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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chicory slices (Laties, 1959) and freshly cut and aerobically circulated carrot slices (L. Dalgarno & L. M. Birt, unpublished work), the addition of malate frequently renders the respiration of the tissue insensitive to malonate.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.

The magnesium salt of ADP was prepared by adding an excess of magnesium carbonate to a solution of the monosodium salt of ADP (100 mM) and standing at 0° until the pH of the supernatant solution was 7. The cytochrome c was Sigma Type III (horse heart). Pyruvate solutions were prepared from the sodium salt, and lactate solutions from DL-lactic acid. Bovine serum albumin was obtained from Commonwealth Serum Laboratories, Melbourne, Australia.

Estimation of malonic acid

Malonic acid was determined colorimetrically by the method described by Stickland (1960) for the determination of oxaloacetate. Stickland found that malonate interfered with the determination of oxaloacetate by this method, giving a colour yield of approx. one-seventh that of the same amount of oxaloacetate. The results using the method at this lower level of sensitivity are in good agreement with those of Stickland, 1 μmole of malonic acid giving an extinction of 0·6–0·7 in 1 cm. cells.

Tissue

Preparation of tissue slices. Preparation of carrot slices was by the method described by Dalgarno & Hird (1960).

Preparation of a mitochondrial fraction from carrot tissue. Mitochondrial fractions were prepared by the methods described by Dalgarno & Birt (1962), except that phosphate buffer (Na and K salts, 0·1 M, pH 7·1) was added to the homogenization medium. The term 'mitochondria' is used with the same qualification as described therein.

Preparation of rat-liver mitochondria. The tissue was homogenized in 0·36 M-sucrose in a Potter–Elvehjem homogenizer; the final concentration of sucrose in both the rat-liver and carrot-tissue homogenates was approximately the same. The homogenate was centrifuged at 600g for 10 min., and the mitochondrial pellet sedimented from the supernatant by centrifuging at 2700g for 30 min. The pellet was resuspended once in 0·36 M-sucrose containing 2·5 mM-EDTA, and finally sedimented at 8500g for 10 min.

Experimental

Measurement of oxygen consumption. A circular Warburg apparatus was used in all experiments. In experiments with slices and 'mitochondria' the procedures adopted by Dalgarno & Hird (1960) and Dalgarno & Birt (1962) respectively were used. In experiments with mitochondria prepared in the absence of albumin the rate of oxidation of substrates has been expressed as the q_{O_2} value (μl. of oxygen consumed/mg. of protein/hr.) on the basis of the first 20 min. of incubation. Those experiments in which q_{O_2} values are calculated on the basis of a different time interval are noted on the appropriate Table. The q_{O_2} values for mitochondria prepared in albumin have been 'corrected' as described by Dalgarno & Birt (1962). Under 'standard' incubation conditions (see Tables), the mitochondrial preparation was incubated in a medium containing 5 mM-$MgCl_2$, 1 mM-DPN, 1 mM-EDTA, 0·3 M-sucrose and phosphate buffer (Na and K salts, 10 mM, pH 7·1). In experiments with slices the rate of oxygen uptake has been expressed as oxygen uptake/g. of fresh weight of tissue/hr.

Measurement of malonic acid uptake. Uptake of malonic acid by the tissue was followed by determining the change in concentration of malonate-reacting material in the medium. There was no net uptake or release of water by tissue during the incubation period, nor was the release of malonate-reacting material sufficient to affect the interpretation of the results. The tissue slices (about 1·2 g.) were weighed and placed in 5 ml. of the incubating solution at pH 5·0 in a stoppered conical flask (50 ml.) and shaken at 150 oscillations/min. in a water bath at 25°. The stopper was removed twice for a few min. during the experiment to allow gas exchange.

Detection of transamination. Approximately 4 mg. of a mitochondrial preparation was incubated in a test tube at 25° with (final concentrations) 0·125 M-phosphate buffer, 0·125 M-L-aspartate or 0·125 M-L-alanine, 0·1 M-sucrose and 2·5 mM-EDTA. After 10 min., α-oxoglutarate (final concentration, 0·125 M) was added, making a total volume of 2·0 ml. Immediately after this addition a sample was taken, spotted on a chromatogram and dried; subsequent samples were taken after 15 min. and 60 min. Control incubations were carried out in which L-aspartate and L-alanine were incubated without α-oxoglutarate, and in which α-oxoglutarate was incubated without either amino acid. A similar procedure was adopted with mitochondria incubated under conditions in which malate is readily oxidized (i.e. 60 mM-malate, 10 mM-phosphate buffer, 0·4 M-sucrose, 5 mM-$MgCl_2$ and mM-DPN). Chromatograms were prepared on Whatman no. 1 paper and developed (descending) with butanol–acetic acid–water (4:1:1, by vol.).

Protein determination. Protein was determined by the method of Dalgarno & Birt (1962).

RESULTS

Malonate sensitivity of respiration of freshly cut and aerobically circulated tissue

Endogenous respiration. The reports of partial inhibition of oxygen uptake with carrot and chicory-root slices (Hanly *et al.* 1952; Laties, 1959) were based on experiments with a relatively narrow range of malonate concentrations (0·01–0·1 M).

