CHARACTERIZATION OF ELECTRON TRANSPORT IN TURNIP MICROSOMES

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Abstract

Electron transport activities in the turnip microsome fraction were characterized. Cytochrome *c*, 2,6-dichlorophenolindophenol (DCPIP), ferricyanide (FeCN), and neotetrazolium were reduced in the presence of NADH and NADPH, NADPH supporting a rate usually less than 10% that with NADH. Cytochrome b_3 was present and implicated in cytochrome *c* reduction. However, cytochrome P-450 was not detected.

The effects of treatment of the microsomes with Triton X-100, trypsin, and *Naja naja* venom on the reductase activities were studied. The treatments resulted in loss of NADH–cytochrome *c* reductase activity but had variable effects on the NADH–DCPIP and –FeCN reductase activities. The effects on the NADPH dehydrogenase activities were also variable, but usually NADPH–cytochrome *c* reductase was inhibited while NADPH–DCPIP and –FeCN reductases were stimulated.

Fractionation of the microsomes by differential centrifuging and centrifuging through sucrose gradients in the presence of ions yielded a fraction rich in NADH dehydrogenases, NADPH–cytochrome *c* reductase, and cytochrome b_3. There was a broader distribution of the other NADPH dehydrogenase activities.

The results indicated the presence of two distinct electron transport chains on the turnip microsomal membranes, one specific for NADH and the other for NADPH. However, the activity of the latter may be partially due to soluble fraction contamination.

I. Introduction

Characterization of microsomal electron transport systems has concentrated on fractions isolated from animal tissue (Dallner 1963; Siekevitz 1963). It is now well established that there are two distinct electron transport schemes on the microsomal membranes. The NADPH electron transport scheme involves cytochrome P-450 and has been implicated in the mixed-function oxidation of a wide range of organic substrates, with oxygen as the final acceptor (Estabrook and Cohen 1969). The NADH electron transport scheme involves cytochrome b_5 but the final acceptor has not been determined (Strittmatter 1965; Dallner, Siekevitz, and Palade 1966).

Work on the plant microsome fraction has concentrated on three aspects:

(1) general characterization in surveys of chemical and enzymic intracellular distribution (Beevers 1961; Loening 1961; Ragland and Hackett 1964),

(2) cytochrome b_3, corresponding to animal microsomal cytochrome b_5 (Morton 1958; Shichi and Hackett 1966), and

(3) evidence for a mixed-function oxidase system involving cytochrome P-450, corresponding to the animal microsomal system (Frear, Swanson, and Tanaka 1969; Murphy and West 1969).

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It would appear that the animal and plant microsomal electron transport systems are similar. Plant microsomes oxidize NADH and NADPH and both cytochrome b_3 and P-450 have been detected. However, no details of the structure of the electron transport systems have been elucidated.

The purpose of this paper is to characterize and elucidate the electron transport systems in the microsome fraction derived from one particular plant tissue. Details of the structure of the systems were determined using inhibitors, membrane fractionation, and disruption techniques.

II. Methods

(*a*) *Preparation of Microsomes*

Commercially obtained white turnip tissue (*Brassica rapa* L.) (400 g) was homogenized in a Braun juice extractor into 30 ml of 0·3M sucrose containing 8 mM sodium ethylenediaminetetraacetate (EDTA) and 0·5 g bovine serum albumin (fraction V powder). The pH was kept at 7·4 during the homogenization by the dropwise addition of 1M tris(hydroxymethyl)aminomethane. The mixture was strained through muslin and centrifuged at 27,000 *g* (maximum) for 15 min to remove cell debris, mitochondria, and the larger mitochondrial fragments. The supernatant was then centrifuged at 150,000 *g* (average) for 45 min to sediment the microsomes. The microsome pellet was washed by resuspending in 0·4M sucrose, with the aid of a Potter–Elvehjem homogenizer, and recentrifuging at 150,000 *g* (average) for 45 min. The final pellet was re-suspended in approximately 10 ml of 0·4M sucrose. All apparatus was prechilled and all operations were performed at 4°C.

The isolation procedure is known to yield active mitochondria showing respiratory control (Millard, Wiskich, and Robertson 1965). It was assumed that this minimized mitochondrial damage and lessened contamination of the microsomal pellet by mitochondrial fragments.

