Preparation and Properties of a Mitochondrial Fraction from Carrot Tissue

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No detailed description of the preparation and properties of mitochondria from storage tissue of the carrot is available, despite the frequent use of this tissue in a variety of metabolic studies.

As a first step in an investigation of the respiratory pathways of the tissue, this paper describes the influence of different preparative procedures, incubation media, cofactors, and respiratory and metabolic inhibitors on the oxidation of different substrates by a mitochondrial fraction isolated from carrot tissue. Carrot mitochondria are shown to possess the full complement of tricarboxylic acid-cycle oxidative enzymes.

MATERIALS AND METHODS

Materials

Apart from those specified below, organic acids, amino acids and coenzymes used were supplied by Sigma Chemical Co., St Louis, Mo., U.S.A. Malonic acid (98% minimum purity) was obtained from Hopkin and Williams, Chadwell Heath, Essex; α-glycerophosphate from L. Light and Co. Ltd., Colnbrook, Bucks.; citric acid (99·5% minimum purity) from May and Baker Ltd., Dagenham, Essex. EDTA (disodium salt), potassium dihydrogen phosphate, disodium hydrogen phosphate and sucrose were standard analytical reagents. The disodium salt of ATP and the monosodium salt of ADP were used. Pyruvate solutions were prepared from the sodium salt. Bovine serum albumin was obtained from Commonwealth Serum Laboratories, Melbourne, Australia.

Preparation of carrot mitochondria

The mitochondrial preparation was isolated from carrots obtained within 24 hr. of the harvest and stored for 16–64 hr. at 0–4° before use. The use of old-season carrots frequently gave inactive preparations and new-season tissue was therefore used whenever possible.

To obtain adequate yields of mitochondrial protein, the relatively drastic disorganization given by a Waring Blendor was required rather than that obtained by grinding with a pestle and mortar or in an homogenizer of the Potter–Elvehjem type. However, the tissue was homogenized for the shortest possible time to minimize the disorganization of mitochondrial structure. As large pieces of diced carrot tissue required a longer homogenization period, cubes of 0·5 cm.^{3} or less were suspended in the blending medium in a ratio of 1:1·1 (w/v). The entire Blendor assembly was rocked round the vertical axis during the homogenization to ensure mixing of the contents.

Although xylem contains less carotene than phloem tissue does, it was not more effective than the whole tissue in giving a mitochondrial fraction free from carotene, which was always present in the homogenate in fragments of chromoplasts of various sizes; consequently all preparations are from almost equivalent amounts of xylem and phloem.

All operations were carried out in a cold room at 0–4° and all apparatus used was chilled to this temperature overnight. The tissue (150 g.) was blended in a Waring Blendor (4-point blade) at maximum speed with 160 ml. of sucrose (0·44 M) containing 5 mM-EDTA (pH 7·2). [A medium of KCl (0·44 M) containing tris–HCl buffer (20 mM, pH 7·2) and $MgCl_2$ (5 mM) caused clumping of the isolated particles and increased the contamination with carotene.] The homogenate was filtered and squeezed through two layers of muslin. The filtrate (total volume approx. 200 ml. with a sucrose concentration of about 0·36 M, calculated from the initial volume of sucrose added and the volume of liquid finally obtained) was centrifuged in a Servall SS34 centrifuge at 3000*g* for 10 min. The resulting pellet was shown by an iodine test and microscopic examination to be mainly starch granules. The supernatant was centrifuged at 12 000*g* for 20 min., the pellet resuspended with a glass homogenizer in sucrose (0·44 M) containing EDTA (2·5 mM), and the suspension centrifuged again at 12 000*g* for 20 min. After the free-flowing carotene (presumably the smallest chromoplast fragments) had been poured off, the pellet was suspended in the required volume (generally 3–8 ml.) of sucrose (0·44 M) containing EDTA (2·5 mM). The final mitochondrial preparation still contained a large proportion of carotene which could be only partly removed by repeated centrifugation. The yield of mitochondrial protein was 6–12 mg./100 g. fresh weight of tissue.

In experiments in which bovine serum albumin was used, the blending medium was sucrose (0·44 M) containing albumin (1%); other uses of albumin are noted in the results.

