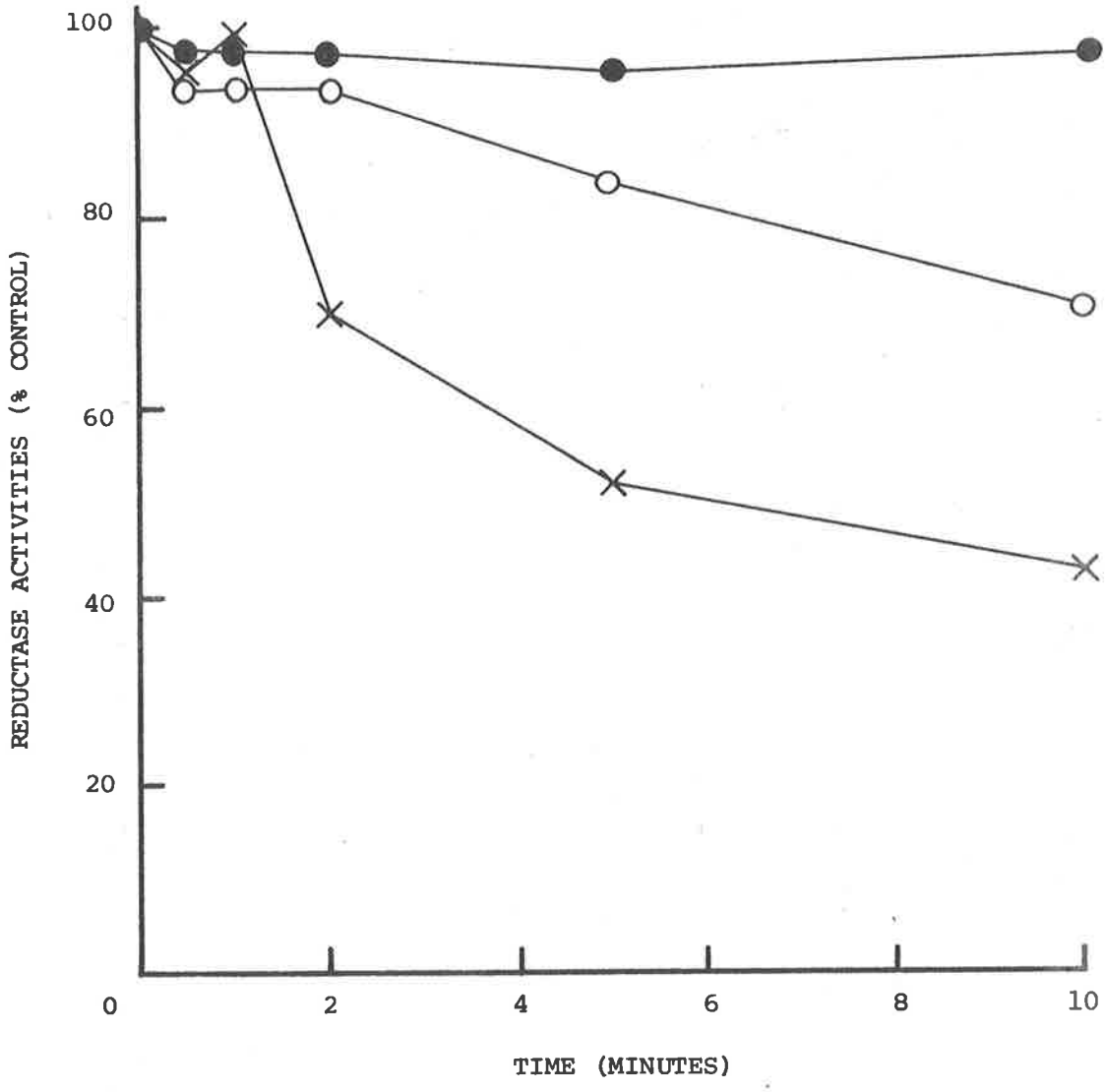


Figure 10. Effect of incubation of microsomes with low concentrations of trypsin on the turnip microsomal dehydrogenase activities. Trypsin incubations are as described in Figure 9 using 5.7 µg trypsin/mg microsomal protein. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of activities in untreated microsomes (given below in µmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.055); NADH-DCPIP (O) (0.460); NADH-FeCN (●) (1.12).

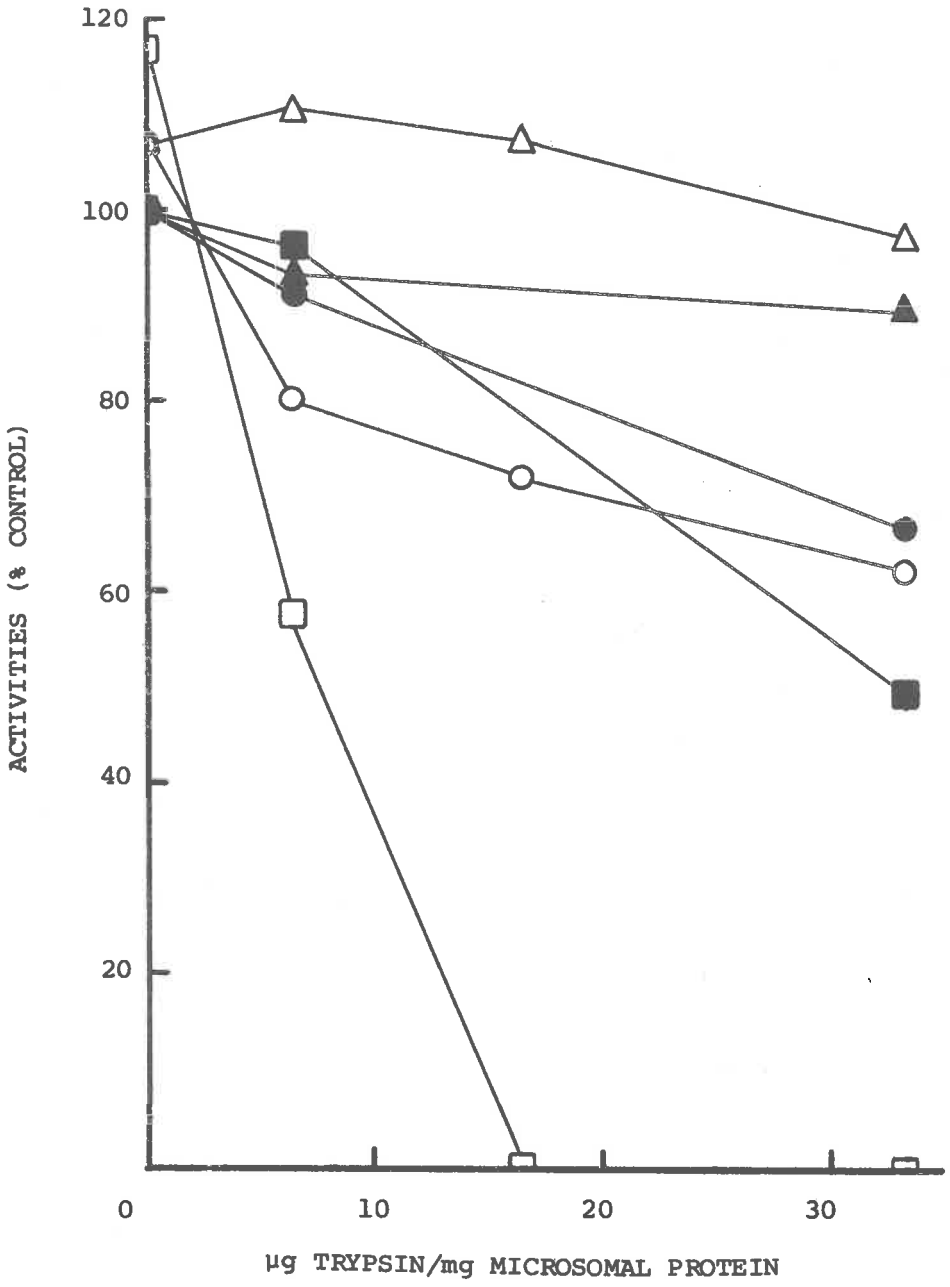


Figure 11. Effect of trypsin incubation in the presence and absence of KCl on the microsomal NADH dehydrogenase activities. Microsomes were incubated with varying amounts of trypsin in the presence (open symbols) and absence (closed symbols) of 70 mM KCl as described in Figure 9. Activities are expressed as a percentage of activities in untreated microsomes (given below in µmoles NADH oxidized/min/mg protein). NADH-cytochrome c (□) (0.072); NADH-DCPIP (○) (0.653); NADH-FeCN (△) (1.62).

The effect of incubating microsomes with *naja naja* venom is shown in Figure 12. The overall effect on the reductase activities was very similar to the effect of trypsin. However, after two hours incubation 20% of the NADH-FeCN reductase activity and 4% of the NADH-DCPIP reductase activity had been solubilized (Table 9).

D. MICROSOMAL SUBFRACTIONATION

1. Differential centrifuging

Microsomes were normally prepared by centrifuging the post-mitochondrial supernatant at 150,000 g for 45 minutes (Chapter II). However, activities were not evenly distributed in the pellets which sedimented at 38,000, 79,000 and 150,000 g for 1 hour and 150,000 g for 5 hours (Table 10). Although the 38,000 g pellet was several times larger than the others, protein was distributed more evenly. The NADPH-cytochrome c reductase was only detected in the 38,000 g pellet, which also contained the majority of the NADH activities. In contrast, the NADPH-FeCN and -DCPIP reductase activities were distributed throughout the four fractions.

2. Discontinuous sucrose gradients

Microsomes could also be subfractioned by centrifuging through discontinuous sucrose gradients in the presence of ions. This method depended on the membranes with bound ribosomes differentially binding Cs^+ and as a result sedimenting faster than those without bound ribosomes. The latter could be further separated into two fractions

Figure 12. Effect of *naja naja* venom incubation on the microsomal NADH-cytochrome c (X), -DCPIP (O) and -FeCN (●) reductase activities. The incubation method of Strittmatter (1967) was used with some variations. Washed microsomes from 50 gm tissue were suspended in 50 ml 50 mM tris-HCl buffer (pH 8.0) + 1 mM EDTA. 7.5 ml the same medium containing 50 mg venom were added to the microsomes in the cold, the pH adjusted to 6.0 with 2N HCl, then the temperature raised to 25°C and samples withdrawn at intervals over 2 hours and assayed immediately. The considerable loss of activities in the control over 2 hours incubation were corrected for. Activities are expressed as a percentage of activities in untreated microsomes (see Table 9).

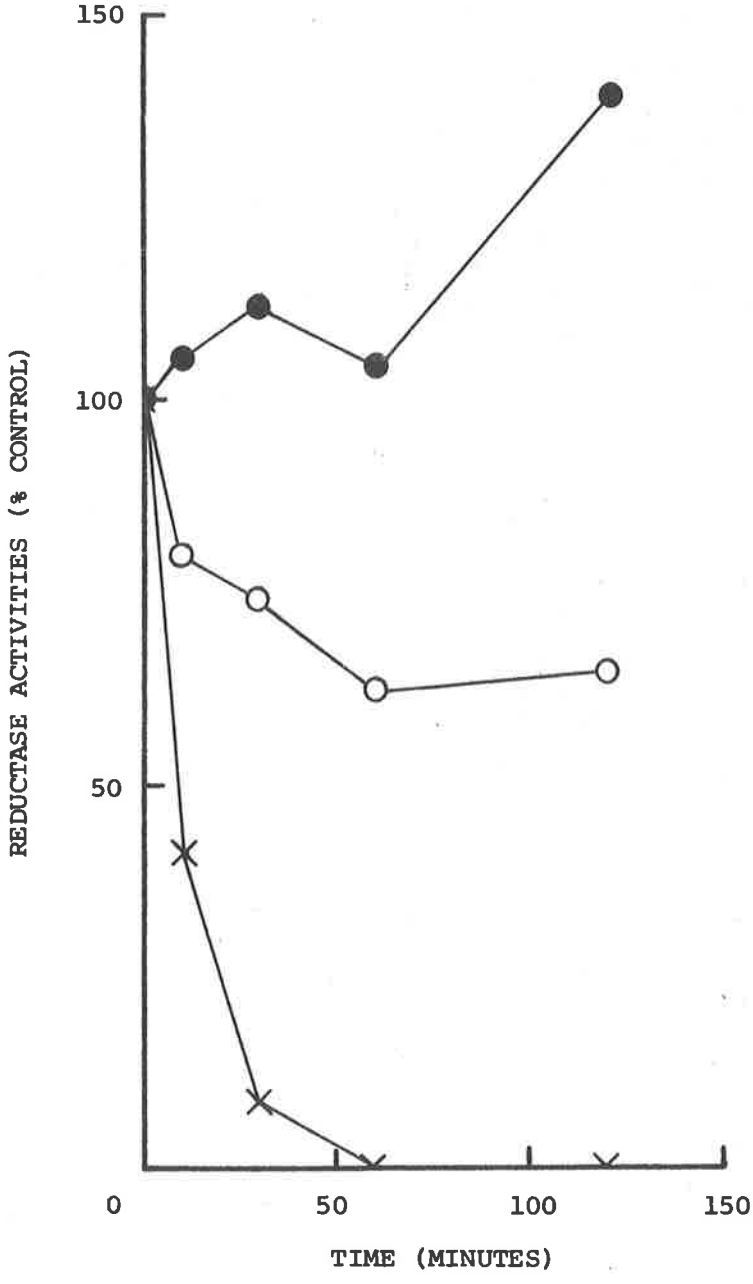

150
100
50
0
50
100
150
REDUCTASE ACTIVITIES (% CONTROL)
TIME (MINUTES)

TABLE 9. Effect of *naja naja* venom on the microsomal NADH dehydrogenase activities. The incubation method is described in Figure 12. After incubation for 120 minutes, the mixture was centrifuged at 27,000 g (average) for 20 minutes and the pellet re-suspended in an equal volume of 50 mM tris-HCl (pH 8.0) + 1 mM EDTA. Assay conditions are as described in Chapter II.

Reductase activities µmoles NADH oxidized/min/ 0.2ml sample	Untreated Microsomes	Incubated		
		Microsomes	Pellet	Supernatant
NADH-cyt.c	0.0482	0	0	0
NADH-DCPIP	0.0515	0.0174	0.0160	0.0007
NADH-FeCN	0.230	0.138	0.102	0.034

TABLE 10. Reductase activities in the turnip microsomal subfractions prepared by differential centrifuging. The post-mitochondrial supernatant was centrifuged successively at the g values and times indicated. The resulting pellets were washed and resuspended in equal volumes. Assay conditions are as described in Chapter II.

Reductase activity (μmoles NADH(P)H oxidized/min/mg protein)	Centrifugation			
	38,000g x 1 hr	79,000g x 1 hr	150,000g x 1 hr	150,000g x 5 hr
NADH-cytochrome c	0.027	0	0	0
NADPH-cytochrome c	0.001	0	0	0
NADH-DCPIP	0.175	0.012	0.007	0.008
NADPH-DCPIP	0.025	0.016	0.011	0.006
NADH-FeCN	0.484	0.057	0.054	0.104
NADPH-FeCN	0.023	0	0.028	0.015
protein (mg/ml)	1.06	0.42	0.44	0.80

depending on their ability to bind Mg^{++} (Dallner, 1963).

(a) Appearance and ultrastructure of subfractions

The first separation in the presence of CsCl resulted in a pellet (fraction 1) and 2 inseparable layers at the sucrose boundary (fraction 2) as reported by Dallner. The pellet contained 30% of the total protein but 80% of the total RNA (Table 11). However, the second separation of fraction 2 in the presence of $MgCl_2$ resulted in 3 layers, rather than a pellet and one layer (as reported by Dallner). The top layer was the largest, light brown, and was suspended in the low sucrose; the middle layer was white and fluffy and at the sucrose boundary; the bottom layer was thin, light brown and suspended in the dense sucrose (see Chapter IV, Fig. 10). The top layer comprised 50% of the total protein of fraction 2 while the RNA was equally distributed in the three subfractions.

Electron micrographs of the turnip microsomal subfractions (Plates 1-4) showed that they were quite different. All contained membranous vesicles and ribosomes, but:

(i) fraction 1 contained predominantly ribosomes with only a few vesicles;

(ii) fraction 2 (top) contained mainly vesicles with only a few ribosomes;

(iii) fraction 2 (middle) contained densely packed vesicles with very few ribosomes;

TABLE 11. Activities in subfractions derived from turnip microsomes by centrifuging through discontinuous sucrose gradients in the presence of ions. The method of subfractioning follows Dallner (1963) and is described in Chapter II. Assay conditions are as described in Chapter II.

	Fraction 1 (pellet in CsCl)	Fraction 2 (layers in $MgCl_2$)		
		Top	Middle	Bottom
NADH-cyt.c reductase {µmoles NAD(P)H oxidized /min/mg protein}	0.016	0.123	0.039	0.057
NADPH-cyt.c. reductase {µmoles NAD(P)H oxidized /min/mg protein}	0	0.008	0	0
NADH-DCPIP reductase {µmoles NAD(P)H oxidized /min/mg protein}	0.043	0.572	0.177	0.328
NADPH-DCPIP reductase {µmoles NAD(P)H oxidized /min/mg protein}	0.026	0.037	0.048	0.043
NADH-FeCN reductase {µmoles NAD(P)H oxidized /min/mg protein}	0.245	1.423	0.655	1.051
NADPH-FeCN reductase {µmoles NAD(P)H oxidized /min/mg protein}	0.122	0.107	0.120	0.210
Peroxidase (µmoles ascorbate oxidized/ min/mg protein)	-	0.280	0	0.241
Cytochrome b_{555} (µmoles/mg protein)	0.17	0.61	0.14	0.36
RNA (mg RNA/mg protein)	0.669	0.056	0.096	0.062
RNA (total mg)	7.500	0.560	0.440	0.320
Protein (total mg)	11.20	10.20	4.60	5.20

Plates 1-4. Ultrastructure of the turnip microsomal subfractions prepared by the method of Dallner (1963).

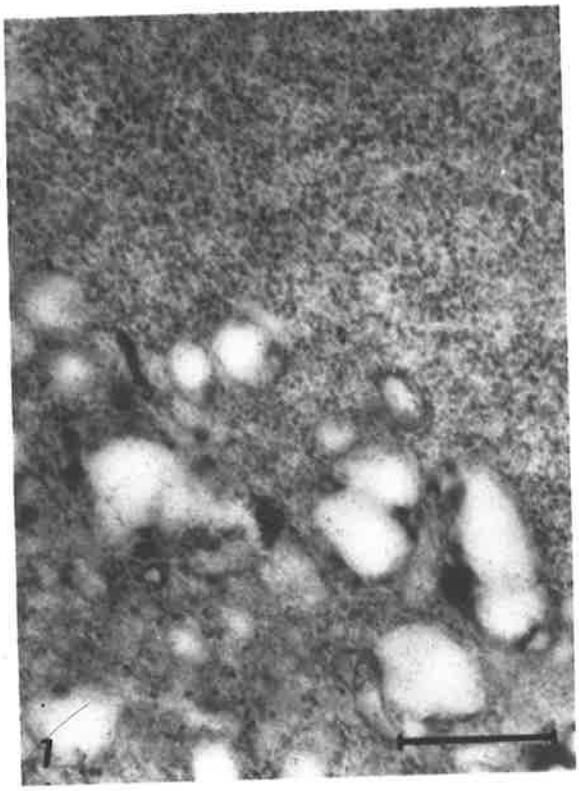
Plate 1 - Fraction 1

Plate 2 - Fraction 2 (top)

Plate 3 - Fraction 2 (middle)

Plate 4 - Fraction 2 (bottom)

Preparation of the subfractions is described in Chapter II, each subfraction being finally pelleted by centrifuging in 0.25M sucrose at 50,000 rpm for 45 minutes so that packing in all pellets is equivalent. The pellets were fixed in glutaraldehyde and osmic acid, and stained in uranyl acetate and lead (see Chapter II). The horizontal bars represent 0.5 μ.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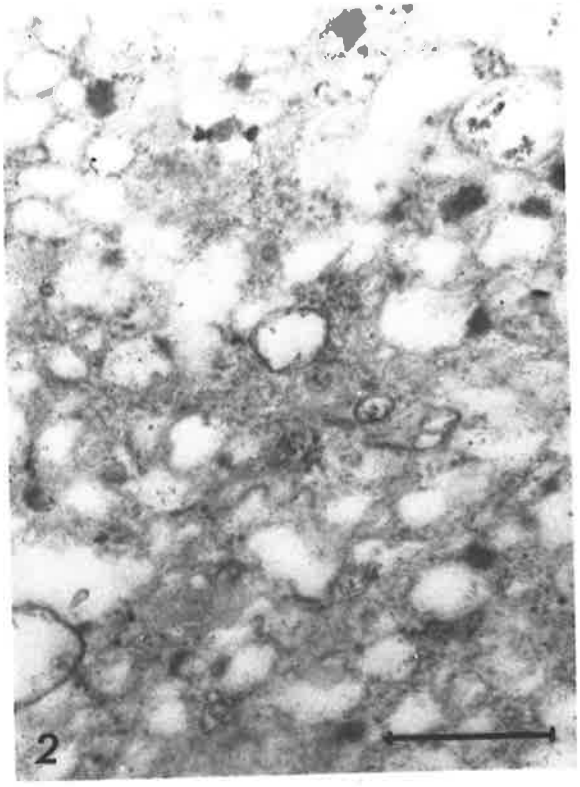
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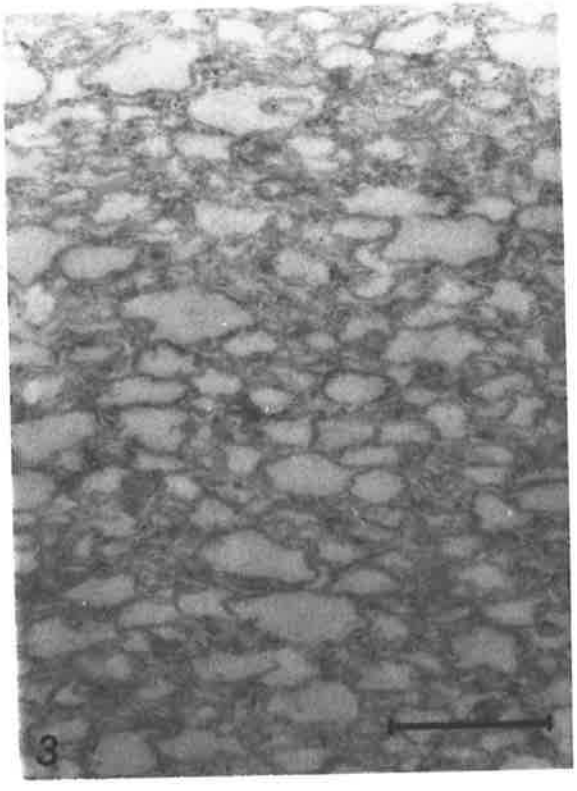
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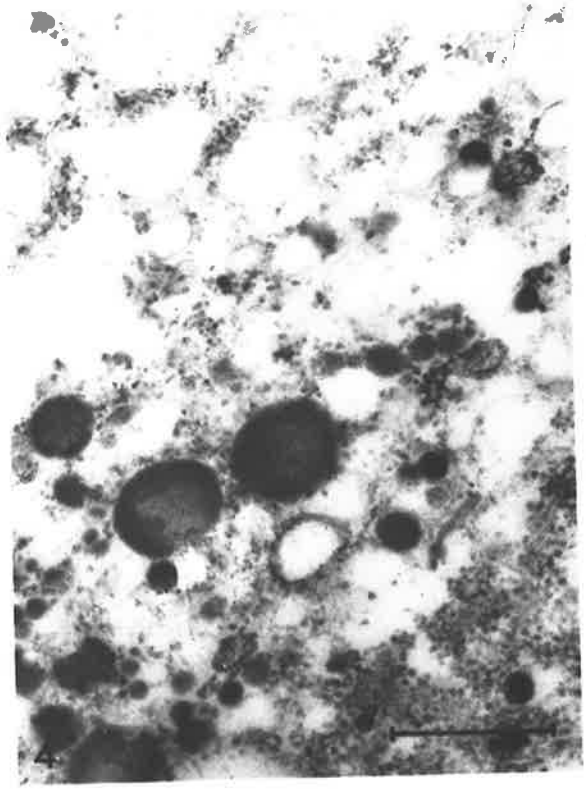

4

(iv) fraction 2 (bottom) contained vesicles, ribosomes and osmiophillic granules.

The vesicles in fractions 1 and 2 (top and bottom) appeared similar, while the vesicles in fraction 2 (middle) may be smaller and less inclined to be round. The majority of ribosomes in fractions 2 (top and bottom) do not appear to be attached to membranes. These may have been caught up with the membranes during subfractionation without actually ever being attached, or may have been released from the membranes at some stage after the fractionation. Osmiophillic granules have often been observed in plant cytoplasm, and this method also provides a way of concentrating them for possible further study.

(b) Enzymic distribution

The majority of the NADH activities, cytochrome b_{555} and peroxidase were concentrated in the top and bottom layers of fraction 2. However, while NADPH - DCPIP and -FeCN reductase activities were evenly distributed throughout the four fractions, NADPH - cytochrome c reductase was only detected in the top layer of fraction 2 (Table 11). This distribution of activities was similar to that given by differential centrifuging (Table 10).

It was later shown (Chapter VII) that lowering the sucrose concentration of the bottom layer in the presence of $MgCl_2$ from 1.15 M to 0.90 M did not affect fraction 2 (middle) but resulted in pelleting of fraction 2 (top) and (bottom). The similar appearance,

enzymic distribution and ability to bind ribosomes (Chapter IV) all suggest that the membranes in fractions 2 (top) and (bottom) are the same.

DISCUSSION

A. NADH ELECTRON TRANSPORT CHAIN

The oxidation-reduction activity most characteristic of the plant microsome fraction was the NADH dehydrogenase. The results indicated that activities were not contaminated with lipoyl dehydrogenase and the mitochondrial reductase. There was a characteristic inhibition by pCMB, which was largely prevented by preincubation of the enzyme with NADH. This demonstrated the presence of an essential sulphydryl group involved in binding NADH to its dehydrogenase. Such protection has been previously reported in animal microsomal and outer mitochondrial membranes. As similar protection was shown with cytochrome c, FeCN and DCPIP, reduction of these acceptors probably occurred by the same enzyme.

Sonication of turnip microsomes released an NADH-FeCN reductase which did not reduce DCPIP or cytochrome c (Table 7). Incubation of turnip microsomes with *naja naja* venom solubilized an NADH dehydrogenase which was 50 times more active in the presence of FeCN than DCPIP, and inactive with cytochrome c (Table 9). As NADH oxidation by the intact microsomes was only 5 times greater with FeCN than DCPIP, solubilization clearly resulted in destruction of both

the NADH-cytochrome c and DCPIP reductase activities. A similar effect has been demonstrated in animal microsomes (Strittmatter, 1967; Takesue and Omura, 1970b). These results are best explained by an electron transport chain in the microsomal membranes where intermediate(s) are required for DCPIP and cytochrome c but not FeCN reduction (Fig. 13). Trypsin-treatment (Fig. 9) and sonication (Table 7) of the microsomes resulted in parallel loss of NADH-cytochrome c reductase activity and cytochrome b_{555} indicating that this cytochrome is the hypothesized intermediate. Reduction of cytochrome c via cytochrome b_5 is well established in animal microsomes (Dallner, 1963; Kuylenstierna *et al.*, 1970; Hara and Minikami, 1971a).

However, incubation of the microsomes with 0.1% Triton completely inhibited cytochrome c reduction but stimulated DCPIP reduction over 100% (Fig. 7). Similarly, concentrations of trypsin (Fig. 9) and *naja naja* venom (Fig. 12) which completely inhibited cytochrome c reduction and released the majority of cytochrome b_{555} resulted in only 40% loss of DCPIP activity. These results may be explained if DCPIP was reduced at a component (X), situated in the electron transport chain between the dehydrogenase and cytochrome b_{555} (Fig. 13). Such a component has been suggested in animal microsomes for a variety of reasons (Chapter I, Fig. 1).

The effects of ions on activities (Fig. 3) and in particular of KCl in facilitating trypsin activity (Fig. 11) suggests structural

changes in the membranes (possibly swelling). The results of Orrenius *et al.* (1969) and Kuylenstierna *et al.* (1970) indicate that KCl induced structural changes in liver mitochondria and microsomes. Results in Chapter IV showed that microsomes isolated from fresh tissue were more susceptible to trypsin than those isolated from aged tissue again indicating that membrane structure is a controlling factor, at least in the action of trypsin. Also results in Chapter VII showed that under certain conditions (sonication) KCl stimulated ATPase activity, probably also due to a structural change.

It has been recently suggested that the reported solubilization of the animal microsomal NADH dehydrogenase by *naja naja* venom (Strittmatter, 1967) may be due to contaminating lysosomes (Takesue and Omura, 1970a; 1970b; Sargent *et al.*, 1970). Loss of NADH dehydrogenase activities from the turnip microsomes by incubation with the soluble supernatant fraction (containing the majority of hydrolytic enzymes) is described in Chapter IV. It could not, however, be shown whether this loss was due to release or inactivation of the enzymes. The plant microsome fraction could well be contaminated with hydrolytic enzymes (Chapter IV) which may release the FeCN reductase. However, the overall effects on microsome activities (Fig. 12) must be due entirely to the *naja naja* venom as losses in the controls were corrected for.

Some of the disadvantages of elucidating the structure of

the electron transport chain by disrupting the membranes are outlined in Chapter I. The results in Table 5 are obtained from intact microsomes and indicate that DCPIP is reduced at a site before cytochrome c and that cytochrome b_{555} is often limiting the rate of cytochrome c reduction.

The function of the NADH electron transport chain was not determined due to the lack of a natural terminal electron acceptor. No cystine or glutathione reduction, ascorbate oxidation or significant transhydrogenase activity was detected. Sterol demethylation and lipid desaturation were, however, not tested for (Chapter I).

B. NADPH ELECTRON TRANSPORT CHAIN

As the NADPH activities were so low, care must be taken in distinguishing them from contaminating activity from the soluble supernatant fraction (Chapter V). There was, however, little or no NADPH-cytochrome c reductase in the soluble fraction (Chapter V), indicating that the corresponding microsomal activity was not likely to be due to contamination. Although protection from pCMB of the NADPH-FeCN reductase was only partial (suggesting partial contamination) the liver microsomal NADPH-cytochrome c reductase was also only partially protected (Williams and Kamin, 1962). However, the lack of protection and low pH optimum of the turnip microsomal DCPIP reductase did resemble the soluble activity indicating contamination. (If this is so then there may also be low contamination of the soluble NADH-

specific FeCN reductase described in Chapter V, and it could be this activity which is solubilized by the disruption treatments.)

The inhibition of NADPH-cytochrome c reductase in turnip microsomes by trypsin, Triton and sonication resembled that of NADH-cytochrome c reductase. However, NADPH-FeCN and -DCPIP reductases were stimulated by both sonication and Triton. The results indicated a separate NADPH dehydrogenase which reduces FeCN and DCPIP directly but cytochrome c via a component (Y) (Fig. 13).

C. MICROSOMAL SUBFRACTIONS

1. Enzymic distribution

While both the methods of fractionation of the turnip microsomes resulted in a broad distribution of the NADPH-DCPIP and -FeCN reductase activities, the NADH reductases appeared to be associated with particular fractions. However, the NADPH-cytochrome c reductase did not sediment with the other NADPH activities but rather followed the NADH-cytochrome c reductase. If the NADPH-cytochrome c reductase did involve a component (Y) as suggested, then it might be expected to be associated (like the NADH-cytochrome c reductase) with the less damaged, membrane fragments. The association could, however, also be explained by the involvement of cytochrome b_{555} in NADPH-cytochrome c reduction (Fig. 13) which has been suggested for animal microsomes (Estabrook and Cohen, 1969; Kamino and Inouye, 1970).

Subfractioning animal microsomes by a variety of methods

usually results in most electron transport activities sedimenting together in the faster sedimenting fractions (Palade and Siekevitz, 1956; Imai *et al.*, 1966; Amar-Costesec *et al.*, 1969) although there are exceptions (Dallman *et al.*, 1969). Dallner (1963), using the CsCl and $MgCl_2$ method, found a concentration of NADH-DCPIP and -cytochrome c reductases in fraction 1, an even distribution of cytochrome b_5, and a concentration of NADPH-DCPIP and -cytochrome c reductases in fraction 1 and the pellet of fraction 2. This differs markedly from the results presented in Table 11. However, slicing turnip tissue induced fraction 2 (top) (which contained the majority of the NADH activities and cytochrome b_{555}) to sediment with fraction 1 (Chapter IV) resulting in a distribution more like that reported by Dallner (1963).

2. Origin of fraction 2 (middle)

Fractions 1 and 2 (top and bottom) are presumably all derived from the ER. This is supported by the binding of ribosomes by fractions 2 (top and bottom) in aging tissue (Chapter IV). However, the results indicated that the origin of fraction 2 (middle) was quite different:

(i) low dehydrogenase activity and cytochrome b_{555} (Table 10);

(ii) different ultrastructure (Plate 3);

(iii) different colour (Chapter III, IV);

(iv) inability to bind ribosomes (Chapter IV);

(v) lack of peroxidase (Chapter VI);

(vi) high ATPase activity (Chapter VII).

Using a continuous sucrose gradient, Glauman and Dallner (1970) showed that the slowest sedimenting liver microsomal membranes were largely derived from the plasmamembranes. Hence, fraction 2 (middle) may also comprise plasmamembranes, the results in Chapter VII suggesting the tonoplast.

D. PROPOSED ELECTRON TRANSPORT SCHEME

The proposed turnip microsomal electron transport system is presented in Figure 13. Both the NADH and NADPH dehydrogenases probably involve flavoproteins although this has not been shown in plants. Due to the difficulty in assaying NT reductase in turnip microsomes this activity was not studied in detail. The results obtained were with beet microsomes where the activity was considerably greater.

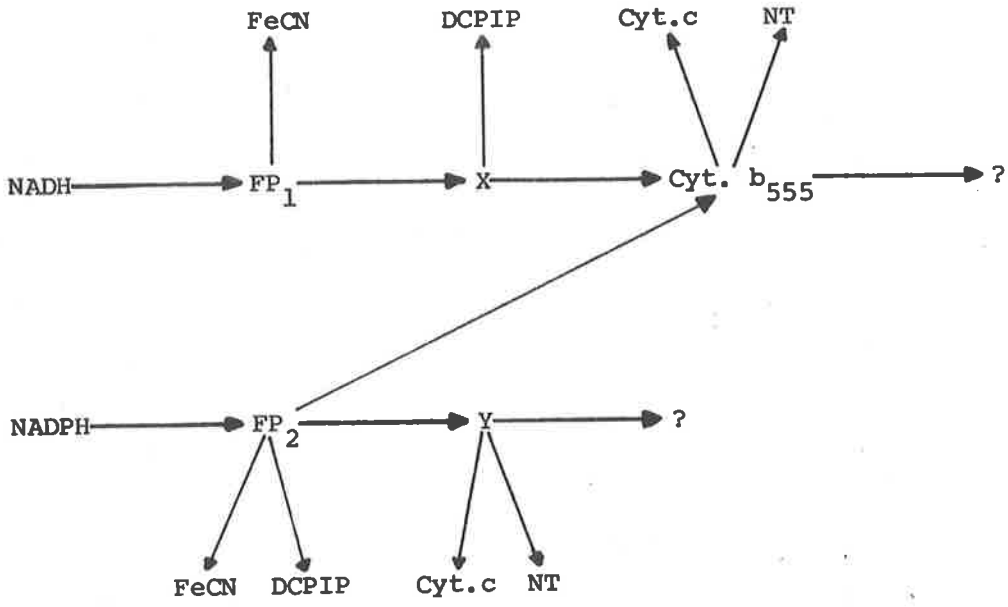


Figure 13. Proposed turnip microsomal electron transport system.

C H A P T E R I V

CHANGES IN MICROSOMAL ELECTRON TRANSPORT INDUCED BY SLICING AND AGING OF TISSUE

INTRODUCTION

Although the major ultrastructural changes in aging tissue slices are in the ER, the corresponding changes in the microsome fraction have not been studied (Chapter I). This Chapter attempts to characterize the changes in the turnip microsomal electron transport system induced by slicing and aging.