(*b*) *Enzyme Assays*

Activities were assayed at 25°C using a Beckman D.B. spectrophotometer with a linear–log 5-in. strip chart recorder. Assay media contained 0·25M sucrose, 20 mM potassium phosphate buffer (pH 7·2), and 0·2 ml microsome suspension in a total volume of 2·5 ml. The following procedures were used as described in the references. NAD(P)H– and sulphite–cytochrome *c* reductase (Joshi *et al.* 1969), NAD(P)H–2,6-dichlorophenolindophenol (DCPIP) reductase, glucose-6-phosphate dehydrogenase, NAD(P)H–acetylpyridine adenine dinucleotide (phosphate) transhydrogenase (Ragland and Hackett 1964), NAD(P)H–ferricyanide (FeCN) and –neotetrazolium (NT) reductase (Takesue and Omura 1970), peroxidase (Gregory 1966), cytochrome b_3 (von der Decken 1967) (assuming that $E^{M}_{425-405}$ for cytochrome b_5 would be similar for cytochrome b_3). NAD(P)H–cystine and –glutathione reductases were assayed by following NAD(P)H oxidation ($E^{M}_{340} = 6{\cdot}2 \times 10^3$) using 0·8 mM cystine and 0·3 mM glutathione. Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as protein standard.

(*c*) *Chemicals*

Cytochrome *c* (type III from horse heart), NADH, NADPH, glutathione, cystine, glucose 6-phosphate, trypsin inhibitor (type IIS from soybean), dicoumarol, antimycin A, and rotenone were obtained from Sigma Chemical Co., St. Louis, U.S.A. Acetylpyridine adenine dinucleotide (APAD) and its phosphate derivative (APADP) were obtained from P. L. Biochemicals, Wisconsin, U.S.A. Hooded cobra (*Naja naja*) venom was obtained from Calbiochem. Inc., California, U.S.A. *p*-Chloromercuribenzoate (pCMB) was obtained from British Drug Houses Ltd., Poole, U.K. Trypsin (powder) was obtained from Difco Laboratories, Michigan, U.S.A. Neotetrazolium was obtained from Aldrich Chemical Co., Inc. Wisconsin, U.S.A. Amytal was obtained from Eli Lilly & Co. Ltd.

III. Results

(a) *General Characterization*

The electron transport activities detected in the turnip microsome fraction are summarized in Table 1. NADH was oxidized in the presence of cytochrome *c*, DCPIP, FeCN, and NT but not janus green B or methyl viologen. Corresponding NADPH activities (usually less than 10% of the NADH activities) were also detected in the presence of the same acceptors. The exceptionally high NADPH : NADH ratio for NT reduction has also been reported in animal microsomes (Williams and Kamin 1962; Dallner, Siekevitz, and Palade 1966).

As NADH–APAD homotranshydrogenase was the only transhydrogenase activity detected, this activity probably occurred as a side reaction of the active NADH dehydrogenase. Both the NADH– and NADPH–homotranshydrogenases were detected in the microsome fraction of etiolated pea stems (Ragland and Hackett 1964) and an NADH–NADP heterotranshydrogenase requiring ATP was detected in wild cucumber endosperm microsomes (Murphy and West 1969).

Table 1

ELECTRON TRANSPORT ACTIVITIES DETECTED IN THE TURNIP MICROSOME FRACTION

Reductase system	Rate*	Reductase system	Rate*	Reductase system	Rate
NADH–cytochrome *c*	0·14	NADH–FeCN	2·49	NADH–NT	0·016 †
NADPH–cytochrome *c*	0·01	NADPH–FeCN	0·35	NADPH–NT	0·005 †
NADH–DCPIP	0·67	NADH–APAD	0·07	Peroxidase	8·57‡
NADPH–DCPIP	0·06				

* Expressed as μmoles nucleotide oxidized/min/mg protein.

† Expressed as change in optical density at 505 nm/min/mg protein.

‡ Expressed as μmoles ascorbate oxidized/min/mg protein.

There was no glucose-6-phosphate dehydrogenase, sulphite–cytochrome *c* reductase, or NADH or NADPH oxidation in the presence of cystine or glutathione detectable in the turnip microsome fraction. NADH–cystine reductase and NADPH–glutathione reductase activities have been reported in plant tissues but their intracellular localization has not been determined (Mapson 1958). Plant glucose-6-phosphate dehydrogenase activity is largely soluble (Ragland and Hackett 1964). Lack of activity was not due to hydrolysis of glucose 6-phosphate by the microsomal acid phosphatase as activity with this substrate was very low (Rungie and Wiskich, unpublished data). Sulphite–cytochrome *c* reductase was predominantly a microsomal enzyme in liver and was considerably stimulated by detergents (Joshi *et al.* 1969). However, this enzyme was not detected in turnip microsomes and up to 0·1% Triton X-100 had no effect.