Although the particulate preparations were never free from carotene, they will be referred to as 'mitochondrial' fractions for simplicity.

Experimental

Protein determination. The method of Cleland & Slater (1953) was used. It was found that the extinction continued to rise over a period of 72 hr. after addition of the biuret reagent. Extinction measurements were therefore made after a standard time of 24 hr. Although albumin precipitated with trichloroacetic acid was removed during the washings used in this method (as shown by the absence of colour reaction finally), incubation of a mitochondrial preparation with albumin increased the final extinction by

a factor depending partly on the ratio of the amounts of albumin and mitochondrial protein. When serum albumin was incubated for 0·5 hr. with the amount of mitochondrial protein used normally, the estimated protein content was approximately doubled, even when the washing of the ppt. in 50% (v/v) ethanol was repeated three times. Where appropriate, results have been corrected on this basis.

Measurement of oxygen consumption. The conventional circular Warburg apparatus was used. The mitochondrial suspension (0·5 or 1·0 ml.) was added to the reaction mixture (pH 7·1), making a total volume of 2·5 ml. The final sucrose concn. was between 0·28 and 0·32 M except in some experiments noted in the text. The centre well contained 0·15 ml. of 2 N-NaOH and filter paper. The bath temperature was 20° or 25°, and the manometers were shaken at 150 oscillations/min. The gas phase in the flasks was air. The suspension was allowed to equilibrate for 5 min. before the first reading. Experiments generally lasted for 1–1·5 hr., readings being taken every 5 min. over the initial 20 min., and every 10 min. thereafter. At the end of the experiment 2 ml. of the suspension was removed for protein determination.

In experiments where albumin was not used, the rates of oxidation of substrates have been expressed as the q_{O_2} values (μl. of O_2 consumed/mg. of protein/hr.) obtained in the first 20 min., or in a later interval as noted in the appropriate Table. In experiments where albumin was used, oxygen uptakes are expressed relative to 'corrected' q_{O_2} values for malate ('corrected' by assuming that one-half of the measured protein in the incubation medium is albumin adsorbed to the mitochondrial pellet).

Estimation of pyridine nucleotides. An approximate estimation of the levels of DPN and TPN remaining after incubation with the 'mitochondria' was obtained by following the increase in the extinction at 340 mμ on a Unicam SP. 500 spectrophotometer, after addition of the appropriate substrates and enzymes. For estimation of DPN, ethanol and alcohol dehydrogenase were added, and, for TPN, glucose 6-phosphate and glucose 6-phosphate dehydrogenase. Results were calculated from the molar extinction coefficient ($6{\cdot}22 \times 10^3$) for the reduced nucleotide (Singer & Kearney, 1954).

RESULTS

In the first experiments only succinate and malate were used as substrates. The oxidation of succinate by a preparation was taken to indicate the presence of mitochondria or mitochondrial fragments, as succinic dehydrogenase of rat liver is considered to be bound exclusively in the mitochondria (Hogeboom, Schneider & Pallade, 1948). Unpublished results (L. Dalgarno & L. M. Birt) indicate extremely low succinoxidase activity in the soluble fraction, probably due to contamination with mitochondrial fragments.

Malate was also used as a test substrate because it frequently promotes a completely malonate-insensitive respiration in washed carrot slices (Dalgarno & Birt, 1962). In addition, malate is the predominant organic acid in freshly cut (Bryant & Overell, 1953) and aerobically circulated (L. Dalgarno & F. J. R. Hird, unpublished work) carrot tissue; the respiration of both types of slice is partly insensitive to 5–20 mM-malonate (Hanly, Rowan & Turner, 1952).

Properties of mitochondria prepared without serum albumin

As oxygen uptake with malate is very low without added DPN (see below), in the early experiments dealing with preparative procedures this coenzyme was always added at a concentration of 1 mM together with 10 mM-phosphate buffer (Na and K salts, pH 7·1), 5 mM-$MgCl_2$ and 1 mM-EDTA. This medium (at a final sucrose concentration of 0·28–0·32 M) is referred to in the text and tables as the 'standard' incubation medium. Endogenous oxygen consumption was very low (q_{O_2} values of the order of 1), and, except where essential to the interpretation of an experiment, it has not been included in the tables.