RESULTS

A. EFFECT ON MICROSOMAL ACTIVITIES IN VIVO

1. Changes in microsomal activities induced by slicing and aging of tissue

Microsomal NADH dehydrogenase activities decreased dramatically on slicing turnip (Fig. 1), swede (Fig. 2) and beetroot (Fig. 3) tissues. While minimum activities were usually detected 3-5 hours after slicing, most of the loss occurred in the first ten minutes. Subsequent to the decline, activities generally increased with further aging, then again decreased. Activities in Figures 1-3 are presented on a protein basis to demonstrate that the induced changes were in character rather than yield (the loss of protein induced immediately by slicing may be a result of less efficient homogenization of disks

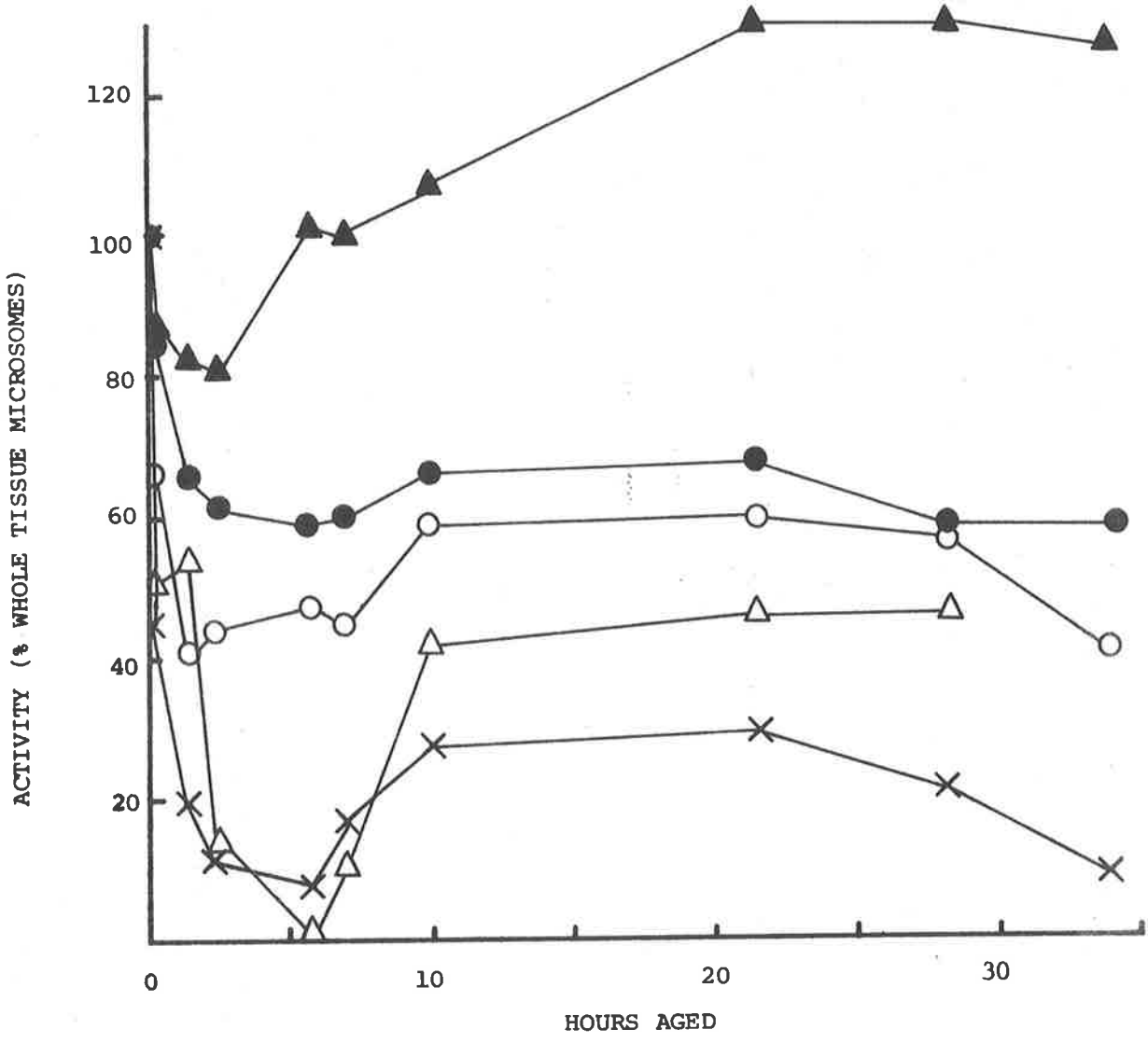


Figure 1. Changes in microsomal reductase activities in 1 mm thick disks of turnip with aging. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the activities in microsomes isolated from whole tissue (given below in μmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.273); NADH-DCPIP (O) (0.393); NADH-FeCN (●) (0.900); cytochrome b_{555} (Δ) (0.35 nmoles/mg protein); protein (▲).

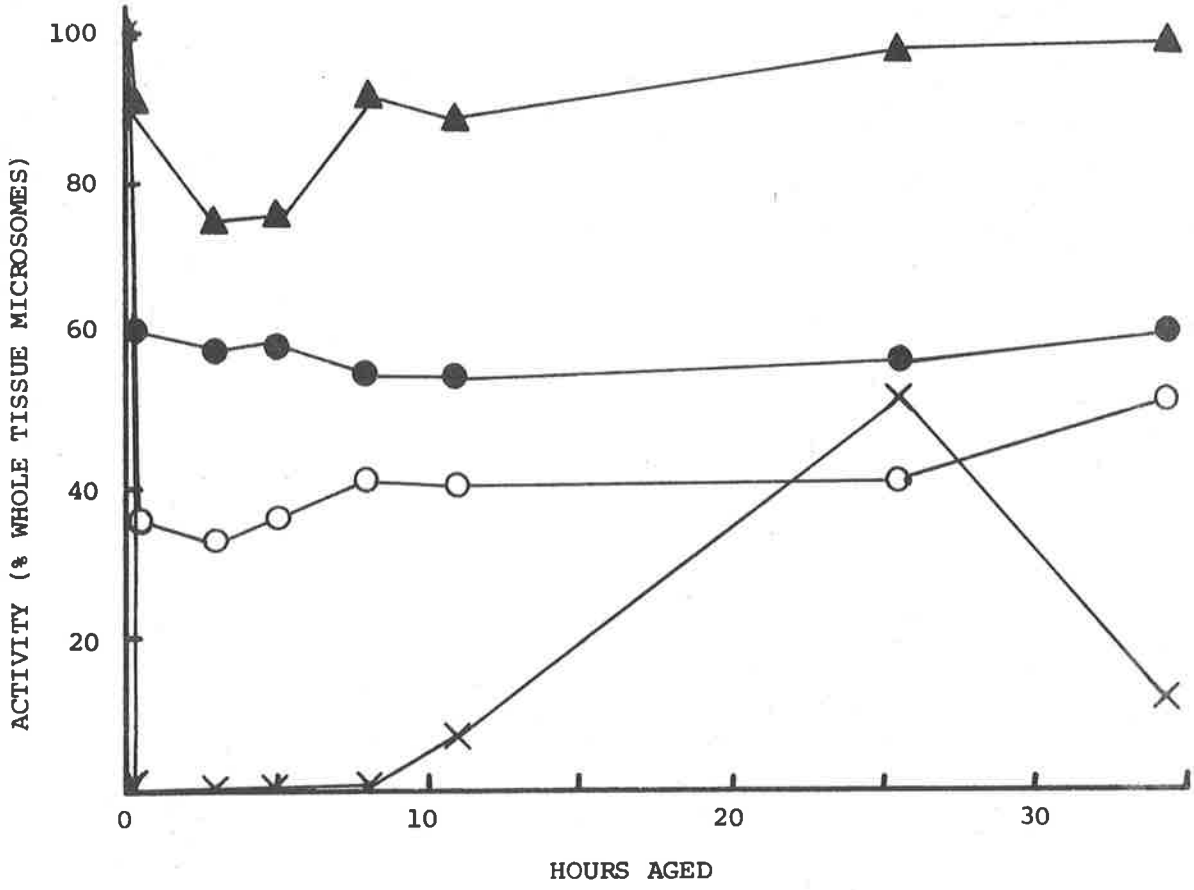


Figure 2. Changes in microsomal reductase activities in 1 mm thick disks of swede with aging. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the activities in microsomes isolated from whole tissue (given below in µmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.045); NADH-DCPIP (O) (0.232); NADH-FeCN (●) (0.320); protein (▲).

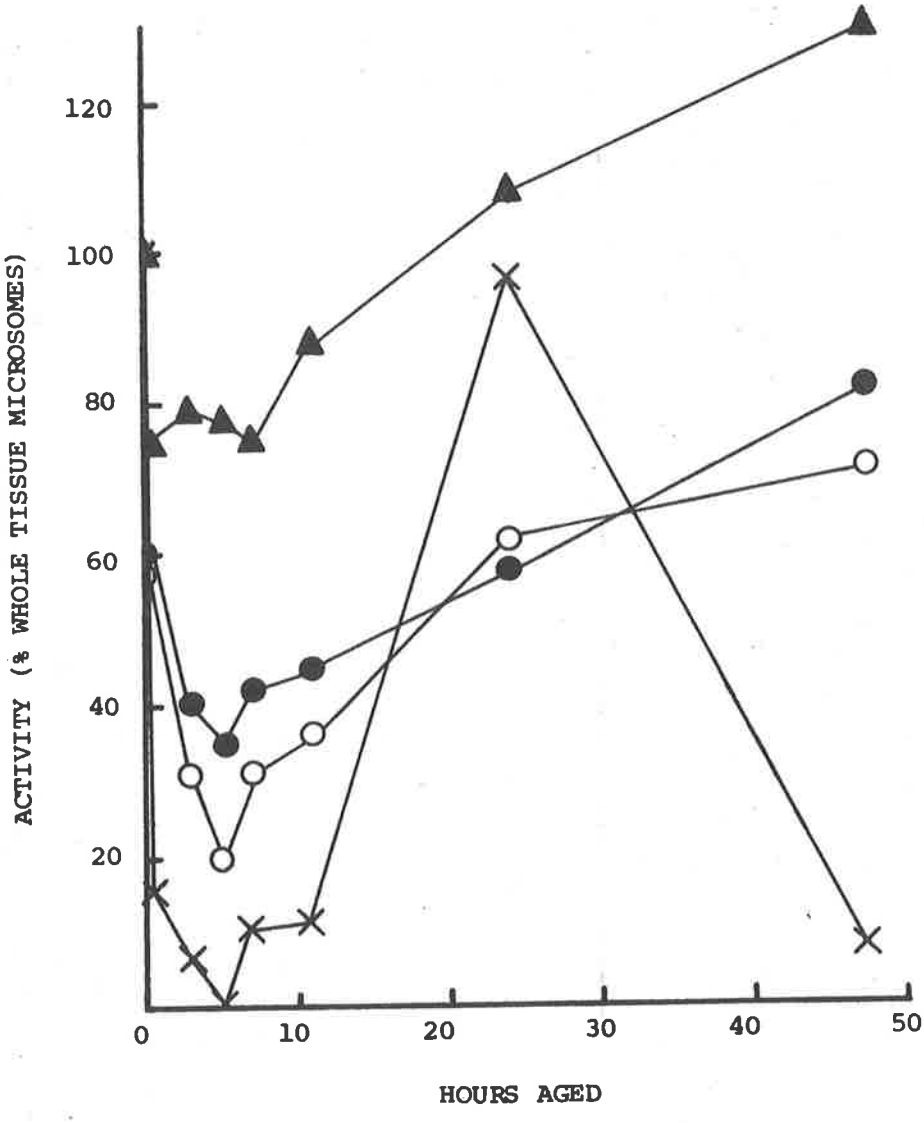


Figure 3. Changes in microsomal reductase activities in 1 mm thick disks of beetroot with aging. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the activities in microsomes isolated from whole tissue (given below in μmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.078); NADH-DCPIP (O) (0.426); NADH-FeCN (●) (1.18); protein (▲).

compared with whole tissue; Chapter II). The cytochrome b_{555} level in the turnip microsome fraction paralleled the NADH-cytochrome c reductase activity. This supported the idea that this pigment was not a peroxidase and that cytochrome b_{555} was the limiting factor in cytochrome c reduction (Chapter III).

2. Relation between induced microsomal changes and RNA and protein synthesis in turnip disks

Figure 4 shows that cycloheximide (10^{-5} M) had no effect on the loss of microsomal activities induced by slicing, but inhibited the subsequent increases. Activity levels in disks aged for 12 hours in the presence of cycloheximide were similar to the minimum levels of activity detected in the absence of inhibitor (Table 1). The minimum level of activities detected after slicing (Fig. 1) cannot, therefore, be due to a balance between inactivation and synthesis. However, 6-methylpurine (at a concentration which did not inhibit respiration, Gayler and Glasziou, 1968) only partially inhibited reductase development (Table 1). Hence the increases in microsomal reductase activities were dependent on protein synthesis but only partially on RNA synthesis.

The net activities measured at various times after slicing were a resultant of the rate of loss and rate of synthesis. An indication of the rate of breakdown could be determined by adding cycloheximide to the disks after resynthesis had become apparent and

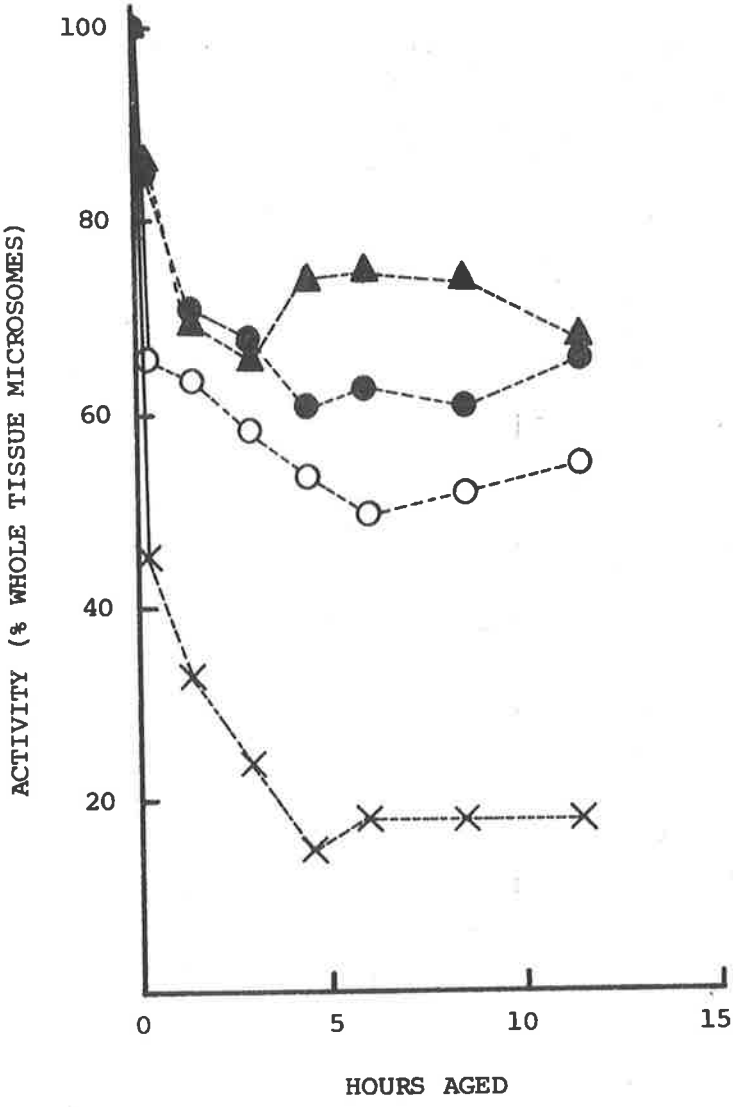


Figure 4. Effect of addition of cycloheximide (10^{-5}M) to turnip disks immediately after slicing on the changes in microsomal reductase activities with aging. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the activities in microsomes isolated from whole tissue (given below in μmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.115); NADH-DCPIP (O) (0.440); NADH-FeCN (●) (0.839); protein (▲).

TABLE 1. Effect of cycloheximide and 6-methylpurine on the development of microsomal activities in aging turnip disks. Inhibitors were added immediately after slicing and disks harvested after 12 hours aging. Manometry and assay conditions are as described in Chapter II. All activities (except respiration) are expressed as a percentage of the activities in microsomes isolated from whole tissue. Respiration is expressed as percentage of the rate in fresh disks.

	Cycloheximide conc. (M)				6-Methylpurine conc. (M)			
	0	0	2.5×10^{-6}	10^{-5}	0	0	10^{-5}	5×10^{-5}
Hours aged	3-5	12	12	12	3-5	12	12	12
NADH-cyt.c reductase	8	22	16	9	8	50	35	21
NADH-DCPIP reductase	44	68	50	51	41	102	91	76
NADH-FeCN reductase	52	76	56	59	59	90	70	81
Protein	76	96	76	78	81	94	83	81
Cytochrome b_{555}	17	50	33	17				
Respiration						115	100	68

then assaying for any subsequent loss of activity in the absence of further synthesis. Addition of cycloheximide after 8 hours aging inhibited further NADH - FeCN and - DCPIP reductase increase, but initiated a rapid loss of NADH-cytochrome c reductase and of microsomal protein (Fig. 5). This rapid drop in microsomal protein had the effect of inflating the activities (on a protein basis) in the microsome fractions isolated from disks aged in cycloheximide relative to the control. However, per gfw the NADH - FeCN and - DCPIP reductase activities remained at the level when cycloheximide was added, while the drop in NADH-cytochrome c reductase was greater than indicated in Figure 5. Cycloheximide added at 12 hours had no effect on reductase activities or protein levels, other than to inhibit further syntheses (Fig. 6). This indicated that 8 hours after slicing NADH-cytochrome c reductase and protein losses were still occurring, although rate of synthesis was sufficiently great for this not to be apparent. However, by 12 hours all losses had ceased.

3. Effect of disk size on induced microsomal changes

The magnitude of reductase losses induced by slicing are such that they could not be attributed to the small percentage of damaged cells on the surface of the 1 mm thick disks (Fig. 1). This suggested that a stimulus was transmitted to the inner cells. Repeating this experiment with 10 mm thick disks resulted in similar rates of breakdown, levels of minimum activities and rates of initial

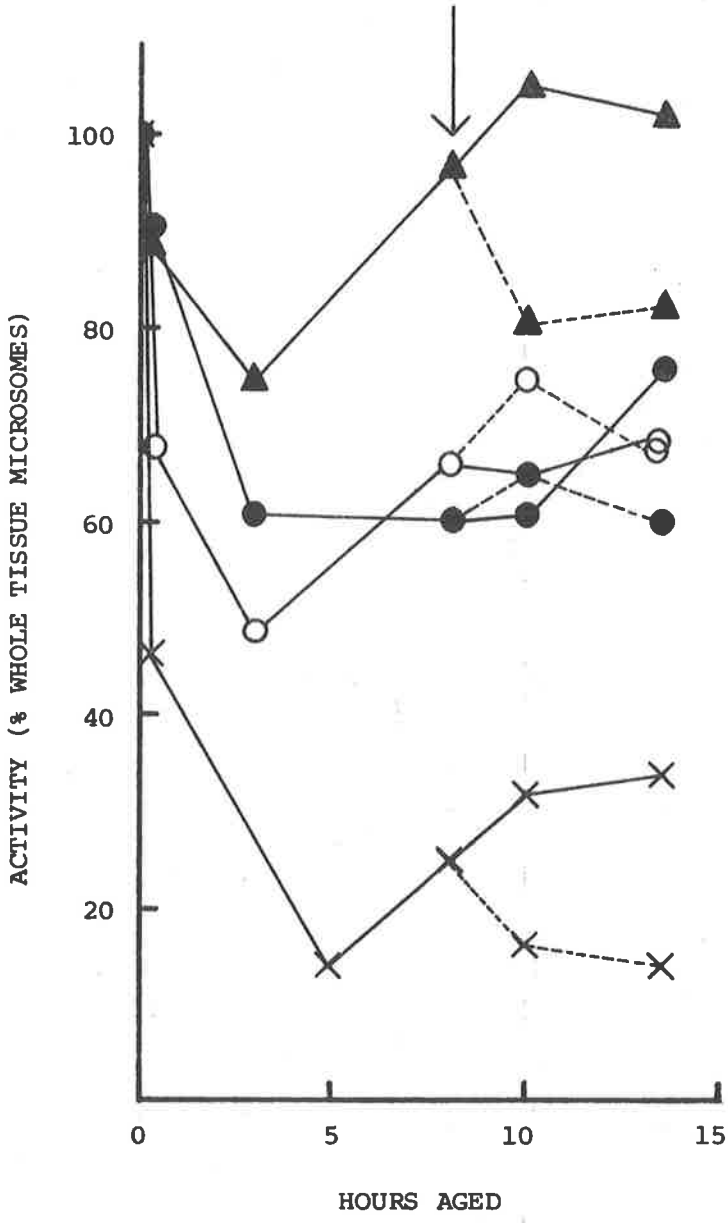


Figure 5. Effect of addition of cycloheximide (arrow) to aging turnip disks 8 hours after slicing on microsomal reductase activities. Disks aged in the absence of cycloheximide are represented by solid lines and in the presence of cycloheximide (10^{-5}M) by broken lines. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the activities in microsomes isolated from whole tissue (given below in μmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.140); NADH-DCPIP (O) (0.460); NADH-FeCN (●) (1.31); protein (▲).

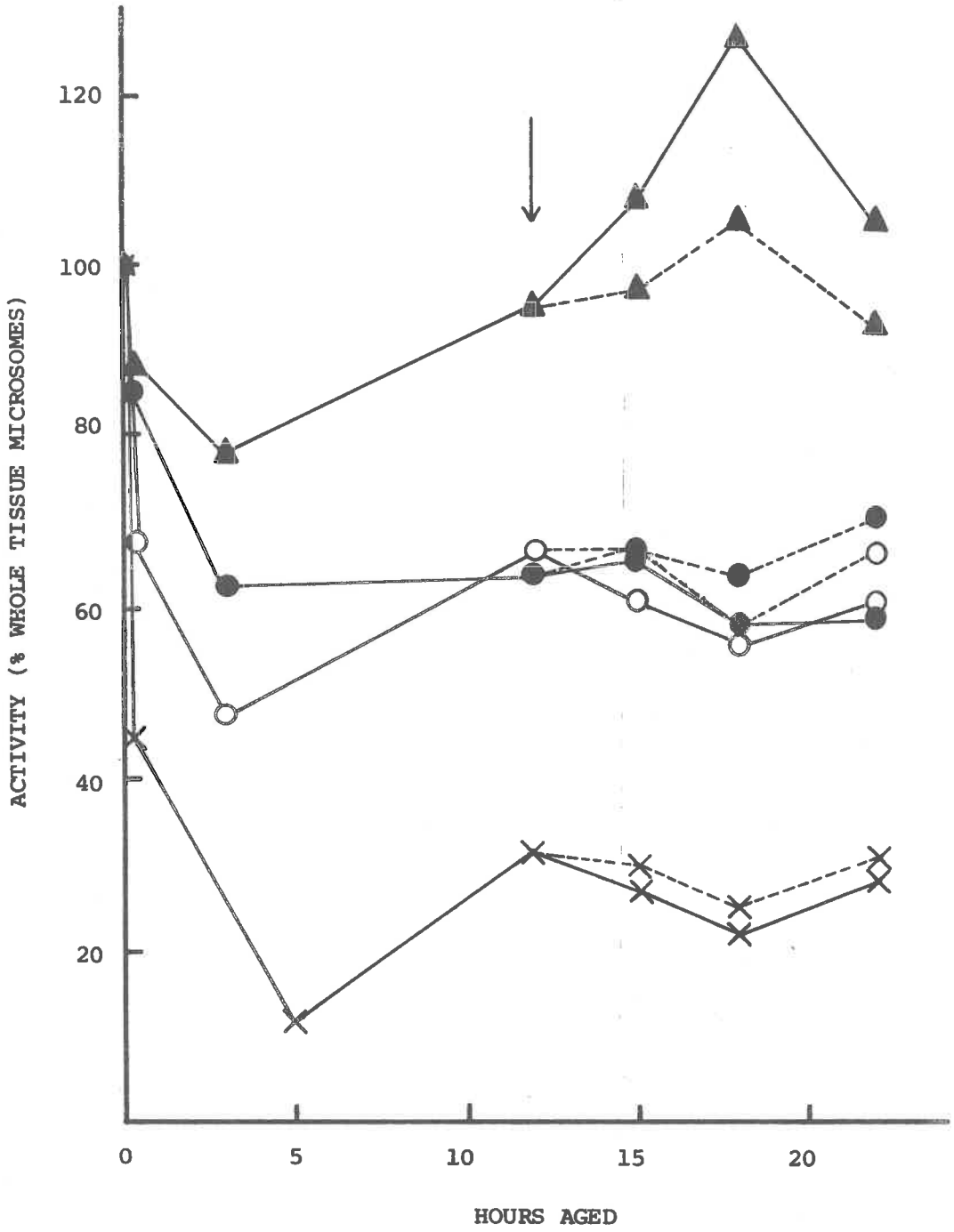


Figure 6. Effect of addition of cycloheximide (arrow) to aging turnip disks 12 hours after slicing on microsomal reductase activities. Disks aged in the absence of cycloheximide are represented by solid lines and in presence of cycloheximide (10^{-5}M) by broken lines. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the activities in microsomes isolated from whole tissue (given below in μmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.124); NADH-DCPIP (O) (0.445); NADH-FeCN (●) (1.03); protein (▲).

resynthesis (Fig. 7). However, after 8 hours aging there was a sudden decline in all reductase activities, particularly NADH-cytochrome c reductase. This apparent inhibition, from which there was no recovery with further aging, resembled the effect of cycloheximide added at 8 hours (Fig. 5). Corresponding to this, respiration in potato became dependent on disk thickness only after 8 hours aging (Laties, 1967). Laties suggested that a diffusable metabolite (inhibiting RNA and protein synthesis) was present in intact tissue and released on slicing. With aging, it again accumulated in the disks - sufficiently to limit respiration by 8 hours.

B. CHARACTERIZATION OF LOSSES OF MICROSOMAL ACTIVITIES

1. Incubation of microsomes with soluble fraction in vitro

The specific loss of microsomal reductase activities on aging could be a result of membrane hydrolysis by hydrolytic enzymes (either permeating from neighbouring damaged cells or released from the intact cells, possibly due to a permeability change). The majority of animal hydrolytic enzymes are contained in lysosomes which may be isolated intact (Novikoff, 1961). Incubation of liver microsomes with the lysosome fraction from the same tissue caused release of NADH-cytochrome b_5 reductase and cytochrome b_5 (Takesue and Omura, 1970a; 1970b; Sargent *et al.*, 1970). It was of interest to test whether incubation of turnip microsomes with the corresponding plant hydrolytic enzymes also resulted in activity losses and, if so, whether

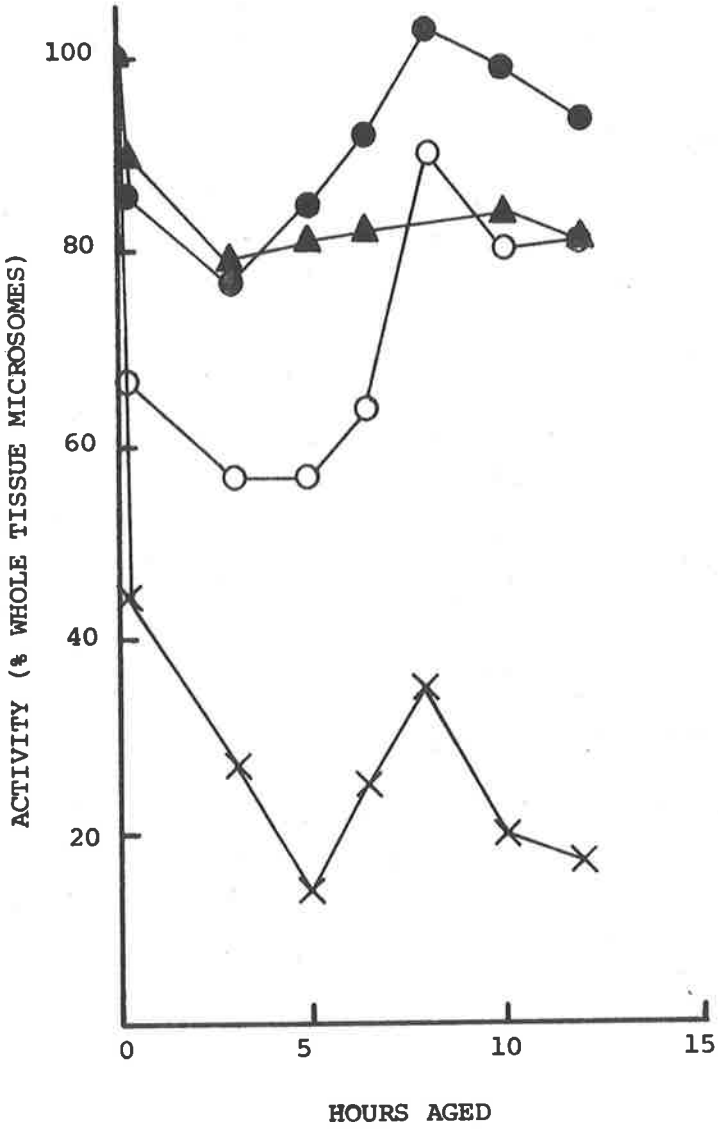


Figure 7. Changes in microsomal reductase activities in 10 mm thick turnip disks with aging. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the activities in microsomes isolated from whole tissue (given below in μmoles NADH oxidized/min/mg protein). NADH-cytochrome c (X) (0.159); NADH-DCPIP (O) (0.413); NADH-FeCN (●) (0.929); protein (▲).

these losses resembled the induced activity losses *in vivo*.

It is now well established that meristematic and differentiating plant cells do contain a variety of small vacuoles which probably contain the hydrolytic enzymes (Gahan, 1965; Frederick *et al.*, 1968; Berjak, 1968; Matile, 1968; Poux, 1970). However, Matile and Moor (1968) have suggested that in mature parenchymatous plant cells the hydrolytic enzymes are likely to be contained in the large central vacuole and hence liberated into the soluble supernatant fraction on tissue homogenization. This is supported by Quarles and Dawson (1969); Clermont and Dounce (1970); Galliard (1970) who have detected little or no sedimentable hydrolytic activity in a wide range of plant tissues. {The hydrolytic activity reported in some mitochondria and microsome fractions (Balz, 1966; Semadeni, 1967; Matile, 1968) may be due to contaminating lysosomes (Matile, 1968) or random absorption of soluble enzymes during homogenization.}

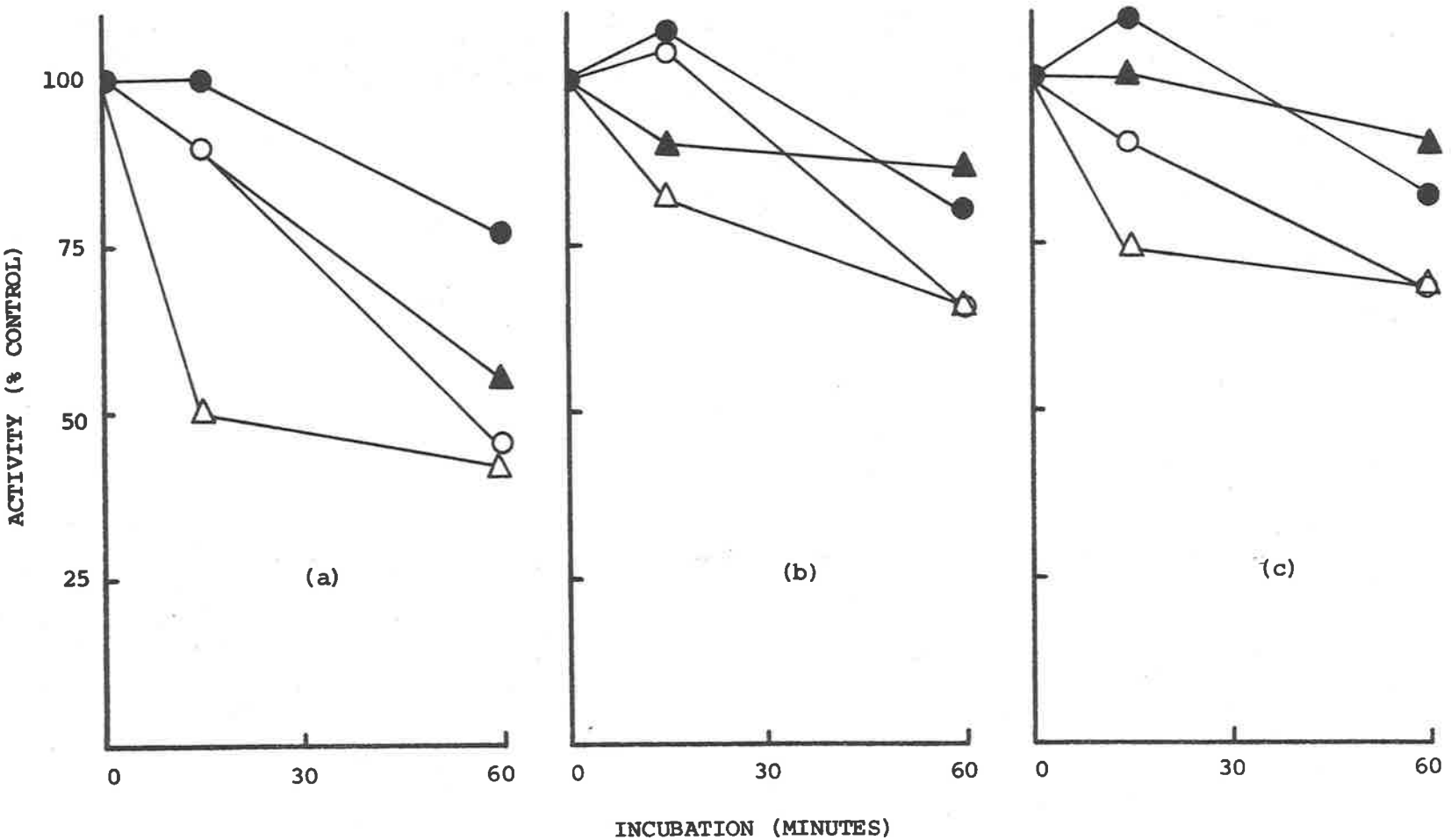
Hence the effect on the microsomal reductases of incubating the isolated microsomes with the soluble fraction was determined (Fig. 8). It was not possible to measure release of reductase components from the membranes to the soluble fraction, due to interference from other soluble reductase (Chapter V) and peroxidase (Chapter VI) activities. Hence activity losses could only be detected by assaying the microsomes after separation from the soluble fraction. As there appeared to be no *in vivo* losses of the microsomal reductase

Figure 8. Effect of incubating turnip microsomes from fresh (open symbols) and 12 hour aged (closed symbols) tissue with the soluble supernatant fraction from fresh (circles) and 12 hour aged (triangles) tissue on the reductase activities. Microsomes from approximately 70 g tissue were incubated with 30 ml undiluted supernatant (previously adjusted to pH 7.2 with 1 M tris) in the presence of 70 mM tris-HCl (pH 7.2), at 25^{o}C with constant shaking. After 15 and 60 minutes, aliquots of the incubation mixture were chilled, centrifuged at 54,000 g for 60 minutes and the supernatant discarded. The pellet was resuspended in 0.4 M sucrose, recentrifuged and the resulting pellet resuspended in the same initial volume of 0.4 M sucrose (2.0 ml). Assay conditions are as described in Chapter II. Corrections have been made for the losses of activity in the controls. Activities are expressed as a percentage of activities in the untreated microsomes (given below in µmoles NADH oxidized/min/mg protein).

a - NADH-cytochrome c (fresh - 0.035), (aged - 0.029);

b - NADH-DCPIP (fresh - 0.102), (aged - 0.102);

c - NADH-FeCN (fresh - 0.313), (aged - 0.265).


ACTIVITY (% CONTROL)
100
75
50
25
(a)
(b)
(c)
0
30
60
INCUBATION (MINUTES)

activities after 12 hours aging (Fig. 6) the effects of soluble fractions on microsomal fractions isolated from both fresh and 12 hour aged disks were determined.

Incubation with the soluble fraction did cause up to 60% loss of microsomal reductase activities (Fig. 8). As this was a time dependent reaction, enzyme hydrolysis rather than inhibition was implicated. The microsomal reductases from aged disks were all significantly less sensitive to the soluble fractions than those from fresh tissue. However, soluble fraction from aged disks inhibited the microsomal reductases more, rather than less, than the soluble fraction from fresh tissue. The inactivating mechanism *in vivo* was presumably not controlled by a rapid turnover, as storage of the soluble fraction for 48 hours at $4^{\circ}C$ did not result in any loss of its inhibitory activity.

The partial development of insensitivity to the soluble fraction was further investigated by incubating microsomes from fresh and 12 hour aged disks with trypsin (Table 2). There did appear to be an increased insensitivity of the NADH-cytochrome c and -DCPIP reductases with aging, particularly the former. (The small difference in trypsin/mg protein between preparations will not significantly affect the trypsin activity, Chapter III.) Table 2 shows that KCl diminished the developed insensitivity of the reductases suggesting that insensitivity was due to a structural change in the membrane

TABLE 2. Effect of trypsin on the reductase activities of microsomes prepared from fresh tissue and 12 hour aged disks. Incubations were for 5 minutes using 35 (fresh) and 45 (aged) µg trypsin/mg microsomal protein and followed the procedure in Chapter III (Fig. 9). Assay conditions are as described in Chapter II.