A cytochrome corresponding to the animal microsomal cytochrome b_5 (but not cytochrome P-450) was detected in the turnip microsome fraction. Absorption maxima were at 554–556; 524–525; 423–425 nm (difference spectra with dithionite). A similar cytochrome has been previously detected in both plant microsomal (cyto-

chrome b_3—Morton 1958) and soluble (cytochrome b_{555}—Shichi and Hackett 1966) fractions. Cytochrome P-450 has only been detected twice in plant microsomes (Moore—see Frear, Swanson, and Tanaka 1969; Murphy and West 1969). Other workers (Frear, Swanson, and Tanaka 1969) have been unsuccessful in detecting this pigment.

As peroxidase activity was detected in the turnip microsome fraction it was necessary to distinguish between cytochrome b_3 and the "cytochrome b type" absorption spectrum of the peroxidase (Kanazawa, Asahi, and Uritani 1967). While the oxidized peroxidase normally absorbed at 403 nm, CN^- (0·1 mM) caused a 10 nm shift in the maximum to 413 nm. However, oxidized cytochrome b_3 absorbed at 413 nm and this was unaffected by CN^-. Low peroxidase activity has been previously detected in plant microsome fractions (Kanazawa, Asahi, and Uritani 1967) but it was not known whether it resulted from contamination.

(*b*) *Properties*

The pH curves of the NADH and NADPH dehydrogenases were broad, usually lacking sharp optima. This appeared to be a consistent property of both plant (Crane 1957) and animal (Strittmatter and Velick 1956) microsomal reductases. The optima were all about pH 7 with the exception of NADPH–DCPIP reductase (Table 2).

TABLE 2

pH OPTIMA AND pCMB SENSITIVITY OF THE TURNIP MICROSOMAL REDUCTASES

pH optima were measured using 20 mM phosphate buffer (pH 6·2–8·0) and 20 mM Tris–HCl buffer (pH 7·2–9·0). pCMB sensitivity was measured by preincubating microsomes with either NADH or 2×10^{-5}M pCMB for 2 min, then assaying immediately, starting the reaction with the acceptor

Reductase system	pH optimum	Inhibition (as % of initial rate)	
		Preincubated with pCMB	Preincubated with reduced nucleotide
NADH–cytochrome *c*	7·0	83	12
NADPH–cytochrome *c*	7·4	—	—
NADH–DCPIP	7·0	95	19 (91*)
NADPH–DCPIP	6·4	27	36
NADH–FeCN	7·0	83	17
NADPH–FeCN	7·0	36	12

* Preincubated with NADPH.

While the reductases were largely insensitive to electron transport inhibitors (Table 7) there was a characteristic inhibition by sulphydryl group poisons (Table 2). pCMB (2×10^{-5}M) and cystine (6×10^{-4}M) inhibited the NADH dehydrogenases 80–100% but the NADPH dehydrogenases by less than 40% (results with the low NADPH–cytochrome *c* reductase were inconsistent). However, preincubation of the

microsomes with NADH but not NADPH largely protected the NADH dehydrogenase from pCMB inhibition, at least initially. Preincubation with NADPH partially protected the NADPH–FeCN reductase but had no effect on the NADPH–DCPIP reductase.

NADH dehydrogenase protection has also been detected in animal submitochondrial particles (Ragan and Garland 1969) and microsomes (Strittmatter 1965) where the effect has been well characterized. The binding of NADH to its dehydrogenase involves a sulphydryl group such that bound NADH prevents the binding of pCMB.

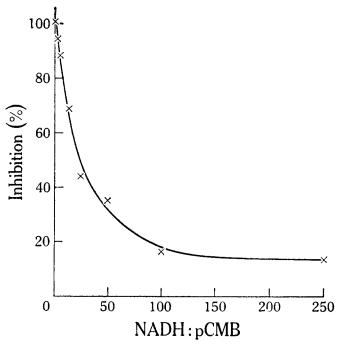


Fig. 1.—Effect of NADH : pCMB ratio on the percentage inhibition of the turnip microsomal NADH–cytochrome *c* reductase. Microsomes were preincubated with pCMB and the reaction started with NADH.