With 0·02, 0·05, 0·1, 0·3 and 0·8 M-malonate, the respiration of freshly cut carrot tissue was inhibited by 26, 48, 81, 95 and 100 % respectively; the respiration of tissue circulated aerobically for 44 hr. was more sensitive to lower concentrations of malonate, being inhibited by 70, 79, 86, 100 and 100 % respectively. (Incubation of slices in mannitol solution—at concentrations from 0·02 to 0·6 M—neither increased nor decreased the respiration.) Apart from the ability of malonate to inhibit completely the oxygen consumption of both freshly cut and circulated tissue, it was noted that, although an increase in malonate sensitivity occurred, the endogenous respiration of freshly cut and aerobically circulated tissue was often almost the same. In those experiments showing a rise in endogenous respiration after aerobic circulation there was an increase, or no change, or even a fall in malonate sensitivity; in those experiments showing a fall in endogenous respiration, there was

sometimes an increase in malonate sensitivity. It is therefore doubtful whether there is any causal relation between an increase in endogenous respiration and any accompanying increase in malonate sensitivity in our preparations.

Effect of preincubation with organic acids and glucose on response to malonate. Laties (1959) has observed a malonate-insensitive stimulation of respiration on adding malate to aerobically circulated chicory-root slices. With aerobically circulated carrot slices and malate (20 mM), the respiration was stimulated by 91% over the 'no substrate' control; with malonate (20 mM) as well, this stimulated respiration was increased slightly relative to changes in the control tissue (Table 1). The inclusion of pyruvate (10 mM) with malate (10 mM) did not alter the response to malonate from that obtained with 20 mM-malate alone. With succinate, α-oxoglutarate and glucose the respiration was stimulated by 186, 130 and 53% respectively; however, with malonate the stimulated respiration is reduced by 45, 15 and 33% respectively. Therefore the oxidation of malate by circulated carrot slices differed from that of all other substrates tested in that it was not inhibited by malonate.

Effect of preincubation with malonate on response to malate and isocitrate. In the experiments described in Table 1, malonate was added after preincubation of the tissue in substrate for 60 min.; in other experiments substrate (20 mM) was added after 45 min. preincubation of the tissue in malonate or in water. In such an experiment Table 2 shows that with aerobically circulated tissue the inhibition (57%) of endogenous respiration by malonate (20 mM) is partly reversed by adding malate (20 mM) but is unaffected by adding isocitrate (20 mM). Malate and isocitrate (both 20 mM) stimulate the respiration in the absence of malonate by 108 and 77% respectively.

The stimulation with malate did not occur after preincubation with 0·05–0·8 M-malonate. It is possible that at high malonate concentrations irreversible effects on cellular organization take place; this may be related to a lack of specificity of malonate inhibition at high concentration (Beevers, 1952; Price, 1953) or more directly to an effect on tricarboxylic acid-cycle-dependent energy production.

Malonate uptake by circulated slices in the presence of organic acids and glucose

As a respiratory stimulation occurs on adding a number of organic acids to carrot slices they must enter the tissue. It is possible that a carrier process transports organic acids into carrot tissue, as with amino acids (Birt & Hird, 1958*a*, *b*), and that competition for entry occurs between a number of organic acids. The malonate-insensitive stimulation of respiration obtained on incubation of the tissue in malate could therefore be explained if the uptake of malonate were selectively inhibited by malate.

Table 1 shows that after incubation for 4 hr.

Table 1. *Effect of organic acids and glucose on malonate sensitivity of respiration, and on uptake of malonate by aerobically circulated carrot slices*

The tissue used had been aerobically circulated for 46 hr.; the concn. of each substrate was 20 mM, except for 'Pyruvate + malate' where the concn. of each was 10 mM; malonate was added from the side arm after 1 hr. giving a final concn. of 10 mM; water was added from the side arm to the controls. Approx. 0·5 g. of tissue was incubated in 2·0 ml. of solution at 25°. The initial pH was 5.

Substrate	Oxygen uptake (μl./g. fresh wt./hr.)											
	None		α-Oxoglutarate		Succinate		Glucose		Malate		Pyruvate + malate	
Duration of incubation (hr.)	*a**	*b**	*a**	*b**	*a**	*b**	*a**	*b**	*a**	*b**	*a**	*b**
0–1·0	85	91	186	165	269	240	128	118	200	193	190	184
1·0–2·0	93	78	215	151	272	134	137	83	200	195	190	188
2·0–3·0	96	80	220	165	269	123	147	90	180	187	173	196
3·0–4·0	93	90	213	173	265	140	147	93	160	195	162	192
Inhibition or stimulation of respiration by malonate (%)†		−17		−15		−45		−33		+11		+13
Uptake of malonate (μmoles/g. fresh wt./4 hr.)		9·4		—		9·6		16·5		15·0		8·7

* *a*, Controls (no malonate added); *b*, malonate added after 1 hr.