Effect of homogenization time on oxidation of malate and succinate by 'mitochondria'. Increasing the time of homogenization from 10 to 60 sec. reduced the oxidation of malate and succinate by 76 and 62% respectively (Table 1). The relatively greater loss of malic-dehydrogenase activity coupled to oxygen uptake is consistent with the greater ease of dissociation of this enzyme from the electron-transport chain in other mitochondria (Mahler, 1953).

An homogenization time of 20 sec. was chosen for all further experiments. Although disintegration of the tissue was not complete, even in this time, almost maximal rates of oxygen uptake were obtained (Table 1).

Effect of homogenization in buffered sucrose solution on oxidation of malate, succinate and citrate. Millerd (1953) reported that an important requirement for the oxidation of α-oxoglutarate and succinate by cytoplasmic particles from mung-bean seedlings is the use of phosphate in the grinding medium. However, Crane (1957) found that

Table 1. *Effect of homogenization time on malate and succinate oxidation*

Incubation was carried out in a medium containing DPN (1 mM), phosphate buffer (pH 7·1, 10 mM), $MgCl_2$ (5 mM), EDTA (1 mM) and sucrose (0·32 M). The substrate concentrations were all 20 mM. The incubation temperature was 25°.

Time of homogenization (sec.)	q_{O_2} values (μl. of O_2/mg. of protein/hr.)	
	Succinate	Malate
10	5·8	16·1
20	5·8	14·9
60	2·2	3·8
300	0·2	0·2

blending in a medium containing phosphate gave less active particles from cauliflower buds than when phosphate was omitted. Accordingly the respiratory activities of 'mitochondria' prepared by homogenizing tissue in sucrose solution, and sucrose solution containing phosphate buffer (Na and K salts, 0·1 M, pH 7·1) were compared, both preparations being resuspended and incubated in sucrose–EDTA medium. The q_{O_2} values of 'mitochondria' prepared in sucrose–phosphate solution and incubated with malate, succinate and citrate were 75, 45 and 50 % higher, respectively, than the corresponding q_{O_2} values for the same substrates and 'mitochondria' prepared in sucrose solution alone. There was no difference in the amount of 'mitochondrial' protein obtained with these two homogenizing media.

Effect of EDTA in the homogenization and incubation media on malate oxidation. 'Mitochondria' were homogenized in the sucrose or the sucrose–EDTA medium; samples of each preparation were resuspended and incubated in media containing either sucrose alone or sucrose–EDTA (final concentration 1 mM).

The q_{O_2} values for malate were 30 % higher with 'mitochondria' prepared by homogenizing in sucrose–EDTA medium rather than in sucrose solution alone, irrespective of the EDTA content of the incubation media.

The q_{O_2} values of 'mitochondria' prepared by homogenizing in sucrose solution were increased by 13 % when incubated in the sucrose–EDTA medium rather than in sucrose solution alone; the q_{O_2} values of 'mitochondria' prepared by homogenizing in the sucrose–EDTA medium were increased by 15 % when incubated in sucrose–EDTA medium rather than in sucrose solution alone. Slater & Cleland (1952) showed a similar effect of EDTA in stimulating α-oxoglutarate oxidation in rat-heart mitochondria.

Effect of sucrose concentration in the incubation medium on malate and succinate oxidation. Considerable variability was found in the relative rates of malate and succinate oxidation by 'mitochondria' incubated in media with final sucrose concentrations of 0·09–0·72 M; the final osmolarities of the media were not equalized, so that the variations were possibly not due to changes in the amount of sucrose present. Malate oxidation decreased rapidly as the sucrose concentration was decreased below 0·3 M, though it was almost unaffected by an increase from 0·3 to 0·7 M. Succinate oxidation was less markedly influenced by lowering the sucrose concentration below 0·3 M. In a typical experiment (Table 2), malate and succinate oxidation decreased by 79 and 59 % respectively as the sucrose concentration was reduced from 0·72 to 0·09 M.

In subsequent experiments, the sucrose concentrations used were in the range 0·28–0·32 M (constant within each experiment). The choice of these concentrations, although not giving maximum q_{O_2} values with the two test substrates, did permit adequate rates of oxygen uptake, and minimized the possibility of complicating later experiments by differential inhibition of oxidative processes at the higher sucrose concentrations (see Ziegler & Linnane, 1958; Johnson & Lardy, 1958).