Figure 1 demonstrates the relationship between inhibition of NADH–cytochrome *c* reductase activity and the NADH : pCMB ratio, where microsomes were not preincubated with NADH. The reductase was inhibited 90–95% when the ratio was 1. The lack of complete protection even at very high NADH concentrations was probably due to other sulphydryl groups not directly involved in substrate binding (Strittmatter 1965). Similar curves were obtained for NADH–DCPIP and –FeCN reductases.

(*c*) *Disruption*

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

Figure 2(*a*) 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. Ultrasonic vibrations, however, resulted in inhibition of all three reductase activities which was greatest for cytochrome *c* and least for FeCN [Fig. 2(*b*)]. It appeared that, while treatment with Triton X-100 and ultrasonic vibrations 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 X-100 concentrations and by ultrasonic vibrations for 30–60 sec (Table 3).

The effect of incubating microsomes with increasing concentrations of trypsin is shown in Figure 3. 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_3 but only an 8% loss of total protein.

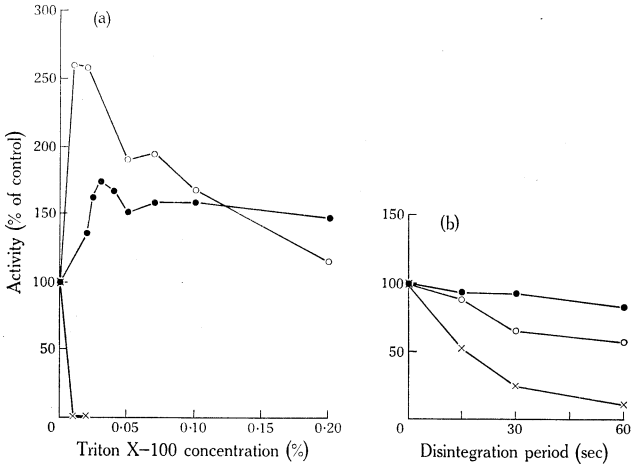


Fig. 2.—Effect of Triton X-100 incubation (*a*) and ultrasonic treatment (*b*) of the turnip microsomes on the NADH dehydrogenase activities. Microsomes were incubated with Triton X-100 for 2 min at room temperature immediately prior to assaying. Microsomes chilled in a salt–ice bath were subjected to ultrasonic disintegration for the times shown, using an M.S.E. 100 W ultrasonic disintegrator with a prechilled probe. Activities are expressed graphically as a percentage of the activities in untreated microsomes, but are given below as μmoles NADH oxidized/min/mg protein:

System	Symbol	Activity	
		Fig. 2(*a*)	Fig. 2(*b*)
NADH–cytochrome *c*	×	0·179	0·663
NADH–DCPIP	○	0·152	0·618
NADH–FeCN	●	0·280	2·81

TABLE 3

EFFECT OF TRITON X-100 AND ULTRASONIC TREATMENT ON NADPH OXIDATIONS OF TURNIP MICROSOMES

Details of methods are described in Figure 3

Triton X-100 concn. (%)	Reductase activity*			Period of ultrasonic treatment (sec)	Reductase activity*		
	NADPH–cyt. *c*	NADPH–DCPIP	NADPH–FeCN		NADPH–cyt. *c*	NADPH–DCPIP	NADPH–FeCN
0	0·002	0·014	0·086	0	0·010	0·037	0·282
0·01	0·002	0·030	—	15	0·005	0·037	0·236
0·02	0	0·030	0·087	30	0·010	0·049	0·328
0·05	0	0·039	0·088	60	0·002	0·049	0·282
0·07	—	0·039	0·117				
0·10	—	0·028	0·119				
0·20	—	0·025	0·092				

* Expressed as μmoles NADPH oxidized/min/mg protein.

Potassium chloride facilitated the action of trypsin on the three NADH oxidations (Fig. 4). As this facilitation was quantitatively different for the three activities and KCl also stimulated the activities in the absence of trypsin, this indicated that KCl may cause a structural change in the membrane rather than an activity change of the trypsin. Possible KCl induced structural changes of animal mitochondrial and microsomal membranes have been reported (Kuylenstierna *et al.* 1970).