	% Inhibition			
	Fresh tissue microsomes		12 hour aged disk microsomes	
	-	70mM KCl	-	70mM KCl
NADH-cyt.c reductase	51	70	31	69
NADH-DCPIP reductase	28	48	19	23
NADH-FeCN reductase	15	12	17	9

TABLE 3. Effect of IAA on microsomal activities in aging turnip disks. IAA was added immediately after slicing and disks harvested after 8 hours aging. Manometry and assay conditions are as described in Chapter II. All activities (except respiration) are expressed as a percentage of the activities in microsomes isolated from whole tissue. Respiration is expressed as a percentage of the rate in fresh disks.

	IAA Concentration (M)					
	0	0	10^{-6}	$6x10^{-6}$	$6x10^{-5}$	$3x10^{-4}$
Hours aged	3-5	8	8	8	8	8
NADH-cyt.c reductase	8	28	31	18	17	17
NADH-DCPIP reductase	41	49	52	55	45	38
NADH-FeCN reductase	59	54	63	58	55	46
Protein	81	107	119	110	109	81
Respiration		127	148	121	130	134

(Chapter III). Jackman and Van Steveninck (1967) showed that the ionic content of beet disks was lowest after 12 hours aging. Hence, insensitivity *in vivo* may be to some extent controlled by ionic strength.

2. Effect of IAA on microsomal activities in vivo

Palmer (1970b) reported that IAA induced an acid phosphatase release from jerusalem artichoke disks, possibly as a result of change in permeability of the lysosome membrane (presumably the tonoplast). Turnip disks were aged in the presence of IAA to test if there was any effect on microsomal breakdown and resynthesis that might be correlated with the suggested increased lysosomal activity. Table 3 shows that at low concentrations (6×10^{-6} M) IAA caused a specific decrease in NADH-cytochrome c reductase activity which was not accompanied by losses of any other parameters. Higher concentrations resulted in a decrease in microsomal protein and of the other reductase activities. This may be due to increased destruction of the enzymes or of the messenger RNA required for production of the enzymes or both (Glasziou *et al.*, 1966). As respiration was unaffected, this effect can presumably not be attributed to cellular degradation. Figure 9 shows the effect of IAA (3×10^{-4} M) on the microsomal activity changes in aging disks under the conditions used by Palmer (1970b) to induce maximum phosphatase activity (i.e. without $CaSO_4$). Loss of NADH-cytochrome c reductase was greater, and of NADH - FeCN

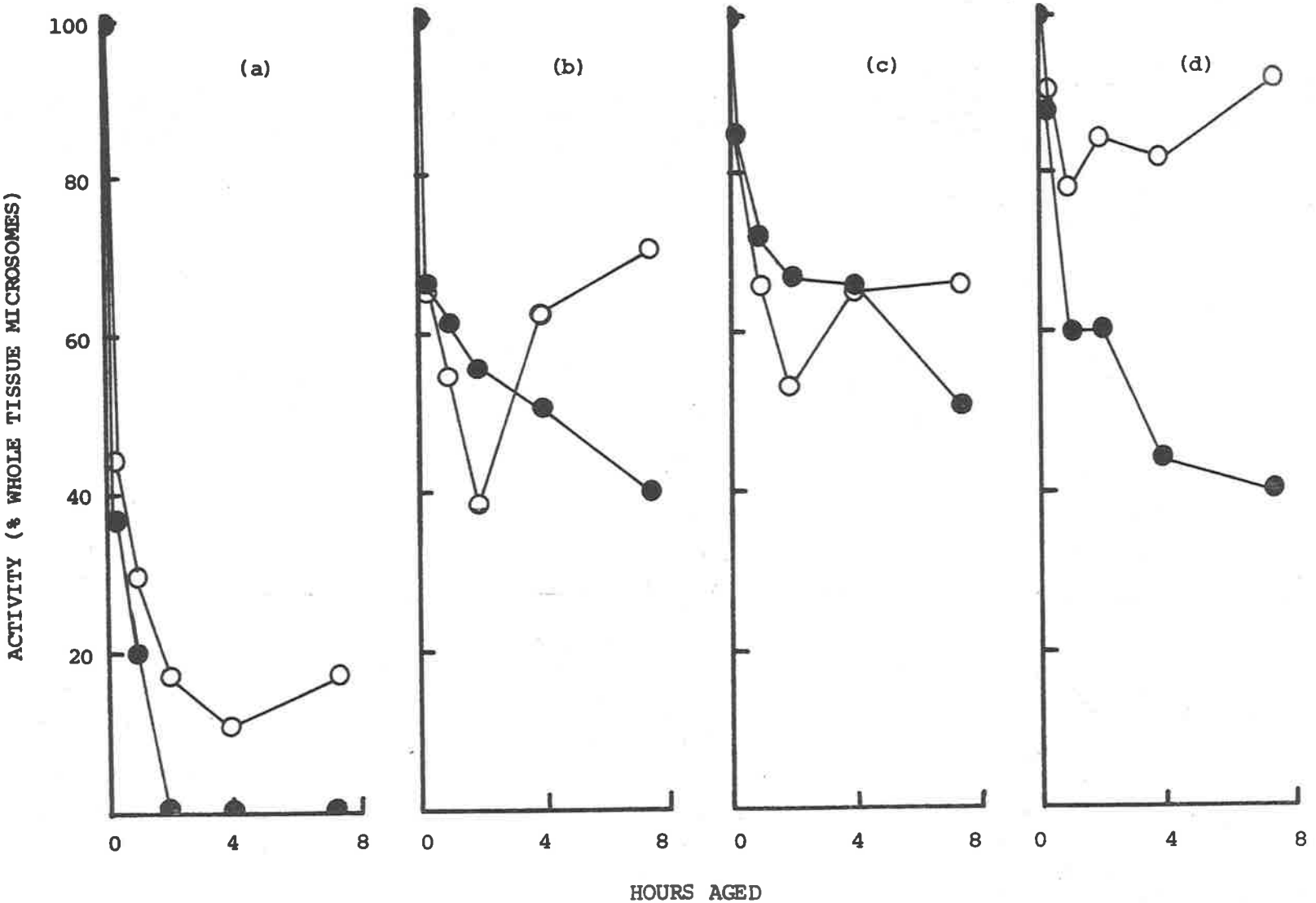
Figure 9. Effect of aging turnip disks in the absence (○) and presence (●) of 3 x 10^{-4}M IAA on microsomal activities. Activities are expressed as a percentage of the activities in microsomes isolated from whole tissue (given below in µmoles NADH oxidized/min/mg protein).

a - NADH-cytochrome c (○- 0.264), (●- 0.174);

b - NADH-DCPIP (○- 0.340), (●- 0.367);

c - NADH-FeCN (○- 0.715), (●- 0.907);

d - protein


100
80
60
40
20
(a)
(b)
(c)
(d)
0
4
8
ACTIVITY (% WHOLE TISSUE MICROSOMES)
HOURS AGED

and -DCPIP reductases less, in IAA than in the control. Decrease in rate of loss of the latter activities was a result of the accompanying high loss of protein (Fig. 9(d)). IAA also prevented the subsequent increases in activities. Hence there does appear to be a correlation between reductase losses and the IAA-induced hydrolytic activity (Palmer, 1970b).

The effect of 10^{-4} M $CaSO_4$ on the action of 3 x 10^{-4} M IAA can be considered by comparing microsomal activities after 8 hours aging in the absence (Fig. 9) and presence (Table 3) of $CaSO_4$. $CaSO_4$ protected against losses of NADH-cytochrome c reductase activity and protein while having no effect on the NADH - DCPIP and -FeCN reductase losses. Van Steveninck (1965) showed that IAA caused leakage of ions from beet cells but that the effect was not reversed by $CaSO_4$. This may suggest that the IAA effect on microsomal reductases was not related to the cell permeability changes reported by Van Steveninck.

3. Effect of slicing and aging in isotonic sucrose

The specificity of activity losses and of the membranes affected in the cell (Jackman and Van Steveninck, 1967; Van Steveninck, 1970) indicated that the slicing-induced microsomal changes were not likely to be due to non-specific osmotic damage as suggested by Kahl (1971). However, as disks accumulated water (increasing in weight) after only short periods of aging some osmotic damage was likely. Disk aging experiments have always been performed

in water, or water saturated air, due to problems of bacterial growth in isotonic media. However, for the short times of aging involved here the use of sucrose as an aging solution was feasible.

Hence turnip disks were sliced and aged (for 4 hours) in isotonic sucrose solution, and the microsomal losses compared with previous experiments performed in water. Sucrose was used at the concentration at which turnip disks neither gained nor lost weight over 4 hours aging (0.35 M). Disks sliced and aged in water were more turgid than those sliced and aged in sucrose, and the lower microsomal yield from the latter (Table 4) was probably due to a less effective homogenization (Chapter II). Activity losses after slicing and aging for 4 hours were equivalent in disks aged in the two media, with the possible exception of NADH-cytochrome c reductase which appeared more stable in the disks aged in sucrose. This was more obvious if activities in 4 hour aged disks were expressed relative to those in fresh disks so that comparisons were made under similar homogenizing conditions (Table 4). Hence the results suggest that there was an equivalent loss of all three reductases induced by slicing and aging and that the greater loss of NADH-cytochrome c reductase may be due to osmotic damage.

C. EFFECT ON DISTRIBUTION OF ACTIVITIES WITHIN MICROSOMAL SUBFRACTIONS

The newly synthesized liver microsomal electron transport components always initially appeared in the rough membranes and were

TABLE 4. Effect of slicing and aging turnip disks in isotonic sucrose (0.35 M) on changes in microsomal activities. All procedures are the same as in Figure 1.

Activities relative to:	Whole tissue microsomes				Fresh disk microsomes	
Aging solution	H_2O		Sucrose		H_2O	Sucrose
Hours disks aged	0	4	0	4	4	4
NADH-cyt.c reductase	45	11	61	40	25	66
NADH-DCPIP reductase	66	63	63	46	96	72
NADH-FeCN reductase	85	65	76	54	78	71
Protein	90	82	78	58	77	75

TABLE 5. Effect of RNA and protein synthesis inhibitors on the change in distribution of protein in the microsomal subfractions with aging. The subfractions were prepared (using a discontinuous sucrose gradient in the presence of CsCl) and assayed as described in Chapter II.

Treatment	Ratio Subfraction $\frac{1}{2\ (\text{total})}$	
	Protein	RNA
0 hour control	0.6	5.7
4 hour control	2.6	5.5
4 hour + 2×10^{-3} M Ethionine	2.4	5.7
4 hour + 10^{-4} M 6-methylpurine	2.2	6.6
4 hour + 7×10^{-6} M Cycloheximide	1.7	4.1

presumably synthesized by the membrane-bound ribosomes (Sargent and Vadlamudi, 1968; Omura and Kuriyama, 1971). It was also stated in Chapter I that slicing plant storage tissue induced a binding of ribosomes to the ER (Fowke and Setterfield, 1968) and microsomal (Sampson and Laties, 1968) membranes. Changes in activities in the turnip microsomal subfractions (separated as described in Chapter III, Table 11) were studied to determine whether the induced syntheses were in the fractions with bound ribosomes.

Figure 10 summarizes the visible changes in the subfractions with aging. There was a rapid disappearance of fraction 2 (top) and an increase in fraction 1; and a slower precipitation of fraction 2 (bottom) to form a pellet. With increased aging, fractions 1 and 2 (bottom) turned dark brown. However, fraction 2 (middle) remained unaltered both in distribution and colour. This is summarized in Figure 11 (a, b) where the protein level dropped in fraction 2 (top) and increased in fraction 1. It would appear that fraction 2 (top) precipitated in fraction 1 probably as a result of binding ribosomes. With further aging there was a small increase in RNA in fraction 2 (bottom) which was presumably sufficient to cause this fraction to pellet in the presence of Mg^{++} but not Cs^{++}. There was no increase in RNA in fraction 2 (middle) which presumably does not bind ribosomes.

Hence, in contrast to Sampson and Laties (1968), there does appear to be some specificity in the binding of ribosomes to the

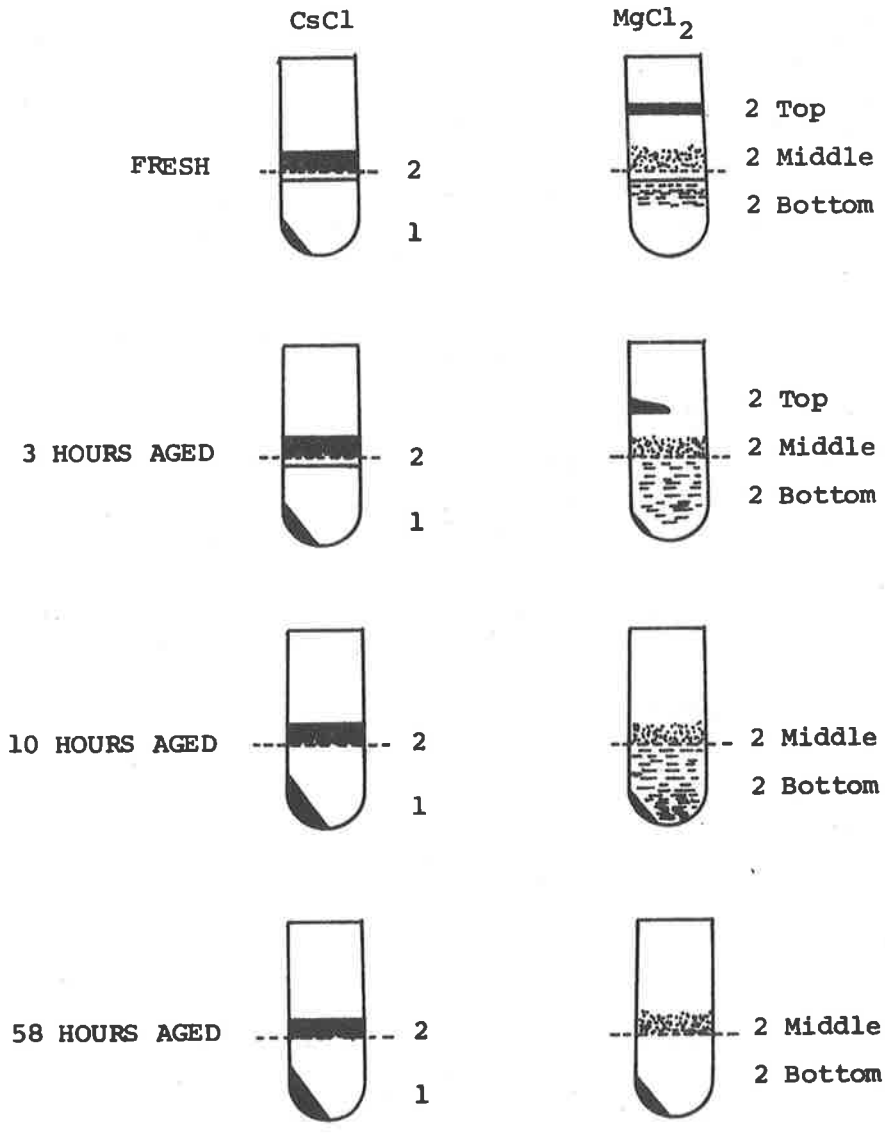


Figure 10. Schematic illustration of the changes in turnip microsomal subfractions with aging. The subfractions were prepared using discontinuous sucrose gradients in the presence of CsCl and then $MgCl_2$ as described in Chapter II. (The broken lines represent the sucrose boundaries.)

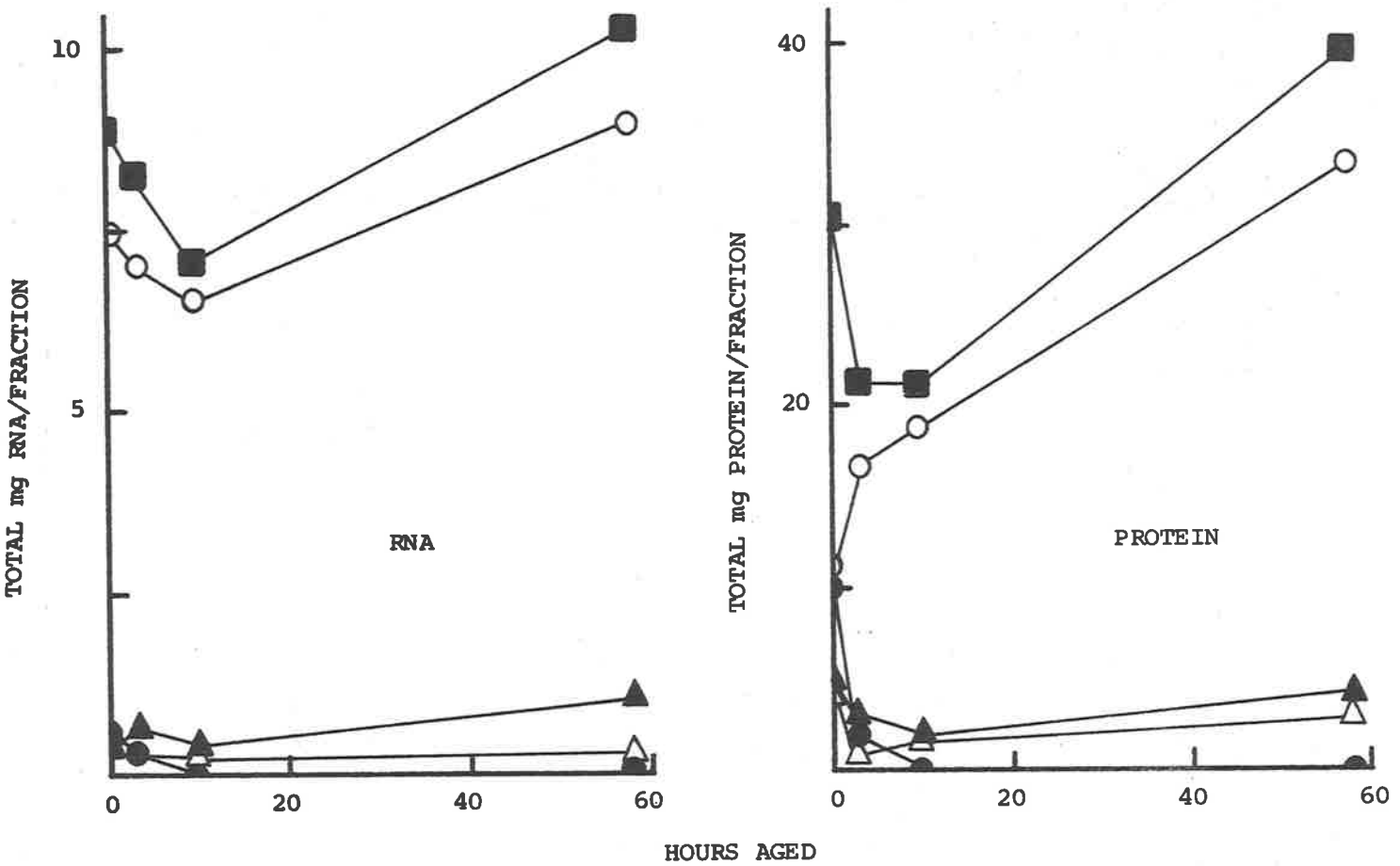


Figure 11. Changes in RNA and protein in turnip microsomal subfractions with aging. The subfractions were prepared (using discontinuous sucrose gradients in the presence of ions) and assayed as described in Chapter II. Fraction 1 (○): fraction 2 top (●); fraction 2 middle (△); fraction 2 bottom (▲); total fractions (■). RNA and protein are expressed as the total (mg)/fraction (from 600g tissue).

membranes. It has not been established why this binding occurs, but as slicing initiates immediate rapid synthesis of both ribosomes (Laties, 1967; Sampson and Laties, 1968) and messenger RNA for polyribosome formation (Leaver and Key, 1967), possibly only new ribosomes or polyribosomes are bound. Both these syntheses are largely complete within 5-10 hours of slicing i.e. the time taken for fraction 2 (top) to completely disappear. This could be tested by aging disks for a short time in cycloheximide, 6-methylpurine or ethionine and testing for lack of protein precipitation in fraction 1 (Table 5). However, none of the treatments altered the protein (or RNA) distribution between fractions 1 and 2 indicating that binding was not dependent on RNA or protein synthesis.

Figure 12 (a, b, c) shows the change in distribution of reductase activities in the microsomal subfractions with aging. There was a drop in all activities just after slicing as expected. The majority of resynthesized activity appeared in membranes subsequent to the binding of ribosomes (i.e. activity increases were in rough membranes). The drop in activities in fraction 2 (middle) over the first three hours aging was probably a result of decreased contamination from the rest of fraction 2 as these separate more clearly in aged tissue preparations (Fig. 10).

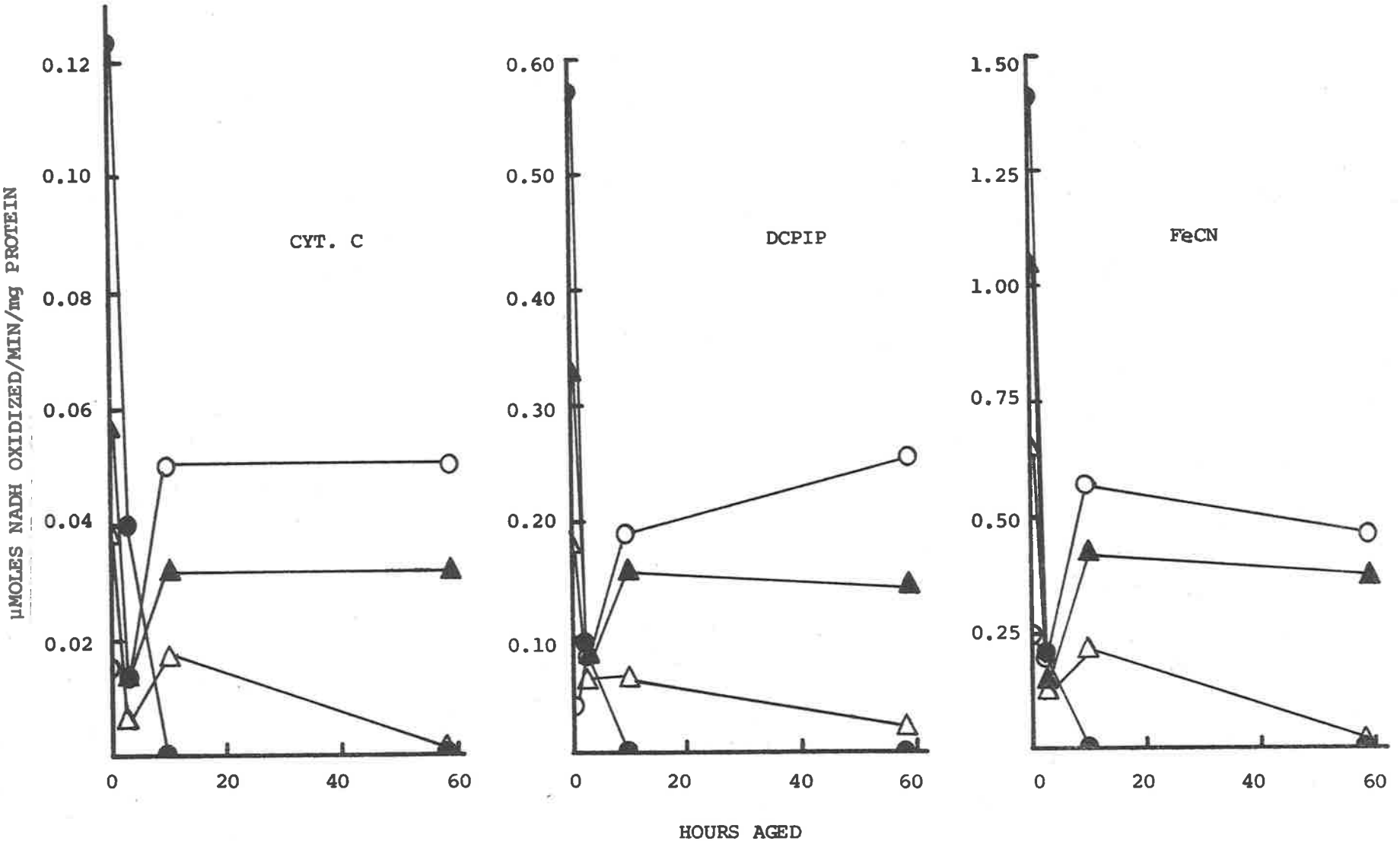


Figure 12. Changes in reductase activities in turnip microsomal subfractions with aging. The subfractions were prepared (using discontinuous sucrose gradients in the presence of ions) and assayed as described in Chapter II. Fraction 1 (○): fraction 2 top (●); fraction 2 middle (△); fraction 2 bottom (▲).

DISCUSSION

A. RELATION TO REPORTED BIOCHEMICAL CHANGES IN MICROSOMES WITH AGING

The rapid synthesis of microsomal phospholipids (and presumably membranes) for approximately 8 hours after slicing (Tang and Castelfranco, 1968) correlated with the period of greatest increase in microsomal protein and NADH - DCPIP and -FeCN reductases. This also corresponded with the period of highest protein synthesis in aging disks (Kahl, 1971). Hackett *et al.* (1960) and Ben Abdelkader (1969) both reported 4-7 fold increases in microsomal reductases after aging potato disks for 24 hours. However, in both cases the comparison was between fresh and aged disks i.e. their controls were the low inactivated levels of fresh disks rather than the higher levels of whole tissue. The equivalent NADH-cytochrome c reductase activities reported by Ben Abdelkader (1969) in fresh disks and disks aged for 24 hours in cycloheximide may be explained by the low concentration of inhibitor used (1.8×10^{-6} M). Table 1 shows that a concentration slightly higher than this only partially inhibited the subsequent increase in NADH-cytochrome c reductase and cytochrome b_{555} in aging turnip disks.

There is increasing evidence that the outer mitochondrial and microsomal membranes in animals are very similar (Sottocasa *et al.*, 1967), although there are differences in trypsin-sensitivity (Kuylenstierna *et al.*, 1970). Corresponding to the increase in

microsomal activities, Hackett *et al*. (1960) also reported a 10-fold increase in the antimycin A-insensitive mitochondrial NADH-cytochrome c reductase in potato disks after aging for 24 hours (which could result from outer membrane activity).

B. RELATION TO REPORTED ULTRASTRUCTURAL CHANGES IN ER WITH AGING

As most of the activity losses occurred immediately on slicing, any associated ultrastructural changes would occur when whole tissue was cut for fixing. Hence these changes would not be detected and the resulting structure of the ER would be interpreted as the natural state in intact tissue. However, slicing tissue into disks induced no change in the ER of swede (Van Steveninck, 1970) but induced vesiculation in the ER of beet (Jackman and Van Steveninck, 1967). Correlated with these observations, there were further losses of reductase activities (after the initial losses) in beet (Fig. 3) but not swede (Fig. 2). The ER strands in beetroot reformed and increased with further aging (Jackman and Van Steveninck, 1967) and there was also an overall increase in the ER of jerusalem artichoke (Fowke and Setterfield, 1968) but not of swede or carrot (Van Steveninck, 1970). The increases in microsomal protein and NADH - FeCN and -DCPIP reductases in beet (Fig. 3) but not in swede (Fig. 2) disks with further aging is in agreement with these ultrastructural changes. Fowke and Setterfield (1968) also showed that with aging there was a binding of ribosomes to the ER, and this coincided with

the increases in activities and protein in the rough microsomal membranes (Figs. 11, 12).

Hence there appeared to be a correlation between changes in NADH - DCPIP and -FeCN reductases and the state of the ER. This correlation did not always hold for total protein or for NADH-cytochrome c reductase which, unlike the other reductase activities, involved the microsomal cytochrome b_{555} which is loosely attached to the membrane and is readily solubilized (Chapter III). Therefore loss of NADH-cytochrome c reductase activity by solubilization of cytochrome b_{555} probably precedes other *in vivo* losses and would not result in any observable ultrastructural change. Table 4 indicates that the greater loss of NADH-cytochrome c reductase may be due to osmotic damage. Hence the osmotic damage caused by slicing and aging tissue in water probably results only in the release of cytochrome b_{555} and does not affect either the other reductases or the ultrastructure of the ER.

Development of ER (Van Steveninck, 1970) and microsomal reductases were both prevented by protein synthesis inhibitors. While RNA synthesis was not involved in the development of the ER (Van Steveninck, 1970), it was partially involved in the development of the reductases particularly NADH-cytochrome c reductase (Table 1). Hence ER development may continue without the full complement of microsomal reductases. Van Steveninck (1970) suggested a lack of

direct correlation between increase in ER and development of the ion accumulation process in aging beet disks as only the latter was sensitive to actinomycin. However, such a correlation may still exist as the development of ion accumulation would be dependent on the electron transport enzymes of the ER rather than the membrane itself.

It has been reported that treatment of plant tissues with certain concentrations of colchicine also causes considerable increases in ER (Mesquita, 1966; Walne, 1967; Wirkus and Meenakski, 1968). This was briefly investigated by germinating peas over aerated water and then transferring to 0.1% colchicine for 15 hours when the roots were about 3 cm long. This resulted in abnormal swelling of the root tips and considerably increased amounts of ER. Microsomes prepared from these roots showed a 200% increase in NADPH-cytochrome c reductase but a 50% loss of NADH-cytochrome c reductase relative to the control.

C. NATURE OF INDUCED MICROSOMAL ACTIVITY LOSSES

1. Nature of slicing-induced stimulus

It has been estimated that slicing induces changes in respiration 2 to 60 cells from the cut surface (see Kahl, 1971, for review). In turnip, the microsomal changes occurred up to 5 mm (approximately 50 cells) from the cut surface. However, the nature of the slicing induced stimulus which results in these changes is

not known.

(i) Kahl (1971) has suggested that polyphenols of injured cells may permeate into neighbouring cells to induce changes. Similarly, released hydrolytic enzymes in the injured cells may also permeate into the neighbouring cells.

(ii) Slicing may induce an unobservable permeability change in the tonoplast which allows release of hydrolytic enzymes. With aging, the membrane may again become impermeable. It is suggested in Chapter VIII that a similar permeability change may occur in the mitochondrial membrane.

2. Nature of the inactivation

The nature of the mechanism which inactivates the microsomal reductases on slicing is also not well understood.

(i) The rapidity of the process indicated that it was not due to cessation of synthesis in the normal turnover of enzymes (the half-lives of the corresponding animal enzymes were about 3 days, Kuriyama *et al.*, 1969).

(ii) Osmotic damage was probably generally not involved as the effects of slicing and aging in isotonic sucrose were similar (Table 4) and the ultrastructural changes appeared to be limited to the ER.

(iii) The *in vivo* effect of IAA (Fig. 9) and *in vitro* effect of the soluble fraction (Fig. 8) on the microsomal reductases suggest

the action of hydrolytic enzymes, although it is not clear whether this results in direct hydrolysis of the microsomal membranes (Takesue and Omura, 1970a), release of inhibitory fatty acids (Dalgarno and Birt, 1963; Galliard, 1970), or both. However, the correlation between ultrastructural change in ER and the change in microsomal reductases indicates hydrolysis of membranes rather than enzyme inhibition. Jones and Wakil (1967) demonstrated a phospholipid requirement for liver microsomal NADH-cytochrome c but not -FeCN reductase activity. Hence, hydrolysis of phospholipids in disks may result in some loss of microsomal activities, and ultrastructural change of the ER. The rapid synthesis of microsomal phospholipids for 8 hours after slicing (Tang and Castelfranco, 1968) may reverse these effects.

(iv) It is also possible that reductase components are actually released from the membranes. An NADH - FeCN reductase in plant- (Chapter V), and b-type cytochromes in plant- and animal-soluble supernatant fractions have been detected (Shichi and Hackett, 1966; Mangum *et al.*, 1970). While these resemble the corresponding microsomal components, it is not known whether they are derived from the microsomal membranes. Slicing does induce a loss of microsomal protein which parallels reductase loss but this may not indicate specific solubilization as the two effects were shown to be only partially related (Fig. 5).

3. Nature of the developed insensitivity

The addition of cycloheximide to disks immediately after slicing resulted in loss of microsomal reductases to a constant level by 2-4 hours (Fig. 4). Similarly, partial insensitivity of the reductases to various disruptive treatments of the isolated microsomes was observed in Chapter III. Ito and Sato (1969) showed that a bacterial protease acted on only the surface of liver microsomal membranes resulting in a structure insensitive to further hydrolysis.

In 12 hour aged tissue there was no loss of resynthesized reductase activities (Fig. 6) suggesting:

(i) that decreased ionic concentrations in the cell (Jackman and Van Steveninck, 1967) may result in lower proteolytic activity;

(ii) inhibition of the inactivating mechanism (possibly by the synthesis of proteolytic inhibitors; Kirsi and Mikola, 1971);

(iii) resynthesis of an insensitive ER structure (possibly as a result of increased phospholipid; Tang and Castelfranco, 1968).

The latter is favoured as partial insensitivity of reductase activities was observed *in vitro* (Table 2; Fig. 8).

C H A P T E R V

SOLUBLE ELECTRON TRANSPORT ACTIVITIES IN FRESH AND AGED TISSUE

INTRODUCTION

The well characterized soluble electron transport enzymes from animal tissues and the lack of information concerning the corresponding plant enzymes are described in Chapter I. The limited reports from plants so far indicate markedly different enzymes to those present in animals. This Chapter endeavours to characterize the soluble electron transport activities from turnip tissue and the subsequent changes on tissue aging. Aging induces small increases in all soluble reductase activities present in fresh tissue as well as rapid development of a soluble NADH-cytochrome c reductase activity often not present in fresh tissue. The majority of characterization has therefore concentrated on the aged tissue soluble fraction where all activities could be considered simultaneously. The marked differences to animal tissues and possible functions are discussed.

RESULTS

A. REDUCTASE ACTIVITIES IN THE CRUDE SOLUBLE FRACTION FROM FRESH AND AGED TISSUE

The dialysed soluble fraction from turnip catalysed the oxidation of NADH and NADPH in the presence of DCPIP, FeCN and

sometimes cytochrome c (but at a much lower rate) (Figs. 1, 2). Low G-6-P dehydrogenase (20 n moles NADP reduced/min/mg protein) and NADH-APAD transhydrogenase (22 n moles APAD reduced/min/mg protein) activities were also detected. Slicing and aging turnip tissue induced an increase in the reductase activities, particularly NADH-cytochrome c reductase (Figs. 1, 2). Maximum activities were usually detected about 60 hours after slicing, although NADH-cytochrome c reductase development was considerably dependent on the temperature of the washing solution (Fig. 1). At 34°C activity developed more rapidly but started to decline after only 12 hours aging. Very low NADH-NT reductase activity was also detected in the soluble fraction from aged but not fresh tissue. (After 52 hours $\Delta E_{505} = 0.0005/10$ min/mg protein; NADPH supported 20% NADH rate.)

NADH-cytochrome c reductase activity was only detected if BSA was present in the isolating medium. The most effective concentration was 2 mg BSA/gfw tissue. The inclusion of cysteine (0.05%) in the isolating medium, however, caused 80-100% inhibition of NADH-cytochrome c reductase activity. This was also noted with reduced glutathione and dithiothreitol. This inhibition could not be reversed by elution through Sephadex G-25, and agrees with the results in Table 4 showing that the cytochrome c reductase was highly sensitive to sulphydryl group poisons. Inclusion of sodium metabisulphite (3 mm) in the isolating medium (Parish, 1968) resulted in a small

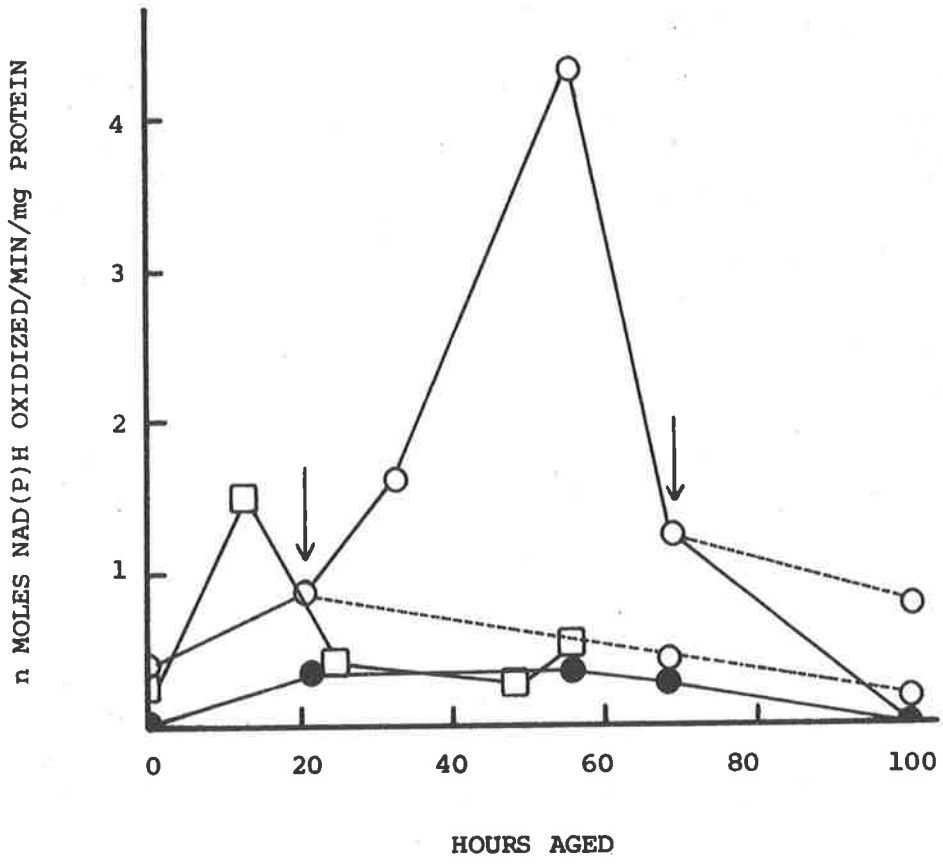


Figure 1. Changes in soluble NADH- (open symbols) and NADPH- (closed symbols) cytochrome c reductase activity in aging turnip disks at 17°C (○); and 34°C (□). The addition of cycloheximide (5×10^{-6}M) is indicated by arrows. Disks aged in the absence of cycloheximide are represented by solid lines and in the presence of cycloheximide by broken lines. Assay conditions are as described in Chapter II.