Effect of magnesium chloride concentration on malate oxidation. Magnesium chloride at concentrations of 1, 5 and 20 mM, with EDTA (1 mM), stimulated oxygen consumption with malate (20 mM) by factors of 8, 16 and 18 respectively over the control. The large stimulation with 1 mM-magnesium chloride is noteworthy, since the presence of 1 mM-EDTA will reduce the effective Mg^{2+} ion concentration to a low level. The rapid decline in oxygen consumption commonly observed during malate oxidation occurred after 50 min. with 20 mM-magnesium chloride, and after 70 min. with 5 mM-magnesium chloride. Endogenous respiration was very low at all concentrations of magnesium chloride but was zero without it.

Effect of cytochrome c on malate and succinate oxidation. A response to cytochrome *c* by plant mitochondria oxidizing succinate has been observed by Pierpoint (1959), though others have found no response (Paquin & Waygood, 1957). The response of our preparation to cytochrome *c* was therefore investigated.

Table 3 shows that horse-heart cytochrome *c* (0·01 mM) stimulated malate and succinate oxidation by 34 and 27 % respectively. Malonate inhibited the oxidation of malate in the presence and absence of cytochrome *c* by 33 and 22 % respectively. The total oxygen uptake on addition of cytochrome *c* to 'mitochondria' with malate is therefore considered not to include an appreciable contribution from a malonate-insensitive pathway, i.e. one other than the tricarboxylic acid cycle.

Table 2. *Effect of sucrose concentration in incubation medium on oxidation of malate and succinate*

Incubation was carried out in media containing DPN (1 mM), phosphate buffer (pH 7·1, 10 mM), $MgCl_2$ (5 mM), EDTA (1 mM) and sucrose at the concn. shown. The substrate concentrations were all 20 mM. The incubation temperature was 25°.

Concn. of sucrose (M)	q_{O_2} values (μl. of O_2/mg. of protein/hr.)	
	Succinate	Malate
0·09	4·5	8·4
0·31	6·3	29·4
0·50	8·7	38·7
0·72	10·8	38·4

Effect of incubation in different concentrations of phosphate buffer on malate oxidation. Phosphate buffer (1 mM) added to the incubation medium almost doubled the rate of oxygen consumption with malate. Increasing the phosphate concentration to 20 mM reduced the q_{O_2} below this maximum by 27 % (Table 4, Expt. 1).

Results with rat-liver mitochondria (Hunter & Ford, 1955) indicate that 20 mM-phosphate inactivates reactions involving DPN and TPN. Kaufman & Kaplan (1960) showed that incubation of animal mitochondria in 20 mM-phosphate results in the loss of pyridine nucleotides. Two further experiments were therefore performed with our preparation to test whether the response to different concentrations of phosphate was compatible with an interaction of this type with endogenous pyridine nucleotides.

Table 3. *Effect of cytochrome* c *on malate and succinate oxidation*

'Standard' conditions of incubation (see text) were used. The concentrations of substrates and of malonate were all 20 mM. The cytochrome *c* concn. was 0·01 mM. Allowance for added cytochrome *c* protein was made in the calculation of q_{O_2} values. The incubation temperature was 25°.

	q_{O_2} values (μl. of O_2/mg. of protein/hr.)	
	Without cytochrome *c*	With cytochrome *c*
Malate	19·5	26·1
Malate + malonate	15·3	17·4
Succinate	9·9	12·6
Succinate + malonate	0·6	0

In the first, the reduction in the oxidation of malate on increasing the phosphate concentration from 1 to 10 mM in the presence of 1 and 0·1 mM-DPN was 45 and 80 % respectively (Table 4, Expt. 2).

In the second, an increase in phosphate concentration from 1 to 10 mM decreased the oxidation of malate by 64 %, but the oxidation of succinate was unaffected (Table 4, Expt. 3).

These results support the view that there is a balance between stimulation and inhibition of malate oxidation by added inorganic phosphate in this preparation. The stimulation by 1 mM-phosphate is probably due to its necessity during electron transport (see Chance, 1959), and the inhibition at higher concentrations (20 mM) is probably due to an interference with the functional binding of intramitochondrial DPN.