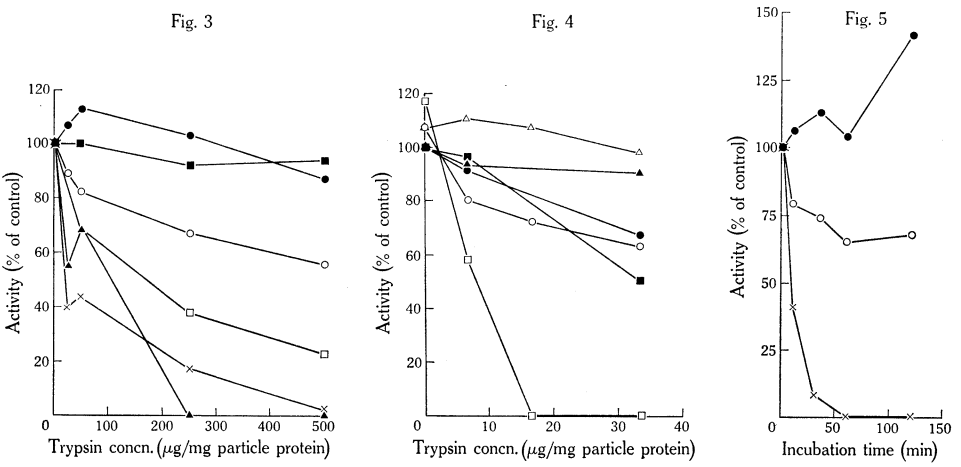


Fig. 3.—Effect of trypsin incubation on the microsomal dehydrogenase activities, protein, and cytochrome b_3. Microsomes (suspended in 0·4M sucrose) were equilibrated at 25°C for 1 min, then varying quantities of freshly made up solution of trypsin added. After 1 min, three times the weight of soybean 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 min and protein and cytochrome b_3 in the pellet assayed. Activities are expressed graphically as a percentage of activities in untreated microsomes, but are here given in μmoles NADH or NADPH oxidized/min/mg protein: NADH–cytochrome *c* (×), 0·140; NADH–DCPIP (○), 0·243; NADH–FeCN (●), 1·19; NADPH–cytochrome *c* (▲), 0·010; cytochrome b_3 (□), 0·29 nmoles/mg protein; protein (■), 0·72 mg/ml.

Fig. 4.—Effect of trypsin incubation in the presence and absence of KCl on the microsomal NADH dehydrogenase activities. Microsomes were incubated in the presence (open symbols) and absence (closed symbols) of 70 mM KCl with varying amounts of trypsin as described in Figure 3. Activities are expressed graphically as a percentage of activities in untreated microsomes, but are here given in μmoles NADH oxidized/min/mg protein: NADH–cytochrome *c* (□,■), 0·072; NADH–DCPIP (○,●), 0·653; NADH–FeCN (△,▲), 1·62.

Fig. 5.—Effect of *N. naja* venom incubation on the microsomal NADH–cytochrome *c* (×), NADH–DCPIP (○), and NADH–FeCN (●) reductase activities. The incubation method of Strittmatter (1967) was used with some variations. Washed microsomes from 500 g of tissue were suspended in 50 ml of 50 mM Tris–HCl buffer (pH 8·0)+1 mM EDTA. 7·5 ml of 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 hr and assayed immediately. The considerable loss of activities in the control over 2 hr incubation were corrected for. Activities are expressed as a percentage of activities in untreated microsomes (see Table 4).

The effect of incubating microsomes with *N. naja* venom is shown in Figure 5. The overall effect on the reductase activities was very similar to the effect of trypsin.

The greater stimulation of NADH–FeCN reductase was probably due to the partial solubilization of the NADH dehydrogenase. After incubation for 2 hr 20% of the NADH–FeCN reductase activity but only 4% of the NADH–DCPIP reductase activity had been solubilized (Table 4).

TABLE 4

EFFECT OF *N. NAJA* VENOM ON THE MICROSOMAL NADH DEHYDROGENASE ACTIVITIES

The incubation method is described in the legend to Figure 5. After incubation for 120 min, the mixture was centrifuged at 27,000 *g* (average) for 20 min and the pellet resuspended in an equal volume of 50 mM Tris–HCl (pH 8·0)+1 mM EDTA

Reductase system	Activity of untreated microsomes*	Activity of fractions*		
		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

* Expressed as μmoles NADH oxidized/min/0·2 ml sample.

