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The Activities of some Particulate and Non-Particulate Oxidative Enzymes in Carrot Tissue

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As part of a study of respiratory pathways in carrot tissue, some aspects of malate metabolism by slices and mitochondria prepared from carrot tissue have already been reported (see Dalgarno & Birt, 1962*a*, *b*). In amplification of this work the enzymic constitution of the extramitochondrial fraction of carrot tissue has been investigated. No comprehensive study of the preparation and properties of a soluble fraction from a similar tissue was available, although James & Richens (1960*a*) have described such a fraction from carrot tissue. However, only one enzyme was assayed (pyruvate decarboxylase) and no general comparisons with soluble fractions prepared by other methods are possible.

This paper describes various procedures for the preparation of soluble fractions from carrot tissue, and the assay of a number of oxidative enzymes in these fractions. The possibility that the soluble malate dehydrogenase is capable of generating reduced nicotinamide nucleotides for oxidation by the mitochondria is discussed in relation to further data on the interaction between extramitochondrial enzymes and the mitochondrial oxidase system.

MATERIALS AND METHODS

Reagents. Apart from those specified below, the organic acids, including DL-isocitrate (trisodium salt), amino acids, coenzymes and enzymes, used were supplied by Sigma Chemical Co., St Louis 18, Mo., U.S.A., or were from the sources described by Dalgarno & Birt (1962*a*, *b*). Spermine was obtained from Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.; glutathione was obtained from S.A.F. Hoffmann-La Roche Co. Ltd., Basle, Switzerland;

the alcohol dehydrogenase used was prepared according to the method of Racker (1950); the cytochrome *c* was a gift from Dr R. Henderson.

Preparation of soluble fractions from carrot tissue

Soluble fractions prepared from tissue disrupted in a blender. The homogenizing procedure used for the preparation of a mitochondrial fraction (see Dalgarno & Birt, 1962*a*) was suitable as a first step in the preparation of a soluble fraction from carrot tissue. All operations were carried out at 0–4° in a cold-room. In experiments with a medium of sucrose (0.44 M) in phosphate buffer (sodium and potassium salts, 0.1 M, pH 7.1) containing 1% (w/v) bovine serum albumin, the homogenate was squeezed through two layers of muslin and the filtrate centrifuged at 0° in a Spinco model L ultracentrifuge at 70 000g for 90 min.; the supernatant liquid was removed, saturated with ammonium sulphate (200 g.) and recentrifuged at 70 000g for 45 min. The considerable precipitate obtained after this centrifuging was resuspended and homogenized in approximately 15 ml. of phosphate buffer (sodium and potassium salts, 10 mM, pH 7.1). In preparations with spermine (20 mM), or without albumin in the homogenizing medium, the procedure was identical except that the ammonium sulphate precipitate (in smaller amount) was most effectively removed by resuspension and filtration of the contents of the centrifuge tubes through Whatman no. 1 paper in a Buchner funnel. The sediment was scraped from the paper and homogenized in approx. 8 ml. of phosphate buffer. Fractions prepared without albumin were dialysed (with stirring) against phosphate buffer (sodium and potassium salts, 10 mM, pH 7.1) for at least 2 hr. before use; continuation of dialysis overnight did not alter the activity of malate dehydrogenase in this fraction. Fractions prepared with albumin were always dialysed overnight. The soluble fractions obtained from the ammonium sulphate precipitates were used immediately, or after a period of storage at –15° (the duration of storage is noted in the

Results section). Malate-dehydrogenase activity in the soluble fractions prepared with serum albumin was less stable during storage at -15° than in soluble fractions prepared without albumin.

Soluble fractions prepared by compression of tissue in a Fischer mincer. Carrot tissue was peeled and diced into cubes of less than 0.5 cm^3 . Portions (8–10 g.) were compressed with a nylon pestle in a stainless-steel Fischer mincer of internal diameter 1.3 in.; the end-plate was perforated by pores of diameter 0.04 in. The yield of soluble fraction was approx. 0.5 ml./10 g. of tissue, and attempts to obtain a greater yield by increasing the compression resulted in the tissue itself being forced through the pores. All operations were carried out at $0-4^{\circ}$ in a cold room and, as it was extracted, the juice was run directly into tubes set in ice.

Preparation of mitochondrial fractions from carrot tissue

Mitochondrial fractions were prepared by the methods described by Dalgarno & Birt (1962a).

Spectrophotometric determination of enzymic activities

The methods employed for the determination of nicotinamide nucleotide-linked enzyme activities were based on those described by Davies (1956), with some modifications. A Unicam SP. 500 spectrophotometer was used in all spectrophotometric estimations. The changes in extinction, E , at $340\text{ m}\mu$ were recorded with the tungsten lamp, with the filter in position 3 and silica cells of 1 cm. light-path. In all determinations the reaction mixtures contained phosphate buffer (sodium and potassium salts, 8 mM, pH 7.1), and the enzyme preparation (50 or $100\text{ }\mu\text{l.}$, depending on the resulting turbidity). Substrates (not more than 0.2 ml.) were usually added last; exceptions are noted below. The addition of spermine (50 $\mu\text{l.}$, 0.2 M) was made either before or after the addition of substrate; the stimulation of a particular enzymic activity was approximately the same under either of these conditions, and the values have been corrected for the comparatively slow rise in E at $340\text{ m}\mu$ which occurs on adding spermine to the enzyme preparation in the absence of substrates; this increase in E was not affected by the addition of either NAD or NADP. The contents of the cell were rapidly stirred with a polythene spoon immediately after the final addition and readings commenced. Care was taken to correct for changes in E due to dilution and absorption by the substrate at $340\text{ m}\mu$. With the undialysed soluble fractions obtained from compressed carrot tissue, the enzyme preparation alone oxidized or reduced the added reduced and oxidized nicotinamide nucleotides respectively (see the Results section). All determinations were carried out at room temperature (about 20°). The activities of NAD- and NADP-dependent dehydrogenases were calculated from the production of NADH_2 and NADPH_2 ; in such assays the initial concentration of NAD and NADP was 1 mM and the conditions described below were employed; all concentrations quoted are the final concentrations in the assay medium. No enzyme preparations reduced NAD in the absence of added substrate; NADP was reduced by undialysed press-juice, but not by other preparations, in the absence of substrates (see Table 4, and text); where

appropriate all NADP-linked activities have been corrected for this reduction.