† The values for inhibition or stimulation of respiration by malonate have been obtained from the average oxygen uptake in the 3 hr. after adding malonate or water, with a correction for the initial difference in oxygen uptake between the duplicates in each case. For example, with α-oxoglutarate, mean oxygen uptake without malonate = 216; mean oxygen uptake with malonate and correction for initial difference = 163 × 186/165 = 184; absolute inhibition of oxygen uptake = (216 − 184) = 32; % inhibition = 100 × 32/216 = 15. Uptake of malonate was determined with tissue from the same batch of circulated slices as used in the respiratory experiment, after incubation for 4 hr. (see Methods section).

Table 2. *Effect of malate and isocitrate on the reversal of malonate inhibition of the respiration of freshly cut and aerobically circulated carrot slices*

Aerobically circulated tissue was washed for 44 hr. Tissue (approx. 0·5 g.) was incubated in either 2 ml. of water or of malonate solution (20 mM) at 25° for 45 min., then equal volumes of water, or malate or isocitrate solutions, were added to the appropriate vessels, the final concn. of each substrate being 20 mM. Oxygen uptake was calculated for the 20 min. periods immediately preceding and immediately following the substrate addition. The initial pH was 5·0.

Treatment of tissue	Period of incubation	Oxygen uptake (μl./g. fresh wt./hr.) No substrate addition to tissue preincubated in: Water	No substrate addition to tissue preincubated in: Malonate	Isocitrate added to tissue preincubated in: Water	Isocitrate added to tissue preincubated in: Malonate	Malate added to tissue preincubated in: Water	Malate added to tissue preincubated in: Malonate
Freshly cut	Before substrate addition	75	68	95	79	110	73
	After substrate addition	98	80	139	79	142	91
Aerobically circulated	Before substrate addition	103	46	102	44	100	41
	After substrate addition	139	52	178	44	208	99

with circulated tissue the disappearance of malonate (10 mM) from the external solution is 9·4 μmoles/g. fresh wt. of tissue. With added malate, glucose or succinate (all 20 mM) the disappearance was 15·0, 16·5 and 9·6 μmoles/g. fresh wt. respectively. The reduction in malonate sensitivity with malate under these conditions (see Table 1) is not therefore the result of any inhibition of malonate uptake; and it is not likely that malonate was inhibiting any enzymes other than succinic dehydrogenase at the concentrations used (see Discussion section).

The uptake of [2-^{14}C]malonate by carrot slices is accompanied by a very small production of $^{14}CO_2$ (L. Dalgarno & L. M. Birt, unpublished work); however, this metabolism is insufficient to affect interpretation of the disappearance of malonate from the medium as uptake by the tissue. Therefore after incubation of tissue with malonate, the final concentration of malonate in the slices is, assuming equal distribution within the tissue, approximately 50% higher than in the external solution (i.e. approx. 15 mM).

Malonate inhibition of malate oxidation by carrot mitochondria

The oxidation of malate by carrot mitochondria was inhibited by malonate; the extent of the inhibition decreased as the concentration of malate increased (see Table 3) and as the concentration of malonate decreased (see below). Malonate inhibition was unaffected by added DPN and ATP. With malate at concentrations greater than 20 mM (at which concentration oxygen consumption was saturated), there was a further decrease in malonate sensitivity. These results are similar to those of

Table 3. *Effect of malate concentration on malonate sensitivity of mitochondria*

'Standard' conditions of incubation (see Methods section) were used, with malonate concn. 20 mM and an incubation temperature of 25°.

Malate concn. (mM)	q_{O_2} values (μl. of O_2/mg. of protein/hr.) Without malonate	With malonate
0	0·8	0·3
1	2·3	0·6
5	10 8	3·0
20	16·5	7·4
50	14·1	10·4

Laties (1957) who used cauliflower-bud mitochondria and reported a large contribution to the respiration by a single-step oxidation on incubation with high concentrations (30–50 mM) of malate and citrate.

With mitochondria prepared in the presence of albumin, malonate at concentrations from 1 to 100 mM was less effective in inhibiting malate oxidation (e.g. 1, 5 and 20 mM-malonate inhibited the oxidation of 20 mM-malate by 0–10, 7 and 12–25% respectively; compare with results in the next section and see also Table 4, Expt. 2). This effect was probably due to the relatively great stimulation of succinoxidase activity (see below).

Comparison of the malonate sensitivity of slices and mitochondria incubated with malate

Malate oxidation, with and without malonate, was compared directly in preparations of freshly cut slices and of mitochondria isolated from a

random sample of the same tissue by the procedure described in the Methods section. The endogenous respiration of freshly cut slices with and without malonate (20 mM) was 46 and 72 μl. of oxygen/g. fresh wt./hr. respectively. In duplicate flasks with and without malonate, to which malate (20 mM) was also added, the q_{O_2} values were 90 and 70 μl. of oxygen/g. fresh wt./hr. respectively. The q_{O_2} values for the oxidation of malate (20 mM) by the mitochondria with 1, 5 and 20 mM-malonate were decreased by 4, 30 and 42% respectively below the control. In this experiment both malate and malonate were present initially in the incubation media and the q_{O_2} values were based on the first 30 min. period of oxygen uptake after preincubation for 10 min. The relative q_{O_2} values did not alter substantially over a period of 1 hr. of incubation.