(*d*) *Fractionation*

Microsomes were normally prepared by centrifuging the post-mitochondrial supernatant at 150,000 *g* for 45 min (Section II). However, activities were not evenly

TABLE 5

REDUCTASE ACTIVITIES IN FRACTIONS DERIVED FROM TURNIP MICROSOMES 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

Reductase system	Activities in fractions*			
	38,000 *g*, 1 hr	79,000 *g*, 1 hr	150,000 *g*, 1 hr	150,000 *g*, 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

* Expressed as μmoles NAD(P)H oxidized/min/mg protein.

distributed in the pellets which sedimented on centrifugation at 38,000, 79,000, and 150,000 *g* for 1 hr and 150,000 *g* for 5 hr (Table 5). 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.

Microsomes could also be fractioned 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 depending on their ability to bind Mg^{2+} (Dallner 1963).

TABLE 6

ACTIVITIES IN FRACTIONS DERIVED FROM TURNIP MICROSOMES BY CENTRIFUGING THROUGH DISCONTINUOUS SUCROSE GRADIENTS IN THE PRESENCE OF IONS

Unwashed microsomes from 600 g turnip tissue were suspended in 90 ml 0·25M sucrose containing 15 mM CsCl and 7·0 ml layered over 4·5 ml 1·3M sucrose containing 15 mM CsCl. Centrifuging at 50,000 r.p.m. (type 50Ti rotor) for 90 min resulted in a pellet (fraction 1) and two inseparable layers (fraction 2) at the sucrose boundary. The total layers were suspended in 90 ml 0·25M sucrose containing 10 mM $MgCl_2$, and 7·5 ml layered over 4·0 ml 1·15M sucrose containing 10 mM $MgCl_2$. Centrifuging at 50,000 r.p.m. for 90 min resulted in three layers at or near the boundary (fractions 2, top, middle, bottom). The four fractions were washed and each resuspended in 10 ml 0·25M sucrose*

System or parameter	Fraction 1 (pellet in CsCl)	Fraction 2 (layers in $MgCl_2$)		
		Top	Middle	Bottom
NADH–cytochrome *c* †	0·016	0·123	0·039	0·057
NADPH–cytochrome *c* †	0	0·008	0	0
NADH–DCPIP †	0·043	0·572	0·177	0·328
NADPH–DCPIP †	0·026	0·037	0·048	0·043
NADH–FeCN †	0·245	1·423	0·655	1·051
NADPH–FeCN †	0·122	0·107	0·120	0·210
Peroxidase‡	—	0·280	0	0·241
Cytochrome b_3 (nmoles/mg protein)	0·17	0·61	0·14	0·36
RNA (mg/mg protein)	0·669	0·056	0·096	0·062
Total RNA (mg)	7·500	0·560	0·440	0·320
Total protein (mg)	11·20	10·20	4·60	5·20

* It has now been shown that lowering the sucrose concentration of the bottom layer in the presence of $MgCl_2$ from 1·15 to 0·90M does not affect fraction 2 (middle) but results in pelleting of fraction 2 (top) and (bottom). It is likely that these two latter fractions are the same.

† Reductase activity expressed as μmoles NAD(P)H oxidized/min/mg protein.

‡ Activity expressed as μmoles ascorbate oxidized/min/mg protein.

The first separation in the presence of CsCl resulted in a pellet (fraction 1) and two 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 6). However, the second separation of fraction 2 in the presence of $MgCl_2$ resulted in three layers rather than a pellet and one layer (as reported by Dallner). The top layer which was light brown was the largest 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. The top layer comprised 50% of the total protein of fraction 2 while the RNA was equally distributed in the three subfractions. The majority of the NADH activities, cytochrome b_3, 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 6). This distribution of activities was similar to that given by differential centrifuging (Table 5).

IV. Discussion

The activity most characteristic of the turnip microsome fraction was the NADH dehydrogenase in the presence of artificial electron acceptors and cytochrome *c*. Similar activities have been detected in a number of other plant and animal tissues. This activity was insensitive to arsenite, dicoumarol, and the mitochondrial electron transport inhibitors. This indicated that Straub's diaphorase (Massey 1963), the animal microsomal DT-diaphorase (Dallner 1963), and the inner membrane mitochondrial reductase (Ikuma and Bonner 1967) were not involved (Table 7). Absence of succinate–cytochrome *c* reductase activity also indicated lack of mitochondrial contamination.