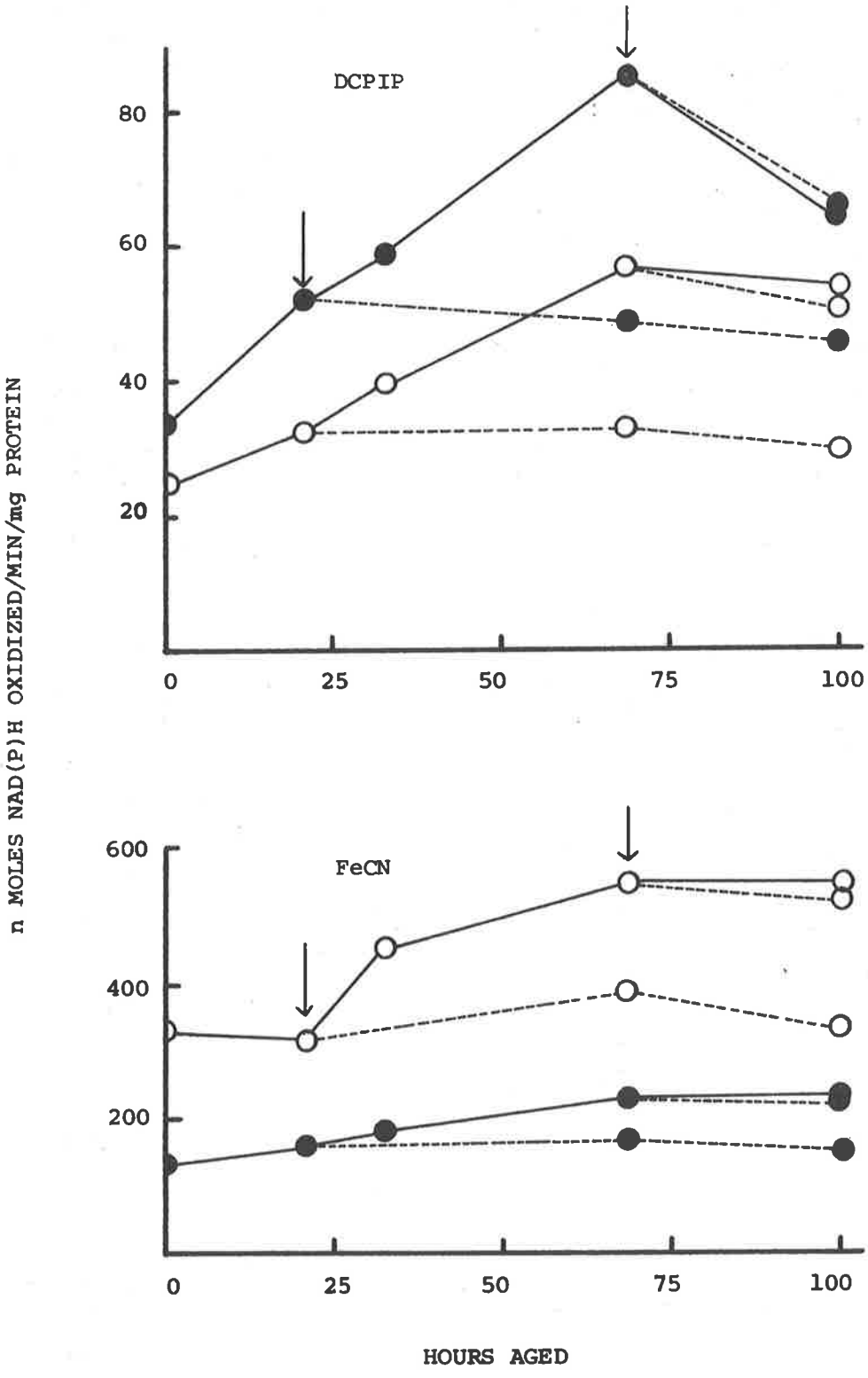


Figure 2. Changes in soluble NADH- (○) and NADPH- (●) DCPIP and FeCN reductase activities in aging turnip disks at 17°C. The addition of cycloheximide (5×10^{-6}M) is indicated by arrows. Disks aged in the absence of cycloheximide are represented by solid lines and in the presence of cycloheximide by broken lines. Assay conditions are as described in Chapter II.

increase in NADH-cytochrome c reductase activity, but polyvinylpyrrolidon (2%) resulted in up to 75% inhibition. These variations had little effect on the other activities assayed.

Dialysis or elution through Sephadex G-25 of the soluble fraction was necessary to remove an endogenous reduction of the acceptors which did not require NAD(P)H. However, dialysis for 24 hours resulted in up to 70% loss of NADH-cytochrome c and 10% loss of NADH-FeCN reductases relative to Sephadex elution. This was probably largely due to the longer times required for dialysis.

B. SOLUBLE REDUCTASE PURIFICATION

1. $(NH_4)_2SO_4$ fractionation

In the experiment presented in Table 1 the majority of the NADH-cytochrome c reductase precipitated in the 30-55% $(NH_4)_2SO_4$ fraction. Some activity, however, was often found in the 55-75% fraction. The 30-55% $(NH_4)_2SO_4$ fraction also contained the majority of the FeCN and DCPIP reductase activity which was apparently not nucleotide specific (but slightly favoured NADPH). The 55-75% fraction contained a FeCN reductase partially specific for NADH. Only NADH-cytochrome c reductase showed increased specific activity and this was not consistent. Further $(NH_4)_2SO_4$ fractionation did not result in increased specific activities or better separation.

Table 2 shows that the NADH-specific reductase activities were characterized by neutral pH optima and protection from pCMB

TABLE 1. $(NH_4)_2SO_4$ fractionation of reductase activities in 60 hour aged turnip soluble fraction. Details of fractionation technique and assay conditions are described in Chapter II.

Reductase (nmoles NAD(P)H oxidized/ min/mg protein)	Crude soluble fraction	$(NH_4)_2SO_4$ Fraction	
		30%-55%	55%-75%
NADH-cyt. c	0.3	2.1	0.1
NADPH-cyt. c	0	0.1	0.1
NADH-DCPIP	131	125	22
NADPH-DCPIP	142	187	26
NADH-FeCN	543	155	201
NADPH-FeCN	206	169	64

TABLE 2. pH optima and pCMB-sensitivity of the reductase activities in the crude soluble fraction and $(NH_4)_2SO_4$ fractions. pH optima were measured using 20 mM phosphate and tris-HCl buffers. pCMB-sensitivity was measured by preincubating the enzyme with either NAD(P)H or pCMB for 2 minutes, then adding pCMB or NAD(P)H (respectively), and assaying immediately. Assays are as described in Chapter II, starting the reaction with the acceptor. (10^{-5} M pCMB with FeCN and DCPIP reductases; 2×10^{-6} M pCMB with cytochrome c reductase.)

Reductase	Fraction	pH Optima NADH	pCMB Inhibition (% of initial rate)			
			Order of Addition			
			pCMB NADH	NADH pCMB	pCMB NADPH	NADPH pCMB
Cytochrome c	Total	7.5	93	53	-	-
	30%-55%	7.5	71	44	-	-
	55%-75%	-	21	0	-	-
DCPIP	Total	<6	27	27	29	35
	30%-55%	<6	17	25	24	23
	55%-75%	<6	50	40	37	34
FeCN	Total	7.0	47	4	24	24
	30%-55%	<6	50	44	23	17
	55%-75%	7.0	61	21	25	13

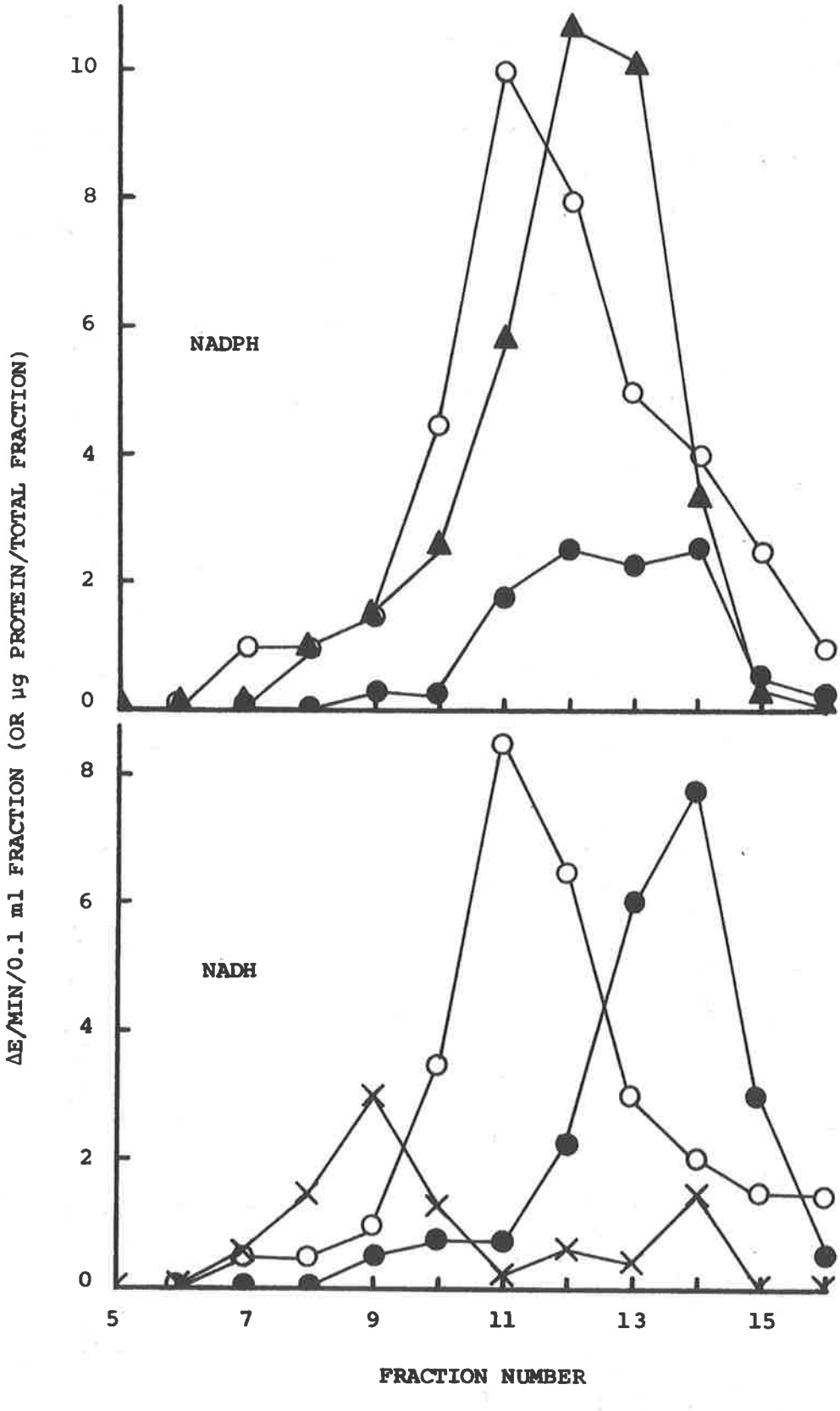
inhibition by preincubation of the enzyme with reduced nucleotide. This is similar to the protection of the microsomal NADH dehydrogenase (Chapter III) which has only been reported for a few specific membrane-bound dehydrogenases and lactate dehydrogenase (Chapter I). Due to the similarity between the NADH specific activities it was possible that cytochrome c reductase activity resulted from interaction of a subunit with the NADH-FeCN reductase. However, recombination of the various $(NH_4)_2SO_4$ fractions did not result in increased NADH-cytochrome c reductase activity.

2. Sephadex elution

Figure 3 shows the sequence of elution through Sephadex G-200 of reductase activities in the soluble fraction from 49 hour aged tissue. The peaks of NADH-cytochrome c, - DCPIP and - FeCN reductases were all separated. The single peaks of the DCPIP and NADH-FeCN reductases indicated that the majority of each of these activities was due to a single enzyme. The broad NADPH-FeCN reductase distribution suggested that the DCPIP reductase also reduced FeCN and that the NADH-FeCN reductase also oxidized NADPH.

Figure 4 shows that with aging of tissue a second peak of NADH-cytochrome c reductase activity developed. This second peak could be separated from the peak of NADH-FeCN reductase using a longer column (120 cm x 1.1 cm) suggesting that these two activities were not related. Corresponding changes in the distribution of other

Figure 3. Sephadex G-200 elution sequence of the NAD(P)H dehydrogenases from 49 hour aged tissue soluble fraction. 10 ml soluble fraction was eluted through column (100 cm x 1.1 cm diam.) and, after a void volume of 25 ml, 5 ml fractions were collected. Sephadex elution and assays are as described in Chapter II. NADH-cytochrome c reductase (X) (ΔE_{550nm}); NAD(P)H-DCPIP reductase (○) (ΔE_{600nm}); NAD(P)H-FeCN reductase (●) (ΔE_{420nm}); protein (▲).


NADPH
NADH
10
8
6
4
2
0
8
6
4
2
0
5
7
9
11
13
15
ΔE/MIN/0.1 ml FRACTION (OR µg PROTEIN/TOTAL FRACTION)
FRACTION NUMBER

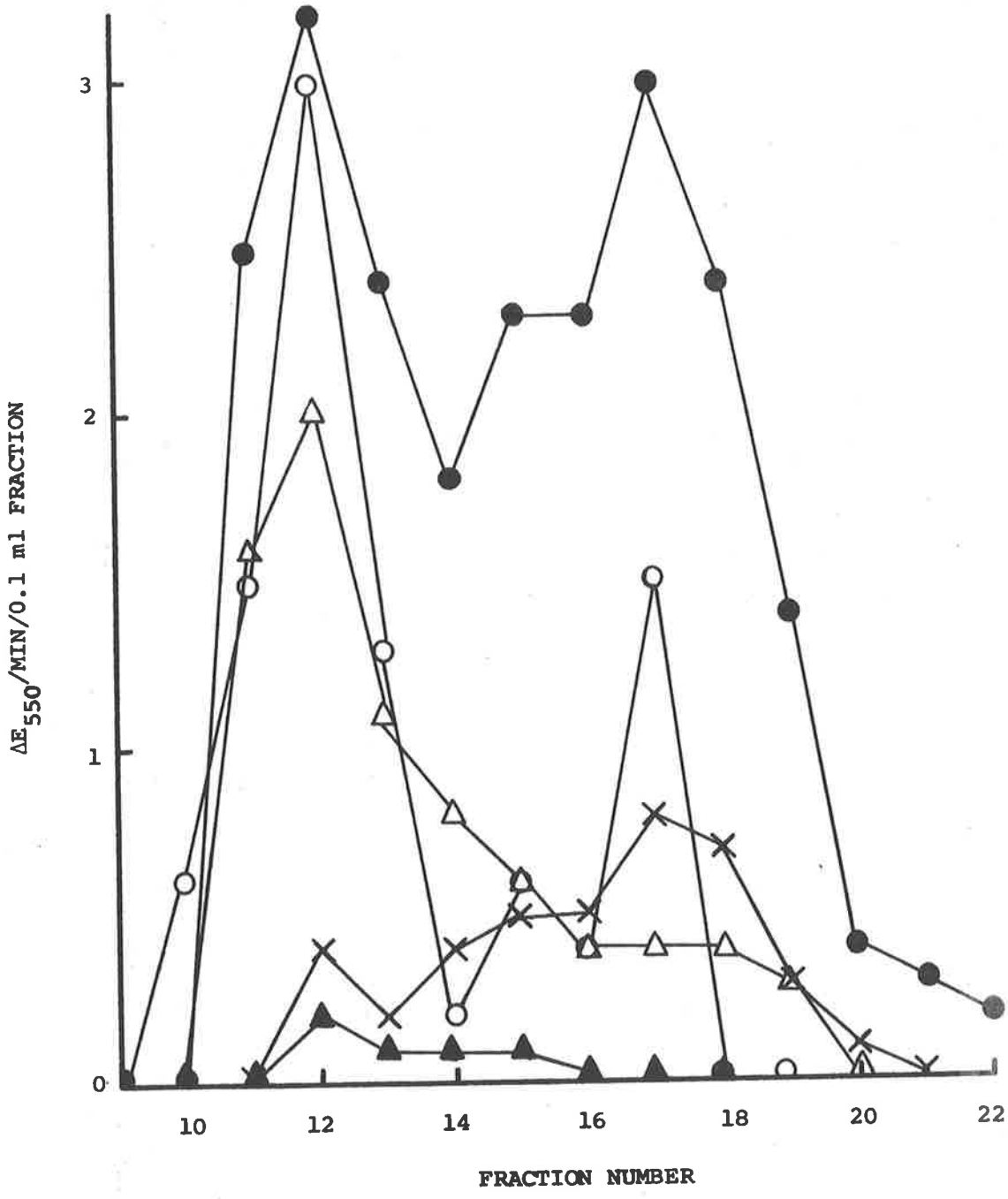


Figure 4. Changing elution of soluble NADH-cytochrome c reductase with aging of tissue. 10 ml of soluble fraction isolated from 0-96 hour aged turnip tissue was eluted through Sephadex G-200 (120 cm x 1.1 cm diam.) and 5 ml fractions collected (after a void volume of 35 ml). The 49 hour aged tissue activity was replotted from Figure 3. 0 hours ▲; 40 hours △; 49 hours ○; 70 hours ● ; 96 hours ✕ .

activities were not detected, indicating that the FeCN and DCPIP reductase activities which develop with aging are the same as those present in fresh tissue. The similarity in NADH specificity and pCMB-sensitivity of the cytochrome c reductases in fractions 12 and 17 (70 hour aged tissue) indicated that the two activities were probably due to the same enzyme. This effect parallels the variable distribution of NADH-cytochrome c reductase in the $(NH_4)_2SO_4$ fractions. The slower elution may be due to the binding of compounds which develop in aging tissue (e.g. polyphenols; Hyodo and Uritani, 1966). However, the addition of polyvinylpyrrolidon (which binds polyphenols during homogenization) did not significantly affect NADH-cytochrome c reductase elution.

Table 3 shows that elution of the crude soluble fraction through Sephadex G-200 resulted in an apparent purification of up to 56-fold of the NADH-cytochrome c reductase (fraction 7) but only 7-fold of the NADH-FeCN reductase (fraction 15). However, both fractions were contaminated with the DCPIP reductase. The results in Table 3 indicate that the NADH-cytochrome c reductase was highly specific for both NADH and cytochrome c. While the FeCN reductase clearly favoured NADH (at least 6-fold), NADPH was probably also oxidized but DCPIP probably not reduced. As DCPIP reduction in both fractions 7 and 15 favoured NADPH, it suggested that this activity did not result from the NADH specific reductases. However, further Sephadex elution of

TABLE 3. Reductase activities of fraction 7 and 15 from elution of the crude soluble fraction through Sephadex G-200 (Fig. 3). The crude soluble fraction was eluted through Sephadex G-25 and assayed immediately. Details of Sephadex elution technique and assays are as described in Chapter II.

Reductase (nmoles NAD(P)H oxidized/min/mg protein)	Crude soluble fraction	Fraction 7	Fraction 15
NADH-cytochrome c	3.7	201	0
NADPH-cytochrome c	0	0	0
NADH-DCPIP	93	412	41
NADPH-DCPIP	102	824	69
NADH-FeCN	896	0	6190
NADPH-FeCN	421	0	1025

the crude soluble fraction and the $(NH_4)_2SO_4$ fractions did not result in better purification and so the extent of the specificity of NADH-FeCN reductase remains unresolved.

3. Properties

The properties of the partially purified NADH-cytochrome c and FeCN reductases are given in Table 4. These activities differed:

(i) in affinity for NADH;

(ii) in sensitivity to pCMB;

(iii) slightly in pH optima;

(iv) in sensitivity to salts.

The cytochrome c reductase was highly sensitive to $CaCl_2$ but the FeCN reductase was only partially sensitive (Fig. 5). Both activities were insensitive to inhibitors of inner mitochondrial membrane electron transport (rotenone, amytal, NOQNO, antimycin A; Ikuma and Bonner, 1967), of lipoyl dehydrogenase (arsenite; Massey, 1963), and of DT diaphorase (dicoumarol; Ernster *et al.*, 1962). Lactate had no effect indicating the absence of lactate dehydrogenase in reducing cytochrome c and FeCN (Slater, 1961). Flavine mononucleotide did not activate the reductases as reported by Horio and Kamen (1962) for a bacterial soluble NADH-cytochrome c reductase. The effect of H_2O_2 and catalase indicated that peroxidase-catalysed reduction of cytochrome c or FeCN was not present (Dolin, 1957). The inhibitory effect of H_2O_2 was variable and was due to a peroxidase-catalysed oxidation of

TABLE 4. Properties of the partially purified soluble NADH-cytochrome c and -FeCN reductases. NADH-cytochrome c reductase was obtained by eluting crude soluble fraction through Sephadex G-200 and pooling fractions 7, 8 and 9 (Fig. 3). NADH-FeCN reductase was obtained by eluting the 55-75% $(NH_4)_2SO_4$ fraction of the crude soluble fraction from fresh tissue (Table 1) through Sephadex G-200 and pooling fractions 14 and 15 (Fig. 3). Assay conditions are as described in Chapter II. (* + = stimulation.)

	NADH-cytochrome c reductase	NADH-FeCN reductase
pH Optima	7.6	7.4
K_M (acceptor)	6.5×10^{-5}	2.5×10^{-4}
K_M (NADH)	1.63×10^{-4}	8.8×10^{-5}
Inhibitor	% Inhibition*	
2×10^{-6}M pCMB	95	36
4×10^{-3}M Arsenite	0	+7
7×10^{-6}M Rotenone	0	15
9×10^{-3}M Amytal	10	0
1.3×10^{-4}M NOQNO	0	7
20mg/ml Antimycin A	+8	0
5×10^{-4}M KCN	0	7
10^{-5}M FMN	0	5
4×10^{-2}M Lactate	0	0
10^{-5}M Dicoumarol	4	7
0.2% Triton X-100	0	0
10^{-2}M $CaCl_2$	100	0
2×10^{-4}M H_2O_2	20	12
Catalase	+50	0

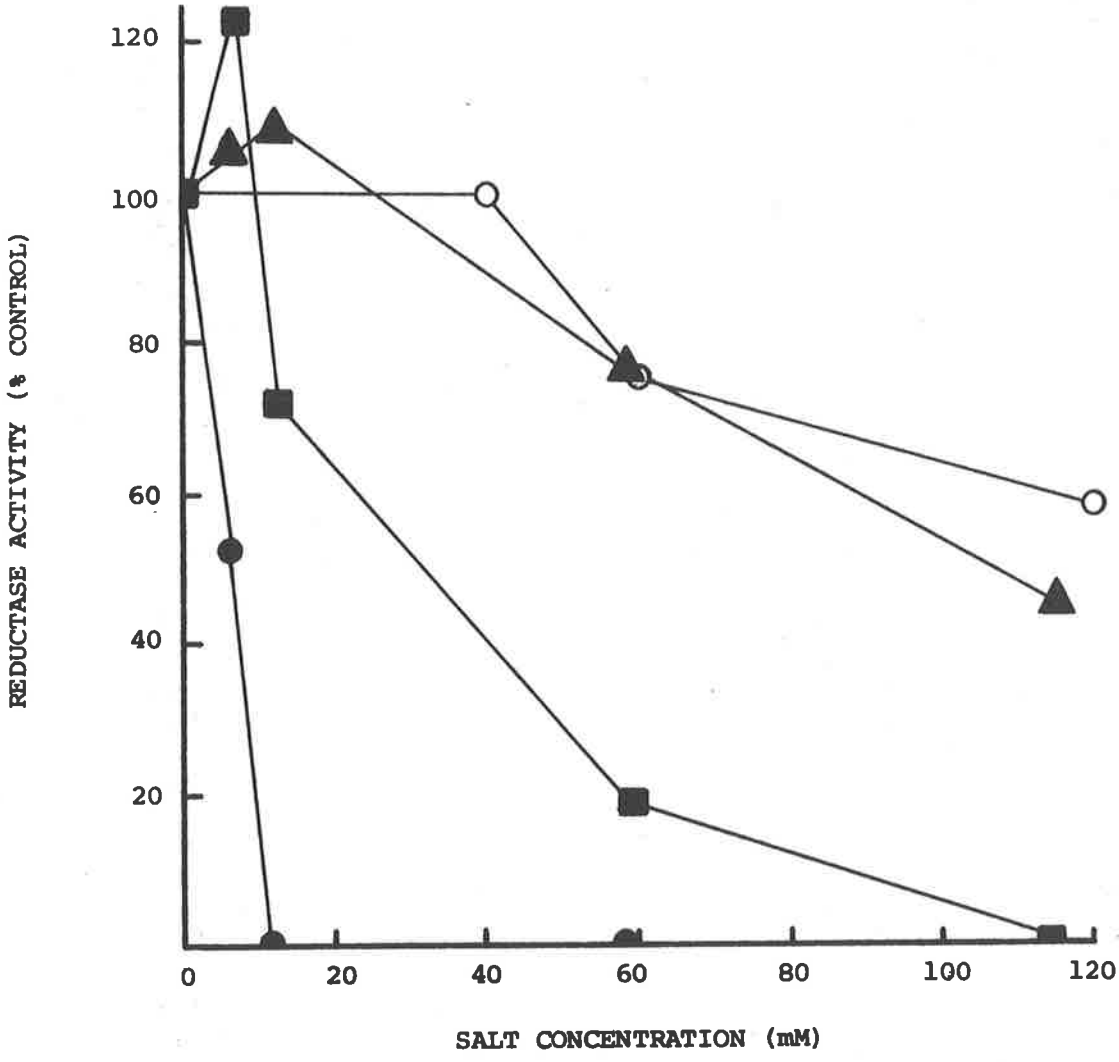


Figure 5. Effect of salts on the partially purified soluble NADH-cytochrome c (closed symbols) and -FeCN (open symbols) reductases from turnip. Assay conditions are as described in Chapter II.
● - $CaCl_2$; ■ - $MgCl_2$; ▲ - KCl.

reduced cytochrome c and FeCN. Catalase inhibited this reoxidation resulting in a stimulated reduction rate. This effect was most noticeable when the activities were low and was routinely allowed for.

C. CHARACTERIZATION OF THE DEVELOPMENT OF THE SOLUBLE REDUCTASE ACTIVITIES

Only the NADH dehydrogenase activities are presented here (the NADPH dehydrogenase activities followed NADH-DCPIP reductase). Increases in reductase activities with tissue aging were inhibited by cycloheximide, puromycin, 6-methylpurine and ethionine but not chloramphenicol (Table 5). None of the inhibitors had a direct effect on the activities of the isolated soluble fraction enzymes. The effect of these inhibitors on respiration was also determined to help differentiate between specific effects on protein and RNA synthesis and non-specific effects of other processes (Glasziou, 1969; Filner *et al.*, 1969):

(i) The inhibition of development of activities by high cycloheximide may be due to limitation of energy supply as it appeared to uncouple respiration.

(ii) 10^{-4} M puromycin was not sufficient to fully inhibit the development of either respiration or the reductase activities. This experiment was not repeated due to the large quantities of puromycin required but did demonstrate the dependence of development of both processes on protein synthesis. MacDonald *et al.* (1966) found that

TABLE 5. Effect of addition of protein and RNA synthesis inhibitors to disks immediately after slicing on the development of the soluble reductase activities and respiration with aging. Manometry and assay conditions are as described in Chapter II. (Respiration expressed in µl O_2/hour/gfw and reductase activities in nmoles NADH oxidized/minute/mg protein.

	Fresh	24-hour aged			
Cycloheximide (M)	0	0	3.6×10^{-6}	7×10^{-6}	1.4×10^{-5}
Respiration	97	121	93	103	139
NADH-cyt.c reductase	0	0.6	0.01	0.01	0.01
NADH-DCPIP reductase	35.6	44.1	27.4	26.6	21.3
NADH-FeCN reductase	213	230	210	210	211
Puromycin (M)	0	0	10^{-4}		
Respiration	65	130	80		
NADH-cyt.c reductase	0.06	0.74	0.27		
NADH-DCPIP reductase	33.0	47.8	43.9		
NADH-FeCN reductase	115	164	136		
Chloramphenicol (M)	0	0	5×10^{-4}	3×10^{-3}	
Respiration	91	151	99	114	
NADH-cyt.c reductase	1.2	3.5	2.5	3.5	
NADH-DCPIP reductase	50.9	65.3	65.3	68.4	
NADH-FeCN reductase	192	322	327	310	
6-Methylpurine (M)	0	0	10^{-5}	10^{-4}	5×10^{-4}
Respiration	64	90	63	28	25
NADH-cyt.c reductase	0.1	0.3	0.2	0.1	0
NADH-DCPIP reductase	13.9	20.5	15.3	13.5	15.3
NADH-FeCN reductase	53	62	54	57	53
Ethionine (M)	0	0	10^{-5}	10^{-4}	10^{-3}
Respiration	83	108	108	95	93
NADH-cyt.c reductase	0	0.9	0	0	0
NADH-DCPIP reductase	16.7	21.3	19.0	15.2	10.6
NADH-FeCN reductase	96	127	96	108	78

10^{-4} M puromycin fully inhibited development of respiration but not invertase in aged beet disks.

(iii) Chloramphenicol did not inhibit activity increases, as expected, but did inhibit development of respiration (ap Rees, 1966).

(iv) The effect of 6-methylpurine at concentrations higher than 10^{-5} M were discounted due to inhibition of respiration (Gayler and Glasziou, 1968).

(v) Low concentrations (10^{-5} - 10^{-4} M) of ethionine inhibit plant RNA synthesis (Stone *et al.*, 1969) but higher concentrations (10^{-3} -5 x 10^{-3} M) decrease ATP content (Atkinson and Polya, 1968). Low concentrations inhibited development of reductases but not respiration, higher concentrations inhibited both.

Hence, increases in dehydrogenase activities appeared to be dependent on both RNA and protein synthesis. There was no evidence for the synthesis or inactivation of an inhibitor with aging. The soluble fractions from 0 and 130 hour aged tissue did not inhibit NADH-cytochrome c reductase in the soluble fraction from 56 hour aged tissue. Similarly, Sephadex elution could not activate any activity in the soluble fractions from 0 and 130 hour aged tissue. Further experiments involved only cycloheximide.

The effect of delayed addition of cycloheximide on the subsequent levels of reductase activities with aging is shown in Figures 1 and 2. Cycloheximide added at 21 hours after slicing inhibited

further development of all reductases. However, there was also a loss of NADH-cytochrome c reductase activity in the presence of cycloheximide. Hence inactivation of this activity had started *in vivo* considerably before it became apparent (i.e. was greater than the rate of synthesis). Addition of cycloheximide 68 hours after slicing had no effect on the FeCN and DCPIP reductases but lessened the inactivation of NADH-cytochrome c reductase. This was not due to inhibition of development of peroxidase (Chapter VI), which can catalyse reduced cytochrome c oxidation, as inclusion of catalase in the assays had no effect.

It is not clear whether cycloheximide inhibition of protein synthesis is reversible (Grollman, 1966; Zucker, 1968). Figure 6 shows the effect on the developing reductases of varying the time of incubation of the tissue slices with cycloheximide. After removal of cycloheximide, aging was continued to give a total of 50 hours. Any activity above that normally detected in the soluble fraction from fresh tissue was presumably a result of removal of the cycloheximide inhibition of protein synthesis. (NADH-cytochrome c reductase activity dropped below the level in the soluble fraction from fresh tissue due to the inactivating mechanism which develops with aging, Fig. 1.)

From the levels of activities in the soluble fraction from fresh disks and disks aged for 50 hours in the absence of cycloheximide an average rate of development of each activity could be calculated.

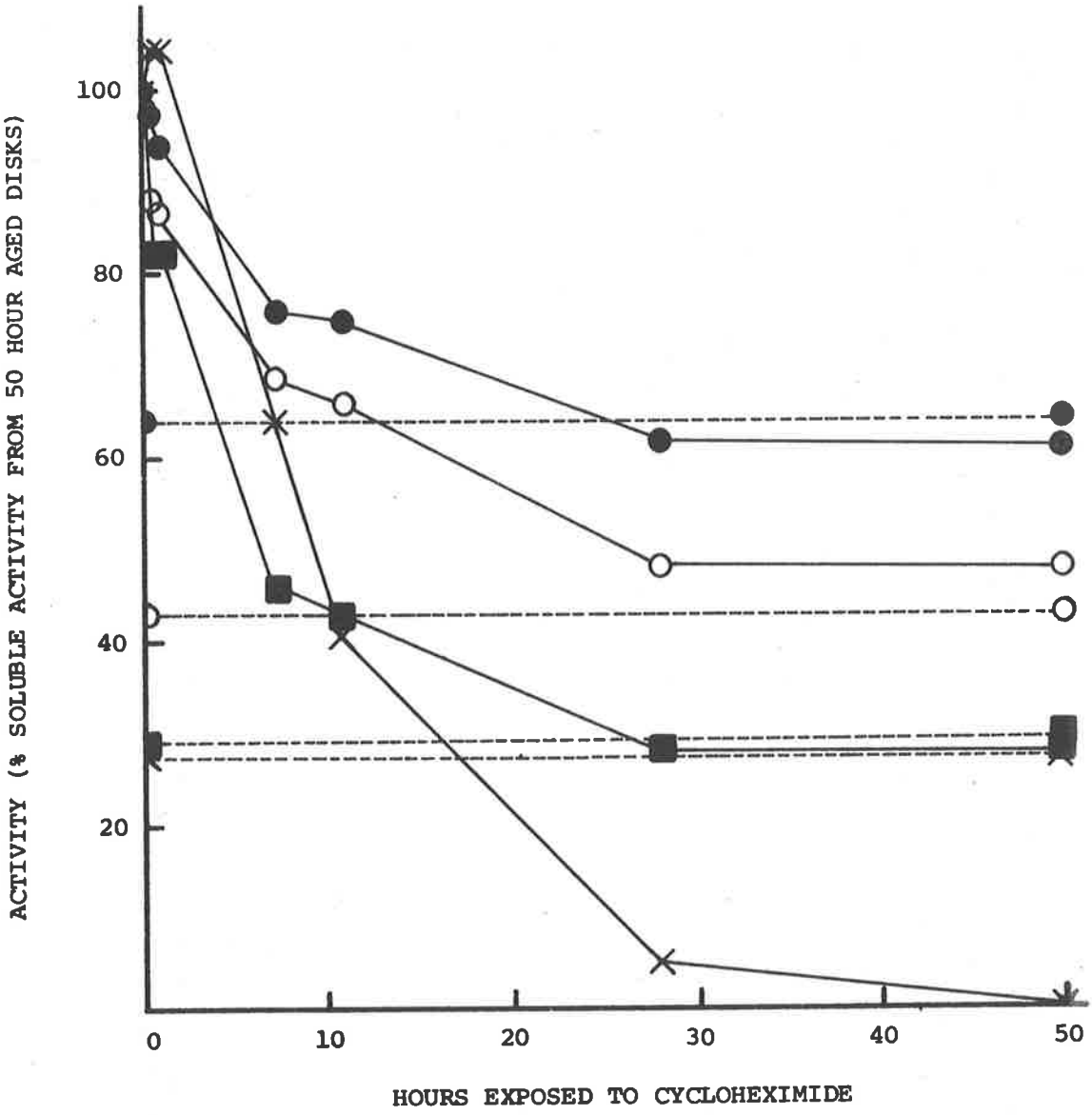


Figure 6. Effect of time of exposure to cycloheximide on development of soluble NADH-cytochrome c (X), -DCPIP (O), -FeCN (●) reductase and peroxidase (■) activities. Freshly cut disks were aged in 5 x 10^{-6}M cycloheximide solution for the times indicated, then washed thoroughly and aged for the remaining period of aging in 10^{-4}M $CaSO_4$ only. After a total of 50 hours disks were harvested and soluble fractions prepared. The broken lines represent the activities in fresh untreated disks. Assay conditions are as described in Chapter II.