Comparison of rates of oxidation of various substrates

The data presented above provide no evidence for any unique insensitivity to malonate of malate oxidation by carrot mitochondria. The general pattern of the malonate inhibition of mitochondrial oxidations is the same as that of rat-liver particles incubated under identical conditions (Table 4, Expts. 1 and 3). Nevertheless, the inhibition of oxygen uptake would not be very marked with the less 'damaged' carrot mitochondria prepared in media containing albumin and exposed to the probable range of concentrations (malate not greater than 20 mM, malonate not greater than 10 mM—see above) occurring in the slice during the first hour of incubation. Thus it seemed possible that, if the mitochondrial malic dehydrogenase had an unusually high activity, a relatively rapid uptake of oxygen could occur with malate even in the presence of malonate (see also Laties, 1957). Accordingly the rates of oxidation of a number of substrates by the mitochondria have been compared in the three types of experiments described below and reported in Tables 4 and 6. Table 4 includes data on malonate inhibition.

In the first type of experiment, mitochondria were prepared in the absence of albumin and incubated with the cofactors previously shown to give the highest rates of oxidation (Dalgarno & Birt, 1962). Malate was oxidized more rapidly than any other substrate (Table 4, Expt. 1). The relatively low oxidation of succinate is especially noteworthy as in other comparable experiments succinate was oxidized even more slowly, i.e. at only about half the rate with malate. The relative rates of succinate and malate oxidation are apparently influenced by the levels of accumulating oxaloacetate (cf. Green, 1936; Das, 1937). Table 5 shows that at low substrate concentration (2 mM) the addition of L-cysteinesulphinate, which removes oxaloacetate (Singer & Kearney, 1956), stimulated succinate oxidation to a greater level than that of malate. However, at a higher substrate concentration (20 mM) the reverse occurred. The addition of glutamate or aspartate did not affect the oxidation of malate although the mitochondrial preparation has been shown to possess glutamate–aspartate–(and glutamate–alanine–)transaminase activity.

Table 4. *Effect of malonate on oxidation of substrates by carrot mitochondria prepared in the presence and in the absence of albumin, and by rat-liver mitochondria*

The 'standard' incubation medium (see Methods section) plus cytochrome *c* (0·01 mM) was used for all substrates. No further cofactors were added with malate as substrate. With other substrates, the following additions were made: succinate, ATP (1 mM); pyruvate, malate (2 mM), CoA (0·5 mM) and thiamine pyrophosphate (2 mM); isocitrate, $MnCl_2$ (2 mM); α-oxoglutarate, CoA (0·5 mM) and thiamine pyrophosphate (2 mM). The concn. of sucrose in the incubation medium was 0·30M. The concn. of each substrate was 50 mM; and that of malonate 100 mM. The incubation temperature was 25°.

	Relative rates of oxygen uptake*					
	Carrot mitochondria					
	Prepared in the absence of albumin (Expt. 1)		Prepared in the presence of albumin (Expt. 2)		Rat-liver mitochondria (Expt. 3)	
Substrate	Without malonate	With malonate	Without malonate	With malonate	Without malonate	With malonate
Malate	100	49	100	59	100	85
Succinate	85	10	109	0	530	62
Pyruvate	47	17	57	4	92	38
Isocitrate	35	48	13	10	154	208
α-Oxoglutarate	16	12	53	3	—	—

* In each experiment oxygen uptake is expressed relative to a value of 100 for malate oxidation without malonate; the q_{O_2} values (μl. of O_2/mg. of protein/hr.) for malate oxidation were 33·3 (Expt. 1), 96·6 (Expt. 2) and 3·9 (Expt. 3). The average content of mitochondrial protein/flask was 4·5 mg. (Expt. 1), 2·7 mg. (Expt. 2, 'corrected' value of mitochondrial protein) and 5·8 mg. (Expt. 3).

With malonate the highest rates of oxidation were obtained with malate and isocitrate (Table 4, Expt. 1).

The second type of experiment was with mitochondria prepared in the presence of albumin and incubated with appropriate cofactors. Both the relative and absolute rates of oxidation were changed by this addition of albumin (Table 4, Expt. 2). The greatest increases in the rates of oxidation relative to malate were with α-oxoglutarate and succinate (cf. Table 4, Expt. 1). The rates of oxidation of succinate and malate (both 2 mM) were unaffected by L-cysteinesulphinate; this presumably results from the more effective removal of oxaloacetate (by oxidation or transamination) in this preparation. Assuming the validity of the correction previously used to estimate mitochondrial protein (Dalgarno & Birt, 1962), the following q_{O_2} values were obtained with preparations made in the presence and in the absence of serum albumin: for malate, 97 and 30 respectively; succinate, 113 and 29; pyruvate, 59 and 16; isocitrate, 14 and 12; α-oxoglutarate, 55 and 5; malate oxidation was the least affected by malonate.