Table 7

Effect of electron transport inhibitors on the turnip microsomal reductase activities

Inhibitor	Concn.	Percentage inhibition		
		NADH–cytochrome *c* reductase	NADH–DCIP reductase	NADH–FeCN reductase
Arsenite	3×10^{-3}M	0	9	0
Dicoumarol	10^{-6}M	7	8	0
Antimycin A	1 mg/ml	0	—	—
Rotenone	3×10^{-6}M	0	—	—
KCN	5×10^{-4}M	0	0	0
Amytal	3×10^{-3}M	29	8	12

The function of this electron transport chain is unknown due to the lack of a natural acceptor (Siekevitz 1965). No reduction of cystine, glutathione, nor any significant transhydrogenase activity was detected, negating these possible functions.

pCMB normally inhibits by binding to reduced sulphydryl groups and it might be expected that the addition of NADH would facilitate this (Tyler *et al.* 1963). The characteristic protection by NADH from pCMB inhibition of the cytochrome *c*, FeCN, and DCPIP reductases indicated that these activities all involved initially the same NADH-binding dehydrogenase. NADPH–FeCN but not –DCPIP reductase was partially protected by preincubation with NADPH. Protection has been reported for the liver microsomal NADPH–cytochrome *c* reductase (Williams and Kamin 1962).

The turnip soluble fraction oxidized NADPH in the presence of DCPIP and FeCN but not cytochrome *c*. This activity was only partially sensitive to pCMB and

was unprotected by NADPH preincubation (Rungie and Wiskich, unpublished data). Thus, the lack of protection of NADPH–DCPIP reductase and the abnormally low pH optimum indicated that most of this activity was probably due to contamination from the soluble fraction. However, the partial protection of NADPH–FeCN reductase demonstrated that an NADPH dehydrogenase was present in the microsomes.

Incubation of turnip microsomes with *N. naja* venom solubilized an NADH dehydrogenase which was 50 times more active in the presence of FeCN than DCPIP, and inactive with cytochrome *c*. As NADH oxidation by the intact microsomes was only five 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 1970). These results are best explained by an electron transport chain in the microsomal membranes where the intermediates required for DCPIP and cytochrome *c* reductions could be released from the chain by disruption of the membranes. In animal microsomes *N. naja* venom solubilized both the NADH dehydrogenase and cytochrome b_5 (Strittmatter 1967) suggesting that the microsomal cytochrome may be the hypothesized intermediate. Trypsin treatment of the turnip microsomes resulted in parallel loss of NADH–cytochrome *c* reductase activity and bound cytochrome b_3. There seems little doubt that in animal microsomes cytochrome *c* reduction does involve the microsomal cytochrome (Dallner 1963; Kuylenstierna *et al.* 1970) and that this relationship also holds for plants.

The concentrations of trypsin and *N. naja* venom which completely inhibited cytochrome *c* reduction, however, resulted in only a 40% loss of DCPIP activity. The remaining 60% was not likely to involve the remaining small amount of cytochrome b_3 as the redox potential values for cytochrome *c* and DCPIP were about the same. These results may be explained if a further component (X) was situated in the electron transport chain between the dehydrogenase and cytochrome b_3 (Scheme 1). The component (X) would be largely responsible for the reduction of DCPIP (and cytochrome b_3) but only partially solubilized by hydrolytic enzymes.

While incubation with 0·01% Triton X-100 was sufficient to completely inhibit NADH–cytochrome *c* reduction and only slightly stimulate FeCN reduction, it stimulated NADH–DCPIP reduction over 100%. This again indicated a site for DCPIP (but not FeCN or cytochrome *c*) reduction which had been exposed. At higher Triton X-100 concentrations this exposed site was destroyed without affecting the stimulated FeCN reduction.

As ultrasonic vibrations fragment the membrane, the probable subsequent reformation of the fragments into smaller vesicles may cause a loss of accessibility and and therefore loss of activity. Ultrasonic disruption also released cytochrome b_3 so that the greater loss of NADH–cytochrome *c* reductase relative to the other reductases was probably due to this. Similarly, loss of NADH–DCPIP reductase activity may have partially resulted from release of the component (X).