Effect of cofactors on oxidation of substrates. (*a*) *Malate.* With DPN (1 mM) and TPN (0·5 mM) respiration was stimulated to give q_{O_2} values of 15·6 and 3·9 respectively; the endogenous q_{O_2} without added cofactors was 1·2. After incubating the preparation separately with DPN and TPN for 90 min., 93 and 50 % respectively of the DPN and TPN were recovered unchanged. After incubation, the final concentration of DPN in the sample to which TPN had been added was 0·07 mM. Half of this was due to an initial content (7 %) of DPN in the TPN used, and the rest probably arose by dephosphorylation of TPN by the 'mitochondria' (Birt & Bartley, 1960). The observed stimulation of malate oxidation could therefore be due to DPN present in the TPN. Reference to Table 4, Expt. 4,

Table 4. *Effect of phosphate buffer and DPN concentrations on malate and succinate oxidation*

The incubation media contained EDTA (1 mM), $MgCl_2$ (5 mM), and substrate at a concn. of 20 mM. The incubation temperature was 25°.

Expt.	Substrate	Concn. of phosphate buffer (mM)	Concn. of DPN (mM)	q_{O_2} values (μl. of O_2/mg. of protein/hr.)
1	Malate	0	1	9·7
		1	1	17·2
		5	1	12·0
		10	1	11·1
		20	1	12·5
2	Malate	1	1	22·2
		1	0·1	15·1
		10	1	12·3
		10	0·1	3·0
3	Malate	1	1	10·8
		10	1	4·8
		100	1	3·9
	Succinate	1	1	3·6
		10	1	4·2
		100	1	3·6
4	Malate	10	0·1	3·0
		10	0·25	16·2
		10	0·5	15·9
		10	2·5	18·3

shows that, with malate, 0·1 mM-DPN gives a lower q_{O_2} than does the preparation (above) incubated with 0·5 mM-TPN. It is therefore probable that the requirement for DPN in malate oxidation can to some extent be replaced by TPN.

When AMP plus ATP were added to incubation media containing DPN or DPN plus TPN, q_{O_2} values were lowered by 27 and 39% respectively; this may be related to an effect on the structure and therefore the permeability of the 'mitochondria' (Table 5, Expt. 1). The simultaneous addition of AMP, ATP, DPN and TPN stimulated the low endogenous respiration, the q_{O_2} values rising from 1·2 to 2·1.

(*b*) *Succinate.* The initial rate of oxidation of succinate was greatly stimulated by ATP (Table 5, Expt. 2). DPN with or without added ATP increased the total oxygen uptake though not the initial rate, i.e. the rate increased during the incubation; this effect is probably related to the stimulation of sequential as opposed to one-step oxidations when DPN is added to the system. A possible explanation of this initial stimulation of succinate oxidation by ATP is discussed by Azzone, Ernster & Klingenberg (1960).

(*c*) *α-Oxoglutarate.* Both CoA and thiamine pyrophosphate were essential for the maximum rate of oxidation of α-oxoglutarate in the presence of DPN (Table 5, Expts. 3*a* and 3*b*; see also Table 7).

(*d*) *Isocitrate.* DPN-linked isocitric-dehydrogenase activity was considerably greater than TPN-linked isocitric-dehydrogenase activity (Table 5, Expt. 4*a*). As was shown also by Davies (1955), Mn^{2+} ions (2 mM) stimulate DPN-linked isocitric dehydrogenase; Table 5, Expt. 4*b*, shows a stimulation of more than 100%.

(*e*) *Pyruvate.* Sustained oxidation of pyruvate was not obtained with DPN unless CoA and malate (2 mM) were also present (Table 6). In the absence of malate, thiamine pyrophosphate inhibits pyruvate oxidation early in the incubation period. Later in the incubation period (30–60 min.) malate (with DPN, CoA and thiamine pyrophosphate) stimulates oxygen uptake with pyruvate to a level above the combined oxygen uptakes for pyruvate (plus DPN, CoA and thiamine pyrophosphate) and for malate (plus DPN). The conditions of incubation (pH and thiamine pyrophosphate concentration) make it unlikely that thiamine pyrophosphate mediates a non-enzymic decarboxylation of pyruvate (see Metzler, 1960); however, the lower oxygen uptake obtained with pyruvate in the presence of thiamine pyrophosphate may result from a stimulation of carboxylase (see Singer &