For malate dehydrogenase, the concentration of malate was 100 mM, except where noted in Table 1; the assay was from 0 to 30 sec. For L-glutamate dehydrogenase, the concentration of L-glutamate was 50 mM; the assay was from 0 to 30 sec. For isocitrate dehydrogenase, the concentration of isocitrate was 40 mM; MnCl_2 (0.3 mM) was also present; the assay was from 1 to 5 min. For glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, the concentration of glucose 6-phosphate and of 6-phosphogluconate was 25 mM; the assay was from 1 to 5 min. For α -oxoglutarate dehydrogenase, the concentration of α -oxoglutarate was 40 mM; CoA (0.2 mM) and thiamine pyrophosphate (0.2 mM) were also present; the assay was from 0 to 3 min. For α -glycerophosphate dehydrogenase and β -hydroxybutyrate dehydrogenase, the concentration of α -glycerophosphate and of β -hydroxybutyrate was 30 mM; the assay was from 1 to 3 min.

Malate-dehydrogenase, glutamate-dehydrogenase and lactate-dehydrogenase activities were also determined from the rate of oxidation of NADH_2 (final concn. 0.06 mM) under the following conditions. For malate dehydrogenase, the concentration of oxaloacetate was 1 mM; the assay was from 0 to 30 sec. For L-glutamate dehydrogenase, the concentration of α -oxoglutarate was 16 mM; ammonium chloride (15 mM) was also present (variation of the ammonium chloride concentration from 3 to 30 mM did not appreciably affect the rate of this reaction).

The activities described below were also determined.

Succinate-dehydrogenase activity was determined from: (i) the decrease in E at $600\text{ m}\mu$ between 1 and 5 min. after the addition of succinate (final concn. 50 mM) to 2,6-dichlorophenol-indophenol solution ($17\text{ }\mu\text{M}$)—the molar extinction coefficient for the oxidized form of 2,6-dichlorophenol-indophenol (referred to as 'indophenol' in the Tables) was taken to be $20.2 \times 10^3\text{ cm}^2$ (Steyn-Parvé & Beinert, 1958); blank values (no succinate added) were not greater than 5% of the rate of dye reduction found with mitochondria in the presence of succinate; and from (ii) the uptake of oxygen in the presence of phenazine methosulphate as described by Singer & Kearney (1957); the phosphate buffer was at pH 7.1 and the concentration of succinate used was 30 mM.

Cytochrome-oxidase activity was determined from the increased rate of oxidation of reduced cytochrome c on the addition of the enzyme preparation (Smith, 1955). Reduced cytochrome c was prepared by treatment of cytochrome c with sodium dithionite, the excess of dithionite being removed by aeration. The molar extinction coefficient for reduced cytochrome c was taken to be $27.2 \times 10^3\text{ cm}^2$ (Henderson & Rawlinson, 1956). Activity (per min.) was calculated from the average change in E between 0 and 10 min. after the addition of the enzyme preparation; a correction was made for autooxidation of reduced cytochrome c over this period.

'Malic enzyme' was assayed by measuring the increase in the rate of change in E at $340\text{ m}\mu$ between 0 and 2 min. after the addition of MnCl_2 (final concn. 0.3 mM) to a solution containing malate (0.1 M) and NADP (1 mM).

NADH_2 -linked and NADPH_2 -linked 2,6-dichlorophenol-indophenol reductases were assayed by measuring the decrease in E at $340\text{ m}\mu$ between 1 and 3 min. after the addition of enzyme preparation ($100\text{ }\mu\text{l.}$) to a solution

containing 2,6-dichlorophenol-indophenol (0.1 mM) and NADH₂ or NADPH₂ (0.1 mM). A correction was made for the small non-enzymic oxidation of NADH₂ and NADPH₂ by 2,6-dichlorophenol-indophenol between 1 and 3 min.

NAD- and NADP-linked reductions of 2,6-dichlorophenol-indophenol with malate were determined by the decrease in *E* at 600 m μ between 1 and 3 min. after the addition of enzyme (100 μ l.) to a solution containing malate (0.1 M), NAD or NADP (1 mM) and 2,6-dichlorophenol-indophenol (0.03 mM).

NAD(P)-transhydrogenase activity was measured by the increase in *E* at 340 m μ between 0 and 3 min. after the addition of glucose 6-phosphate (final concn. 20 mM) to a solution containing NAD (1 mM) and NADP (a trace).

Oxaloacetate carboxylase and pyruvate decarboxylase were assayed by the method of Davies (1956) with minor modifications. The initial rate of CO₂ production was determined when the enzyme preparation was added to substrate (30 mM), phosphate buffer (sodium and potassium salts, 40 mM, pH 7.1), thiamine pyrophosphate (2 mM), MnCl₂ (0.2 mM) and glutathione (freshly prepared, 0.2 mM). The total volume was 2.5 ml. and the gas phase N₂+CO₂ (95:5). The rate of CO₂ production before the addition of enzyme was negligible, and with pyruvate the rate of CO₂ production after the addition of enzyme was a measure of pyruvate-decarboxylase activity (Singer & Pensky, 1952; James & Richens, 1960*a*), although non-enzymic decarboxylation may have contributed in part (see the Results section); CO₂ production with oxaloacetate results from the oxaloacetate-carboxylase component of 'malic enzyme' (Ochoa, Mehler & Kornberg, 1948) and also may be due in part to non-enzymic decarboxylation. The increase in CO₂ production on the addition of ATP or ITP (final concn. 4 mM) represents oxaloacetate carboxylase (Utter, Kurahashi & Rose, 1954; Mazelis & Vennesland, 1957).

Analytical methods

Protein determination. Mitochondrial protein was estimated as described by Dalgarno & Birt (1962*a*); protein in the press-juice was determined by adding biuret reagent (see Cleland & Slater, 1953) directly, and reading the *E* after 1 hr.

Measurement of oxygen consumption. The apparatus and methods used for measuring oxygen consumption were the same as those described by Dalgarno & Birt (1962*a*).

Detection of transamination. The experimental procedure and chromatographic methods were the same as those described by Dalgarno & Birt (1962*a, b*), except that no EDTA or sucrose was present in the incubation medium; 0.2 ml. of press-juice, dialysed overnight against phosphate buffer (sodium and potassium salts, 10 mM, pH 7.1), was added to the incubation medium.