As the NADPH dehydrogenase activities were so low it was difficult to detect significant changes and to verify that activities were not due to soluble fraction contamination. The presence of an NADPH dehydrogenase in animal microsomes (Williams and Kamin 1962) would suggest the presence of a similar system in plant microsomes. The inhibition of NADPH–cytochrome *c* reductase by trypsin, Triton

X-100, and ultrasonic disruption resembled that of NADH–cytochrome *c* reductase. However, NADPH–FeCN and –DCPIP reductases were stimulated by both ultrasonic vibrations and Triton X-100. This was further evidence for a separate NADPH dehydrogenase which reduced FeCN and DCPIP directly but cytochrome *c* via a component (Y) (Scheme 1). Due to the lack of soluble NADPH–cytochrome *c* reductase the microsomal NADPH–cytochrome *c* reductase activity can be regarded as being quite valid. However, both the distribution and stimulations of the other NADPH activities must be interpreted carefully due to the possibility of soluble contamination.

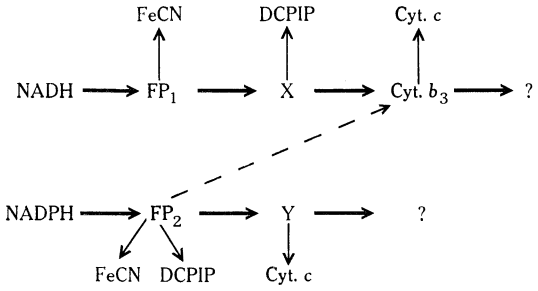
While both the methods of fractionation of the turnip microsomes resulted in a broad distribution of the NADPH–DCPIP and –FeCN reductase activities, the NADH reductases appeared to be associated with particular fractions. However, the NADPH–cytochrome *c* reductase did not sediment with the other NADPH 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_3 in NADPH–cytochrome *c* reduction which has been suggested for animal microsomes (Estabrook and Cohen 1969; Kamino and Inouye 1970). The latter authors actually proposed two NADPH flavoproteins, one reducing cytochrome *c* directly and the other via cytochrome b_5.

Fractionation of animal microsomes by a variety of methods has generally resulted in separation of activities. Electron transport activities are usually concentrated in the faster-sedimenting fractions (Palade and Siekevitz 1956; Dallman *et al.* 1969) and NADH– and NADPH–cytochrome *c* reductase activities usually sediment together (Imai, Ito, and Sato 1966; Amar-Costesec *et al.* 1969) although there are exceptions (Dallman *et al.* 1969). Dallner (1963), using the CsCl and $MgCl_2$ method, found NADH–DCPIP and –cytochrome *c* reductases concentrated in fraction 1, an even distribution of cytochrome b_5, and NADPH–DCPIP and –cytochrome *c* reductases concentrated in fraction 1 and the pellet of fraction 2. This differs markedly from the results presented in Table 6. However, slicing turnip tissue induced the fraction 2 (top) layer (which contained the majority of the NADH activities and cytochrome b_3) to sediment with fraction 1, probably due to an induced binding of ribosomes (Rungie and Wiskich, unpublished data). The origin of the fraction 2 (middle) layer was unknown. The lower dehydrogenase and peroxidase activities, cytochrome b_3 level and the colour all indicated that it was not related to the other fractions.

The proposed turnip microsomal electron transport system is presented in Scheme 1. Both the NADH and NADPH dehydrogenases probably involve flavoproteins (FP) although this has not been shown in plants.

For various reasons, unknown components (X) between FP_1 and cytochrome b_5 and (Y) between FP_2 and cytochrome P-450 have been proposed in animal microsomes (Siekevitz 1965; Dallner, Siekevitz, and Palade 1966; Estabrook and Cohen 1969). Takesue and Omura (1970) and Strittmatter (1967) have shown that the solubilized animal microsomal NADH flavoprotein reduced cytochrome b_5. However, associated with this solubilization was a stimulation of the rate of cytochrome b_5 reduction and an increase in the redox potential for cytochrome b_5 (Strittmatter 1968). This indicated that a new site for cytochrome b_5 reduction had been exposed. As this site was not

available in intact microsomes, cytochrome b_5 reduction probably involved the component (X). While the NADH chain is similar to that found in animal micro-



Scheme 1

somes, the activity of the NADPH chain was lower and cytochrome P-450 could not be detected.

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VI. References

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