If it was assumed that, on the removal of cycloheximide, there was a finite washing out period followed by a period of normal (i.e. average) reductase development, then this washing out period could be calculated for each of the varying times of incubation of the disks in cycloheximide. These washing out or delay times are presented in Table 6. If disks were in contact with cycloheximide for one hour or less then the delay time was short. If disks were in cycloheximide for 7½ hours or longer then the delay time increased to a constant level (approx. 20 hours).

Hence turnip disks had to be in contact with 7×10^{-6} M cycloheximide for over one hour for maximum protein synthesis inhibition, and 20 hours of washing with numerous changes of the washing solution were required to completely reverse this inhibition.

D. SOLUBLE REDUCTASE ACTIVITIES IN OTHER TISSUES

Reductase activities similar to those present in turnip soluble fraction were detected in the dialysed soluble fraction from potato, carrot, parsnip, radish, swede and beetroot storage tissues and pea roots (Table 7). Some development of NADH-cytochrome c reductase activity was detected on aging disks of all the storage tissues except radish. However, often the corresponding NADPH-cytochrome c reductase activities were high and increases in the FeCN and DCPIP reductases with aging were not detected. The potato tuber soluble fraction was atypical in that the undialysed form did not

TABLE 6. Effect of time of exposure of disks to cycloheximide on the calculated time required to recover normal rate of reductase development. These times were calculated from Fig. 6 as described in the text. Assay conditions are as described in Chapter II.

	Calculated time (hours) to recover normal rate of reductase development					
Hours exposed to 5×10^{-6}M cycloheximide	0	½	1	7½	11¼	28¼
NADH-cytochrome c reductase	0	0	0	11	17¾	19¼
NADH-DCPIP reductase	0	10½	12	22½	21¼	21¼
NADH-FeCN reductase	0	2½	8	23	20¾	19¼
Peroxidase	0	12	11½	31½	30	>22

TABLE 7. Reductase activities in the crude soluble fractions from fresh and aged plant tissues. Soluble fractions were prepared as in Chapter II and dialysed for 24 hours (except potato). Reductase activities are expressed in nmoles NAD(P)H oxidized/min/0.1 ml soluble fraction.

Tissue	Carrot		Parsnip		Radish		Swede		Beet		Potato	
Hours aged	0	65	0	66	0	66	0	60	0	65	0	25
NADH-cyt.c reductase	0.5	2.2	2.3	3.6	4.5	0.5	2.3	2.7	0.6	2.7	3.6	11.3
NADPH-cyt.c reductase	0	0.6	1.3	-	0.8	0	1.6	0.7	-	-	3.2	9.5
NADH-DCPIP reductase	5.0	1.7	25.9	15.0	44.5	19.8	23.1	31.4	-	-	40.2	136.5
NADPH-DCPIP reductase	5.8	2.5	32.5	25.6	77.5	38.0	32.2	44.6	-	-	41.7	133.5
NADH-FeCN reductase	122	18.6	585	214	142	58	173	226	-	-	211	278
NADPH-FeCN reductase	68	18.6	185	68	167	72	130	155	-	-	135	318

reduce the acceptors in the absence of NAD(P)H. The undialysed soluble fraction reduced cytochrome c but in the presence of either NADH or NADPH. This activity was only partially sensitive to pCMB and not protected by preincubation with the reduced nucleotide, the FeCN reductase being the only activity to show such protection (Table 8). However, the cytochrome c reductase was more pCMB-sensitive than the DCPIP reductase.

Sephadex elution (and similarly dialysis) resulted in 15% loss of NAD(P)H-DCPIP, 30% loss of NADPH-FeCN, 50% loss of NADH-FeCN, and 98% loss of NAD(P)H-cytochrome c reductase activities. {Loss of NADH-cytochrome c reductase activity upon dialysis of potato soluble fraction has been previously reported (Hackett, 1958).} Table 9 shows that the NADH-cytochrome c reductase could be partially recovered by combining the Sephadex G-25 fraction containing the DCPIP reductase with a slower eluting fraction. Hence all cytochrome c and possibly some FeCN reduction in the potato soluble fraction can be attributed to an interaction between a dialysable factor and the NAD(P)H-DCPIP reductase.

The nature of the factor was not determined. However, the results in Table 8 suggest that it involves a sulphydryl group. It could not mediate electron flow between the turnip DCPIP reductase and cytochrome c (Table 9). The NADPH-cytochrome c reductase activities in the dialysed soluble fractions from a number of the

TABLE 8. pCMB-sensitivity of the reductase activities in the crude, undialysed soluble fraction from fresh potato. Sensitivity was measured by preincubating the soluble fraction with either NAD(P)H or pCMB for 2 minutes, then adding pCMB or NAD(P)H (respectively) and assaying immediately. Assay conditions are as described in Chapter II, starting the reaction with the acceptor.

Reductase	% Inhibition of initial rate			
	Order of Addition			
	pCMB NADH	NADH pCMB	pCMB NADPH	NADPH pCMB
Cytochrome c	45	41	38	35
DCPIP	7	7	15	11
FeCN	49	12	16	8

TABLE 9. Effect of recombination of (0.1 ml) Sephadex G-25 fractions (5 ml) of the soluble fraction from fresh potato on NADH-cytochrome c reductase activity. Fractions 6+7 contained the majority of protein and reductase activities. Fractions eluting after fraction 9 showed no NADH-cytochrome c reductase activity. Sephadex elution and assays are as described in Chapter II.

Sephadex G-25 (25x1cm) fraction	NADH-cytochrome c reductase activity (nmoles NADH oxidized /min/0.1ml fraction 6+7)
Crude soluble fraction (not eluted)	3.5
(6+7)	0.1
(6+7) + 10	0
(6+7) + 11	0
(6+7) + 12	0.1
(6+7) + 13	1.3
(6+7) + 14	1.1
(6+7) + 15	0.3
Dialysed soluble fraction from turnip (46 hour aged)	1.3
Dialysed soluble fraction from turnip + potato fraction 13	0.7

tissues in Table 7 may result from remnants of such a factor. The relatively low NADPH-cytochrome c reductase in the dialysed soluble fraction from turnip and the lack of interaction with the potato factor would suggest the absence of such a factor in turnip.

On aging potato disks, all activities in the undialysed soluble fraction increased (Table 7). The percentage increase in NAD(P)H-DCPIP and -cytochrome c reductases were comparable and much greater than the increase in NADH-FeCN reductase. This suggested an increase in the reductase rather than the factor. It is not clear (even after elution through Sephadex) whether there was any NADH-specific cytochrome c reductase in the soluble fraction from either fresh or aged tissue.

DISCUSSION

A. SUMMARY OF THE SOLUBLE ACTIVITIES

Although the properties of the soluble NADH-cytochrome c and -FeCN reductases were very similar, the NADH-cytochrome c reductase was characteristic in it's:

(i) requirement for the presence of BSA and absence of cysteine in the isolating medium;

(ii) greater sensitivity to pCMB and some ions;

(iii) pH optimum;

(iv) affinity and specificity for NADH.

No attempt was made to purify the DCPIP reductase which had an acid pH

optimum and was not specific for either nucleotide or acceptor. These electron transport activities appeared to be characteristic at least of the non-photosynthetic plant tissues.

B. COMPARISON WITH REPORTED SOLUBLE ACTIVITIES FROM PLANTS

The reductase activities reported by Wosilait and Nason (1954a) and Ragland and Hackett (1964) were probably due to the NADH-FeCN reductase. The activities reported by Martin and Morton (1956a), Hackett (1958) and Marré *et al.* (1962) were probably due to the unspecific DCPIP reductase. The nucleotide specificity and properties of the soluble NADH-cytochrome c reductase in cauliflower buds (Crane, 1957) were not determined. As the soluble fraction was not dialysed this activity may be similar to that detected in potato.

C. COMPARISON WITH REPORTED SOLUBLE ACTIVITIES FROM ANIMALS

There was surprisingly little similarity between the soluble reductase activities from turnip and the corresponding activities from bacteria and animals. In particular, on the basis of the criteria given in Chapter I there appeared to be no activity corresponding to the widespread DT diaphorase in animals. There was also no evidence for an NADPH specific dehydrogenase (Akeson *et al.*, 1963) or an NADH-glutathione reductase which could catalyse the reduction of cytochrome c (Froede and Hunter, 1970).

D. COMPARISON WITH SOLUBILIZED MEMBRANE-BOUND ACTIVITIES

A variety of NADH-dehydrogenases have been solubilized from

membrane fractions:

(i) the NADH lipoyl dehydrogenase with diaphorase activity which forms part of the α-ketoglutarate dehydrogenase complex (Massey, 1963);

(ii) the initial NADH-flavoprotein from the mitochondrial inner membrane (Ringler *et al.*, 1963);

(iii) the NADH-cytochrome b_5 (or b_{555}) reductase from the outer mitochondrial (Sottocasa *et al.*, 1967) and microsomal (Takesue and Omura, 1970b) membranes.

The lipoyl dehydrogenase, which has been isolated from plants (Basu and Burma, 1966), is characterized by (Massey, 1963):

(i) specificity for NADH;

(ii) reduction of FeCN (pH optimum 4.8) and DCPIP but not cytochrome c;

(iii) high sensitivity to 5 mM arsenite.

This does not resemble any of the soluble activities from turnip. The solubilized inner mitochondrial membrane reductase is somewhat similar to the NADH-FeCN reductase but does not show protection from pCMB inhibition by preincubation with NADH (Tyler *et al.*, 1965; Ragan and Garland, 1969). However, the solubilized microsomal (and probably outer mitochondrial membrane) reductase does resemble the NADH-FeCN reductase in substrate specificities and inhibitor sensitivities including protection from pCMB.

There is no evidence to suggest that any of these enzymes

are soluble in tissue homogenates. However, it has been shown that on slicing turnip tissue there was an immediate loss *in vivo* of all microsomal NADH dehydrogenase activities possibly due to their release from the membranes (Chapter IV). Hence the soluble fraction would contain reductases released from the microsomal (and possibly other) membranes. Such an *in vivo* solubilization is probably caused by hydrolytic enzymes released from the large central vacuole (Matile and Moor, 1968) after tissue slicing and probably also occurs on homogenizing tissue. The corresponding hydrolytic enzymes of animal cells are situated in lysosomes (Takesue and Omura, 1970a) and are not generally released during tissue disruption. Hence such a soluble NADH-FeCN reductase would not be present in animal tissue homogenates.

E. FUNCTIONS OF THE SOLUBLE ACTIVITIES

The low pH optimum of the DCPIP reductase indicates a vacuolar origin. This enzyme could correspond to the animal DT diaphorase but adapted to the acid conditions in plant cells. The pH optimum and NADH-specificity of the cytochrome c and FeCN reductases indicate that these enzymes may normally be active in the cytoplasm of the intact cell, though not necessarily in the soluble form. The NADH-FeCN reductase may be solubilized from the membranes when the tissue is homogenized. However, the majority of membrane bound NADH-cytochrome c reduction involves more than one component and cannot therefore be similarly solubilized. A single enzyme NADH-cytochrome c

reductase has been reported in liver microsomes (Schulze *et al.*, 1970) but there was no evidence for this activity in turnip microsomes (Chapter III). Hence the NADH-cytochrome c reductase may be soluble *in vivo*. The K_m for cytochrome c (Table 4) indicates that this acceptor may well be the natural substrate. The very low activities (relative to the other reductases) may indicate a highly specific function in the disks. A possible function may be to feed cytoplasmic NADH to the inner mitochondrial membrane electron transport chain at about cytochrome c. It would require that cytochrome c (or the natural acceptor) be available to the reductase which would be outside the outer membrane. If so the resulting respiration would be insensitive to antimycin A and NOQNO but remain sensitive to azide and cyanide (Chapter VIII, Fig. 5). Chapter VIII shows that such a respiration does develop in aging turnip disks corresponding to the development of the soluble NADH-cytochrome c reductase. However, the results suggest that the two phenomena are not related.

F. DEVELOPMENT OF THE ACTIVITIES WITH AGING

Increase in the cytochrome c, DCPIP and FeCN reductases depended on both RNA and protein synthesis. The apparent reversibility of cycloheximide inhibition contrasted with the results of Zucker (1968) using potato disks and may be a reflection of tissue differences. Figure 1 showed that addition of cycloheximide 20 hours after slicing promoted NADH-cytochrome c reductase loss, but at 68 hours retarded

loss. Addition of cycloheximide at 68 hours probably prevents a rapid synthesis of degradative enzymes which may occur in old degenerating disks. Similar effects have been observed with a variety of compounds and treatments (Glasziou, 1969). These results probably also explain the results of Pan *et al.* (1970) who reported that addition of cycloheximide to corn leaf sheathes at time of excision promoted, inhibited or did not affect the rates of loss of different activities.

C H A P T E R V I

MICROSOMAL AND SOLUBLE OXYGENASES IN FRESH AND AGED TISSUE

INTRODUCTION

The initial aim of this section was to test for and characterize any mixed function oxidation (MFO) activity in turnip microsomes similar to that detected in animal microsomes. Since completion of this work two reports of likely plant microsomal MFO activity have been published (Chapter I, Table 1). An activity was detected, and partially characterized, in the turnip microsome fraction which required O_2, NAD(P)H and an organic substrate. However, the predicted organic products and involvement of cytochrome P-450 could not be detected. Also, sensitivity to inhibitors and other treatments which affect membranes varied from the animal system. Activity did somewhat resemble the animal microsomal lipid desaturation and sterol demethylation (cyanide-sensitive) processes and the reported plant microsomal MFO activities.

Subsequently it was shown that this activity was largely or completely due to peroxidase (by the mechanism described in Chapter I) and was not restricted to the microsomal membranes (the majority of activity was detected in the soluble supernatant fraction). Peroxidase was then assayed in a specific way (Gregory, 1966) by following ascorbate oxidation in the presence of peroxide. The NAD(P)H-requiring

oxygenase activity in turnip microsomes is therefore considered with respect to the reported animal and plant microsomal MFO as well as purified horse radish peroxidase catalysing NAD(P)H oxidation as described in Chapter I.

RESULTS

A. MICROSOMAL NAD(P)H OXIDATION REQUIRING O_2 AND ORGANIC SUBSTRATE

1. Characterization of the reaction

The reaction was assayed by measuring both NAD(P)H oxidation (340 nm) and O_2 consumption (oxygen electrode or Warburg manometer). Organic substrates used were dimethylaniline (DMA), aniline and phenol all of which support MFO activity in animal microsomes yielding the products shown in Table 1. Figure 1 shows the simultaneous requirement for O_2, NADPH and organic substrate (aniline) for activity with turnip microsomes. Similar traces were obtained with DMA. The NADPH:O ratio could be calculated if a known amount of NADPH was added to the reaction (rather than an NADPH-generating system). However, this ratio varied both within (0.3-1.3) and between (0.3-1.9) assays. (The ratio for both animal microsomal MFO and for horse radish peroxidase is 0.5.) NADH usually supported 60-80% of the NADPH rate. This was much higher than the NADPH-specific animal microsomal MFO (Cohen and Estabrook, 1971) but comparable with the animal microsomal cyanide-sensitive oxidations, plant microsomal MFO and horse radish peroxidase (Chapter I).

TABLE 1. Reported products of animal microsomal MFO activity with DMA, aniline, and phenol as substrates.

Substrate	Product	Reference
DMA	DMA-N-oxide ↓ Methylaniline + Formaldehyde	Ziegler and Pettit (1964); Smuckler *et al.* (1967)
Aniline	p-Aminophenol	Mitoma *et al.* (1956); Imai and Sato (1959); Kampffmeyer and Kiese (1965); Anders (1968)
Phenol	Catechol + Hydroquinone	Posner *et al.* (1961)

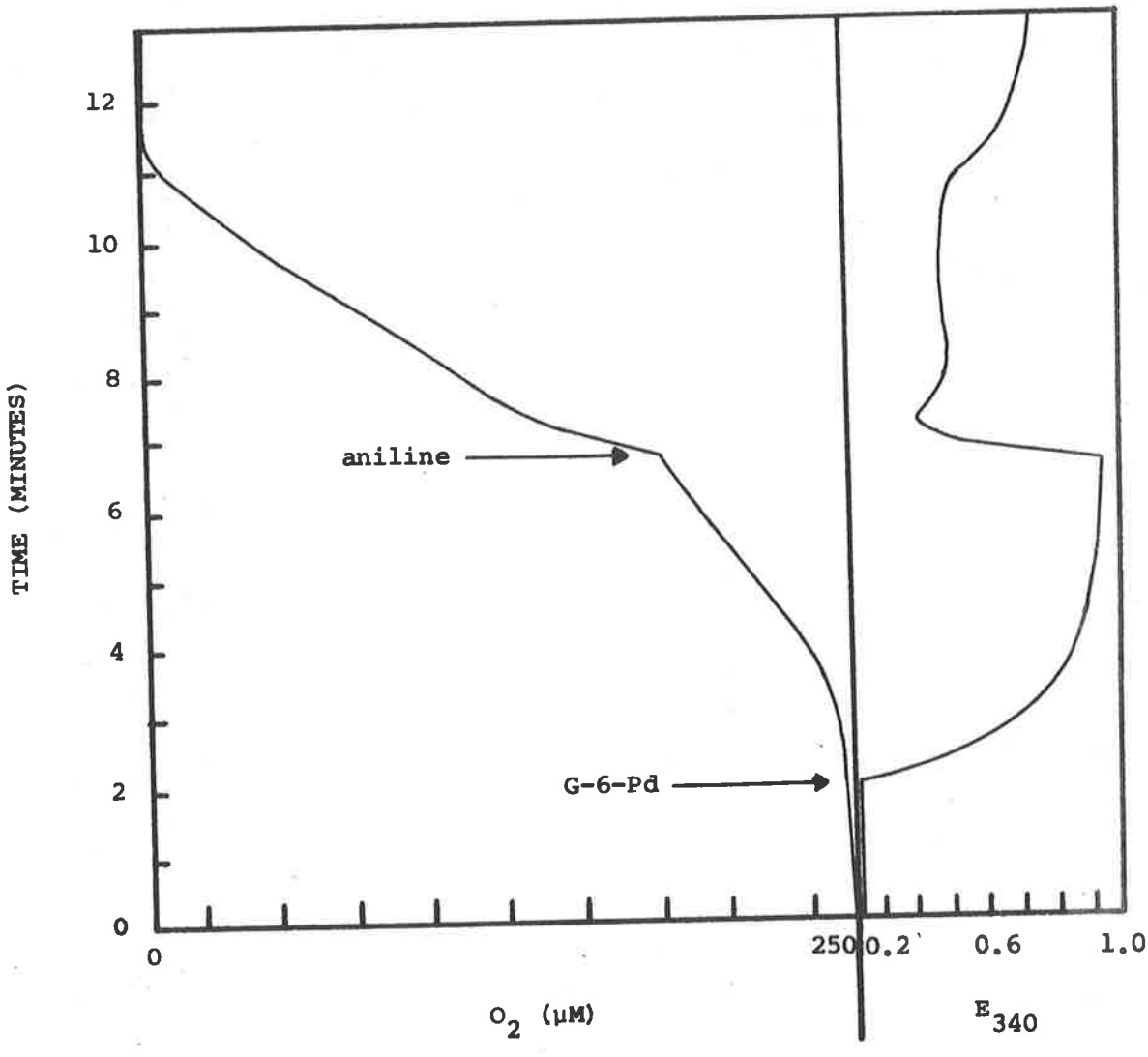


Figure 1. Oxygen consumption and NADPH oxidation by turnip microsomes in the presence of aniline. Oxygen uptake (measured polarographically) and NADPH oxidation (measured spectrophotometrically) were assayed simultaneously as described in Chapter II. The assay system contained 20 mM phosphate buffer (pH 7.2); 0.25 M sucrose; 10 mM G-6-P; 0.3 mM NADP; 0.5 ml microsomes in a total volume of 2.5 ml. 0.02 ml G-6-P dehydrogenase and 0.05 ml 100 mM aniline were added as indicated.

A variety of organic substrates were tested for activity on the basis of their activity with animal microsomes or their possible involvement in plant metabolism (Table 2). A number of the substrates that were inactive supported liver microsomal MFO. Aniline and phenol activated horse radish peroxidase (no reference to the other compounds; Akazawa and Conn, 1958). The NADPH-aniline oxidase activity had a sharp pH optimum at 5.8 (Fig. 2) which was comparable with horse radish peroxidase (Gamborg *et al.*, 1961) but lower than animal MFO (8.0-8.5; Imai and Sato, 1959). Similar activities were detected in microsomes isolated from pea and wheat roots but not from beetroot or cotton and pea leaves.

2. Sensitivity to inhibitors and activators

MFO and peroxidase activities can be distinguished by testing sensitivity to effectors of electron transport, cytochrome P-450 and peroxidase (Table 3).

(a) Electron transport effectors

Inhibition by electron transport inhibitors may indicate MFO as this activity involves an electron transport chain. Inhibition by amytal and electron acceptors (which divert electron flow from cytochrome P-450) indicated reductase involvement. Cyanide (but not azide) inhibited strongly in contrast to the insensitivity of both animal and plant MFO. However, both animal microsomal lipid desaturation (Oshino *et al.*, 1966) and sterol demethylation (Gaylor and

TABLE 2. Specificity of the turnip microsomal NAD(P)H oxidase for the organic compound. Assay conditions are as described in Chapter II. Activities are expressed as a percentage of the NADPH oxidation rate with aniline (0.7 µmole NADPH oxidized/min/mg protein).

Substrate (2mM)	Relative Activities	
	NADH	NADPH
aniline	75	100
DMA	70	90
N-methylaniline	43	76
phenol	-	14
p-aminobenzoic acid	-	6
p-chloroaniline	-	31
m-chloroaniline	-	5
o-chloroaniline	-	9

The following compounds gave no activity: cinnamic acid; azobenzene; alcohol; trimethylamine; gibberellic acid; amphetamine sulphate; indole; IAA; formic acid; colchicine; 8-hydroxyquinoline; p-dichlorobenzene; p-aminophenol; dichloromethyl urea; paraquat; diquat; phenacetin; tryptamine; p-phenylenediamine HCl; nitrobenzene; phenylalanine; tyrosine; toluene; o-aminobenzoic acid; acetanilide; catechol; diphenylamine; benzene; α naphthylacetate; phenylurethane; tryptophan.

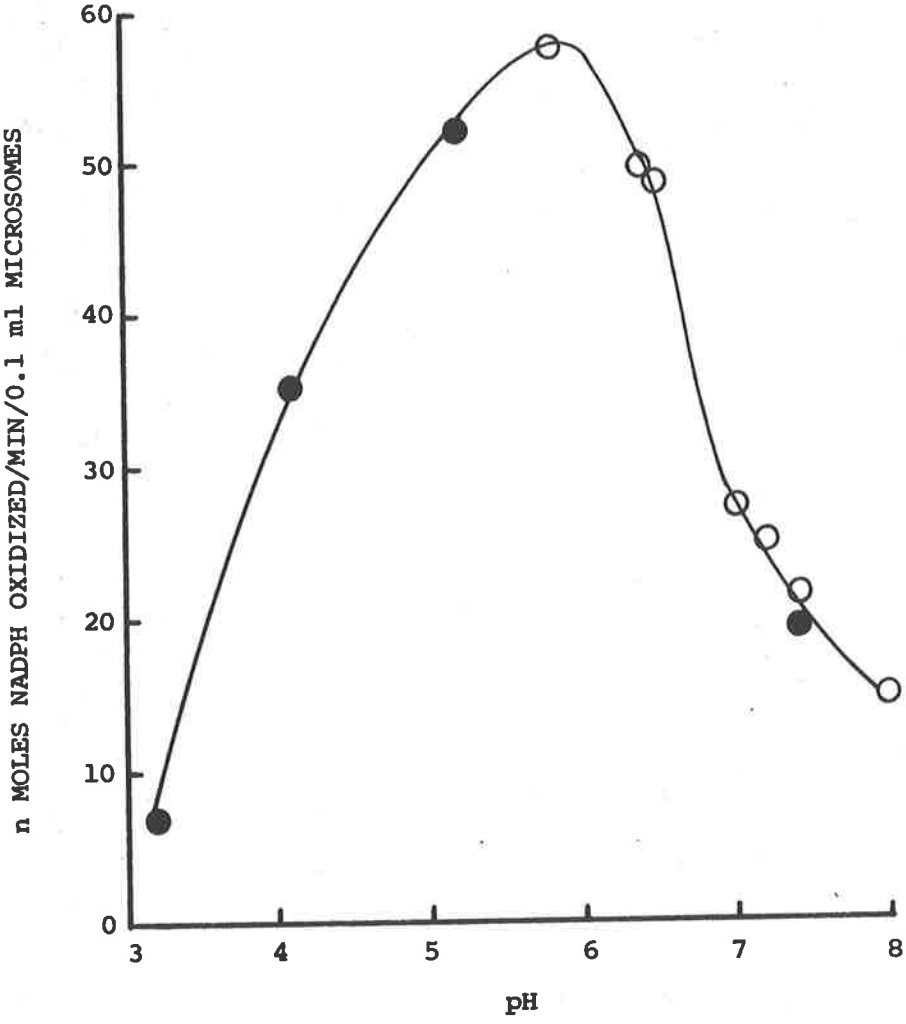


Figure 2. pH optimum of the turnip microsomal NADPH-aniline oxidase. Activity was assayed spectrophotometrically (340 nm) as described in Chapter II using 0.3 mM NADPH and 20 mM tris-acetate (●) and phosphate (○) buffers.

TABLE 3. Characterization of the turnip microsomal (and soluble) NADPH-aniline oxidase reaction. Assay conditions are as described in Chapter II. (* + = stimulation)

MICROSOMAL ENZYME

Compound	% Inhibition*		Compound	% Inhibition*	
	NADPH oxidation (340nm)	O_2 consumption		NADPH oxidation (340nm)	O_2 consumption
$2x10^{-5}$M pCMB	8	13	$5x10^{-6}$M CCCP	0	-
CO	22	47	10^{-3}M DNP	0	-
10^{-3}M SKF525A	29	30	10^{-3}M NH_4Cl	0	-
$3x10^{-3}$M Amytal	21	48	10^{-3}M $(NH_4)_2SO_4$	+14	-
1µg/ml Antimycin A	-	13	10µl Alcohol	0	0
10^{-4}M Cyanide	86	82	$8x10^{-3}$M EDTA	+11	2
10^{-4}M Azide	-	23	$2x10^{-4}$M $CuCl_2$	-	70
$5x10^{-4}$M Cyt. c	100	96	$2x10^{-4}$M $Pb(NO_3)_2$	-	0
$4x10^{-5}$M DCPIP	100	100	$2x10^{-4}$M $MgCl_2$	-	0
$8x10^{-4}$M FeCN	-	100	$2x10^{-4}$M KCl	-	+14
0.05% Triton X-100	+38	+49	$2x10^{-4}$M $ZnCl_2$	-	0
Boil (2 mins)	100	100	$2x10^{-4}$M $FeCl_3$	0	+20
$5x10^{-4}$M $MnCl_2$	+100	-	10^{-3}M ADP	0	-
10^{-5}M H_2O_2	+72	-	$2x10^{-4}$M $FeCl_3$) + 10^{-3}M ADP)	0	-
Catalase	95	-			

SOLUBLE ENZYME

$5x10^{-4}$M $MnCl_2$	+100	-	10^{-4}M Cyanide	+5	-
10^{-5}M H_2O_2	+440	-	$2x10^{-5}$M pCMB	0	-
Catalase	95	-			

Mason, 1968), and horse radish peroxidase (Gamborg *et al.*, 1961) are cyanide-sensitive (cyanide-sensitivity of the peroxidase varies depending on the affinity of the particular state of the peroxidase for cyanide; Yamazaki *et al.*, 1967). pCMB strongly inhibited both animal and plant microsomal reductases (Chapter III) and MFO (Chapter I) but inhibited the turnip microsomal NADPH-aniline oxidase only 10%. Cyanide inhibition could be completely reversed by pCMB. This effect could be repeated several times in the same assay and was observed both in NADH oxidation and oxygen consumption (Fig. 3). Cyanide inhibition could be similarly relieved with $HgCl_2$ but not with $MgCl_2$, $CaCl_2$ or choline chloride. The ratio of cyanide : pCMB (or $HgCl_2$) for complete recovery from inhibition was about 10 : 1. These results suggest a peroxidase reaction in which pCMB destroys the cyanide-peroxidase complex in a similar manner to its effect on the para-peroxidase (Yamazaki *et al.*, 1966).

(b) Cytochrome P-450 effectors

As CO binds both with cytochrome P-450 (Omura *et al.*, 1965) and (variably) with peroxidase (Nicholls, 1962), the partial inhibition of the NADPH-aniline oxidase is inconclusive. Triton X-100 stimulated activity but inhibited both animal (Mason *et al.*, 1965) and plant (Frear *et al.*, 1969) microsomal MFO, by altering (in animal microsomes) the structure of cytochrome P-450 and the non-haem iron protein (Chapter I, Fig. 1). Triton X-100 also inhibited the turnip

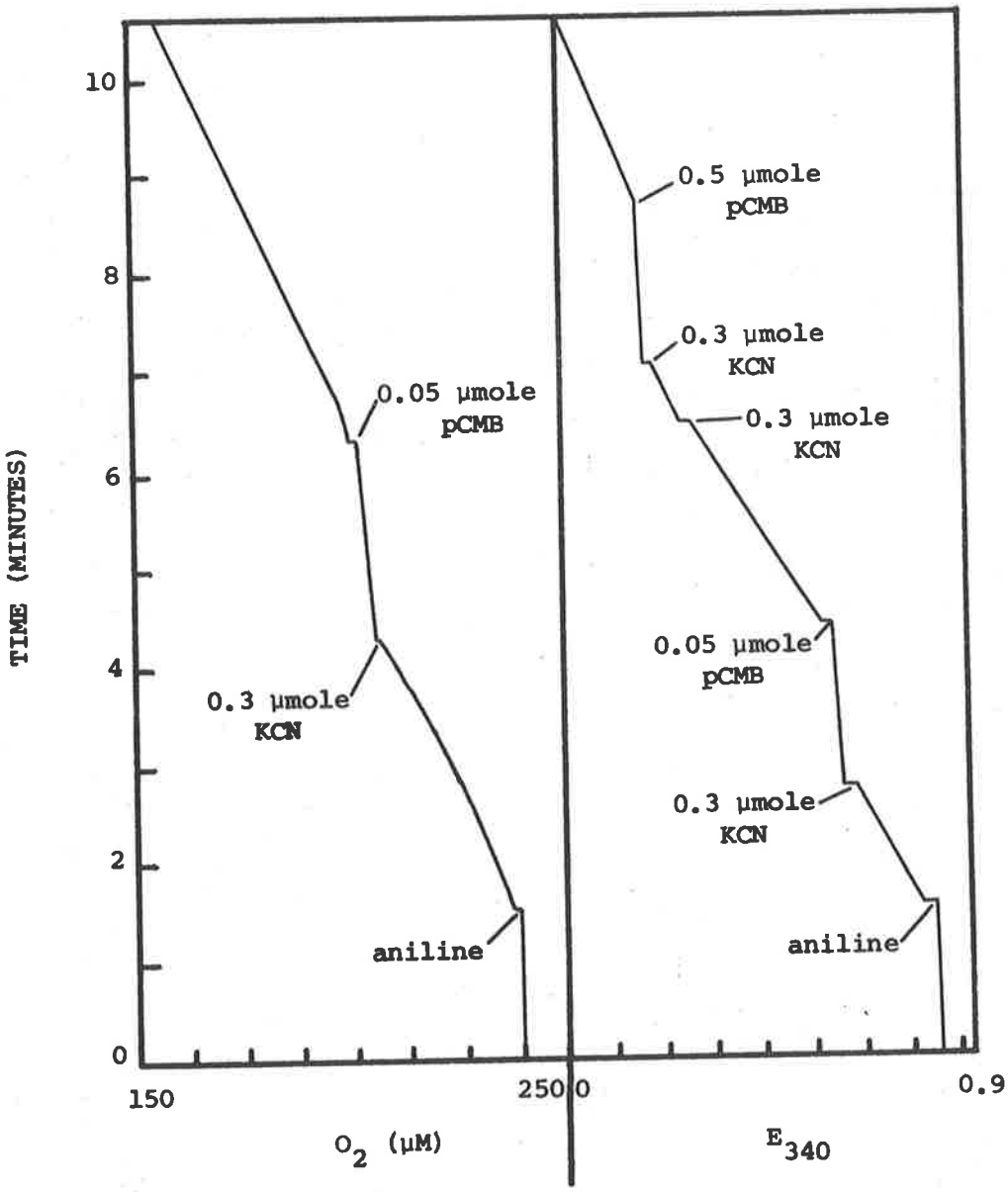


Figure 3. Effect of cyanide and pCMB on oxygen consumption and NADH oxidation by turnip microsomes in the presence of aniline. Oxygen uptake (measured polarographically) and NADH oxidation (measured spectrophotometrically) were assayed as described in Chapter II. The assay system contained 20 mM phosphate buffer (pH 7.2); 0.25 M sucrose; 0.3 mM NADH; 0.5 ml microsomes; 2 mM aniline (to start the reaction) in a total volume of 2.5 ml. Cyanide and pCMB were added as indicated.

microsomal NADPH-cytochrome c reductase (Chapter III).

(c) Peroxidase effectors

The peroxidase activators, Mn^{++} and peroxide (Nicholls, 1962), stimulated the turnip microsomal NADPH-aniline oxidase, while catalase inhibited. There was normally a lag at the start of the NADPH-aniline oxidase reaction. This was probably due to synthesis of catalytic amounts of peroxide (Gamborg *et al.*, 1961) as it could be eliminated by the addition of peroxide. The catalase inhibition indicated that, even in the absence of added peroxide and Mn^{++} the majority of activity was due to peroxidase.

(d) Others

The only heavy metal to significantly inhibit the turnip microsomal activity (Cu^{++}) also inhibited cotton microsomal N-demethylation (Frear *et al.*, 1969). The stimulation of O_2 consumption by $FeCl_3$ indicated lipid peroxidation (Hochstein and Ernster, 1963). However, there was no corresponding effect on NAD(P)H oxidation in presence or absence of aniline and ADP (Chapter I, Fig. 1). SKF 525A is a characteristic inhibitor of microsomal MFO in animals (Orrenius, 1965) but not plants (Frear *et al.*, 1969). It consistently inhibited the turnip microsomal activity 15-30%. In testing for product inhibition, the predicted products of the reactions given in Table 1 did not inhibit the reactions. Instead it was found that there was 100% inhibition of the reactions with DMA, aniline and phenol by

o-aminophenol and o-aminobenzoic acid. This may indicate hydroxylation at the ortho rather than the para position.

3. Products

While the organic substrate is consumed in MFO reactions it is normally recycled by the peroxidase and only hydroxylated under exceptional circumstances (Buhler and Mason, 1961). Hence detection of products (and loss of substrate) may indicate a MFO reaction - even in the presence of high peroxidase activity. However, this is complicated by the results of Murphy and West (1969) who showed that the plant and animal systems did not yield the same products from a particular substrate. The expected products of animal microsomal mixed function oxidation of DMA, aniline and phenol are given in Table 1.

(a) DMA

After 15 minutes incubation 0.3 µmole O_2 had been consumed but only 0.02 µmole formaldehyde and 0.004 µmole DMA-N-oxide were formed. (Corresponding to this 0.06 µmole cytochrome c was reduced by NADPH.)

(b) Aniline

No p-aminophenol could be detected using microsomes with active NADPH-aniline oxidase activity. Incubating microsomes with p-aminophenol resulted in no change demonstrating that p-aminophenol was not further metabolised. Also, no significant change could be detected

in total aniline concentration. Using three paper chromatography methods (Bratton and Marshall, 1939; Posner *et al.*, 1961; Gram *et al.*, 1968) aniline and p-, m-, and o-aminophenol could all be separated. However, on termination of the reaction only aniline could be detected indicating that none of these products were formed.

(c) Phenol

After one hour incubation there was no change in phenol concentration and no indication of any of the predicted products.

B. SOLUBLE NAD(P)H OXIDATION REQURING O_2 AND ORGANIC SUBSTRATE

A similar NAD(P)H oxidation was detected in the soluble supernatant fraction, requiring O_2 and DMA, aniline or phenol. On a protein basis it was 5-10 x more active than the microsomal enzyme. It closely resembled the purified horse radish peroxidase (Chapter I) but differed in some aspects with the turnip microsomal activity (Table 3). The soluble enzyme showed greater stimulation with H_2O_2 and was cyanide-insensitive. These results again indicate a predominance of peroxidase activity, the difference in cyanide-sensitivity resulted from different states of the same enzyme (Nicholls, 1962).

C. MICROSOMAL AND SOLUBLE PEROXIDASE

1. Characterization

In this section the presence of peroxidase activity in the microsomal and soluble fractions was tested using an assay specific for peroxidase (Gregory, 1966). Some of the properties of the

peroxidase using this method were compared with the properties of the NADPH-aniline oxidase to establish their synonomy. Experiments were also performed in an attempt to establish whether the microsomal peroxidase was specific to this fraction or randomly bound during homogenization.