RESULTS

Tables 1, 2 and 4 refer by name and also by number to a variety of enzymes; for convenience these are quoted in the text with the appropriate number(s) in parentheses after the particular enzyme.

Enzymic activities in a soluble fraction prepared without albumin

The enzymes of relatively high activity in the soluble fraction prepared without albumin included NAD-linked malate dehydrogenase (5, 6, 7, 8, 9, 11), NAD-linked lactate dehydrogenase (12) and NADP-linked isocitrate dehydrogenase (13);

Table 1. *Enzymic activities of mitochondrial and supernatant fractions from carrot tissue, prepared without albumin in the homogenizing medium*

For details of measurements see the Materials and Methods section; all activities refer to determinations on the supernatant fraction, except for 1 and 4 which refer to a mitochondrial fraction.

Enzyme system	Time of storage at -15° (hr.)	Direction of reaction	Activity (μ moles of product/mg. of protein/hr.)
1 Succinate dehydrogenase	0	Indophenol reduction	0.7
2 Succinate dehydrogenase	0	Indophenol reduction	0.0
3 Cytochrome oxidase	92	Reduced cytochrome <i>c</i> oxidation	0.015
4 Malate dehydrogenase	0	NAD reduction	6.5
5 Malate dehydrogenase	0	NAD reduction	2.9
6 Malate dehydrogenase	24	NAD reduction	2.2
7 Malate dehydrogenase	24	NAD reduction (malate 200 mM)	1.7
8 Malate dehydrogenase	24	NADH ₂ oxidation	20
9 Malate dehydrogenase	72	NAD reduction	2.0
10 Malate dehydrogenase	72	NADP reduction	0.2
11 Malate dehydrogenase	92	NAD reduction	1.4
12 Lactate dehydrogenase	24	NADH ₂ oxidation	4.1
13 Isocitrate dehydrogenase	92	NADP reduction	0.4
14 Isocitrate dehydrogenase	96	NAD reduction	0.0
15 α -Oxoglutarate dehydrogenase	96	NAD reduction	0.0
16 L-Glutamate dehydrogenase	24	NAD reduction	0.0
17 NADH ₂ -linked indophenol reductase	72	NADH ₂ oxidation	3.3
18 NADPH ₂ -linked indophenol reductase	72	NADPH ₂ oxidation	4.0
19 Malate-NAD-linked indophenol reductase	72	Indophenol reduction	0.5
20 Malate-NADP-linked indophenol reductase	72	Indophenol reduction	0.2

Table 2. *Enzymic activities of mitochondrial and supernatant fractions from carrot tissue, prepared with albumin in the homogenizing medium*

For details of measurement see the Materials and Methods section; all activities refer to determinations on the supernatant fraction except for 22 and 24 (mitochondria).

Enzyme system	Time of storage at -15° (hr.)	Direction of reaction	Activity (μ moles of product/0.1 ml. of enzyme/hr.)
21 Succinate dehydrogenase	56	Indophenol reduction	0.0
22 Succinate dehydrogenase	Nil	Phenazine methosulphate reduction	11.0
23 Succinate dehydrogenase	56	Phenazine methosulphate reduction	0.0
24 Cytochrome oxidase	Nil	Reduced cytochrome c oxidation	0.024*
25 Cytochrome oxidase	96	Reduced cytochrome c oxidation	0.0005*
26 Malate dehydrogenase	56	NAD reduction	0.85-1.16
27 Malate dehydrogenase	72	NAD reduction	0.77
28 Malate dehydrogenase	72	NADP reduction	0.12
29 Lactate dehydrogenase	72	NADH ₂ oxidation	1.62
30 Isocitrate dehydrogenase	56	NADP reduction	0.65-0.96†
31 Isocitrate dehydrogenase	56	NAD reduction	0.0
32 Glucose 6-phosphate dehydrogenase	56	NADP reduction	0.91
33 Glucose 6-phosphate dehydrogenase	56	NAD reduction	0.0
34 6-Phosphogluconate dehydrogenase	56	NADP reduction	0.12
35 6-Phosphogluconate dehydrogenase	56	NAD reduction	0.0
36 α -Oxoglutarate dehydrogenase	56	NAD reduction	0.0
37 L-Glutamate dehydrogenase	56	NAD reduction	0.0-0.12
38 L-Glutamate dehydrogenase	56	NADH ₂ oxidation	0.03
39 α -Glycerophosphate dehydrogenase	72	NAD reduction	0.0
40 β -Hydroxybutyrate dehydrogenase	72	NAD reduction	0.0
41 Transhydrogenase	72	NAD reduction	0.0

* For purposes of comparison the activity of cytochrome oxidase in mitochondrial and soluble fractions is expressed as μ moles of product/g. fresh wt. of original tissue/hr.

† The relatively greater NADP-linked isocitrate-dehydrogenase activity present in this preparation (cf. Table 1, no. 13) is due to the inclusion of MnCl_2 (0.3 mM) in the reaction mixture during the estimation; this addition is also made for the assay of NAD-linked isocitrate dehydrogenase.

no NAD-linked glutamate-dehydrogenase (16) activity was detected in this preparation although this enzyme was found in other fractions prepared with or without albumin (Table 1; cf. Tables 2 and 4). No NAD-linked isocitrate-dehydrogenase (14) or α -oxoglutarate-dehydrogenase (15) activity was detected. The soluble fraction contained no succinate-dehydrogenase (2, cf. 1) activity and only a trace of cytochrome-oxidase (3) activity, as estimated by methods which have been shown readily to detect such activities in mitochondrial preparations (cf. also Table 2). In plant tissues both succinoxidase (Pierpoint, 1959) and cytochrome oxidase (James & Richens, 1960b) are regarded as being exclusively associated with the mitochondrial fraction; it is likely therefore that the soluble fraction was free from substantial contamination by mitochondria or mitochondrial fragments.

The demonstration of a malate dehydrogenase in the soluble fraction recalls the report of a similar enzyme in pea-stem tissue (Price & Thimann, 1954; Davies, 1961). The soluble malate dehydrogenase in carrot tissue reduced NADP at one-tenth the rate of NAD (9, 10, Table 1; cf. Brummond & Burris, 1954). The activity of NAD-linked

malate dehydrogenase was lowered by 22 % when the concentration of malate was increased from 0.1 to 0.2 M (6, 7), perhaps as a result of substrate inhibition by malate. Malate-dehydrogenase activity determined by NAD reduction was one-ninth that determined by NADH₂ oxidation (6, 8).