Table 5. *Effect of cofactors on oxidation of malate, succinate, α-oxoglutarate and isocitrate*

Incubation media contained phosphate buffer (10 mM), EDTA (1 mM), $MgCl_2$ (5 mM); the concentrations of substrates and of malonate were all 20 mM, except that of isocitrate in Expt. 4*b* which was 40 mM. Other concentrations were: $MnCl_2$, 2 mM; AMP, 1 mM; ATP, 1 mM; DPN, 1 mM; TPN, 0·5 mM; thiamine pyrophosphate (TPP), 2 mM; CoA, 0·2 mM. The incubation temperature was 25°. The duration of Expt. 2 was 80 min.

Expt.	Substrate	Cofactors added	q_{O_2} values (μl. of O_2/mg. of protein/hr.) Without malonate	With malonate	Total O_2 uptake (μl./mg. of protein)
1	Malate	—	1·2	—	—
		AMP	2·4	—	—
		ATP	4·2	—	—
		TPN	3·9	—	—
		DPN	15·6	—	—
		DPN + TPN	16·2	—	—
		AMP + ATP + DPN	11·4	—	—
		AMP + ATP + DPN + TPN	9·9	—	—
2	Succinate	—	5·7	—	9·1
		DPN	4·8	—	12·8
		ATP	10·5	—	13·1
		ATP + DPN	10·2	—	20·8
3*a*	α-Oxoglutarate	—	0	—	—
		DPN + CoA	6·3	—	—
		DPN + CoA + TPP	14·1	5·4	—
3*b*	α-Oxoglutarate	DPN + TPP	3·0	—	—
		DPN + TPP + CoA	5·7	—	—
4*a*	Isocitrate	—	0·6	0·6	—
		DPN	6·0	4·8	—
		TPN	1·2	0·6	—
4*b*	Isocitrate	DPN	6·0	—	—
		DPN + Mn^{2+}	12·6	—	—

Pensky, 1952), the acetaldehyde produced reacting preferentially with $DPNH_2$ derived from the action of pyruvic oxidase to form ethanol. This explanation is consistent with the observation of Rehak & Truitt (1958) that acetaldehyde inhibits oxygen uptake in rat-brain mitochondria metabolizing pyruvate.

(*f*) L-*Glutamate.* L-Glutamate (50 mM) incubated in the 'standard' medium was oxidized with and without added cytochrome *c* (0·01 mM), CoA (0·5 mM) and thiamine pyrophosphate (2 mM). During the initial 40 min. of incubation there was no stimulation by these cofactors (q_{O_2} values both approx. 7); but in the period from 80 to 100 min. the q_{O_2} values were increased from 4·5 to 8 in the presence of cofactors. Malonate (20 mM) inhibited glutamate oxidation in the presence of cofactors over the entire incubation period (q_{O_2} approx. 1); the oxidation of glutamate must therefore proceed beyond the formation of α-oxoglutarate.

Properties of mitochondria prepared in the presence of serum albumin

Serum albumin influenced the oxidative properties of preparations only when present in the blending medium; the optimum concentration was 1%. Addition of albumin to the resuspension and incubation media had no effect on the properties of mitochondria prepared in either the presence or the absence of albumin in the blending medium.

Influence of added cofactors and ions. Albumin diminished the dependence on added cofactors (Table 7). Stimulation of malate oxidation by magnesium ions was still apparent, but with 1 and

Table 6. *Effect of cofactors on oxidation of pyruvate*

Incubation media contained sucrose (0·28M), phosphate buffer (10 mM), EDTA (1 mM) and $MgCl_2$ (5 mM). The concn. of pyruvate was 20 mM. Other concentrations were: malate, 2 mM; thiamine pyrophosphate (TPP), 2 mM; and CoA, 0·125 mM. The incubation temperature was 25°. Unbracketed q_{O_2} values are based on the 20 min. period after the first reading; bracketed q_{O_2} values are based on the 30–60 min. interval.