Using Gregory's method, there was 9 x more peroxidase in the soluble fraction than the microsomes (on a protein basis) (Fig. 6). The microsomal peroxidase activity was considerably less cyanide-sensitive (16% inhibition) than the NADPH-aniline oxidase (Table 3) probably due to increased peroxide in the former assay medium. The soluble activities were both completely cyanide-insensitive.

The turnip soluble fraction was also rich in the peroxidase 'b'-type haem which has absorption maxima at 403 (oxidized) and 422, 553 and 582 nm (dithionite reduced). Similar spectra were observed in the soluble fractions of parsnip and swede but not beet. Cyanide caused a 10 nm shift in the γ peak of the oxidized but not reduced peroxidase (Table 4). This was reversed by pCMB in a similar manner to the cyanide inhibition of the NADH-aniline oxidase (Fig. 3) indicating that this activity was due to peroxidase.

Care must be taken in differentiating between this peroxidase and cytochrome b_{555} in the microsomes (Chapter III). In turnip microsomes absorption maxima were at 424, 525 and 555 nm (dithionite reduced). The oxidized γ peak was at 413 nm and was unaffected by either cyanide or pCMB (Chapter III). Hence the majority of this

TABLE 4. Properties of the absorption spectrum of the soluble peroxidase from turnip. Determination of absorption peaks is described in Chapter II.

Treatment	Absorption Maxima (nm) α	γ
oxidized	524	403
oxidized + 10^{-4}M KCN	525	413
oxidized + 10^{-5}M pCMB	524	403
oxidized + 10^{-4}M KCN + 10^{-5}M pCMB	524	400
reduced (dithionite)	553	422
reduced + 10^{-4}M KCN	553	422

TABLE 5. Peroxidase activity in microsomal subfractions from fresh and aged turnip tissue. The method of subfractioning follows Dallner (1963) as described in Chapter II. Distribution of other activities are in Chapter III (Table 11). Peroxidase was assayed by the method of Gregory (1966) as described in Chapter II and expressed in μmoles ascorbate oxidized/min/mg protein.

Hours Aged	Fraction 1 (pellet in CsCl)	Fraction 2 (layers in $MgCl_2$) Top	Middle	Bottom
0	-	0.28	0	0.24
58	10.06	0	0.39	3.38

absorption was attributed to the microsomal cytochrome.

2. *Specificity of membrane binding*

The inability to solubilize a membrane bound activity has been used as a criterion against random absorption of the activity (Hallaway *et al.*, 1970). Figure 4 shows that no turnip microsomal peroxidase activity could be solubilized by either sonication or Triton X-100. This was further investigated by testing the random absorption of turnip soluble peroxidase onto beet microsomes. Beet microsomes have been shown to be enzymically similar to turnip microsomes Chapter III) but have no NADPH-aniline oxidase or peroxidase activity, whether isolated from fresh or aged tissue. Figure 5 shows that there was absorption of turnip peroxidase onto the beet microsomes which could only be partially removed by washing and sonication. Hence microsomal membranes do to some extent randomly absorb peroxidase which cannot be subsequently completely solubilized i.e. the inability to solubilize need not necessarily indicate a specific membrane-bound enzyme.

Fractionating the microsomes by the method of Dallner (1963) (Chapter II) resulted in a number of subfractions, one of which had no peroxidase activity (Table 5). With tissue aging, very little activity developed in this subfraction (in contrast to the others). Results in Chapters III, IV and VII indicate that this may be the only microsomal subfraction not originating from the ER. Hence, either different

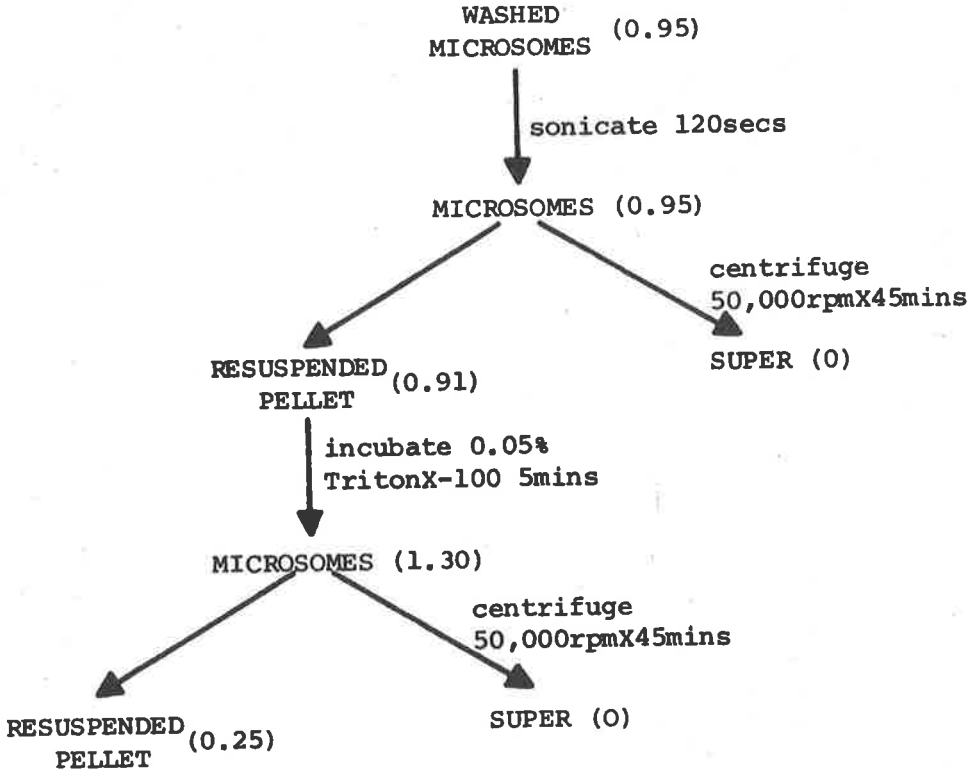


Figure 4. Attempted solubilization of turnip microsomal peroxidase. Washed microsomes were prepared as described in Chapter II and treated as indicated in the Figure (after centrifuging, pellets were resuspended in an equal volume). The figures in parentheses are the peroxidase activities (in μmoles ascorbate oxidized/min/0.05 ml) assayed as described in Chapter II (Gregory, 1966).

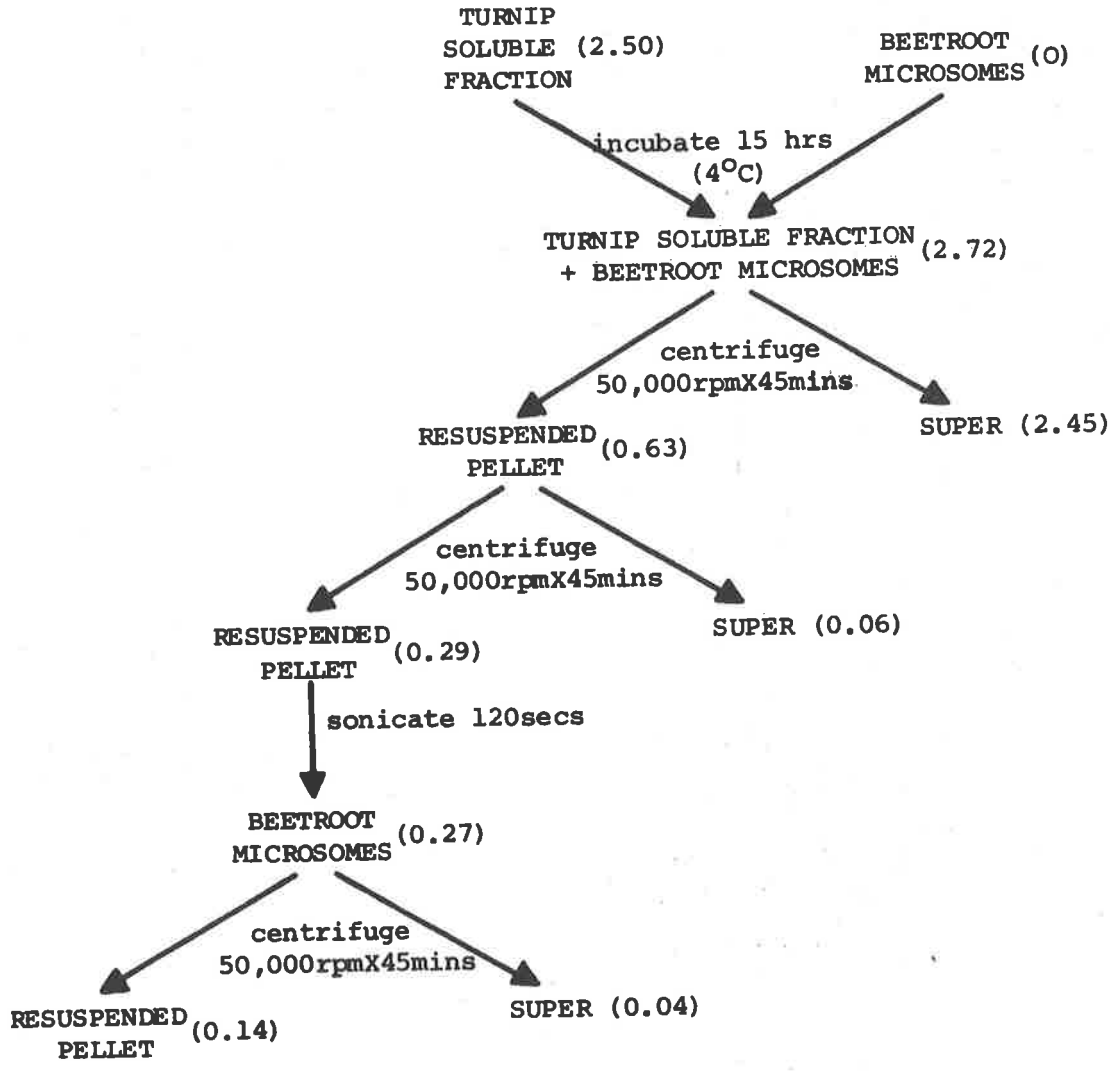


Figure 5. Absorption and attempted solubilization of turnip soluble peroxidase on beetroot microsomes. Beetroot microsomes and turnip soluble fraction were prepared as described in Chapter II and treated as indicated in the Figure. The figures in parentheses are the peroxidase activities (in μmoles ascorbate oxidized/min/0.05 ml) assayed as described in Chapter II (Gregory, 1966).

membranes differ in their ability to absorb soluble peroxidase *in vitro*, or the binding of peroxidase is specific for certain membranes *in vivo*.

D. AGING INDUCED CHANGES IN MICROSOMAL AND SOLUBLE NAD(P)H-ANILINE OXIDASE AND PEROXIDASE

As aging induces increases in the ER (Chapter IV), changes with aging in the microsomal NADPH-aniline oxidase and peroxidase activities were studied (i.e. corresponding to the drug-induced increases in MFO activity and ER in liver). Changes in the soluble fraction were also followed to determine whether they paralleled changes in the microsomes or whether there were specific changes in these fractions.

Figure 6 shows that, after the first 20 hours of aging, peroxidase activity started to increase at a much greater rate in the microsome than the soluble fraction. Over 70 hours aging there was a corresponding increase in the microsomal NADPH-aniline oxidase (10-fold). This was clearly not related to the microsomal NADPH-cytochrome c reductase (Chapter IV) which increased only 2-fold over the same period and could not be detected after 130 hours. The unproportional increase in microsomal peroxidase relative to the soluble peroxidase (Fig. 6) is not likely to result from increased binding, and strongly indicated specific synthesis within this fraction. The results of Kanazawa *et al.* (1967) also indicated specific synthesis of peroxidase

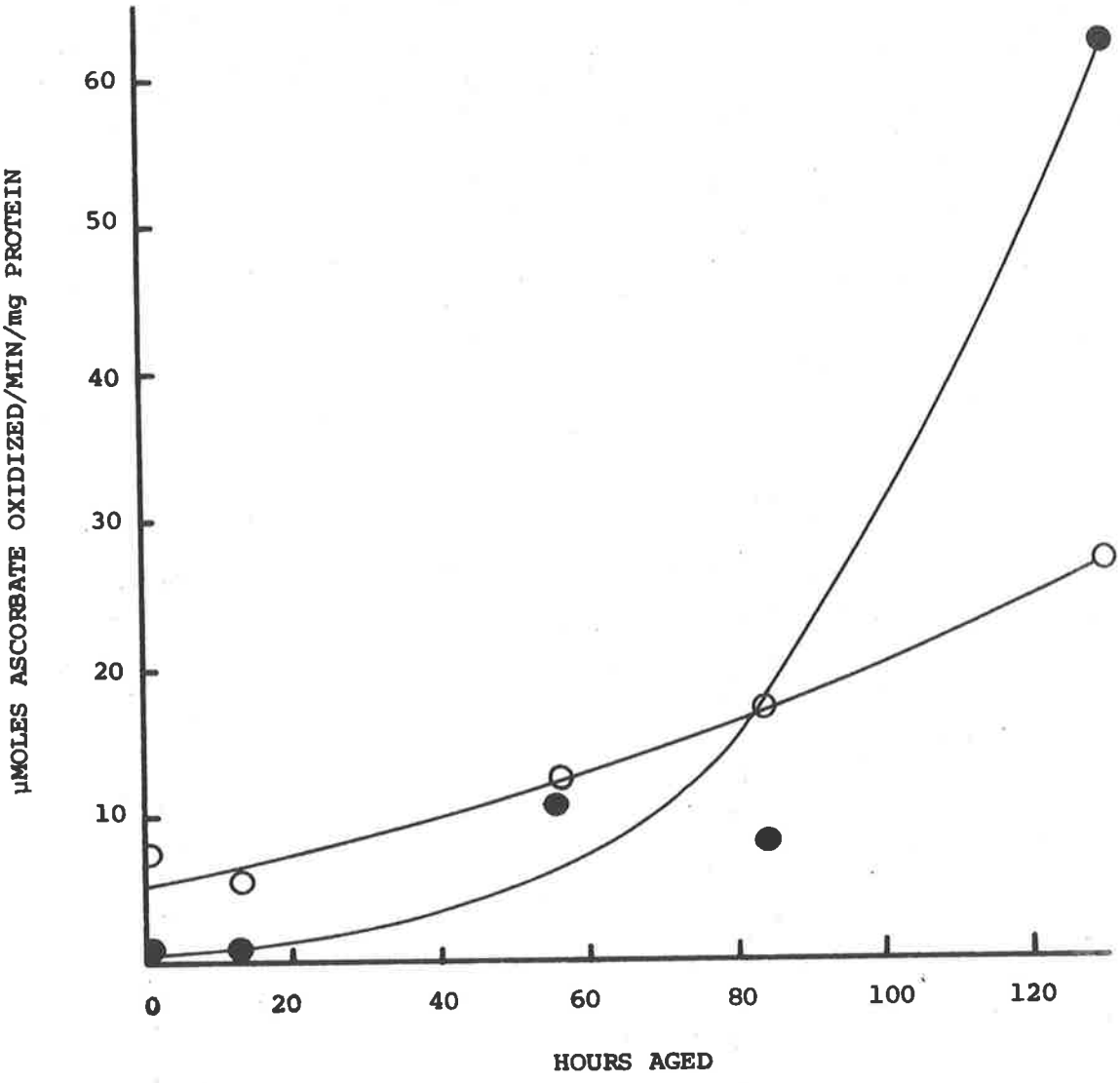


Figure 6. Aging-induced increase in turnip microsomal (●) and soluble (○) peroxidase. Disks were aged and peroxidase assayed (Gregory, 1966) as described in Chapter II.

in the mitochondrial, microsomal and soluble fractions in aging sweet potato disks. Kanazawa *et al.* (1967) further showed that the increase in peroxidase activity in the microsome fraction over the first 24 hours aging was not accompanied by an increase in the haem group. This is important when considering changes in cytochrome b_{555} with aging (Chapter IV).

Sephadex (G-200) elution of the crude soluble fraction yielded a single peak of peroxidase activity (Fig. 7) coinciding with the protein peak (Chapter V, Fig. 3). Aging induced an increase in this peak as well as the development of a second faster-eluting peak (Fig. 7). This resembled the development with aging of a second slower eluting peak of soluble NADH-cytochrome c reductase activity (Chapter V, Fig. 4). Both peaks of peroxidase were detected in fresh pea root soluble fraction in agreement with Janssen (1970).

Increases in soluble peroxidase under various conditions have been shown to be generally sensitive to both protein and RNA synthesis inhibitors (Kanazawa *et al.*, 1967; Bastin, 1968; Gayler and Glasziou, 1968; Parish, 1968; Asahi and Majima, 1969). Soluble peroxidase development was inhibited in aging turnip disks by 3.6×10^{-6} M cycloheximide and 10^{-3} M ethionine (although the latter effect may be due to limitation of ATP; Chapter V). Figure 6 (Chapter V) shows the effect of incubating turnip disks in cycloheximide for a short time on the subsequent development of soluble peroxidase activity. It

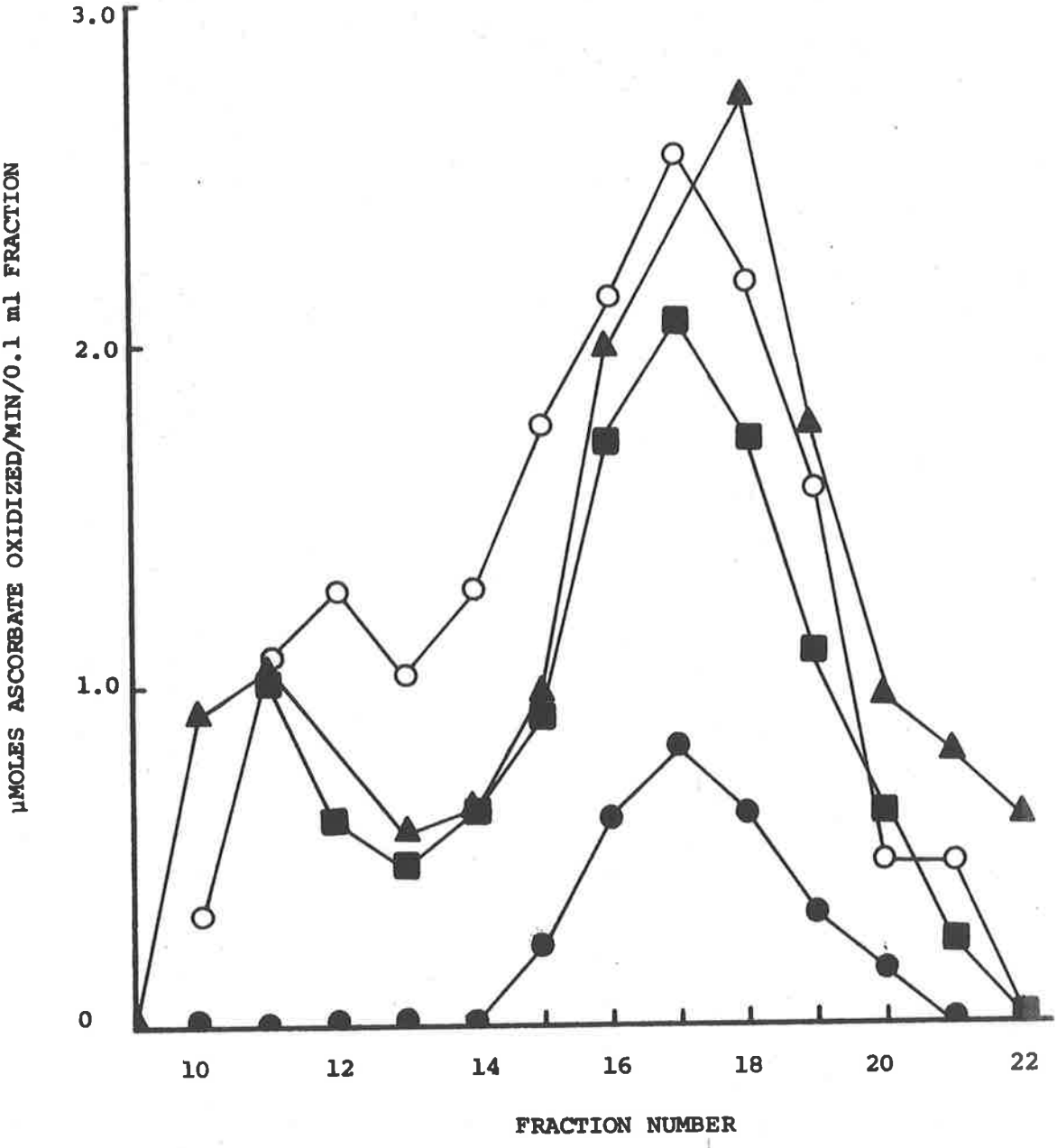


Figure 7. Sephadex G-200 elution sequence of soluble peroxidase from pea root and fresh and aged turnip tissue. 10 ml soluble fraction was eluted through column (100 cm x 1.1 diam.) and, after a void volume of 25 ml, 5 ml fractions were collected. Sephadex elution and peroxidase assay (Gregory, 1966) are as described in Chapter II. Pea root (○); fresh turnip (●); 40 hour (■) and 70 hour (▲) aged turnip.

appeared that cycloheximide inhibition of peroxidase was similar to that of the soluble reductases i.e. inhibition was reversible but there was a finite washing out time required. However, in this case the washing out time was longer (about 30 hours instead of 20 hours) (Chapter V, Table 6). This longer time may be due to inhibition of the peroxidase activity itself by cycloheximide (Chinnadurai and Govindaswamy, 1970).

DISCUSSION

The results indicate that the majority of the NADPH-aniline oxidase activity is due to peroxidase rather than MFO. However, there may be low MFO activity indicated by the low (but significant) level of formaldehyde detected after reaction with DMA. If so then it certainly did not correlate with the rates of O_2 uptake and NAD(P)H oxidation. However, there was a low catalase-insensitive oxygen uptake possibly indicating a second oxygen consuming reaction. Such an oxidase may still be a MFO of the type reported by Murphy and West (1969) as the products of the plant microsomal oxidation of kaurenoic acid were quite different to those of the animal microsomal oxidation. Hence the absence of the predicted products of aniline and phenol oxidations does not necessarily indicate lack of MFO activity. The NAD(P)H-aniline oxidase reaction does, however, resemble the animal microsomal lipid desaturation and sterol demethylation reactions (Chapter I, Fig. 1) in nucleotide specificity and cyanide sensitivity.

The problem of differentiating peroxidase activity in animal microsomes has rarely occurred although some workers have noted low contaminating activity (Gillette *et al.*, 1957; Das *et al.*, 1968). Peroxidase is generally associated with the lysosome fraction and therefore low activity may be associated with the smooth microsomes but not rough microsomes or mitochondria (Sedar, 1969).

The properties of the reported plant microsomal MFO (Chapter I, Table 1) are somewhat similar to the NADPH-aniline oxidase but quite different to the corresponding animal MFO. The differences between the reported animal and plant microsomal MFO (i.e. products formed, reduced nucleotide-specificity, SKF 525A-sensitivity) suggest quite a different process in plants to that of animals.

In plants the peroxidases may perform synthetic and detoxifying reactions equivalent to the MFO in animals. (Estabrook and Cohen (1969) have noted the similarity in mechanism of the MFO and peroxidase.) Lieb and Still (1969) have suggested that plant susceptibility to the herbicide proponil may depend on the substrate specificity of the peroxidase i.e. the peroxidase may be detoxifying compounds *in vivo*. Gage (1968) has demonstrated NADPH oxidation and O_2 consumption by liver microsomes in the presence of the herbicides paraquat and diquat, in which O_2 consumption was stimulated by peroxide. This activity may also be detoxifying but could not be demonstrated in turnip microsomes. While these sorts of reactions are feasible, there

is no indication of the likely function of the NAD(P)H-aniline oxidase which does not metabolize the organic compound required for activity. This reaction may:

(i) not take place in intact tissue;

(ii) serve to oxidize cytoplasmic NAD(P)H;

(iii) react with other components (e.g. flavoprotein).

The specific association of peroxidase with particular microsomal membranes (Table 5), and the disproportional increase in the microsome fraction with aging (Fig. 6) indicate specific peroxidase binding rather than a random absorption during homogenization. Attachment may be a result of synthesis by the membrane bound ribosomes (with subsequent release of the peroxidase into the soluble fraction). However, there may be reasons for incorporation of peroxidase into the membranes e.g. interaction between the peroxidase and the flavoprotein (Galston *et al.*, 1953). Weliky *et al.* (1969) showed that horse radish peroxidase bound to carboxymethylcellulose and that this altered its cyanide- and azide-sensitivity. However, the changes were quite different to those apparently conferred upon the membrane bound peroxidase from turnip. These complex reactions are not fully understood (Nicholls, 1962) and were not considered further.

CHAPTER VII

MICROSOMAL AND SOLUBLE PHOSPHATASES IN FRESH AND AGED TISSUE

INTRODUCTION

The aim of this Chapter was to attempt to establish the presence of specific G-6-Pase activity in the turnip microsome and soluble fractions (Thompson, 1969) and ATPase activity in the microsome fraction (Chapter I). The acid phosphatase activities have been previously described in plants (Morton, 1961; Atkinson and Polya, 1967) and are only briefly considered here (mainly in distinguishing the other activities).

RESULTS

A. ACID PHOSPHATASE

Figure 1 shows that fresh turnip microsomal and soluble fractions contain approximately equal acid phosphatase activity (on protein basis). Table 1 shows that the acid phosphatase (activity with NaBGlP at pH 5.0) was highly sensitive to NaF and Na_2MoO_4 but only partially to tartrate (inhibitors of acid phosphatase; Dawson *et al.*, 1959; Hubscher and West, 1965; Gahan and McLean, 1967; Thompson, 1969) and EDTA (inhibitor of alkaline phosphatase; Hubscher and West, 1965; Thompson, 1969).

Figure 1 shows that with aging of tissue the acid phosphatase activity rapidly increased in the microsomes then subsequently

TABLE 1. Inhibitor sensitivity of turnip microsomal and soluble phosphatase activity with NaBGlP and G-6-P at varying pH. Assay conditions are as decribed in Chapter II.

Substrate	NaBGlP			G-6-P		
pH	5.0	6.5	8.0	5.0	6.5	8.0
	µmoles Pi/min/mg protein					
Microsomes	9.8	6.2	0	3.3	2.3	0
Soluble	26.5	16.3	2.2	15.0	10.6	2.9
	% Inhibition					
Microsomes 10mM NaF	99	66	-	100	100	-
Soluble						
5mM NaF	78	-	-	84	68	-
10mM NaF	86	62	43	-	61	54
50mM NaF	92	94	-	99	100	-
5mM EDTA	11	-	-	-	0	-
5mM EDTA + 5mM NaF	65	72	-	-	15	-
20mM Tartrate	25	-	-	-	46	-
5mM Na_2MoO_4	100	-	-	-	100	-

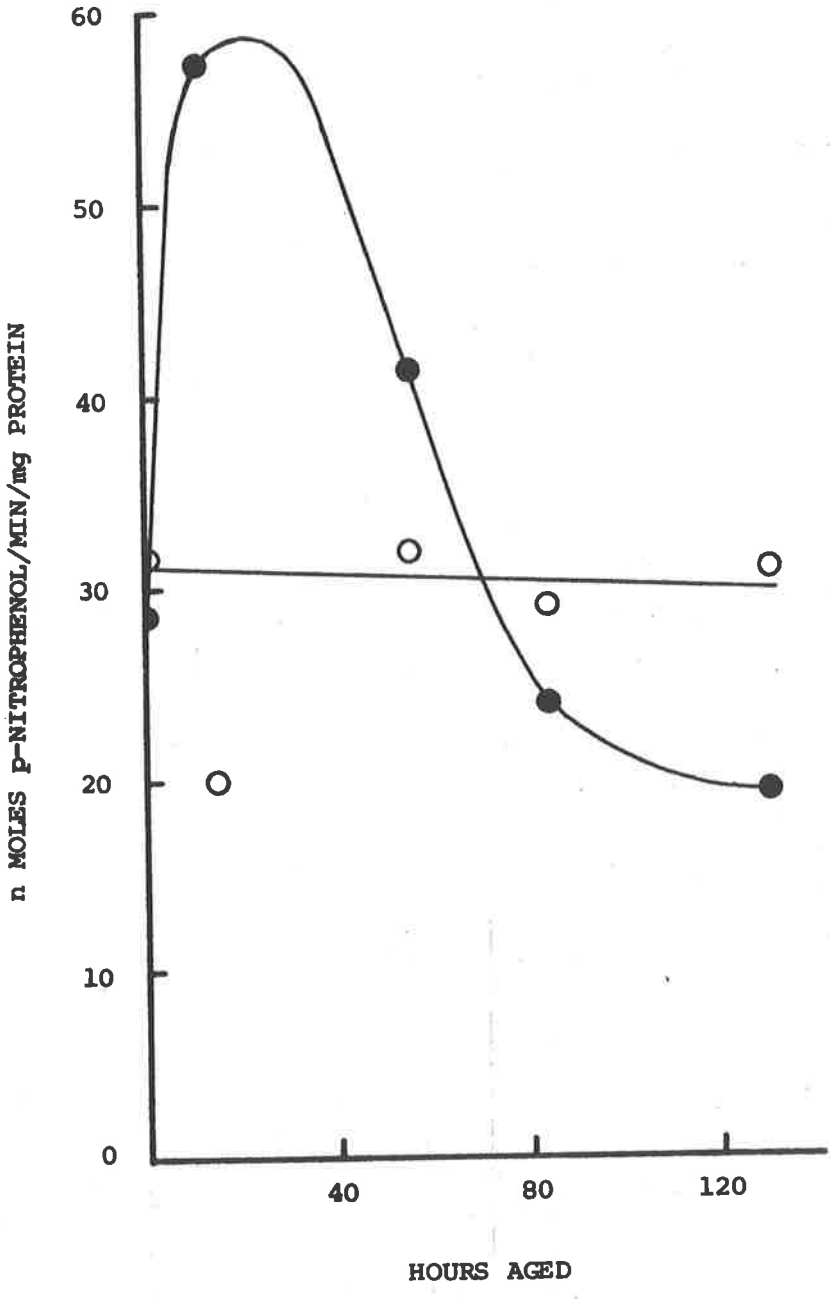


Figure 1. Aging induced changes in turnip microsomal (●) and soluble (○) acid phosphatase activity. Assay conditions are as described in Chapter II using p-nitrophenylphosphate as the substrate.

declined, only dropping below the fresh level after 80 hours. However, the soluble activity appeared to remain constant throughout the aging period. The specific increase in microsomal acid phosphatase indicated specific synthesis on the ER suggesting that the microsomal activity is not due to random absorption of soluble enzyme. The subsequent decline in microsomal activity may be a result of enzyme release. The expected increase in the soluble fraction could not, however, be detected due to the much higher (total) soluble acid phosphatase level.

B. G-6-PASE

The aim of this section was to determine if a specific G-6-Pase activity could be distinguished from the acid phosphatase in either the microsomes or soluble fraction. This was done by comparing the effects of inhibitors on the activities with NaBGlP (pH optimum 5.0) and G-6-P (pH optimum 6.5; Thompson, 1969) at varying pH (Table 1). Inhibitor sensitivities did appear to change with pH (i.e. relatively less sensitive at higher pH) but were the same for the two substrates. This suggested that activity with G-6-P was probably completely due to acid phosphatase.

Thompson (1969) assayed bean microsomal and soluble G-6-Pase at pH 6.5 in the presence of 4 mM NaF + 4 mM EDTA. However, NaF did not completely inhibit turnip soluble acid phosphatase at this pH, and EDTA appeared to relieve this inhibition with G-6-P but not NaBGlP. Hence it appeared that all phosphatase activity with G-6-P at pH 6.5

in the presence of 4 mM NaF + EDTA cannot be attributed to specific G-6-Pase.

C. MICROSOMAL ATPASE

1. Elimination of acid phosphatase activity

The pH profiles for microsomal phosphatase activity with ATP, in the presence of $MgCl_2$ and $MgCl_2$ + KCl are shown in Figure 2. At low pH, activity was probably due to the acid phosphatase which was partially inhibited by $MgCl_2$ (Atkinson and Polya, 1967). Only at or above pH 7.8 where the microsomal acid phosphatase was inactive (Table 1) did a KCl-stimulated $MgCl_2$-ATPase become evident. At pH 7.5, $MgCl_2$ had no effect as inhibition of the acid phosphatase and stimulation of the ATPase were equal. All subsequent reactions were assayed at pH 7.8. Similar activity was detected in artichoke but not beetroot microsomes.

2. Subfractionation of microsomal membranes

Table 2 shows the distribution of this ATPase in the various microsomal subfractions. Activity was present in all fractions but was concentrated in the smooth membranes. Hence in all ATPase assays the total smooth fraction 2 was used.

3. Conditions for optimum activity

Table 2 also shows that there was a requirement for both $MgCl_2$ and KCl i.e. it was only in the presence of $MgCl_2$ that KCl stimulated the ATPase activity. Figure 3 shows that 2 mM $MgCl_2$ was

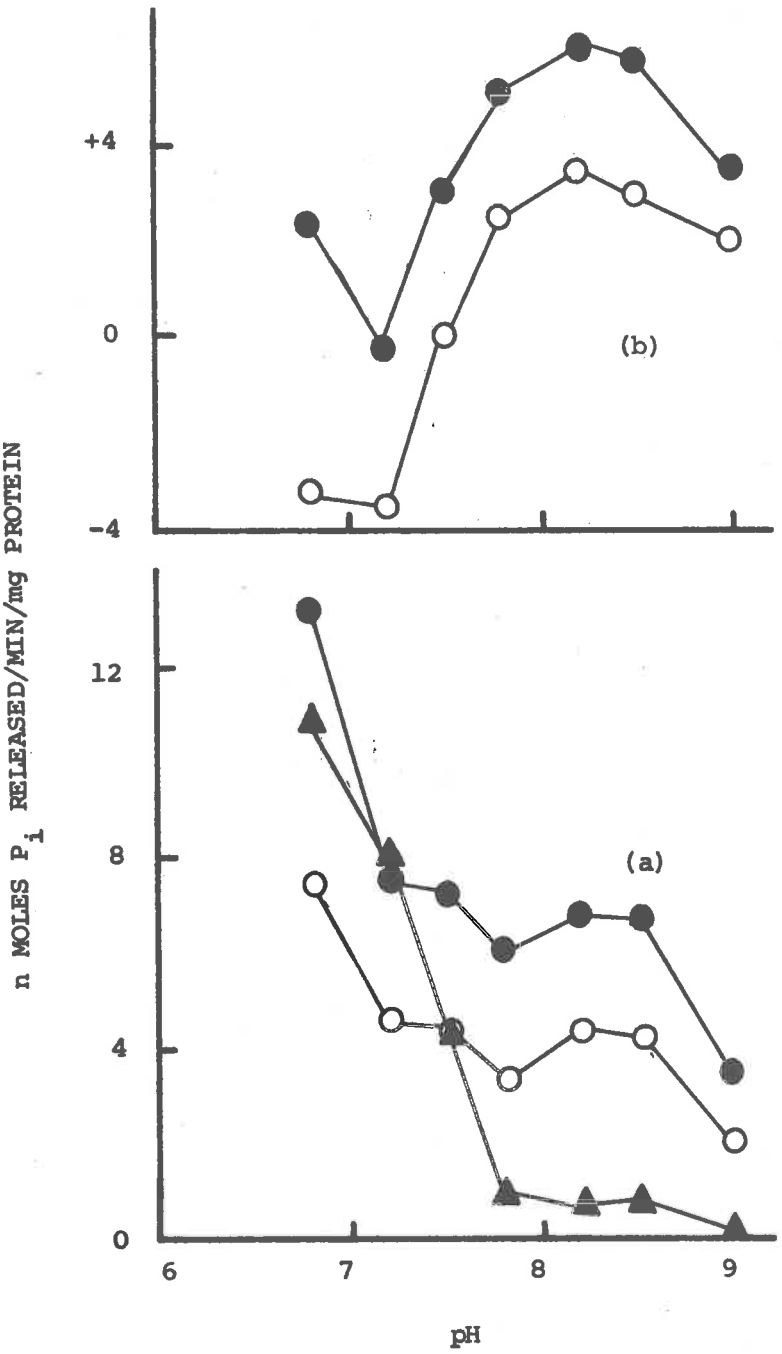


Figure 2(a). Effect of pH on the smooth, turnip microsomal ATPase activity in the absence of ions (▲) and in the presence of 2 mM $MgCl_2$ (○) and 2 mM $MgCl_2$ plus 50 mM KCl (●).

Figure 2(b). Effect of pH on the ATPase activity in the presence of ions with the control in the absence of ions subtracted. Assay conditions are as described in Chapter II.

TABLE 2. ATPase distribution in the microsomal subfractions from turnip. The method of subfractioning follows Dallner (1963) and is described in Chapter II. Assay conditions are described in Chapter II.