The demonstration of similar levels of NADH₂- and NADPH₂-linked 2,6-dichlorophenol-indophenol-reductase activity (17, 18) is consistent with the report by Sisler & Evans (1958) that, in soluble fractions from roots of tobacco plants, nicotinamide nucleotide-linked diaphorases were equally active with either NADH₂ or NADPH₂ as electron donor. With malate providing hydrogen equivalents in this system (19, 20), the activity of the NADPH₂-linked diaphorase was low relative to the NADH₂-linked diaphorase; this was probably due to the rate limitation imposed by NADP-linked as compared with NAD-linked malate dehydrogenase. NAD-linked malate-dehydrogenase activity was reduced by 23, 31 and 52 % after storage of the soluble fraction at -15° for 24, 72 and 92 hr. respectively (5, 6, 9, 11).

The addition of mitochondria and soluble fractions (both prepared without albumin) to oxaloacetate and pyruvate solutions, with thiamine

pyrophosphate (2 mM) and manganous chloride (0.2 mM), led to a decarboxylation of these organic acids at twice the rate induced by boiled fractions (see the Materials and Methods section for the conditions of the incubation). The addition of ATP (final concn. 4 mM) stimulated decarboxylation twofold only with the unboiled preparation; ITP had no marked effect on the rate of decarboxylation.

Enzymic activities in a soluble fraction prepared with albumin

Mitochondria isolated from carrot tissue dispersed in a medium containing albumin had different properties from those prepared without albumin (Dalgarno & Birt, 1962*a*). It was therefore of interest to determine whether dispersion of carrot tissue in a medium containing albumin (1 %) altered the properties of the resulting soluble fraction (Table 2). NAD-linked and NADP-linked malate-dehydrogenase (26, 27, 28), NAD-linked lactate-dehydrogenase (29) and NADP-linked isocitrate-dehydrogenase (30) activities were detected in this fraction. NAD-linked malate-dehydrogenase activity was 6.5 times as great as that of NADP-linked malate dehydrogenase (27, 28). NAD-linked glutamate-dehydrogenase activity was assayed by both forward and reverse reactions (27, 28). There was no succinate-dehydrogenase activity as estimated by the reduction of either 2,6-dichlorophenol-indophenol (21) or phenazine methosulphate (23, cf. 22, with mitochondria); the activity of cytochrome oxidase in the soluble fraction was approximately one-fiftieth that in the mitochondrial fraction (24, 25), expressed on the basis of the fresh weight of original tissue used for each preparation. There was no NAD-linked isocitrate-dehydrogenase (31) or α -oxoglutarate-dehydrogenase (36) activity. The glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase had absolute requirements for NADP (32, 33, 34, 35). The demonstration of these latter enzymes in the soluble fraction is consistent with the report by Ap Rees & Beevers (1960) that part of the respiration of carrot slices involves the pentose phosphate pathway. No NAD-linked α -glycerophosphate-dehydrogenase (39), NAD-linked β -hydroxybutyrate-dehydrogenase (40) or NAD(P)-transhydrogenase (41) activities were detected in this fraction. The absence of detectable transhydrogenase activity recalls the low transhydrogenase activity in the soluble fraction of rat liver (Stein & Kaplan, 1959).

As albumin was present in large amounts in this preparation (approximately 125 mg./ml.), activities were expressed as μ moles of product/0.1 ml. of enzyme solution/hr., rather than on a protein basis (Table 2). Since 1 ml. of soluble fraction prepared with or without albumin was derived from either approximately 10 g. or 20 g. fresh wt. of tissue

respectively, an approximate comparison can be made of various activities in the two fractions. On this basis there was no marked difference in the activity of malate dehydrogenase (or of lactate dehydrogenase) in the two preparations.

Enzymic activities detected in a soluble fraction prepared in a medium containing sucrose, phosphate buffer and spermine

Bernfeld, Bernfeld, Nisselbaum & Fishman (1954) reported the activation of diluted purified β -glucuronidase by 0.3 mM-spermine, and the results of Tabor (1960) indicate that spermine or spermidine (0.3 mM) inhibit the succinate-induced swelling of freshly prepared rat-liver mitochondria, cause agglutination of mitochondria in suspension and inhibit leakage of ultraviolet-absorbing material from these particles. As these data suggested that spermine may protect mitochondria from damage and facilitate their sedimentation, it was added to the homogenizing medium (final concn. 20 mM) during the preparation of both soluble and mitochondrial fractions from carrot tissue. The following enzymes were detected in the soluble fraction: NAD-linked and NADP-linked malate dehydrogenases, NAD-linked lactate dehydrogenase, NADP-linked isocitrate dehydrogenase, NAD-linked glutamate dehydrogenase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and 'malic enzyme'.

On a fresh weight basis the activities of all the soluble enzymes prepared from the spermine-containing homogenate except NADP-linked isocitrate dehydrogenase were substantially less than those obtained from a homogenate prepared with albumin (Table 3; cf. Table 2). The inclusion of spermine (final concn. 3 mM) in the assay medium (see the Materials and Methods section) increased the activity of NAD-linked malate dehydrogenase 4.6-fold, glucose 6-phosphate dehydrogenase 1.2-fold, and lactate dehydrogenase 1.4-fold, but not the activity of NAD-linked glutamate dehydrogenase (further information concerning the addition of spermine during the assay is included in the next section).

Mitochondria isolated from tissue homogenized in a medium containing spermine oxidized both malate and succinate more rapidly than particles prepared without spermine; q_{O_2} values with malate were increased 1.5- to 6-fold, and with succinate 10- to 13-fold.

Enzymic activities in press-juice from carrot tissue

The application of sufficient pressure with a Fischer mincer to supply 0.4 ml. of press-juice from 10 g. of carrot tissue yielded a fraction exhibiting NAD-linked malate-dehydrogenase (44),

Table 3. *Comparison of the enzymic activities of soluble fractions prepared by homogenizing in the presence of albumin or spermine*

All data are calculated on the basis of the fresh weight of the disrupted tissue. For details of measurement see the Materials and Methods section.