In the third type of experiment, mitochondria were prepared by homogenizing and resuspending in media containing albumin and incubated with substrate, albumin and cofactors including ADP. The oxidation of all substrates tested was increased by adding 10 mM-ADP (Table 6). Increases of 105, 191, 47 and 767% were obtained with malate, succinate, isocitrate, and α-oxoglutarate respectively; with pyruvate, detectable oxygen uptake occurred only in the presence of added ADP. The very great stimulation obtained with α-oxoglutarate is probably related to the requirement for ADP in the deacylation of succinyl-CoA. ADP also promoted a further alteration in the relative rates of oxidation, succinate being oxidized 42% more rapidly than malate and α-oxoglutarate, which were oxidized at almost equal rates. The considerable increase in the relative rate of isocitrate oxidation without ADP (compare Tables 4 and 6) was probably due to the use of a sample of L$_s$(+)-isocitrate. These data indicate that ADP limits the rate of oxidation of these substrates. Confirmatory evidence was obtained using malate (50 mM), with which oxygen uptake was increased by 25% on addition of 2,4-dinitrophenol.

The increased rates of oxygen uptake with ADP or 2,4-dinitrophenol were obtained only with mitochondria prepared and incubated in serum albumin. Omission of DPN and magnesium chloride had no effect on the ADP-induced stimulation of malate oxidation, but depressed that of succinate by 30%.

Thus with only one type of preparation was malate oxidation consistently greater than that of other substrates. This was with mitochondria prepared in the absence of albumin, where the greatest losses of potential activities occurred (see Dalgarno & Birt, 1962). Thus it seemed as though malate oxidation was the most resistant to disruption of mitochondrial organization. Accordingly the possibility of contamination of this preparation with non-mitochondrial malic dehydrogenases was examined.

Assessment of contamination of the mitochondrial fraction with non-mitochondrial malic dehydrogenases

Particulate contaminants. The relatively high malate oxidation could result from a contamination of the mitochondria with chloroplasts (or pro-

Table 5. *Effect of* L-*cysteinesulphinate on oxidation of succinate and malate*

The 'standard' conditions of incubation (see Methods section) were used, with an incubation temperature of 25°. The q_{O_2} values are based on the interval from 30 to 50 min.

L-Cysteinesulphinate concn. (mM)	q_{O_2} values (μl. of O_2/mg. of protein/hr.) Succinate concn. (mM) 2	Succinate concn. (mM) 20	Malate concn. (mM) 2	Malate concn. (mM) 20
0	1·9	13·6	3·1	16·9
4	7·9	14·4	5·6	23·7
10	8·7	14·6	6·4	27·5

Table 6. *Effect of adenosine diphosphate on mitochondrial oxidations*

The 'standard' incubation medium containing 0·2% bovine serum albumin was used in all flasks with no other cofactors present initially with malate. The other substrates had the same cofactors as in Table 4, except that no malate was added with pyruvate. The final sucrose concn. in the incubation medium was 0·28M. The magnesium salt of ADP (see Materials section) and substrates were added from the side arm after equilibration for 10 min. The final concentrations of ADP and substrates were 10 and 100 mM respectively. Rates of oxygen uptake were determined during the first 10 min. after adding the magnesium salt of ADP and substrates. L(+)-Isocitrate was used (see Materials section). The incubation temperature was 25°.

Substrate	Relative rates of oxygen uptake* Without ADP	With ADP
Malate	100	205
Succinate	100	291
Pyruvate	0	2
Isocitrate	68	100
α-Oxoglutarate	24	208

* In each experiment oxygen uptake is expressed relative to a value of 100 for malate oxidation. The 'corrected' q_{O_2} for malate oxidation without ADP was 76 μl. of O_2/mg. of protein/hr. Average 'corrected' content of mitochondrial protein was 3 mg./flask.

plastids) similar to those isolated from spinach leaves by Zelitch & Barber (1960). These oxidize only malate, glycollate and glyoxalate. In some, though not all, experiments our preparation did oxidize glycollate (20 mM), the maximum q_{O_2} values obtained being about 3 both in the presence and absence of DPN. It is therefore likely that in some experiments the preparation did contain plastids, because mitochondria do not contain glycollate oxidase (Zelitch & Barber, 1960). These workers also showed that in spinach chloroplasts malate oxidation was completely dependent on the addition of an external hydrogen acceptor and proceeded at about twice the rate of glycollate oxidation. Even if the mitochondria in our preparations were accepting hydrogen derived from the oxidation of malate by plastids, the relatively high rates of malate oxidation could not be accounted for by contamination with plastids, as q_O values obtained with glycollate were very low.

'Soluble' contaminants. DPN-linked malic-dehydrogenase activity has been observed in the supernatant obtained after centrifuging an homogenate at 70 000*g* for 90 min. (results on enzymes from the 'soluble' fraction of carrot tissue will be reported in a future paper). It is possible that this enzyme, adsorbed on the mitochondria, may account for the high malate oxidation relative to succinate oxidation in carrot mitochondria. In the routine mitochondrial preparations, lactic-hydrogenase and glucose 6-phosphate-dehydrodegenase activities were present; as both these enzymes are commonly regarded as being extra-mitochondrial, they have been used as 'markers' for contamination by a 'soluble' malic dehydrogenase.