	Fraction 1 (pellet in CsCl)	Fraction 2	
		Layer in $MgCl_2$	Pellet in $MgCl_2$
Control) ATPase nmoles Pi/min/mg protein	0.8	2.3	3.0
+ 2mM $MgCl_2$)	2.6	7.0	8.8
+ 50 mM KCl)	1.6	3.8	5.4
+ (2mM $MgCl_2$ + 50mM KCl)	5.6	12.3	13.8

TABLE 3. Effect of divalent cations on the smooth microsomal (Fraction 2) ATPase activity. The divalent cations were added as chloride salts. Assay conditions are as described in Chapter II.

Divalent cation	ATPase activity (nmoles Pi/min/mg protein)	
	-	50mM KCl
-	2.06	4.46
2mM Mg	8.41	17.00
2mM Ca	3.26	3.09
2mM Mn	2.06	1.03
2mM Mg + 2mM Mg	11.51	15.98
2mM Mg + 2mM Ca	8.93	11.82
2mM Mg + 2mM Mn	2.92	2.75

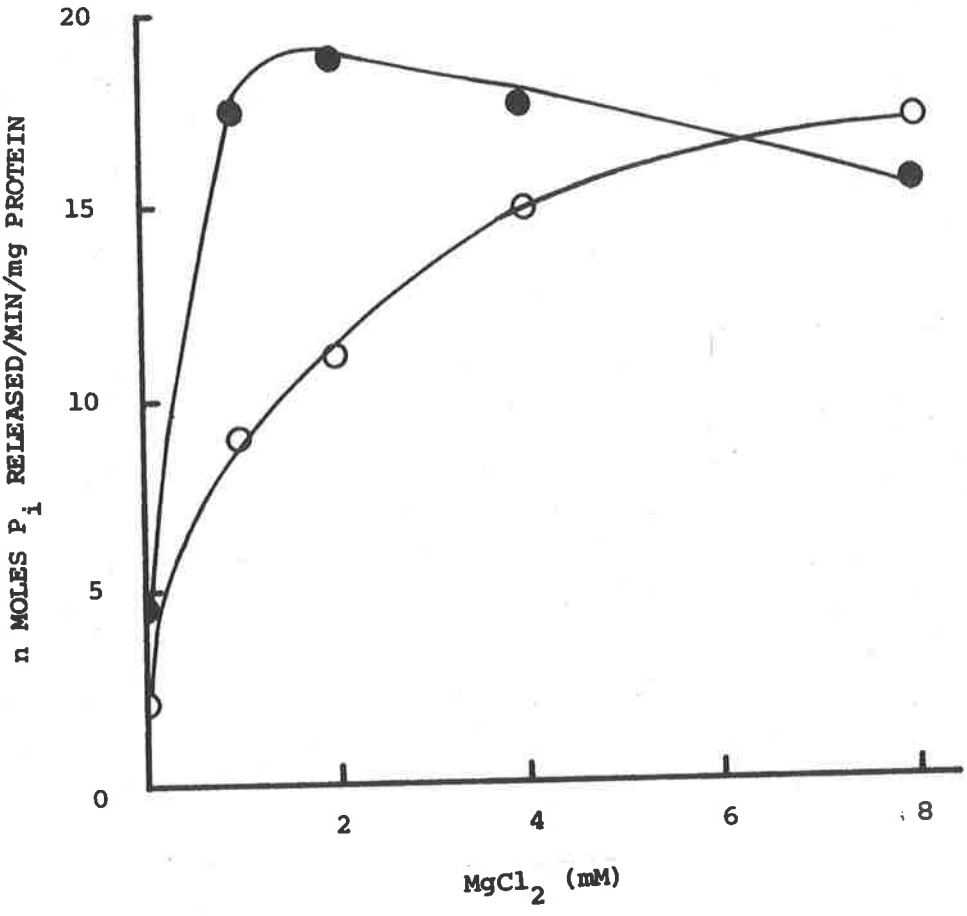


Figure 3. Effect of $MgCl_2$ on the smooth, turnip microsomal ATPase activity in the absence (○) and presence (●) of 50 mM KCl. Assay conditions are as described in Chapter II.

the most effective concentration for activation in the presence of 50 mM KCl. In the absence of KCl the increase in activity with higher concentrations of $MgCl_2$ was probably due to the increasing concentrations of Cl^- (Fig. 4). Ca^{++} and Mn^{++} did not substitute for Mg^{++} (Table 3). Also Ca^{++} partially inhibited, and Mn^{++} severely inhibited the KCl-stimulated activity in the presence of Mg^{++} (Table 3). Figure 4 shows that 50 mM was the most effective concentration of KCl in the presence of 2 mM $MgCl_2$. At higher concentrations there was considerable inhibition. Optimum ATP concentration in the presence of 2 mM $MgCl_2$ and 50 mM KCl was about 6 mM (Fig. 5).

4. Specificity of salt stimulation

The specificity of salt (50 mM) in stimulating the Mg^{++}-ATPase is shown in Table 4 (a, b, c). With the exception of NH_4Cl and CsCl, there was very little difference in the effects of the various cations (with Cl^- as the anion) (Table 4). Even choline and tris chorides were as effective as KCl. The smaller stimulation by Na^+ than by K^+ was not reproducible (see Table 7) and there was no significant synergistic effect between Na^+ and K^+. Table 4(b), however, shows that there was considerable variation in activity with different anions (with Na^+ and K^+ as the cations). This indicated that it was the anion rather than the cation species that was important in salt stimulation. Table 4(c) shows that a range of similar four-carbon organic anions all stimulated the Mg^{++}-ATPase and, with the exception of succinate,

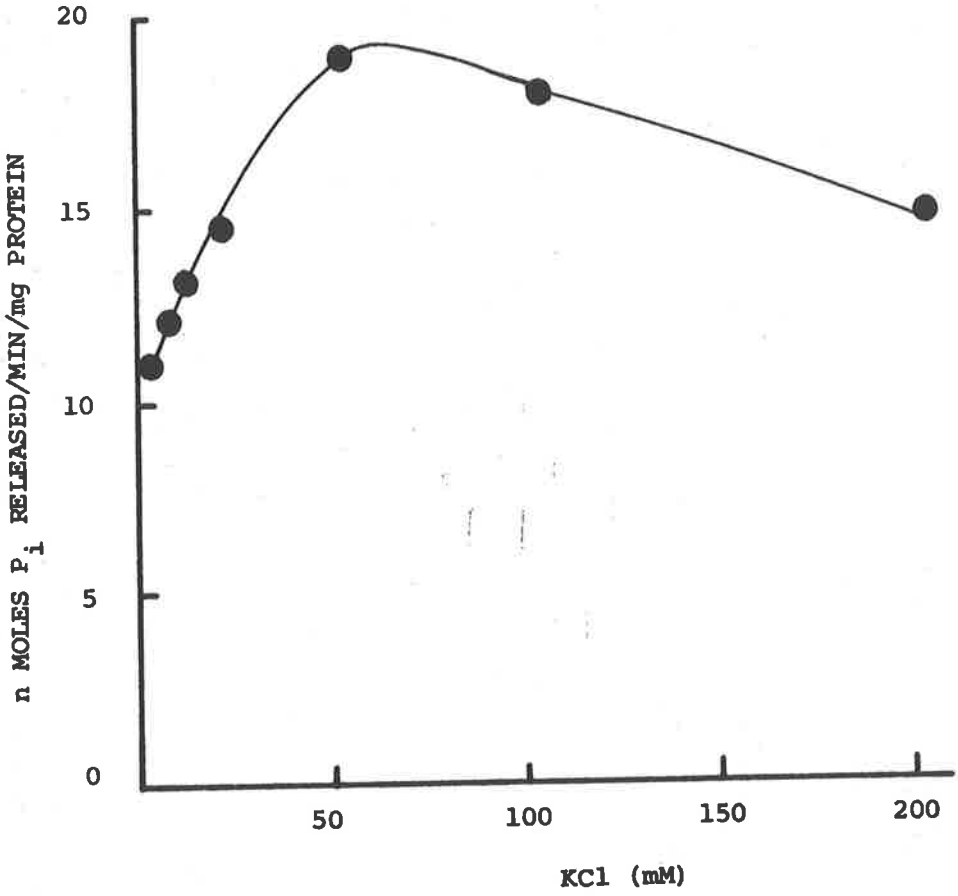


Figure 4. Effect of Cl^- on the smooth, turnip microsomal ATPase activity in the presence of 2 mM $MgCl_2$ (Cl^- added as KCl except for 4 mM added with the Mg^{++}). Assay conditions are as described in Chapter II.

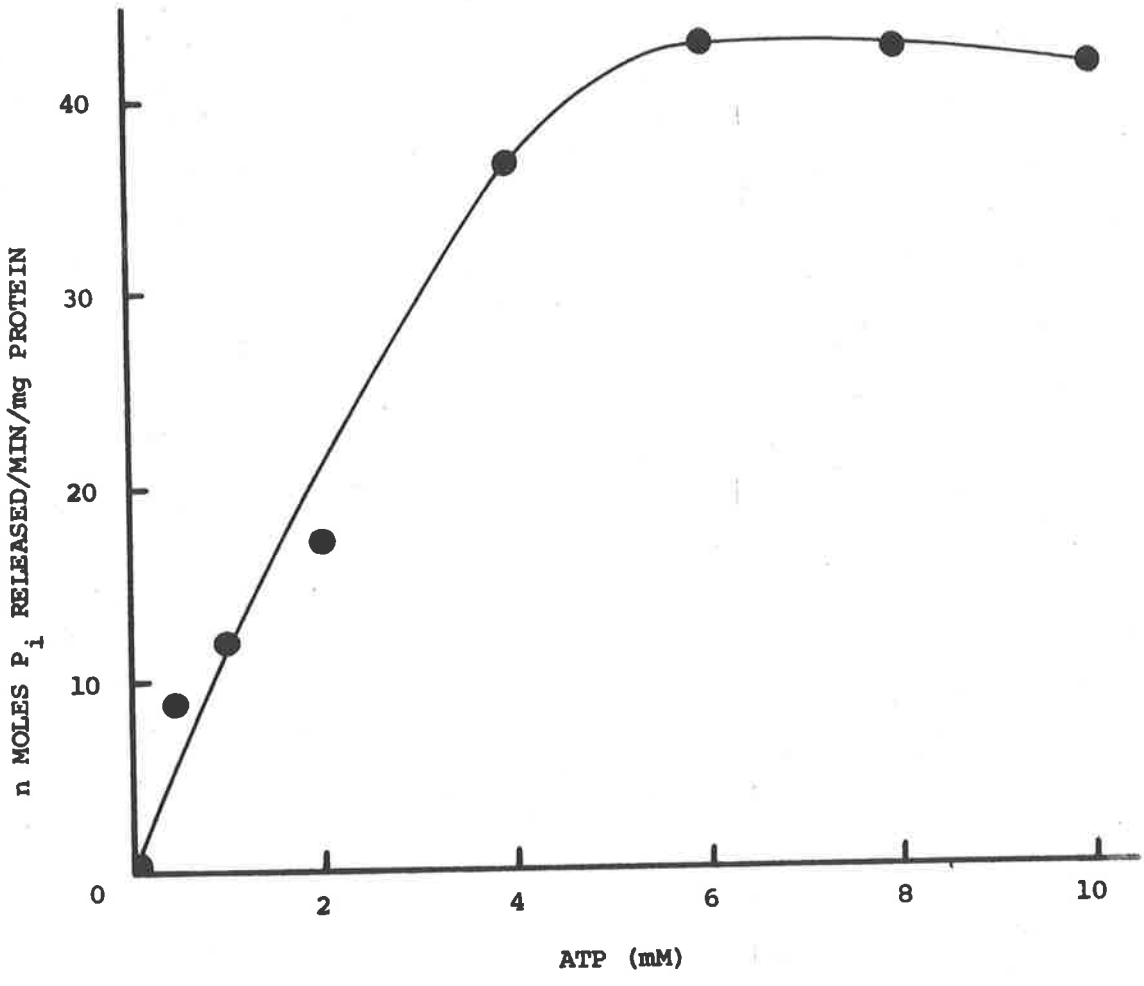


Figure 5. Effect of ATP concentration on the smooth, turnip microsomal ATPase activity in the presence of 2 mM $MgCl_2$ and 50 mM KCl. Assay conditions are as described in Chapter II.

TABLE 4. Effect of salts on the smooth microsomal (Fraction 2) ATPase activity in presence and absence of 2mM $MgCl_2$ (a) varying cation, (b) varying anion, (c) varying 4-carbon organic anion. The assay conditions are as described in Chapter II (* - = inhibition)

	ATPase activity (nmoles Pi/min/mg protein)			% Stimulation by salt in presence of Mg*
	-Mg	+Mg	Net	
(a)				
-	0.64	6.05	-	-
KCl	0.64	9.09	+3.04	49
NaCl	0.11	8.23	+2.18	35
NaCl + KCl	0.38	9.47	+3.42	56
Tris-HCl	0.78	9.47	+3.42	56
CholineCl	1.13	9.19	+3.14	51
CsCl	0.64	7.46	+1.41	23
RbCl	0.87	9.09	+3.04	49
LiCl	0.27	8.44	+2.39	39
NH_4Cl	1.13	14.01	+7.96	130
(b)				
-	2.73	5.71	-	-
KCl	3.40	8.99	+3.28	57
NaBr	3.14	7.31	+1.60	28
KI	1.09	3.66	-2.05	-36
$NaHCO_3$	1.99	8.47	+2.76	48
K_2SO_4	1.79	5.39	-0.32	-6
KNO_3	1.79	3.20	-2.50	-44
$NaC_6H_5SO_3$	1.48	3.91	-1.80	-32
KCH_3COO	3.59	7.76	+2.05	36
NaF	2.12	6.86	+1.15	20
(c)				
-	1.13	5.17	-	-
KCl	0.97	8.90	+3.73	72
Na Malate	0.97	12.46	+7.29	141
Na Succinate	0.65	7.76	+2.59	50
Na Fumarate	1.29	10.20	+5.03	97
Na Tartrate	1.29	11.81	+6.64	129
Na Aspartate	1.29	9.55	+4.38	85
Na Oxaloacetate	1.29	16.18	+11.01	213

to a greater degree than KCl.

5. Effect of membrane disruption

Electron micrographs (Chapter III) showed that fraction 2 microsomes consisted of smooth, single membrane vesicles. The effect of the detergents, digitonin and Triton X-100 (Fig. 6) and of sonication (Table 5) of the microsomes was tested to determine if such a structure limited ATPase activity. While very low concentrations of Triton stimulated activity six-fold, slightly higher concentrations inhibited. Digitonin, however, only slightly stimulated at the same concentrations demonstrating the different action of these two detergents on microsomal membranes. As the acid phosphatase activity was unaffected by Triton X-100, it was possible to further minimize acid phosphatase contamination by assaying with a very low concentration of microsomes in the presence of Triton. Under these conditions the pH optimum was 8.5 (Fig. 7). Sonication only slightly stimulated the ATPase. However, this effect increased if microsomes were sonicated in the presence of KCl.

6. Relation to other ATPases

Table 6 lists the sensitivity of the KCl-stimulated Mg^{++}-ATPase to a number of compounds. The anions which inhibited the Mg^{++}-ATPase {Table 4 (b)} also partially inhibited (at 5 mM) the KCl-stimulated Mg^{++}-ATPase. The marked inhibition by ADP (not observed with the acid phosphatase) supports the idea of a specific ATPase. The

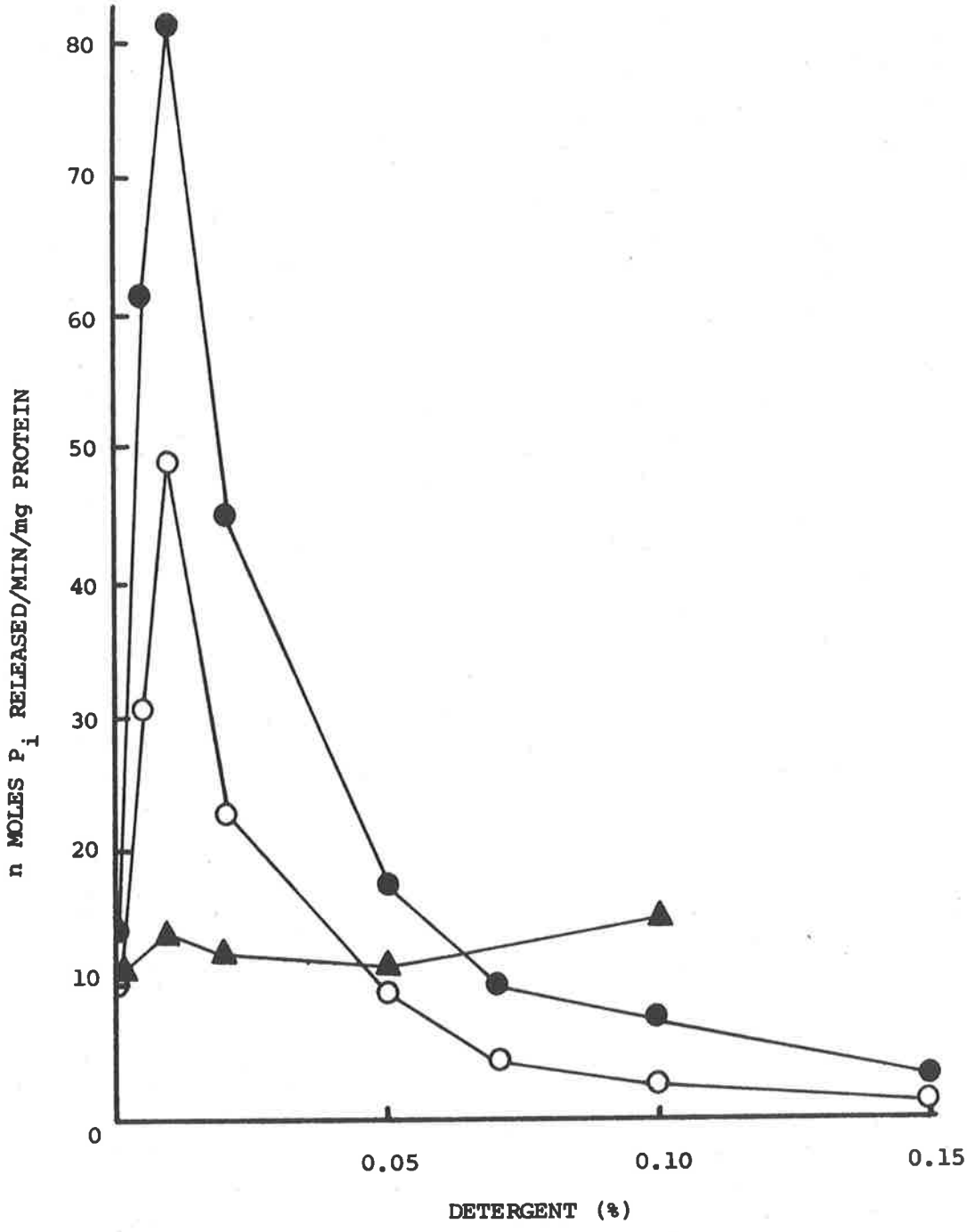


Figure 6. Effect of Digitonin in the presence of 50 mM KCl (▲) and Triton X-100 in the absence (○) and presence (●) of 50 mM KCl on smooth, turnip microsomal ATPase activity in presence of 2 mM $MgCl_2$. Assay conditions are as described in Chapter II. The detergents are included in the assay system.

TABLE 5. Effect of sonication of the smooth microsomes (Fraction 2) in the presence and absence of KCl on the ATPase activity. Microsomes were sonicated and then immediately assayed in the presence of 2mM $MgCl_2$ plus 50mM KCl as described in Chapter II.

Addition	Sonication time (secs.)					
	0	15	30	45	60	90
-	12.06	12.06	13.29	15.10	14.88	16.42
50mM KCl			22.35			
330mM KCl			25.65			

Activities are expressed in nmoles P_i/min/mg protein.

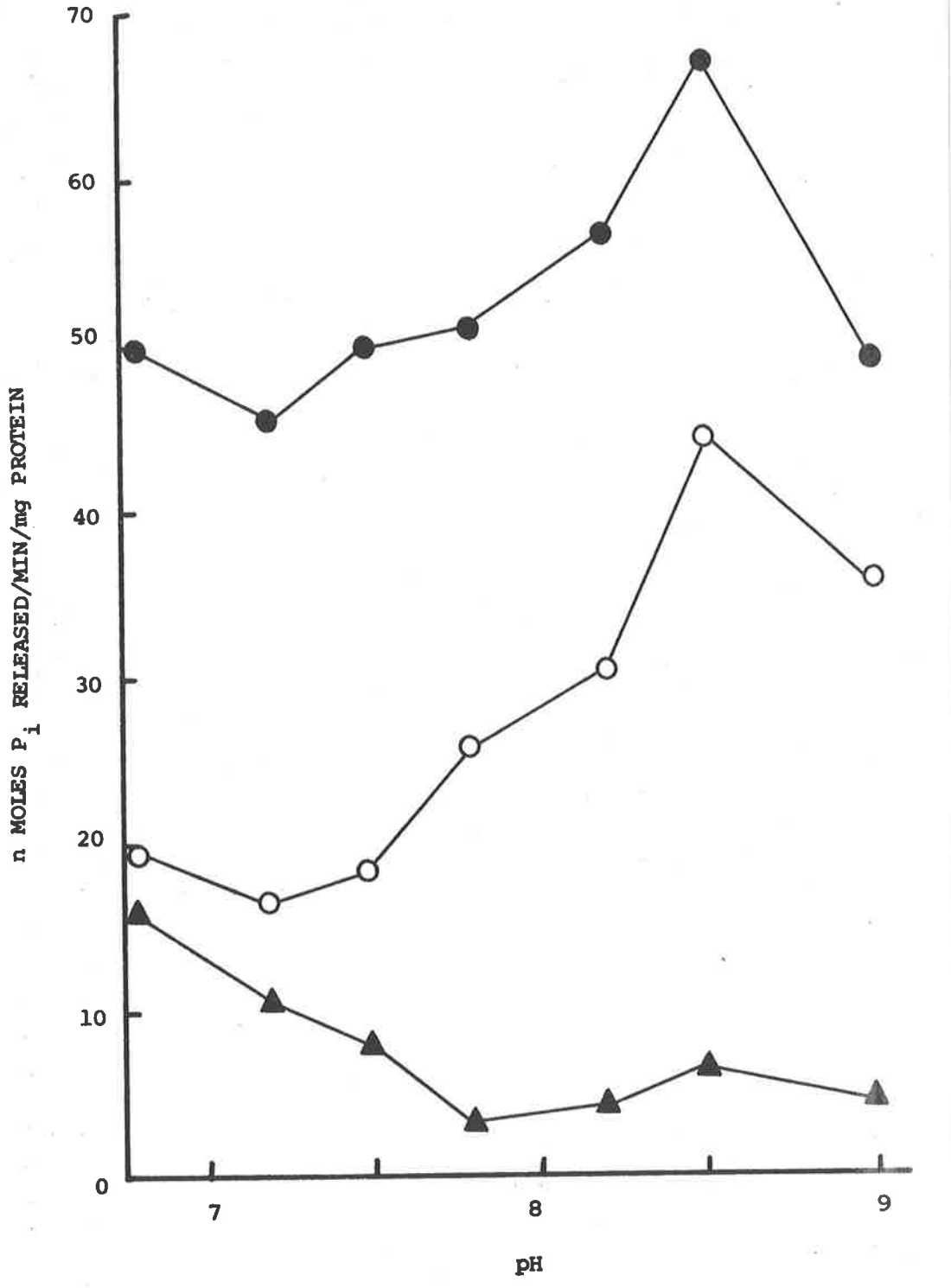


Figure 7. Effect of Triton X-100 on the pH profiles of the smooth, turnip microsomal ATPase activity in the absence of ions (▲) and in the presence of 2 mM $MgCl_2$ (○) and 2 mM $MgCl_2$ plus 50 mM KCl (●). Assay conditions are as described in Chapter II.

TABLE 6. Sensitivity of the smooth microsomal (Fraction 2) ATPase to various compounds. Activity was assayed in the presence of 2mM $MgCl_2$ plus 50mM KCl as described in Chapter II.

Compound		% Inhibition (-) or Stimulation (+)
5×10^{-3}M	K_2SO_4	-8
5×10^{-3}M	$NaHCO_3$	-5
5×10^{-3}M	KNO_3	-22
5×10^{-3}M	Na $C_6H_5SO_3$	-12
5×10^{-3}M	Na succinate	-2
7×10^{-4}M	NADH + NADP	-5
2×10^{-3}M	ADP	-57
4×10^{-3}M	ADP	-75
10^{-6}M	Mersalyl	-44
10^{-5}M	Ouabain	-6
5×10^{-5}M	Atractylate	-13
1 µg/ml	Oligomycin	+12
6×10^{-4}M	Phlorizin	0
5×10^{-4}M	DNP	+19
2×10^{-6}M	CCCP	+51
10^{-6}M	Valinomycin	+11
1.75×10^{-8}M	Nigericin	+44
1%	Alcohol	-26

lack of effect of NADH + NADP indicated that the plant microsomal ATP-dependent NADH-NADP transhydrogenase was not involved (Murphy and West, 1969). The activity was insensitive to ouabain.

The ATPase was also insensitive to inhibitors of the mitochondrial (oligomycin) and chloroplast (phlorizin) ATPases but was stimulated by the uncouplers CCCP and DNP as well as valinomycin and nigericin. Table 7 shows that there was a synergistic effect between CCCP and valinomycin such that the stimulation due to their combination was greater than the sum of the individual effects. The partial specificity for K^+ over Na^+ with valinomycin indicated that this was a specific effect rather than a non-specific detergent action on the membrane (Fig. 6).

7. *Changes with tissue aging*

Attempts were made to detect changes in ATPase activity in aging disks that could be correlated with the well established increase in ion accumulation (Laties, 1967). In turnip disks KCl accumulation increased 8-fold to a maximum after approximately 60 hours aging (Chapter VIII). However, there was not a corresponding change in the ATPase activity which only increased from 15.7 n moles Pi/min/mg protein (in fresh disks) to 25.7 (in 60 hour aged disks).

DISCUSSION

A. RELATION OF TURNIP MICROSOMAL ATPASE TO OTHER PLANT MICROSOMAL ATPASES

The results indicate that the turnip microsomes have a

TABLE 7. Effect of CCCP and valinomycin on the smooth microsomal (Fraction 2) ATPase in the presence of 2mM $MgCl_2$ plus either 50mM KCl or 50mM NaCl. Assay conditions are as described in Chapter II.

Salt	Control	2×10^{-6} M CCCP	10^{-6} M Valinomycin	CCCP + Valinomycin
KCl	11.25	16.97	12.47	24.95
NaCl	11.45	-	12.59	21.80

Activities are expressed in nmoles P_i/min/mg protein.

specifically bound acid phosphatase (which is also present in the soluble fraction) and an ATPase, but no G-6-Pase. Previously, microsomal ATPase activity had only been detected in tissues which were apparently low in acid phosphatase (Hansson and Kylin, 1969; Sexton and Sutcliffe, 1969; Fisher and Hodges, 1969; Kylin and Gee, 1970; Fisher *et al.*, 1970; Lai and Thompson, 1971). In tissues with high acid phosphatase, specific ATPase activity could not be detected at pH 4.5 (Atkinson and Polya, 1967), 7.2 (Brown and Altschul, 1964), and 7.5 (Bonting and Caravaggio, 1966). However, in the turnip microsome fraction specific ATPase activity can be assayed in the presence of high acid phosphatase activity by working at or above pH 7.8. The high pH optimum of the turnip microsomal ATPase is in agreement with the activities from young barley roots (Fisher and Hodges, 1969) and bean cotyledons (Lai and Thompson, 1971) and suggests alkaline phosphatase activity. However, lack of activity with NaBGlP (Table 1) and inhibition by ADP (Table 6) and by activators of alkaline phosphatase (Ca^{++} and Mn^{++}; Dixon and Webb, 1958) (Table 3) does not support this. The distribution of ATPase activities (Table 2) would also indicate no relation with the ribosomal ATPase reported in pea seedlings (Matsushita and Raacke, 1968).

In characterizing the plant particulate ATPase, other workers have tended to concentrate on the effects of monovalent cations with no attempt to test for the effect of anions. However, the results in

Table 4 indicate that the salt stimulation of the turnip microsomal Mg^{++}-ATPase is specific for anions rather than cations. In fact, most workers have assayed for salt stimulation in the presence of tris-HCl buffer which itself could activate the ATPase and minimize further effect of salts. This may explain the lack of salt stimulation of pea root ATPase (Sexton and Sutcliffe, 1969). The activity reported by Fisher and Hodges (1969) in barley roots most closely resembles the activity in turnip microsomes. However, the two activities differ markedly in their sensitivity to NH_4Cl, $MnCl_2$ and oligomycin. This may be explained by the fact that, although ATPase activity was detected in cell wall, mitochondrial and microsomal fractions, it was the non-vesicular cell wall activity which Fisher and Hodges characterized. In fact they showed that the vesicular microsomal activity was considerably less oligomycin sensitive but this activity was not further characterized.

With some exceptions (e.g. NO_3^-, I^-) the anions (inorganic and organic) which stimulate the ATPase are those which are generally accumulated by intact plant cells, while those that inhibit the ATPase are not accumulated (Table 4). This suggests that the ATPase could well be involved in anion accumulation by plants.

B. RELATION TO "MITCHELL" ATPASE

Electron micrographs (Chapter III) show that the fraction studied here consists of membranous vesicles. Further, the effect of

the uncouplers (proton carriers) and the ionophorous antibiotics (cation carriers) would suggest that ion movements across a membrane may be coupled to, and limit the ATPase activity. This is reminiscent of the energy-conserving ATPases of mitochondria and chloroplasts which can use electron transport energy or ATP to move ions across their respective membranes. However, the possibility of these fractions being sub-mitochondrial particles can be eliminated because:

(i) oligomycin had no effect on the ATPase;

(ii) there was no succinate-cytochrome c reductase in spite of the presence of an active NADH-cytochrome c reductase;

(iii) the NADH-cytochrome c reductase was insensitive to antimycin A (and therefore probably not of the inner membrane type).

If this is an ion-translocating ATPase (Mitchell, 1966) there should be a sidedness to the membranes with respect to site of ATP hydrolysis and pH gradient. The stimulation of activity with NH_4Cl would suggest a parallelism to the ATPase of chloroplast thylakoids (Robertson, 1968) i.e. the ATP reacts on the outside and, in effect, protons are moved into the internal space whilst the outside goes alkaline. The inward movement of anions (Cl^-) would maintain electrical neutrality. Subsequent exchanges of cations (e.g. K^+ for H^+) would remove the pH gradient (Fig. 8). So compounds which promote these cation exchanges (CCCP, DNP for H^+, valinomycin for K^+ or nigericin for both) should stimulate the ATPase activity - hence the

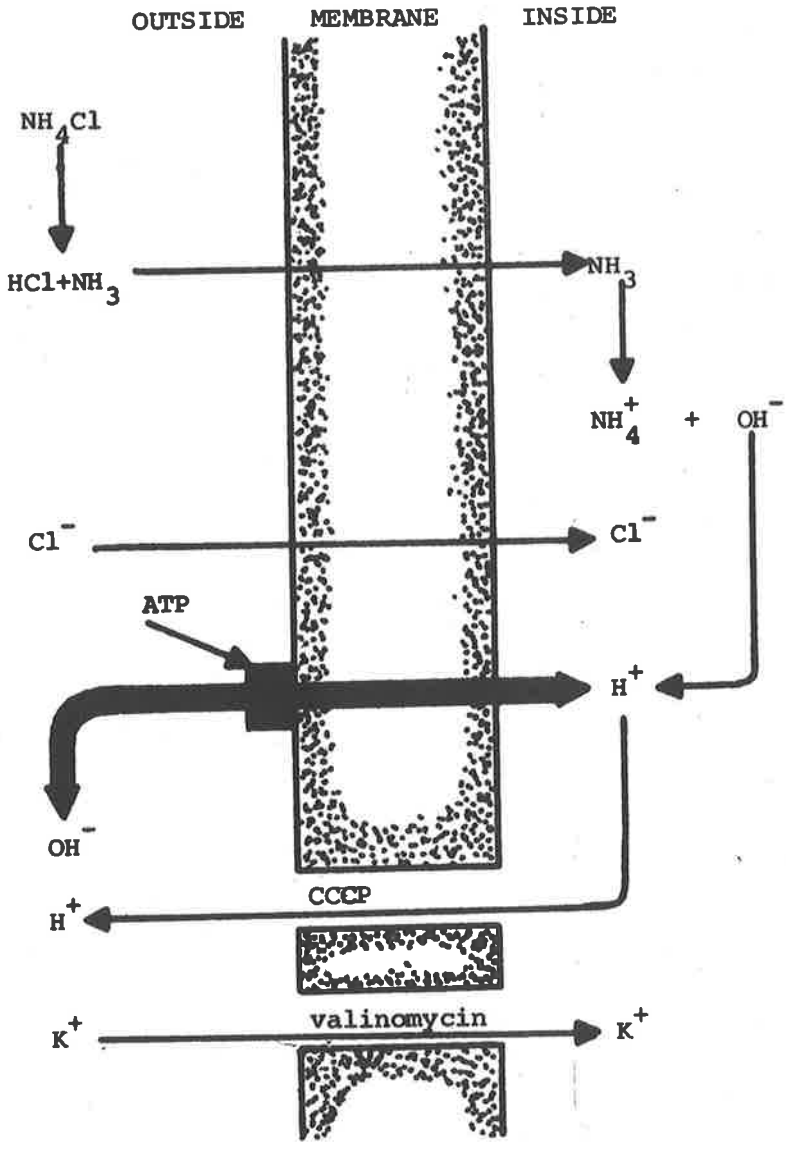


Figure 8. Proposed ion translocating function of the ATPase on the turnip microsomal vesicle showing the effect of NH_4Cl, CCCP and valinomycin.

synergistic effect of CCCP and valinomycin (Table 7). However, this activity did differ from the chloroplast ATPase in that it was insensitive to phlorizin. The pH optimum of the turnip microsomal ATPase was also similar to the chloroplast ATPase (Avron, 1962). However, as activity of the "Mitchell" ATPase depends on the pH on each side of the membrane, this probably has little meaning.

The effects of detergents and sonication demonstrated that there were severe structural limitations on the activity of the ATPase. Attempts to destroy the pH gradient with Triton X-100 and therefore inhibit anion stimulation were unsuccessful presumably due to a number of different effects of the detergent. The cause of increased activity by sonication in the presence of KCl is not clear. It is possible that during sonication a site on the membrane may be exposed to KCl which in turn causes a structural change facilitating ATPase activity similar to the KCl facilitation of trypsin action on liver microsomes (Chapter III).

Attempts were made to measure microsomal swelling, and both K^+ and Cl^- accumulation by the vesicles (by following decrease in outside ion concentration using K^+ and Cl^- electrodes). This was unsuccessful presumably due to leaky membranes and low rates of ion movement (despite the resistance to K^+ movement indicated in Table 7). Attempts to measure H^+ accumulation during electron flow (monitored directly on a pH meter) were also unsuccessful.

C. ORIGIN OF THE ATPASE

It was suggested in Chapter III that, of the smooth microsomes, fractions 2 (middle) may be derived from the plasmamembranes and the rest from the ER. Results in this Chapter suggest that fraction 2 (middle) may be derived from the tonoplast as the ATPase in this fraction:

(i) was probably on the outside of the membrane, i.e. the cytoplasmic side of the tonoplast;

(ii) showed specificity for anions (Poole, 1971) and was maximally stimulated by organic anions likely to be accumulated in the vacuole;

(iii) was saturated at high salt concentrations (50 mM) in accordance with the (system 2) ion pump proposed on the tonoplast (Laties, 1967).