	$\frac{\text{Activity of the enzyme from the homogenate containing spermine}}{\text{Activity of the enzyme from the homogenate containing albumin}} \times 100$	
	(%)	
	Expt. 1	Expt. 2
NAD-linked malate dehydrogenase	17	21
NADP-linked malate dehydrogenase	50	25
NAD-linked lactate dehydrogenase	3	9
NADP-linked isocitrate dehydrogenase	85	109
NAD-linked glutamate dehydrogenase	63	22
NADP-linked glucose 6-phosphate dehydrogenase	14	29
NADP-linked 6-phosphogluconate dehydrogenase	40	—

Table 4. *Enzymic activity of press-juice from carrot tissue*

For details of measurement see the Materials and Methods section.

Enzyme system	Direction of reaction	Activity (μ mole of product/mg. of protein/hr.)
42 Succinate dehydrogenase	Indophenol reduction	0.0
43 Cytochrome oxidase	Reduced cytochrome c oxidation	0.0
44 Malate dehydrogenase	NAD reduction	0.17
45 Isocitrate dehydrogenase	NAD reduction	0.0
46 Isocitrate dehydrogenase	NADP reduction	0.05*
47 Lactate dehydrogenase	NADH ₂ oxidation	0.06*
48 L-Glutamate dehydrogenase	NAD reduction	0.05
49 L-Glutamate dehydrogenase	NADP reduction	0.0
50 Glucose 6-phosphate dehydrogenase	NADP reduction	0.0
51 6-Phosphogluconate dehydrogenase	NADP reduction	0.0
52 α -Glycerophosphate dehydrogenase	NAD reduction	0.0
53 β -Hydroxybutyrate dehydrogenase	NAD reduction	0.0

* The enzyme fraction without added substrate reduced NADP, but not NAD, at the rate of 0.007 μ mole/mg. of protein/hr.; the addition of MnCl₂ (0.3 mM) increased the rate to 0.014 μ mole/mg. of protein/hr. (see text). The activity of NADP-linked isocitrate dehydrogenase has been calculated from the increase in the rate of NADP reduction on the addition of isocitrate. The enzyme fraction without added substrate also oxidized NADH₂ at the rate of 0.02 μ mole/mg. of protein/hr. Lactate-dehydrogenase activity has been calculated from the increase in rate of NADH₂ oxidation with pyruvate.

NADP-linked isocitrate-dehydrogenase (46), NAD-linked lactate-dehydrogenase (47) and NAD-linked glutamate-dehydrogenase (48) activities (Table 4). No succinate-dehydrogenase (42), cytochrome-oxidase (43), NAD-linked isocitrate-dehydrogenase (45), NADP-linked glutamate-dehydrogenase (49), α -glycerophosphate-dehydrogenase (52) or β -hydroxybutyrate-dehydrogenase (53) activities were detected.

This preparation also reduced NADP but not NAD without the addition of any substrate (see Table 2, footnote), and the rate of reduction was increased twofold with manganous chloride (0.3 mM); it was concluded that endogenous substrates capable of reducing NADP were present in the enzyme fraction. This was confirmed by the use of press-juice dialysed overnight against phosphate buffer (sodium and potassium salts, 10 mM, pH 7.1); with this preparation the rate of reduction

of NADP was very low without added substrate (about 10 % of the original rate). The absence of glucose 6-phosphate-dehydrogenase (50) and 6-phosphogluconate-dehydrogenase (51) activities is noteworthy as the fraction contained other typically soluble enzymic activities; as NADP-linked isocitrate dehydrogenase was readily detected, the failure of glucose 6-phosphate or 6-phosphogluconate to increase the rate of NADP reduction was not due to the saturation of the NADP-reducing system by endogenous substrate. It seems possible therefore that these two enzymes are not in free solution in the cytoplasm of the cell (cf. Newburgh & Cheldelin, 1956).

The stimulation by manganous chloride of the endogenous formation of NADPH₂ by the soluble fractions suggested that this reduction was due either to NADP-linked isocitrate dehydrogenase or to 'malic enzyme'. In other press-juice fractions

'malic enzyme' activity was low relative to those of NADP-linked isocitrate dehydrogenase and NAD-linked malate dehydrogenase; as isocitrate is the third most abundant organic acid in carrot tissue (Bryant & Overell, 1951) the reduction of NADP by press-juice alone was probably due to endogenous isocitrate. 'Malic enzyme' activity was present (cf. Anderson, Stafford, Conn & Vennesland, 1952), and the rate of reduction of NADP by this enzyme was approximately one-tenth the rate of reduction of NAD by malate dehydrogenase under the same conditions.

Dialysed press-juice also contained an active glutamate-oxaloacetate transaminase; approximate calculations of activity indicated that the dialysed press-juice from 1 g. fresh wt. of tissue transaminated 4 μ moles of aspartate to glutamate/hr.

The addition of spermine (3 mM) during the assay of press-juice dialysed for 3 hr. stimulated the activity of NAD-linked malate dehydrogenase 5-fold, that of NADP-linked malate dehydrogenase 3.5-fold, that of isocitrate dehydrogenase 1.2-fold, that of 'malic enzyme' 3.5-fold, and that of L-glutamate dehydrogenase 3-fold. The addition of spermine did not inhibit the activity of any enzymes studied. No glucose 6-phosphate-dehydrogenase or succinate-dehydrogenase activity was detected in the presence of spermine. With 0.3, 0.9, 3.0 and 9.0 mM-spermine in the assay medium the activity of NAD-linked malate dehydrogenase was increased by factors of 1.05, 1.1, 5.1 and 7.1 respectively. The presence of bovine serum albumin in the assay medium (final concentrations of 1.0 and 7.5 mg./ml.) did not alter the activity of NAD-linked malate dehydrogenase.

Further experiments on the subcellular localization of malate dehydrogenase

Work described by Dalgarno & Birt (1962b) indicated that the malonate-insensitive respiration of carrot slices in the presence of malate was not due to the oxidation of malate by a malonate-insensitive respiratory pathway in the mitochondria. On the basis of detecting malate-dehydrogenase activity in the soluble fraction of the tissue, it was suggested that the extramitochondrial dehydrogenase may be capable of generating hydrogen for oxidation by a pathway not directly involving the tricarboxylic acid cycle. Although it has not been possible to prepare soluble fractions which do not contain an active malate dehydrogenase, the work of Bendall & de Duve (1960) indicated that the disruption of rat-liver mitochondria in a blender may release 'latent' malate dehydrogenase and glutamate dehydrogenase. Therefore a direct attempt has been made to determine the extent to which the malate-dehydro-

genase and glutamate-dehydrogenase activities observed in the extramitochondrial fraction are likely to be derived from mitochondria.