	Additions	q_{O_2} values (μl. of O_2/mg. of protein/hr.)
Without pyruvate	DPN + malate	22·7 (7·8)
With pyruvate	DPN	0·4 (0·4)
	DPN + CoA	16·2 (2·8)
	DPN + CoA + TPP	0·9 (2·4)
	CoA + TPP	0·9 (4·2)
	DPN + malate	12·6 (13·2)
	DPN + CoA + TPP + malate	24·4 (22·0)
	CoA + TPP + malate	0·9 (5·7)

Table 7. *Influence of serum albumin on the oxidative properties of carrot mitochondria*

The concn. of each substrate was 20 mM; other concentrations were: DPN, 1 mM; TPN, 1 mM; ATP, 1 mM; CoA, 0·3 mM; thiamine pyrophosphate (TPP), 2 mM; albumin, 1% in blending medium. The q_{O_2} values were calculated for the first 20 min. of incubation at 25°.

		$\frac{O_2 \text{ uptake with added cofactor(s)}}{O_2 \text{ uptake with minimal cofactor requirement*}}$	
Substrate	Cofactors added	Without albumin	With albumin
Malate	—	1·0	1·0
	DPN	8·7	1·4
	TPN	2·2	1·0
	ATP	2·3	1·1
Succinate	—	1·0	1·0
	ATP	1·9	1·3
	DPN	0·9	1·1
Isocitrate	—	1·0	1·0
	DPN	10	2·2
	TPN	2·0	1·9
α-Oxoglutarate	DPN + CoA	1·0	1·0
	DPN + CoA + TPP	2·1	1·0

* Oxidation of α-oxoglutarate was not measurable without added DPN for mitochondria prepared in the absence of serum albumin; with serum albumin the 'corrected' q_{O_2} values for α-oxoglutarate with no additions, DPN, DPN plus CoA, and DPN plus CoA plus TPP were 2·7, 5·7, 8·1 and 8·4 respectively. The values in the Table are expressed relative to the rate with DPN plus CoA.

5 mM-magnesium chloride, the increases were only 2- and 3-fold respectively. Manganese chloride increased DPN-dependent isocitrate oxidation 1·7 times with albumin and 2·1 times without albumin.

Influence on q_{O_2} values. Despite the difficulties of assessing mitochondrial-protein content (see Methods section) it was apparent that q_{O_2} values were increased by serum albumin. Even without 'correction', q_{O_2} values for malate, succinate, α-oxoglutarate, isocitrate and pyruvate were of the order of 40, 40, 10, 15 and 15 respectively (cf. Table 5). As it is certain that the protein values on which these figures depend are too high, it is clear that there has been an increase in the q_{O_2} values for each substrate.

Influence on relative rates of oxidation for different substrates. The addition of albumin enhances the oxidation of some substrates relative to others (cf. Dalgarno & Birt, 1962).

DISCUSSION

Carrot tissue has been disrupted in a blender and a fraction which exhibits all the oxidative steps of the tricarboxylic acid cycle has been isolated from it by standard procedures of differential centrifuging. Although the fraction isolated did not represent a pure preparation (it always contained carotene) and required the addition of cofactors for high and sustained rates of oxidation, the existence of the complete sequence of tricarboxylic acid-cycle oxidative reactions indicates the presence of mitochondrial particles (Green & Hatefi, 1961).

The ability of the preparation to oxidize succinate in the absence of cofactors is similar to the results of Beevers & Walker (1956), who have shown that succinate oxidation by *Ricinus* particles is far higher than the oxidation of any other substrate in the absence of added cofactors. These results indicate that the succinoxidase of these plant particles is a complete structural unit without readily dissociable or replaceable cofactors. It is likely that the relatively low rates of oxidation of the other substrates without added cofactors are due to the ease of dissociation of coenzymes (especially the pyridine nucleotides) from the mitochondrial enzymes.

This finding is supported by the results with serum albumin, which is known to enhance the oxidation and phosphorylation of insect mitochondria (Wojtczak & Wojtczak, 1960), probably by binding unsaturated fatty acids (Priegnitz & Wojtczak, 1961).