(*a*) *Lactate and glucose 6-phosphate oxidation by mitochondria.* DL-Lactate (100 mM) was oxidized at a low rate in the presence and in the absence of DPN (q_{O_2} values 1–3), but oxygen uptake was consistently about twice the initial rate of endogenous respiration. The rate of lactate oxidation was unaffected by CoA or thiamine pyrophosphate; in one experiment DPN stimulated the low oxygen consumption and in another it had no effect. The oxidation was not due to the action of glycollate oxidase which may oxidize lactate at about half the rate of glycollate (Zelitch & Ochoa, 1953); with one preparation lactate was oxidized appreciably whereas glycollate was not oxidized at all.

The oxidation of glucose 6-phosphate (20 mM) had an absolute requirement for TPN; with TPN (1 mM) the rate of oxidation was 2–6 times that of lactate. The oxidation of glucose 6-phosphate is coupled, like that of malate and succinate, through the electron-transport system to cytochromes *a* and a_3, as is indicated by the inhibition of oxygen uptake by 2 mM-azide, which reduced the q_{O_2} from 5·1 to 2·7. Glucose 6-phosphate oxidation was unaffected by malonate.

(*b*) *Effect of resuspension of mitochondria in sucrose solution or in sucrose solution followed by saline on lactic-dehydrogenase and glucose 6-phosphate-dehydrogenase activities.* To determine whether lactic dehydrogenase and glucose 6-phosphate dehydrogenase were attached firmly to the mitochondria, their activities were compared with that of succinic dehydrogenase in a standard mitochondrial preparation (resuspended twice) and in similar preparations resuspended seven times either in sucrose solution or saline (Table 7). The small initial lactic-dehydrogenase activity was lost completely on resuspension in either medium. Glucose 6-phosphate-dehydrogenase activity falls to one-quarter and to one-half its initial activity relative to succinic dehydrogenase after resuspen-

Table 7. *Effect of resuspension in sucrose and saline on endogenous respiration and on oxidation of succinate, lactate and glucose 6-phosphate*

The 'standard' incubation medium (see Methods section) was used with 'no substrate', succinate and lactate; for glucose 6-phosphate 1 mM-TPN was added, instead of 1 mM-DPN. The concn. of each substrate was 20 mM. The concn. of the sucrose resuspension medium was 0·44 M; it contained 2·5 mM-EDTA. The saline resuspension medium contained 0·23 M-KCl, phosphate buffer (0·02 M, pH 7·1) and 2·5 mM-EDTA, and was calculated on the basis of the dissociation constant for KCl of 0·86 (*Handbook of Chemistry and Physics*, 33rd ed. p. 1505, 1951–52) to be iso-osmotic with the sucrose resuspension medium. The final resuspension before incubation was in sucrose–EDTA in all cases. All resuspensions were carried out at 0–4° and the incubation temperature was 25°.

	q_{O_2} values (μl. of O_2/mg. of protein/hr.)			
	Substrate			
Resuspension medium	None	Succinate	Lactate	Glucose 6-phosphate
Sucrose (×2)	1·5	8·4	3·3	4·8
Sucrose (×7)	0	18·0	0	2·7
Sucrose (×2), saline (×4), sucrose (×1)	0	15·0	0	5·1

Table 8. *Effect of resuspension in sucrose and saline on malate and succinate oxidation and on malonate inhibition*

The 'standard' incubation medium was used (see Methods section); malate and succinate concentrations were 10 mM, and that of malonate was 20 mM. The sucrose and saline resuspension media were of the same composition as that given in Table 7. Protein values are of a 1 ml. sample of 'mitochondria' obtained after the final resuspension in a standard volume of medium. The incubation temperature was 25°.

Expt.	Resuspension medium	q_{O_2} values (μl. of O_2/mg. of protein/hr.) Malate: Without malonate	Malate: With malonate	Succinate: Without malonate	Succinate: With malonate	Protein (mg.)
1	Sucrose (× 1)	6·0	3·9	2·1	0·3	19
	Sucrose (× 5)	15·0	6·9	3·0	0	9
	Sucrose (× 10)	26·4	9·9	6·6	0	4
2	Sucrose (× 3)	49·5	21·3	22·2	0·9	10
	Saline (× 2), sucrose (× 1)	21·3	10·5	7·5	0·3	5

sion in sucrose solution and saline respectively. Succinoxidase activity (per mg. of protein) rises on resuspension, presumably owing to the removal of contaminant protein from the mitochondrial pellet.

Servattaz (1956, quoted by Hackett, 1959) reported the presence of pentose phosphate-cycle enzymes in mitochondrial extracts of pea stems; Davies (1956) noted the presence of lactic-dehydrogenase activity in mitochondrial extracts of the same tissue. The ready dissociation of lactic-dehydrogenase and glucose 6-phosphate-dehydrogenase activities from the terminal oxidase system on resuspension of the preparation indicates that our results, and probably those of Servattaz and of Davies, are the result of adsorption of these 'soluble' enzymes to the mitochondria. A useful comparison may therefore be made between the response of these activities to resuspension and that of the high malic-dehydrogenase activity.