Relative activity of malate dehydrogenase and glutamate dehydrogenase in press-juice fractions obtained with increasing compression. Press-juice was obtained from carrot tissue compressed extremely gently (10 g. of tissue producing 0.15 ml. of liquid); the compression was increased and two fractions were taken from the subsequent 0.35 ml. of liquid. Table 5 indicates that no marked change occurred in the ratio of NAD-linked glutamate-dehydrogenase and NAD-linked malate-dehydrogenase activities with increasing compression; in the three fractions glutamate-dehydrogenase activity was 28, 21 and 25 % respectively of the malate-dehydrogenase activity. The maximum compression employed was the same as that used for preparing the soluble fraction described in Table 4; no succinate-dehydrogenase or cytochrome-oxidase activity was present in such a preparation.

Release of malate dehydrogenase from mitochondria incubated at 0° and 25°. Mitochondria were prepared by the standard procedure of Dalgarno & Birt (1962a) except that 150 g. of tissue was used. One half of the preparation was incubated at 25° with NAD (1 mM), malate (20 mM), manganous chloride (5 mM) and sucrose (0.3 M). The other portion was incubated at 0° under identical conditions. After 70 min. the suspensions were centrifuged (12 000g for 10 min.) and the supernatant fluids tested for malate-dehydrogenase activity under standard conditions (i.e. reduction of NAD). The supernatant fluids derived from mitochondria incubated at 0° and 25° respectively contained malate-dehydrogenase activities of 0.56 and 1.78 μ -moles of NADH₂/mg. of protein/hr. These activities

Table 5. *Enzymic activities in press-juice obtained from carrot tissue with increasing compression*

Fractions 1, 2 and 3 respectively represent the first 1-3, 4-8 and 9-11 drops (inclusive) of soluble fraction squeezed from 10 g. of diced tissue as described in the Materials and Methods section. To obtain sufficient soluble fraction, eight lots of tissue (10 g.) were used, and each fraction was pooled after extraction. The activities of malate dehydrogenase and glutamate dehydrogenase have been determined from 0 to 30 sec. after the addition of malate or glutamate to solutions containing NAD (1 mM).

Fraction	Activity (μ mole of product/mg. of protein/hr.)		Protein/ml. of soluble fraction (mg.)
	Malate dehydrogenase	Glutamate dehydrogenase	
1	0.61	0.17	5.2
2	0.92	0.17	4.0
3	0.93	0.23	4.2

represented 8.6 % and 27 % respectively of the total malate-dehydrogenase activity of a mitochondrial suspension similarly prepared without albumin (i.e. 6.5 μ moles/mg. of protein/hr., see Table 1). The amount of protein in the supernatant obtained after incubation of the mitochondrial suspension at 0° and 25° was 12 and 13 mg. respectively; calculations based on relative activities indicate that approximately 10 % of the total activity in the supernatant fractions prepared either with or without albumin could be ascribed to contamination with the mitochondrial enzyme. A q_{O_2} value of 21 was recorded during incubation of the mitochondrial suspension at 25°.

Oxidation of 'external' reduced nicotinamide nucleotide coenzymes by carrot mitochondria prepared with and without albumin

Since there are a number of enzymes present in the soluble fraction of carrot tissue which are capable of generating reduced nicotinamide nucleotides, malate oxidation by the soluble dehydrogenase may contribute to the malonate-insensitive respiration of carrot slices with malate (Dalgarno & Birt, 1962*b*) if the hydrogen could be transferred from the coenzymes to a suitable terminal oxidase. As Bonner & Voss (1961) have concluded that a number of preparations of plant mitochondria are capable of oxidizing extramitochondrial $NADH_2$, the rate of oxidation of both $NADH_2$ and $NADPH_2$ by the carrot particles has been estimated.

Both $NADH_2$ derived from NAD, ethanol and alcohol dehydrogenase, and $NADH_2$ added as such (0.3 mM), were oxidized by carrot mitochondria prepared without albumin (Table 6). The addition of cytochrome *c* increased the rate of oxidation of generated $NADH_2$ by 83 %, and of added $NADH_2$ oxidation by 44 % (cf. Humphreys & Conn, 1956). The oxidation of $NADH_2$ (1 mM) by a similar preparation was 67 % more rapid than that of $NADPH_2$ at the same concentration (q_{O_2} values of 9.0 and 5.4 respectively). $NADPH_2$ generated from NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase was also oxidized. Malonate (20 mM) did not consistently affect the rate of oxidation of generated $NADH_2$ or $NADPH_2$ by these preparations.

Carrot mitochondria prepared in the presence of albumin are 'intact' by one criterion since they show respiratory control with ADP (Dalgarno & Birt, 1962*b*; cf. Bellamy & Bartley, 1960). However, q_{O_2} values for $NADH_2$ oxidation by carrot mitochondria prepared with and without albumin were 28 ('corrected' value) and 18 respectively. Carrot mitochondria prepared with and without spermine gave q_{O_2} values of 19 and 6 respectively during the oxidation of $NADH_2$ (1 mM); with ADP (10 mM) the values were 25 and 4.5 respectively.

Although 'external' $NADH_2$ was oxidized readily by carrot mitochondria prepared both with and without albumin, it was possible that a 'redox carrier', of one of the types proposed by Klingenberg & Bucher (1960), Sacktor & Dick (1961) and Racker (1961), may facilitate such $NADH_2$ oxidation if it functioned by assisting the transfer of extramitochondrial hydrogen into the mitochondrion.

However, the oxidation of generated $NADH_2$ by mitochondria prepared without albumin was not altered by α -glycerophosphate or malate (both 1 mM); the oxidation of added $NADH_2$ (2 mM) by mitochondria prepared with albumin was not altered by malate, α -glycerophosphate, β -hydroxybutyrate, glycollate or lactate (all 2 mM).