A protection by bovine serum albumin of succinoxidase in particles isolated from etiolated cotton-seedling hypocotyls has been reported by Throneberry (1961). With carrot mitochondria, decreased dependence on added cofactors possibly indicates not only the removal of deleterious compounds from the tissue homogenate, but also a definite mechanical protection of the particles. Even in the absence of albumin, however, incubation in different concentrations of sucrose and of phosphate buffer induced different relative changes in malate and succinate oxidation, suggesting that the mitochondrial organization and therefore perhaps the structure were not entirely disrupted.

Mitochondrial fractions prepared by the methods described have been found satisfactory for use in studies of certain aspects of the respiratory metabolism of this tissue with special reference to malate (Dalgarno & Birt, 1962).

SUMMARY

1. A mitochondrial fraction has been isolated from carrot-root tissue disrupted in a blender; the preparation catalysed all the oxidative steps involved in the tricarboxylic acid cycle after addition of appropriate cofactors.

2. The effects of homogenization time and of various homogenization and incubation conditions upon the oxidation of several substrates by the preparation have been determined.

3. Succinate oxidation was less sensitive than was malate oxidation to decreases in sucrose concentration below 0·3 M in the incubation medium; malate oxidation decreased by over 70 % when the sucrose concentration was lowered from 0·3 to 0·09 M, while the corresponding change in succinate oxidation was less than 30 %.

4. Phosphate buffer (1 mM) added to the incubation medium almost doubled the rate of oxygen consumption with malate. Increasing the phosphate concentration to 20 mM reduced the q_{O_2} value below this maximum by 27 %.

5. Malate oxidation was stimulated 16-fold by the addition of 5 mM-magnesium chloride to the incubation medium.

6. Cytochrome *c* stimulated the rates of oxidation of both malate and succinate by about 30 %.

7. The effect of added cofactors on the oxidation of malate, succinate, α-oxoglutarate, isocitrate, pyruvate and glutamate is described. These additions stimulated the oxidation of all substrates though succinate oxidation was less affected by the absence of cofactors than was the oxidation of other substrates.

8. Serum albumin (1 %) added to the blending medium decreased the dependence on added cofactors and ions, and increased the q_{O_2} values obtained with all the substrates tested.

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Aspects of Malate Metabolism by Slices and Mitochondria from Carrot Tissue

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Laties (1959) observed that the respiration of freshly cut chicory-root slices is 'almost entirely malonate-resistant', and that the respiratory increment which arises on aerobic circulation of the tissue in water is 'qualitatively distinct from the initial respiration', i.e. is malonate-sensitive. Hanly, Rowan & Turner (1952) conclude that the basal respiration of carrot-tissue slices is 'mediated by enzyme systems not inhibited by malonate, cyanide or carbon monoxide'. However, with both aerobically circulated chicory slices (Laties, 1959) and freshly cut and aerobically circulated carrot slices (L. Dalgarno & L. M. Birt, unpublished work), the addition of malate frequently renders the respiration of the tissue insensitive to malonate.

All these results, and the observation that malate is the predominant organic acid in freshly cut carrot tissue (Bryant & Overell, 1953), suggested that an investigation of pathways of malate metabolism in slices and subcellular fractions from carrot tissue may assist the study of malonate sensitivity and insensitivity in this storage tissue.

This paper describes aspects of the oxidative metabolism of such preparations and indicates some distinctive features of malate metabolism.

MATERIALS AND METHODS

Materials

Apart from those specified below, organic acids, amino acids and coenzymes used were supplied by Sigma Chemical Co., St Louis, Mo., U.S.A. Malonic acid (98% minimum purity) was obtained from Hopkin and Williams, Chadwell Heath, Essex; α-glycerophosphate from L. Light and Co. Ltd., Colnbrook, Bucks.; citric acid (99·5% minimum purity) from May and Baker Ltd., Dagenham, Essex; L-cysteinesulphinic acid from Mann Research Laboratories Inc., New York, U.S.A. In all experiments with isocitrate, the purified (DL+allo) trisodium salt (Sigma Chemical Co.) was used, except in the one experiment described in Table 6, where potassium dihydrogen L(+)-isocitrate (Vickery & Wilson, 1958) was used. EDTA (disodium salt), potassium dihydrogen phosphate, disodium hydrogen phosphate and sucrose were standard analytical reagents. The disodium salt of ATP was used.