(c) *Effect of resuspension of mitochondria in sucrose solution and saline on malic-dehydrogenase activity and malonate sensitivity.* The retention of malic-dehydrogenase and succinoxidase activities and the extent of malonate inhibition were measured after resuspension of the mitochondria ten times in sucrose (Table 8, Expt. 1). On resuspension ten times, q_{O_2} values for malate and succinate oxidation increased by 440 and 310% respectively. After one, five and ten resuspensions, malonate inhibited malate oxidation by 35, 47 and 62% respectively. This increase in malonate sensitivity is possibly related to the removal of endogenous substrate from the mitochondria on resuspension, or to an increase in the permeability of the mitochondria to malonate.

Resuspension in saline instead of sucrose solution resulted in a decrease in q_{O_2} values for both malate and succinate oxidation, and a greater loss of protein from the pellet (Table 8, Expt. 2). The reduction in protein, and the loss of ability to oxidize succinate and malate in saline-resuspended mitochondria compared with that of mitochondria resuspended in sucrose solution were 50, 63 and 57% respectively. There was therefore a rough correspondence between the greater loss of protein on resuspension in saline and the decreased rates of oxidation of malate and succinate. Other workers (Hogeboom, Schneider & Pallade, 1948) have reported that saline media are less effective in some conditions in preserving mitochondrial activity. This would also seem to apply to the carrot preparations used here. Malonate sensitivity of malate oxidation after resuspension in sucrose solution and saline was 57 and 51% respectively.

Thus there was no evidence that the dehydrogenases either of plastids or of the 'soluble' fraction contributed appreciably to malate oxidation by the mitochondria.

Other oxidative systems detected in the mitochondria

Mitochondria were capable of oxidizing β-hydroxybutyrate, α-glycerophosphate, butyrate, acetate, octanoate and formate. Although serum albumin enhanced the oxidation of the first three substrates, the q_{O_2} values obtained were always low. Representative 'corrected' q_{O_2} values are: β-hydroxybutyrate, 5·4; α-glycerophosphate, 4·2; butyrate, 0·8; butyrate (with ATP and CoA), 4·9.

DISCUSSION

Conditions for specific inhibition by malonate

At low malonate concentrations (5–20 mM), Hanly *et al.* (1952) regard the partial inhibition of carrot-slice respiration as being due to a specific

inhibition of succinic dehydrogenase; at higher concentrations (40–50 mM), although complete inhibition was not obtained, malonate was considered to inhibit other enzymes. As malonate at high concentrations inhibits enzymes other than succinic dehydrogenase in other plant tissues (Beevers, 1952; Price, 1953), the complete inhibition of freshly cut and circulated carrot slice respiration by 0·8 and 0·3 M-malonate respectively was probably a result, in part at least, of this non-specific action.

Thus malonate may inhibit respiration completely, partially, or not at all, depending on the conditions of incubation. Discussions of malonate sensitivity must therefore include a definition of incubation conditions (substrate and buffer used, and malonate concentration) and also precise information about oxidative pathways operating in the tissue.

Oxidative pathways in various types of carrot-tissue preparation

Slices. The data reported in this paper suggest that there is a pathway for malate oxidation in carrot-tissue slices which does not depend on the unimpaired functioning of succinic dehydrogenase. In summary, the evidence for this conclusion is: (*a*) Malonate added after preincubation for 1 hr. inhibited the endogenous respiration and the oxygen uptake with α-oxoglutarate, succinate and glucose, but stimulated that with malate and malate plus pyruvate (Table 1); (*b*) the inhibition of respiration of tissue preincubated in malonate was partly reversed by malate but not by isocitrate (Table 2); (*c*) the respiration of mitochondria incubated with malate was inhibited by malonate even though that of the tissue from which the particles were isolated was stimulated by malonate under similar conditions of incubation (see Tables 1, 3 and 4).

Moreover, the unique behaviour of malate in this regard may account for the incomplete malonate inhibition of respiration of freshly cut carrot tissue which contains high levels of malate (Bryant & Overell, 1953).

Mitochondria. Mitochondrial oxidation of malate was observed under the same conditions as that of the other tricarboxylic acid-cycle intermediates. Moreover, even at high concentrations (50 mM), oxygen uptake with all these substrates was partly inhibited by 100 mM-malonate (Table 4), indicating that none of these oxidations is entirely a one-step reaction (see Laties, 1959). The only striking features of malate oxidation by these particles were: (*a*) The *relative* insensitivity to malonate under conditions permitting maximum oxygen uptake (Table 4, Expt. 2), a feature shown more markedly with isocitrate. However, this similarity in the response to malonate with these two substrates was not found with slices (see Table 2). (*b*) The retention of a relatively active malic dehydrogenase by mitochondria prepared without albumin. As these particles are depleted of co-factors (Dalgarno & Birt, 1962), and do not respond to ADP, their properties are probably less representative of the intact mitochondria than are those of preparations with albumin.