Interaction of soluble and mitochondrial fractions from carrot tissue in the oxidation of malate

Since the soluble fraction can generate reduced $NADH_2$ in the presence of malate, and carrot mitochondria even in a relatively 'intact' state are capable of oxidizing exogenous $NADH_2$, the malonate-sensitivity of malate oxidation by the soluble fraction plus mitochondria has been compared with that by the mitochondria described above. The extent to which the respiration was

Table 6. *Oxidation of 'external' reduced nicotinamide-adenine dinucleotide by carrot mitochondria*

Mitochondria were prepared and incubated without albumin. Oxygen uptake was measured in Warburg manometers. NAD or $NADH_2$ was added from the side arm after 10 min. equilibration, giving final concentrations of 1 and 0.3 mM respectively. The $NADH_2$ -generating system comprised ethanol (25 mM), alcohol dehydrogenase (6 mg.) and NAD. Malonate (final concn. 20 mM) was added 30 min. after readings started; the incubation medium contained $MgCl_2$ (5 mM), phosphate buffer (sodium and potassium salts, 10 mM, pH 7.1) and sucrose (0.32 M) in a total volume of 2.5 ml. Allowance was made, in the calculation of q_{O_2} values, for added alcohol dehydrogenase and cytochrome *c* (1 mg.). The incubation temperature was 25°. Unbracketed q_{O_2} values are calculated for the 30 min. period after the addition of NAD or $NADH_2$; the q_{O_2} values in parentheses are for the period 30–70 min. after the addition of NAD or $NADH_2$. ADH, alcohol dehydrogenase.

	q_{O_2} values (μ l. of O_2 /mg. of protein/hr.)	
	Without cytochrome <i>c</i>	With cytochrome <i>c</i>
$NADH_2$	1.6 (0.4)	2.3 (1.0)
$NADH_2$ -generating system:		
Complete	2.3 (3.4)	4.2 (4.2)
Without NAD	—	1.4 (0.5)
Without ADH	—	0.4 (0.3)
Without ethanol	—	0.0 (0.3)
With malonate	3.9 (3.2)	3.4 (3.8)

depressed by malonate depended on (i) whether the mitochondria and soluble fraction had been prepared with albumin, and (ii) whether L-cysteine-sulphinic acid was present during the incubation.

Soluble and mitochondrial fractions prepared without albumin. When mitochondria and soluble fraction were combined and incubated with malate (2 mM) and NAD (1 mM), the oxygen uptake was 60 % greater than that of mitochondria alone with malate and NAD. No oxygen uptake was detected when the soluble fraction was incubated with malate and NAD. Malonate (20 mM) decreased the oxygen uptake of mitochondria alone by 50 %, whereas with mitochondria plus soluble fraction the respiration was not depressed at all. Cysteine-sulphinic acid (4 mM) increased the oxygen uptake of mitochondria alone by 167 %, and of soluble fraction plus mitochondria by 317 %. With higher concentrations of malate (5–20 mM), complete insensitivity to malonate was not obtained, although the extent of inhibition by malonate was always lowered.

Soluble and mitochondrial fraction prepared with albumin. When similar experiments were performed with fractions prepared in the presence of albumin, incubation of soluble fraction with mitochondria decreased oxygen uptake below that obtained with mitochondria alone, and malonate-sensitivity was unaffected. Cysteine-sulphinic acid stimulated the respiration only slightly; it decreased considerably, but did not abolish, the inhibition of respiration by malonate.

This model system indicates the possibility of co-operation between subcellular compartments in determining the respiratory behaviour of the intact tissue. However, without further data, the many complex factors which influence the interaction of the systems *in vitro* preclude precise interpretation of the results and any rigorous extrapolation to the respiratory behaviour of the 'intact' carrot slice.

DISCUSSION

The data on the enzymic constitution of the soluble fractions, considered in conjunction with the results presented by Dalgarno & Birt (1962*a*, *b*), permit an evaluation of the localization of a number of enzymes in carrot tissue.

Particulate enzymes. The absence of succinate-dehydrogenase activity and the absence or very low level of cytochrome-oxidase activity in all the soluble fractions prepared is consistent with the reports (Pierpoint, 1959; James & Richens, 1960*b*) that these enzymes are located almost entirely, if not exclusively, in the mitochondria of plant tissues. No activities corresponding to the following enzymes were detected in any soluble fraction:

AD-linked isocitrate dehydrogenase, NAD-linked

α -oxoglutarate dehydrogenase, NAD-linked β -hydroxybutyrate dehydrogenase and NAD-linked α -glycerophosphate dehydrogenase. Since such activities have been detected in mitochondrial preparations (Dalgarno & Birt, 1962*a*, *b*) it is likely that these enzymes are also exclusively located in the mitochondria.

Soluble enzymes. A primarily extramitochondrial localization for NAD-linked lactate dehydrogenase, NADP-linked glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase is suggested by the results; the relative ease of dissociation of the two former enzymes on resuspension of mitochondria (Dalgarno & Birt, 1962*b*), and the absence of NADP-linked 6-phosphogluconate-dehydrogenase activity in mitochondria prepared with albumin (L. Dalgarno & L. M. Birt, unpublished work), supports this conclusion.

Enzymes in both the soluble and particulate fractions. NADP-linked isocitrate dehydrogenase, NAD-linked glutamate dehydrogenase and NAD-linked and NADP-linked malate dehydrogenase appear to be located in both mitochondrial and soluble fractions (see the Results section; and Dalgarno & Birt, 1962*a*, *b*).

NADP-linked isocitrate dehydrogenase was found in high activity in all three soluble fraction preparations, and was also present in the mitochondrial fraction. Resuspension of mitochondria ten times in sucrose caused some loss of NADP-linked isocitrate dehydrogenase relative to both succinate-dehydrogenase and NAD-linked isocitrate-dehydrogenase activities (L. Dalgarno & L. M. Birt, unpublished work). This was not as marked as the loss of lactate-dehydrogenase and glucose 6-phosphate-dehydrogenase activities on resuspension of carrot mitochondria (Dalgarno & Birt, 1962*b*), and the resuspended mitochondria retained appreciable NADP-linked isocitrate dehydrogenase. This suggested that, although there was some NADP-linked isocitrate-dehydrogenase activity adsorbed to the particles, at least part of this enzyme activity resided in the mitochondria themselves. Hogeboom & Schneider (1950) reported that 20 % of the total NADP-linked isocitrate-dehydrogenase activity of mouse liver was present in the mitochondria; however, in the work with carrot preparations different methods were used for measuring mitochondrial and soluble dehydrogenases and no direct comparison of total activities in the two fractions is possible.