The relationship of such an ion-translocating ATPase to the cell, and in particular to the ion translocating pump (probably an ATPase) at the plasmalemma of Chara (Smith, 1970) is shown in Figure 9. The ATPase is on the cytoplasmic side of each membrane separating H^+ to the outside of the cell or the vacuole and leaving OH^- on the inside (cytoplasm). The plasmalemma pump is saturated at 1 mM and probably shows a specificity for cations over anions (Poole, 1971). However, the tonoplast pump is saturated at 50 mM and shows specificity for anions (i.e. organic in particular) over cations (already selected

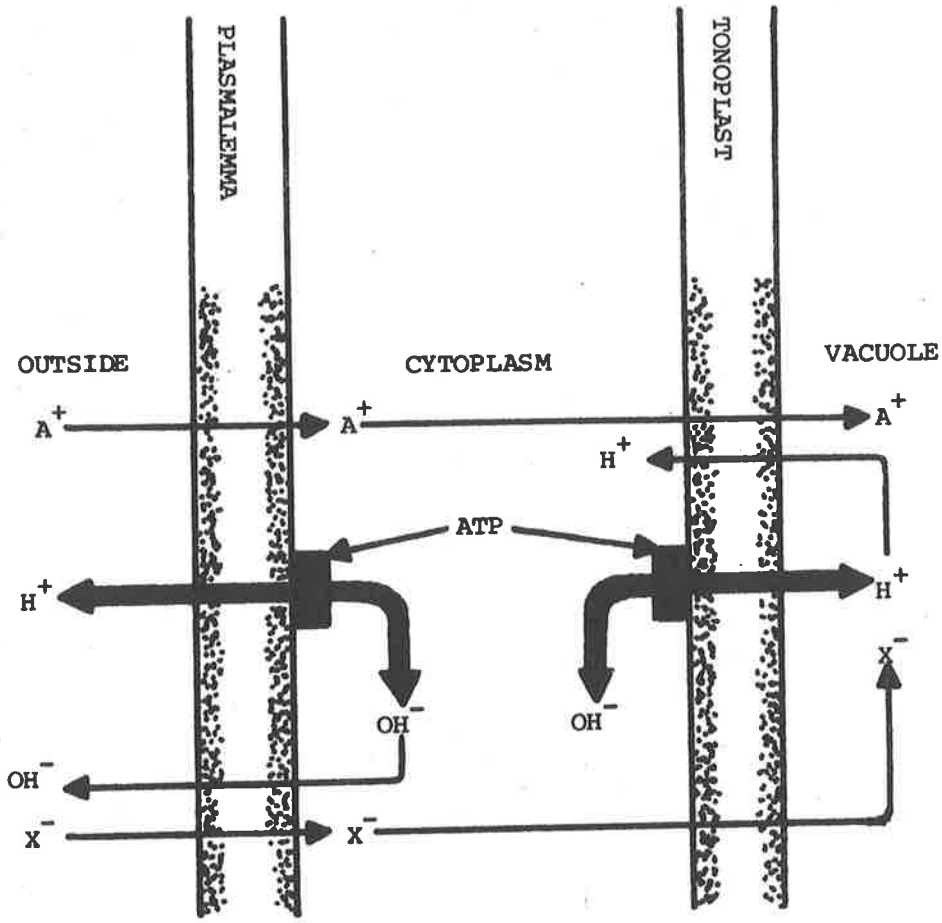


Figure 9. Relation between proposed ion pump at the plasmalemma in Chara (Smith, 1970) and the proposed ion pump at the tonoplast in turnip.

at the plasmalemma).

Whatever the origin of fraction 2 (middle), it is of interest that equivalent activity was also found in the endoplasmic reticulum. This may support the movement of ions across the cytoplasm in small endoplasmic reticulum vesicles (MacRobbie, 1969).

As little change in turnip microsomal ATPase was observed with aging of tissue, the development of ion accumulation is probably not due to the synthesis of such an ion pump. Table 4(c) shows that the ATPase favours organic anions and may normally move such ions in intact tissue. Tissue aging induces a depletion of the organic substrates by increased respiration (Chapter VIII) and washing out. Under these conditions of low organic anion concentration the cell may accumulate inorganic anions.

C H A P T E R V I I I

SLICING AND AGING INDUCED PHYSIOLOGICAL CHANGES

INTRODUCTION

Correlation of the biochemical with the physiological changes induced by slicing and aging may help to indicate functions of the enzymes or reasons for the physiological changes. Although the latter have been well characterized in a variety of tissues (Laties, 1967; Kahl, 1971), little information is available for turnip (Berry and Steward, 1934; Davies, 1961; 1964; 1966). It is also desirable to correlate biochemical and physiological changes under the same conditions.

RESULTS

Slicing and aging turnip tissue induced the respiratory rise (Fig. 1) characteristic of most plant tissues (ap Rees, 1966) although the increase was somewhat less than for many other plant tissues. Maximum respiration developed after about 50 hours aging (at 17°C). DNP, by stimulating respiration at all times, indicated that ADP was limiting. Azide strongly inhibited respiration in fresh disks but with aging an azide-resistant respiration developed which was unaffected by DNP (Fig. 1). Cyanide had a similar effect on respiration, although the inhibition tended to be greater than for the same concentration of azide.

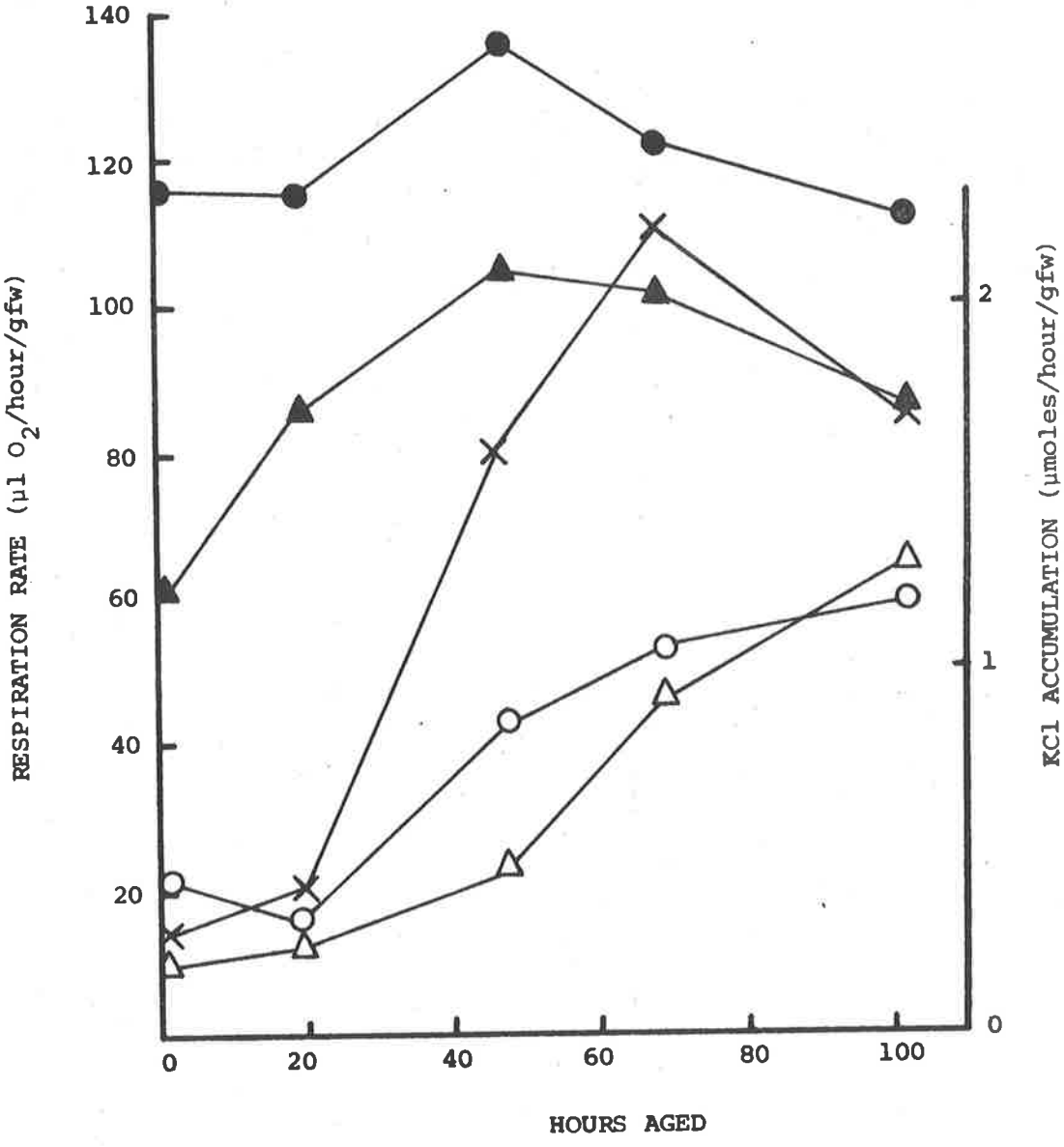


Figure 1. Induced respiration in aging turnip disks (▲) and the effect of 2 x 10^{-4}M DNP (●), 10^{-4}M azide (△) and DNP + azide (○). Induced ion accumulation (×) in aging turnip disks. Details of conductivity and manometry are described in Chapter II.

Figure 2 shows the effect of NOQNO on the respiration of turnip disks over 100 hours. (NOQNO, like antimycin A, inhibits mitochondrial electron flow between cytochromes b and c, Fig. 5.) Fresh tissue respiration was highly NOQNO-sensitive, but by 58 hours was completely insensitive to low concentrations (10^{-6} M). However, it was not until 112 hours that respiration became completely insensitive to higher concentrations (by which time respiration was largely azide-resistant).

Fresh tissue respiration was also highly sensitive to antimycin A, but complete insensitivity to low concentrations developed by 25 hours (Fig. 3). Disks were routinely incubated in antimycin A for one hour, prior to determining respiration rate, to ensure entry (Hackett *et al.*, 1960). With both fresh and aged disks there was an equivalent loss of antimycin A (measured at 232 nm) from the incubation solution (after allowing for control losses). This indicated that, at least, the developed antimycin A-insensitive respiration probably did not result from a permeability change which prevented antimycin A entry into the disks. The rate of development of NOQNO (10^{-5} M) - insensitive respiration increases with temperature (Fig. 4). However, at all temperatures it precedes the development of azide (10^{-4} M) - resistant respiration suggesting that these two effects may not be related.

Table 1 compares the effect of DNP on the NOQNO-insensitive

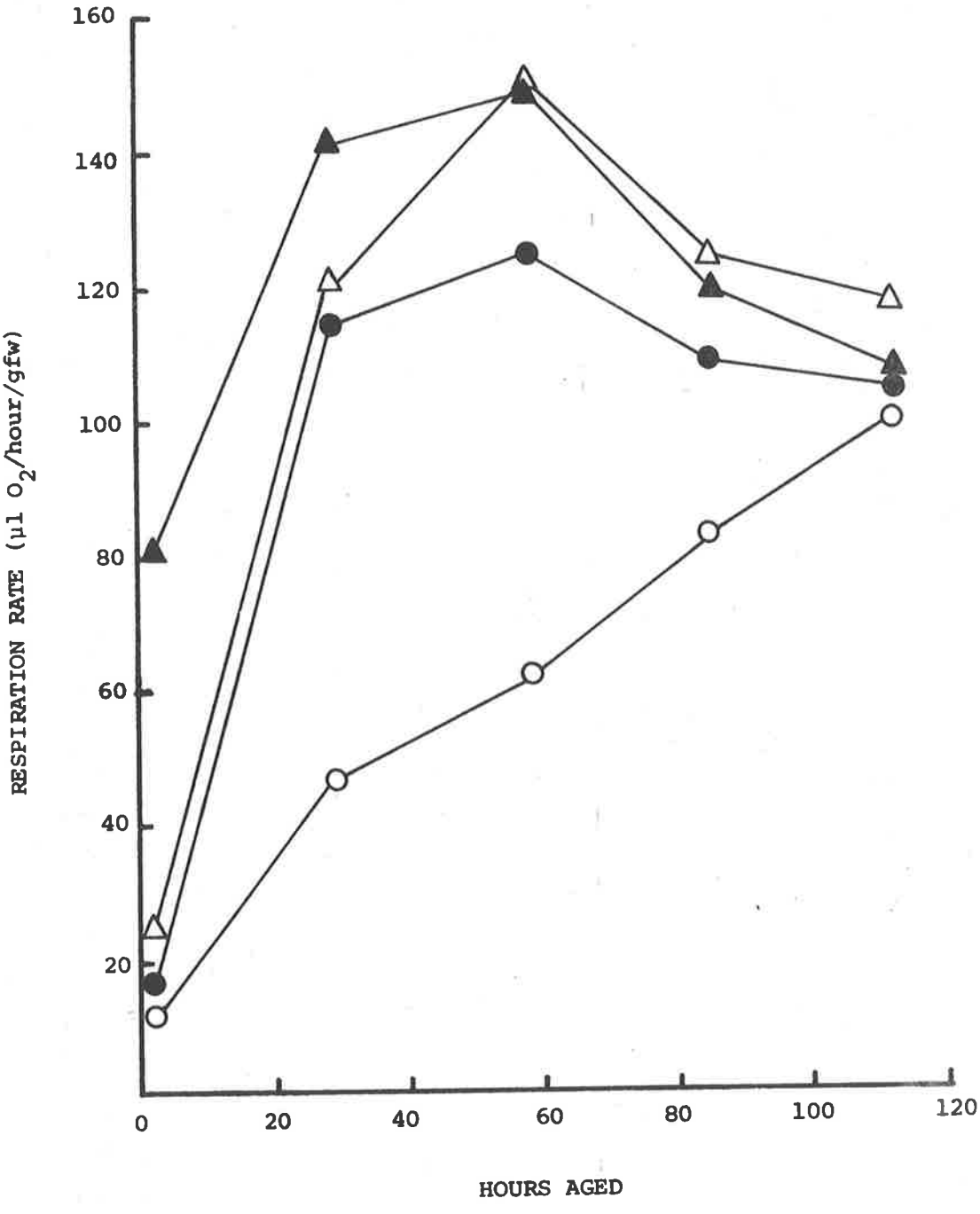


Figure 2. Effect of NOQNO on the aging-induced respiration of turnip disks. Details of manometry are described in Chapter II. ▲ - control; △ - 10^{-6}M; ● - 10^{-5}M; ○ - 10^{-4}M NOQNO.

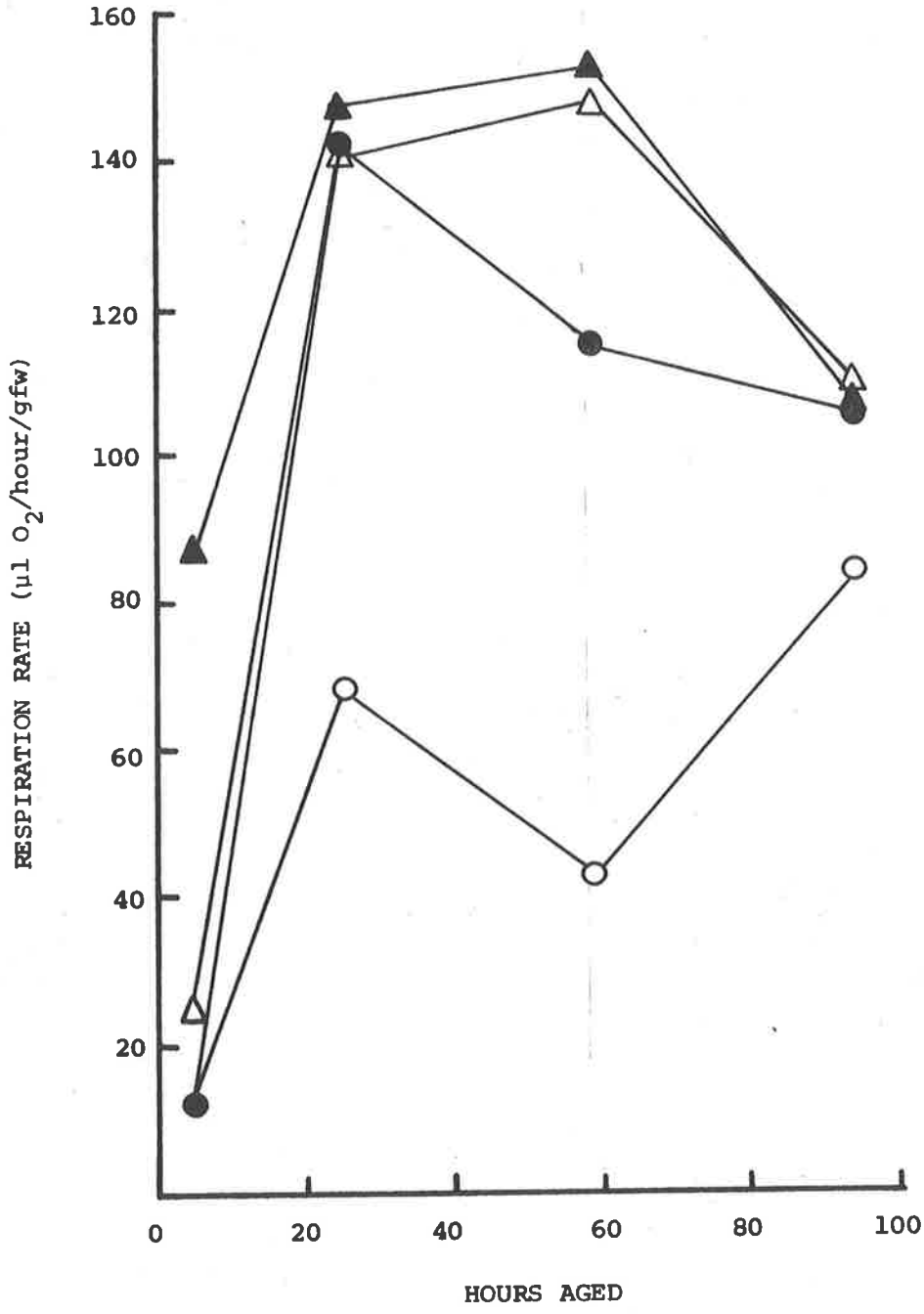
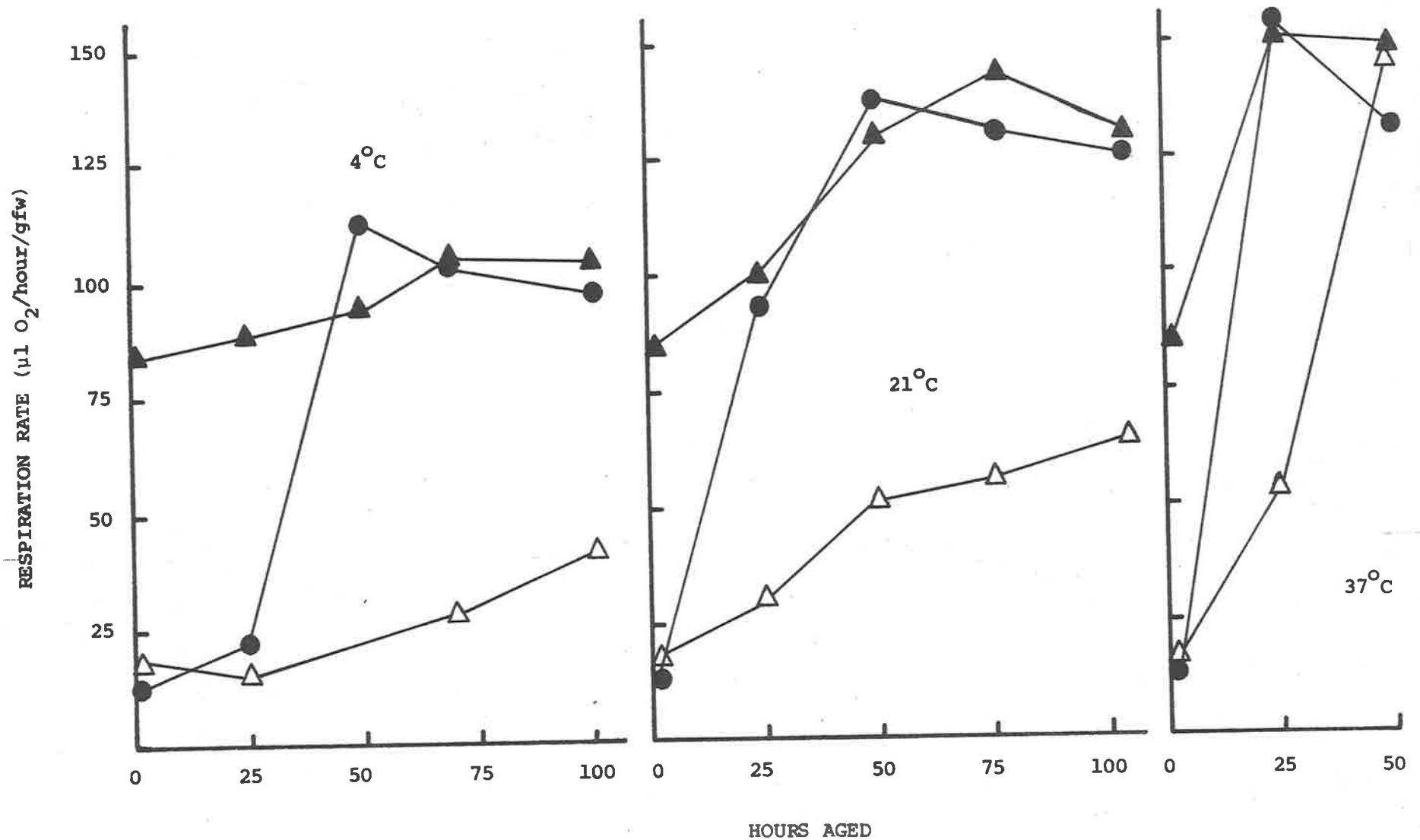


Figure 3. Effect of antimycin A on the aging-induced respiration of turnip disks. Details of manometry are described in Chapter II. Disks were preincubated for one hour in antimycin A before determining its effect on respiratory rate. ▲ - control; △ - 0.1 µg/ml; ● - 1.0 µg/ml; ○ - 10 µg/ml antimycin A.

Figure 4. Effect of temperature of aging of turnip disks on the development of azide- and NOQNO-insensitive respiration. Details of manometry are as described in Chapter II. ▲ - control; ● - 10^{-5}M NOQNO; Δ - 10^{-4}M azide.


RESPIRATION RATE (µl O_2/hour/gfw)
150
125
100
75
50
25
4°C
21°C
37°C
0
25
50
75
100
0
25
50
75
100
0
25
50
HOURS AGED

respiration in fresh and aged disks. The low NOQNO-insensitive rate in fresh disks was unaffected by DNP. In aged disks respiration was insensitive to 10^{-6} and 10^{-5} M NOQNO and this was stimulated by DNP in a manner similar to the control. However, aged tissue respiration was only partially insensitive to 10^{-4} M NOQNO and this was unaffected by DNP.

The 10^{-4} M NOQNO inhibition resembled the azide inhibition in both the effect of DNP and the development of an insensitive respiration. However, Table 2 shows that at 37°C (rather than 25°C) fresh disk respiration was largely azide-insensitive but remained sensitive to all concentrations of NOQNO. This may suggest that the mechanisms by which respiration becomes insensitive to NOQNO and to azide are not the same. Davies (1964) showed that increasing the assay temperature from 26° to 38° decreased the ethionine inhibition of fresh turnip disk respiration (i.e. similar to azide).

Figure 4 shows that low temperature (4°C) largely inhibited the development of NOQNO-insensitive respiration over the first 24 hours after slicing. The increase in antimycin A-insensitive respiration was inhibited by cycloheximide (Table 3) but not chloramphenicol. However, the inhibition of development of antimycin A-insensitive respiration by cycloheximide decreased as the temperature of the washing solution increased. This suggested that the effect was indirect rather than preventing the synthesis of a protein specifically

TABLE 1. Effect of DNP (2×10^{-4} M) on NOQNO-insensitive respiration of fresh and aged turnip disks. Details of manometry are described in Chapter II.

	Respiration (μl O_2/hr/gfw)			
	Fresh		21 hour aged	
	-	DNP	-	DNP
Control	83	189	114	179
10^{-6}M NOQNO	14	12	113	188
10^{-5}M NOQNO	15	17	108	152
10^{-4}M NOQNO	15	17	60	57

TABLE 2. Effect of azide and NOQNO on the respiration of fresh turnip disks at 25° and 37°C. Details of manometry are described in Chapter II.

Temperature	Respiration (μl O_2/hr/gfw)					% Inhibition			
	-	10^{-4}M Azide	10^{-6}M NOQNO	10^{-5}M NOQNO	10^{-4}M NOQNO	10^{-4}M Azide	10^{-6}M NOQNO	10^{-5}M NOQNO	10^{-4}M NOQNO
25°C	98	17	34	12	9	83	65	88	91
37°C	109	92	58	30	27	16	47	72	75

TABLE 3. Effect of cycloheximide on the development of antimycin A-insensitive respiration in aged turnip disks. Details of manometry are described in Chapter II.

	Respiration (μl O_2/hour/gfw)		% Inhibition
	Control	Antimycin A* (1 μg/ml)	
Fresh	83	11	87
24 hour aged	156	165	+6
24 hour aged in 3.6×10^{-6} M cycloheximide	84	33	61

* Disks were preincubated for 1 hour in antimycin A at 25°C before determining respiration rate.

required for insensitive respiration.

Under similar aging conditions, salt accumulation increased to an 8-fold maximum about 60 hours after slicing (Fig. 1).

DISCUSSION

A. INDUCED RESPIRATION

Slicing initiates a number of synthetic processes (Chapters IV, V, VI) which presumably require ATP and the resulting ADP would allow increased respiration. Hence the development of induced respiration was inhibited by cycloheximide, puromycin and 6-methylpurine (Chapter V). Adams (1970) supported the action of DNP as an uncoupler of respiration by showing that the addition of ADP to carrot disks had a similar effect. As respiration increases in turnip disks with aging so the DNP stimulation diminishes, and is minimal when respiration is at a maximum. Hence, it appears that at all times (except in very old disks) respiration is controlled by the ADP level.

Hackett *et al.* (1960) showed that mitochondria isolated from aged potato disks remained cyanide-sensitive. There also appears to be no conclusive evidence for the development of an alternate extramitochondrial oxidase. Peroxidase does develop at about the same time as the azide-resistant respiration (Chapter VI). However, these are probably not related as the cyanide-insensitive peroxidase is probably contained within the vacuoles and therefore unavailable,

while the microsomal peroxidase may be active in the tissue, but is largely azide-insensitive but highly cyanide-sensitive. Also the development of cyanide-insensitive respiration, unlike peroxidase, appears not to be inhibited by cycloheximide. Hence, it seems likely that respiration continues in aged tissue through cytochrome oxidase, and that resistance develops due to changing conditions in the cell such that the cytochrome "sees" a less effective concentration of azide or cyanide.

B. DEVELOPMENT OF NOQNO- AND ANTIMYCIN A-INSENSITIVE RESPIRATION

This effect may be explained either by the development of a reductase which by-passes the point of inhibition or the development of a resistance similar to the effect with azide and cyanide.

1. Development of a reductase

The development of the NOQNO-insensitive respiration parallels the development of the soluble NADH-cytochrome c reductase described in Chapter V. It was suggested that this enzyme may feed cytoplasmic NADH into the mitochondrial chain at about cytochrome c (Fig. 5). This would result in an antimycin A- and NOQNO-insensitive but cyanide- and azide-sensitive respiration. However, such a respiration may also result from the development of an outer mitochondrial membrane NADH-cytochrome c reductase of the type well established in animals (Sottocasa *et al.*, 1967). The development of an antimycin A-

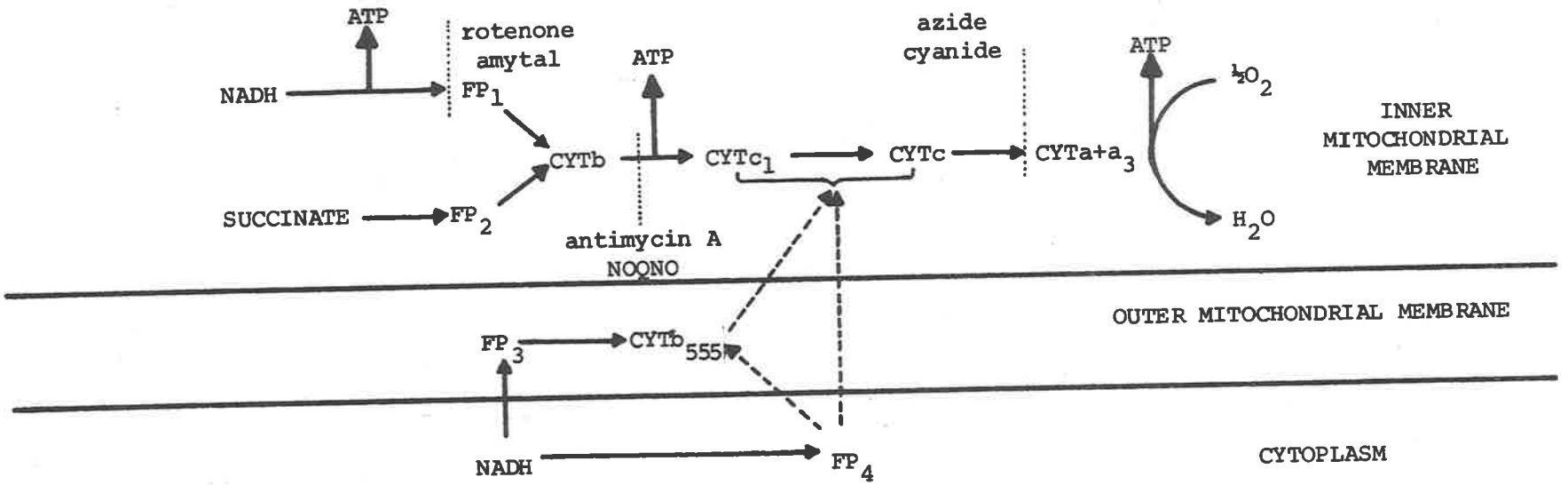


Figure 5. Scheme of mitochondrial electron transport and possible relation to the cytoplasm.

insensitive NADH-cytochrome c reductase in the mitochondria from aged potato disks (Hackett *et al.*, 1960) may be due to the development of such a reductase. The development of both a soluble and membrane bound enzyme would appear to be unnecessary duplication. However, they may be present under different conditions in the cell, be available to different pools of cytoplasmic NADH, or be able to enter at different sites on the mitochondrial electron transport chain.

If this hypothesis is correct, then increasing the concentration of NOQNO should not significantly increase the inhibition of respiration (in aged tissue). Hence, the effect of 10^{-4} M NOQNO would be explained by a second site of inhibition at high concentrations.

2. Development of a resistance

The suggested cause of development of azide- and cyanide-resistant respiration may also apply to antimycin A and NOQNO, i.e. respiration continues through the usual mitochondrial electron transport chain but that cell conditions change such that the components of the chain "see" a lower concentration of inhibitor. If so, the higher the concentration of inhibitor added to the disks, the greater the inhibition, and therefore the slower the development of resistance with aging. Such a resistance may result from a change in membrane permeability preventing the entry of the inhibitor. As cycloheximide appears to enter freely and inhibit in aged disks (Chapter V), this may suggest that it is the mitochondrial permeability which changes.

However, incubating fresh disks at high temperature (Table 2) indicates that azide- and NOQNO-resistance may be controlled by different factors, and therefore more than just the permeability of the mitochondrial membrane may be involved.

3. Discussion of two hypotheses

The partial inhibition of development of NOQNO-insensitive respiration by cycloheximide does parallel the inhibition of the soluble reductase. However, despite the apparent lack of dependence of development of cyanide-resistant respiration on protein synthesis, the development of NOQNO-insensitivity could well be indirectly dependent on this process. The subsequent decline in soluble reductase but continued increase in NOQNO-insensitive respiration at 17^{o} and 37^{o}C (Fig. 4 and Chapter V), does not support the involvement of this enzyme. The effect of DNP on resistant respiration (Table 1) would depend on just how much inhibitor the enzyme "sees". DNP will not stimulate respiration if the inhibitor is already limiting electron flow (10^{-4} M NOQNO) and will only stimulate to the point where the inhibitor starts to limit electron flow (10^{-5} M NOQNO). If a by-pass enzyme is present, two sites of phosphorylation are eliminated (Fig. 5) which may increase the effective ADP concentration to the third. Small stimulations of respiration in the presence of NOQNO have been sometimes observed. Hence, DNP will only stimulate if ADP is still limiting electron flow and this effect should be the same at

all NOQNO concentrations. The lack of DNP effect in the presence of 10^{-4} M NOQNO can only be explained again by a secondary effect at this concentration.

While the results could be explained by a combination of hypotheses, it seems most likely that with aging the tissue develops a resistance to NOQNO and antimycin A as it does to cyanide and azide. This may well result from a change of permeability of the mitochondrial membrane preventing the entry of these inhibitors.

C. INDUCED ION ACCUMULATION

The rate of salt accumulation by turnip disks increases 8 x with aging of disks to a maximum after 60 hours (Fig. 1). It was suggested in Chapter IV that this may correlate with the increase in microsomal electron transport, although not with the cytochrome c reductase. It is not known whether there was any relation between the coincidence of maximum ion accumulation and soluble NADH-cytochrome c reductase. The ATPase described in Chapter VII was probably involved in ion accumulation. However, its activity had only doubled at the time of maximum ion accumulation (it was not determined if this was dependent on protein synthesis). The development of ion accumulation in aging disks is now considered to be only partially dependent on protein synthesis and probably does not depend directly on the synthesis of a pump. As suggested in Chapter VII, the overall decrease in organic anions (as substrates for increased respiration) may create an environment in which the inorganic anions may be accumulated.

C H A P T E R I X

GENERAL DISCUSSION AND FUTURE WORK

The ubiquity of the NADH and NADPH electron transport chains in the microsomal, golgi, nuclear and outer-mitochondrial membranes of most animal tissues apparently also holds for plants where these activities were detected in microsome fractions isolated from a variety of tissues. The results suggest that the two chains are intrinsically part of the same membrane and probably interact, rather than existing separately to carry out their own particular functions. However, at this stage, many plant (and animal) microsome fractions apparently lack cytochrome P-450 suggesting that reduction of this cytochrome for MFO may be only one of many functions of the two chain complex. However, in liver microsomes, phenobarbital induces increases in both NADPH-cytochrome c reductase and cytochrome P-450 but not NADH-cytochrome c reductase or cytochrome b_5 (Orrenius *et al.*, 1965). This indicates a control between cytochrome P-450 and the NADPH chain, but not the NADH chain. This is despite the results of Estabrook and Cohen (1971) showing that reduction of cytochrome P-450 requires electrons from both chains. Clearly the requirements for MFO in plants will often be quite different to animals, and a detailed characterization of both the properties and distribution (within and between cells) of cytochrome P-450 and MFO activity in plants is needed.

The existence of three quite distinct soluble reductases in plants contrasts markedly to the single soluble reductase in animals (DT diaphorase; Ernster *et al.*, 1962). One of the plant reductases may correspond to the DT diaphorase, but adapted to the acid conditions in plant cells. This activity is completely insensitive to dicoumarol, and the corresponding lack of dicoumarol-sensitivity of the plant microsomal reductases suggests that the partial sensitivity in animals (Dallner, 1963) is a result of contamination from the soluble enzyme. The functions and properties of all three soluble reductases need further clarification and, in particular, the synonomy of the soluble NADH-FeCN reductase and the solubilized NADH microsomal dehydrogenase needs to be better established.

The involvement of an essential sulphydryl group binding NADH is an interesting common feature of the microsomal and the two soluble NADH-dehydrogenases. This has also been reported in animals for the microsomes (Strittmatter, 1965), outer mitochondrial membrane (Ragan and Garland, 1969) and recently for lactate dehydrogenase (Holbrook and Stinson, 1970). This may be an evolutionary relationship possibly indicating a common source or function (e.g. Fondy and Holohan, 1971).

The presence of the ion (anion-specific) - stimulated ATPase in plant microsomes, if involved in ion transport, is a predictable difference to animal cells. The results suggest that this enzyme is

on the ER and the tonoplast, the latter membrane being largely absent from animal cells. However, as only one turnip microsomal subfraction does not bind ribosomes in aging tissue, it suggests that this fraction must contain the plasmalemma as well as the tonoplast. Hence, further subfractioning of plant microsomes, and in particular of the fraction 2 (middle), is desirable.

The well characterized $Na^+ - K^+$ - ATPase involved in ion movement in animal cells is concentrated in the plasmalemma fraction (Jorgensen *et al.*, 1971). However, physiological experiments indicate that this enzyme is not present in plants, and recent work favours an ATPase at the plasmalemma in plants similar to that at the tonoplast but with specificity for the cation and saturating at 1 mM (Packer *et al.*, 1970; Smith, 1970). However, if the plasmalemma folds the same way as the tonoplast on tissue homogenization, then the ATPase will be on the inside of the vesicle possibly resulting in a different pH range over which activity can be assayed. Any treatment to aid ATP entry will destroy the intactness of the vesicle required for salt stimulation. It may be necessary in studying the plasmalemma to work with meristematic tissue with low acid phosphatase and poorly developed tonoplast.

Hence, the results suggest that in the energy-expending processes in the post-mitochondrial fractions the electron flow and phosphatase activities are not related, and support quite different

processes. The purely energetic processes appear to be supported by ATP. However, the synthetic processes appear to be supported by NAD(P)H and are concentrated in the internal cell membranes and soluble fraction (i.e. low in plasmamembranes). Such a differentiation is sensible as there is a constant supply of ATP in the cell for continuous maintenance of ion concentrations (for example) while there is probably no such source of cytoplasmic NAD(P)H (possibly unnecessary for the less demanding synthetic processes). In fact, it is still not clear just how important the reductases are - they may be largely involved in simply maintaining a balance between oxidized and reduced nucleotides, cystine, ascorbate etc. in the cytoplasm.

Aging of tissue induced some change in all fractions although a correlation with physiological and ultrastructural changes was not always found. The changes in microsomal reductases did correlate quite well with reported changes in ultrastructure of the ER. However, there was no physiological difference in the 3-5 hour aged disks (compared with 24 hour aged disks) that could be attributed to lack of microsomal reductase. In fact, the physiological changes in disks seemed to be best explained by general changes in cell conditions (i.e. ATP and ion concentrations and membrane permeability) rather than specific changes in enzymes.

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