It has been concluded from these data that the mitochondrial fraction alone was not responsible for the observed malonate insensitivity of carrot tissue oxidizing malate [compare results for tobacco-leaf particles obtained by Pierpoint (1959)].

'Soluble' fraction. Malic-dehydrogenase activity was detected in the 'soluble' fraction of carrot tissue. A soluble malic dehydrogenase from rat liver has already been reported (Christie & Judah, 1953) and described (Wieland, Pfleiderer, Haupt & Worner, 1959). Dehydrogenation of malate by such enzymes will result in oxygen uptake only if hydrogen removed from the substrate can be passed to a suitable terminal oxidase whether in the mitochondrial or soluble portion of the cell. Various proposals for the transport of extramitochondrial hydrogen to mitochondrial cytochrome oxidase via the cytochrome chain of insect and animal particles have been made; these systems involve either α-glycerophosphate dehydrogenase (Klingenberg & Bucher, 1960) or β-hydroxybutyrate dehydrogenase (Devlin & Bedell, 1960). There is evidence for the presence of both these enzymes in the carrot preparations, though the activities are relatively low. It is not known whether a soluble α-glycerophosphate dehydrogenase, required for hydrogen transport according to the hypothesis of Klingenberg & Bucher (1960), also occurs in this tissue. Nevertheless, these observations suggest the possibility that cytochrome oxidase, which is probably located exclusively in the mitochondria of higher plants (see James & Richens, 1960), is a terminal oxidase involved in this hydrogen transport from malate.

There is also evidence that ascorbic acid oxidase permits an extramitochondrial hydrogen transfer from $DPNH_2$ to oxygen in the soluble fraction of maize-root tips (Mertz, 1961). Further investigation of the properties of the soluble fraction of the carrot is being undertaken to clarify the problem of the oxidation of $DPNH_2$ produced in the soluble part of the cell.

The soluble fraction also contains a TPN-linked isocitric dehydrogenase, but no DPN-linked enzyme (L. Dalgarno & L. M. Birt, unpublished work); the inability of isocitrate to stimulate respiration in the presence of malonate may be a consequence of this requirement for a different codehydrogenase.

Control of the respiratory rate of carrot mitochondria by ADP

Although mitochondria prepared from many plant slices have been shown to oxidize tricarboxylic acid-cycle intermediates, only one detailed report of the control of the rates of substrate oxidation (succinate and citrate) by ADP has been published (Hackett, Rice & Schmid, 1960). However, the preparation methods described above permit the isolation of carrot mitochondria which not only catalyse the tricarboxylic acid-cycle oxidations at rapid rates, but also exhibit a control of each oxidative step by ADP. The stimulation by ADP compares favourably with the data obtained with preparations of animal mitochondria (see Bellamy & Bartley, 1960).

SUMMARY

1. The endogenous respiration of freshly cut and aerobically circulated carrot slices is completely inhibited by 0·8 and 0·3 M-malonate respectively.

2. Changes in endogenous respiration on aerobic circulation of the slices do not appear to bear any relation to changes in malonate sensitivity.

3. Malate, succinate, α-oxoglutarate and glucose stimulated the respiration of aerobically circulated carrot tissue by 91, 186, 130 and 53 % respectively; malonate (20 mM) did not inhibit the respiration with malate but inhibited respiration by 45, 15 and 33 respectively with the other substrates.

4. Malate stimulated the respiration of freshly cut and aerobically circulated tissue preincubated in 20 mM-malonate, but not at higher concentrations; isocitrate did not stimulate the respiration of circulated tissue preincubated in 20 mM-malonate.

5. Malate and glucose stimulated the uptake of malonate by aerobically circulated slices by 59 and 75 % respectively; succinate did not affect this uptake.

6. Malonate inhibited by 44 % the oxidation of malate by mitochondria isolated from a sample of tissue slices in which malate respiration was insensitive to malonate.

7. Mitochondria prepared in the absence of serum albumin had q_{O_2} values for the oxidation of malate, succinate, pyruvate, isocitrate and α-oxoglutarate of 34, 29, 16, 12 and 5 respectively. When serum albumin (1 %) was added to the blending medium, the corresponding values were 97, 106, 55, 13 and 15 respectively.

8. The addition of 10 mM-ADP to mitochondria prepared and incubated in a medium containing albumin increased rates of oxygen uptake with malate, succinate, isocitrate and α-oxoglutarate by 105, 191, 47 and 767 % respectively.

9. The mitochondria slowly oxidized glycollate, formate, lactate, glucose 6-phosphate, α-glycerophosphate, β-hydroxybutyrate and butyrate.

10. The lactic-dehydrogenase and glucose 6-phosphate-dehydrogenase activities, but not the malic-dehydrogenase activity, were dissociated from the terminal oxidase system on resuspension of the mitochondria.

11. It is concluded that an extramitochondrial metabolism of malate is involved in the malonate-insensitive respiration of carrot-tissue slices.

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