The presence of glutamate-dehydrogenase activity in the soluble fraction recalls the work of Bendall & de Duve (1960), who found that, after rat-liver mitochondria had been homogenized in a Waring Blendor, 'activation' of glutamate dehydrogenase and malate dehydrogenase occurred with the liberation of these enzymes into solution.

In 'graded osmotic activation' experiments, the 'activation' curves for these enzymes followed each other closely; the 'activation' of glutamate dehydrogenase was maximal only after homogenization for 15 min. at full speed in 0.25 M-sucrose. After homogenization of rat-liver tissues for the same time as was used in the preparation of both soluble and mitochondrial fractions from carrot tissue (20 sec.), the increase in glutamate-dehydrogenase activity was approximately 5% of the maximum value. Thus it seemed possible that the results obtained with soluble fractions were due to contamination with enzymes lost from the mitochondria during the homogenizing of the tissue, or during the extraction of the press-juice. However, in one experiment with press-juice where very gentle pressure was used initially (insufficient to contaminate the preparation with the particle-bound enzymes succinate dehydrogenase and cytochrome oxidase), activity corresponding to both enzymes was obtained; an increase in pressure yielded fractions with a relatively constant ratio of the activities of malate dehydrogenase and glutamate dehydrogenase; it appears unlikely, therefore, that substantial glutamate-dehydrogenase or malate-dehydrogenase activity was derived from mitochondria. Moreover, there are other reports of glutamate dehydrogenase in the soluble fraction of plant tissues (cf. Estamann, Conn & McLaren, 1959; Rautanen & Tager, 1955, quoted by Estamann *et al.* 1959; Sanwal, 1961), and of malate dehydrogenase in the soluble fractions of plant (Price & Thimann, 1954; Davies, 1961), mammal (Christie & Judah, 1953; Delbruck, Schimmassek, Bartsch & Bucher, 1959) and insect (Delbruck, Zebe & Bucher, 1959) tissues.

Oxidation of 'external' reduced nicotinamide nucleotide coenzymes by mitochondria. No evidence was obtained for the operation of any of the enzyme sequences proposed as hydrogen carriers from soluble reduced nicotinamide nucleotides to the mitochondrial terminal oxidase (for a discussion of these systems see Klingenberg & Bucher, 1960). Indeed, the results support the contention of Bonner & Voss (1961) that a characteristic feature of plant mitochondria is the relative ease with which they oxidize external NADH_2 itself. Nevertheless, the assessment of this observation as 'one of the marked differences between plant and animal tissues' is complicated by the changes occurring in mitochondrial properties with changing conditions of isolation and incubation. Thus Stickland (1961), using polarographic methods, has shown the importance of NADH_2 concentration and of preliminary shaking on the oxidation of NADH_2 by pea-root mitochondria, and Deshpande, Hickman & Von Korff (1961) described preparations of heart-muscle mitochondria which will couple the rela-

tively rapid oxidation of exogenous NADH_2 to the formation of ATP.

The results also re-emphasize the difficulties of making general assessments of the 'intactness' of isolated mitochondria. It is commonly considered that 'intact' particles exhibit a considerable dependence on ADP in respiration and a very low rate of oxidation of exogenous reduced nicotinamide nucleotides (see Klingenberg & Bucher, 1960; Linnane & Ziegler, 1958; Lehninger, 1951). However, the carrot mitochondria prepared with albumin oxidize external NADH_2 at relatively rapid rates although they also show considerable respiratory control by ADP (Dalgarno & Birt, 1962b), and give P:O values of 2-3 during the oxidation of malate (L. Dalgarno & L. M. Birt, unpublished work).

SUMMARY

1. Soluble fractions, substantially free of mitochondrial contamination, have been prepared from carrot tissue by homogenizing with and without albumin in the blending medium and by compressing the tissue in a Fischer mincer.

2. No marked differences were observed in the enzymic constitution of soluble fractions prepared with and without albumin.

3. Activities corresponding to the following enzymes have been found in the soluble fraction prepared with albumin: NAD-linked and NADP-linked malate dehydrogenase, NAD-linked lactate dehydrogenase, NADP-linked isocitrate dehydrogenase, NAD-linked glutamate dehydrogenase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase; there was no succinate-dehydrogenase, NAD-linked isocitrate-dehydrogenase, α -glycerophosphate-dehydrogenase, β -hydroxybutyrate-dehydrogenase or transhydrogenase activity, and only a trace of cytochrome-oxidase activity.

4. Activities corresponding to the following enzymes have been found in press-juice: NAD-linked malate dehydrogenase, NADP-linked isocitrate dehydrogenase, NAD-linked lactate dehydrogenase, NAD-linked glutamate dehydrogenase, 'malic enzyme' and glutamate-oxaloacetate transaminase; there was no succinate-dehydrogenase, cytochrome-oxidase, NAD-linked isocitrate-dehydrogenase, NADP-linked glutamate-dehydrogenase or glucose 6-phosphate-dehydrogenase activity.

5. Press-juice extract from tissue compressed extremely gently contained malate-dehydrogenase and glutamate-dehydrogenase activity; the ratio of these activities did not alter markedly in press-juice fractions obtained with higher compression.

6. Both NADH_2 and NADPH_2 added as such, and NADH_2 and NADPH_2 formed from a gener-

ating system, were oxidized by carrot mitochondria prepared without albumin. NADH_2 is oxidized 67% more rapidly than NADPH_2 , and malonate (20 mM) does not consistently affect the oxidation of either NADH_2 or NADPH_2 .

7. The oxidation of added NADH_2 by carrot mitochondria prepared with and without albumin gave q_{o_2} values of 28 and 18 respectively; the corresponding values for mitochondria prepared with and without spermine were 19 and 6.

8. The oxidation of added NADH_2 (2 mM) by carrot mitochondria prepared with albumin is not stimulated by malate, α -glycerophosphate, β -hydroxybutyrate, glycollate or lactate (all 2 mM).

9. The incubation of carrot mitochondrial and soluble fractions together sometimes lowered the malonate-sensitivity of the respiration; the extent to which this occurred depended on (i) whether the mitochondria and soluble fraction had been prepared with albumin, and (ii) whether L-cysteine-sulphinic acid was present during the incubation.

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