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The Distribution of Succinate Dehydrogenase and Malate Dehydrogenase among Components of Tobacco-Leaf Extracts

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(Received 18 January 1963)

In a study of the localization of malate dehydrogenase and succinate dehydrogenase [L-malate-NAD oxidoreductase, EC 1.1.1.37, and succinate-(acceptor) oxidoreductase, EC 1.3.99.1] in etiolated pea stems, Price & Thimann (1954) concluded that the succinate dehydrogenase was almost exclusively in the mitochondrial fraction, whereas the malate dehydrogenase was distributed between the mitochondrial fraction and the supernatant fluid. The 'plastid' fraction appeared to contain neither enzyme. Davies (1961) confirmed the presence of malate dehydrogenase in at least two fractions of pea-epicotyl extracts, and found small differences between the two. Similarly, Hiatt (1961) confirmed the presence of succinate dehydrogenase in mitochondrial preparations of roots and leaves.

However, there is evidence that malate dehydrogenase occurs in mature and immature chloroplasts. Sorokin & Thimann (1960) demonstrated it histologically in the prolamellar bodies of prochloroplasts of *Avena* coleoptiles, and the enzyme was detected in concentrated and well washed preparations of chloroplasts by Leech & Ellis (1961) and Zelitch & Barber (1960). There is similar evidence for the occurrence of succinate dehydrogenase in chloroplasts (Sorokin & Thimann, 1960; Sissakian, 1958), although in this case there are also contradictory claims (Zelitch & Barber, 1960).

There seemed therefore good cause to extend the work of Price & Thimann (1954) to extracts of mature leaves, by using methods of leaf disintegra-

tion and fractionation by gradient centrifuging that are known to separate chloroplasts, smaller chlorophyll-bearing fragments and mitochondria (Pierpoint, 1962). It seemed desirable to use a method of estimating succinate dehydrogenase in which phenazine methosulphate was the electron acceptor. This, unlike the method used by Price & Thimann, would be expected to measure the enzyme in both free and bound form, and not only the amount attached to electron-transfer factors probably specific to the mitochondria (Singer, Kearney & Massey, 1957).

EXPERIMENTAL

Leaf preparations. Chloroplasts and mitochondrial preparations were made from tobacco leaves by the methods and materials previously described (Pierpoint, 1962). In experiments where the washing procedure was altered, details are given. The preparations were suspended in either a small volume of the washing medium, or, when they were to be centrifuged in a sucrose gradient, in 15 ml. of a solution containing sucrose (0.15 M), tris (5 mM, adjusted to pH 7.4 with HCl) and EDTA (1 mM).

Centrifuging in sucrose gradients. Sucrose gradients were made by layering buffered sucrose solutions of decreasing densities in 100 ml. glass centrifuge tubes, and allowing the interfaces of the layers to become smooth by standing for 5 hr. in the cold (0–5°). Details of constructing the gradients, centrifuging and fractionating the tube contents were described by Pierpoint (1962). The densities (g./ml.) of fractions 1–9 obtained in a typical experiment were 1.017, 1.021, 1.036, 1.045, 1.074, 1.127, 1.141, 1.162 and 1.199 (Pierpoint, 1962).

Succinate dehydrogenase. This was measured spectrophotometrically by the reduction of 2,6-dichlorophenol-indophenol in the presence of phenazine methosulphate (Ells, 1959; Hiatt, 1961). The contents of the experimental cuvette were: NaH_2PO_4 (0.2 m-mole, adjusted to pH 7.6 with KOH); sodium succinate (0.12 m-mole); sodium cyanide (30 μmoles); phenazine methosulphate (0.3 mg./ml.); 2,6-dichlorophenol-indophenol (90 $\mu\text{m-moles}$); enzyme and water, 3 ml. The dichlorophenol-indophenol was omitted from the optical blank. The decrease in $E_{600\text{ m}\mu}$ was followed at room temperature (22–26°) over a period of 8 min. in a Unicam SP. 500 or for 3–5 min. in an Optica CF4 recording spectrophotometer. During these times the extinction decreased linearly with time. The rates of reduction were always corrected for the partly non-enzymic reduction that occurred without added succinate. The magnitude of this correction differed greatly in different fractions of tobacco-leaf extracts. It was usually 10–15% of the succinate-dependent rate with most mitochondrial preparations, but was considerably more in diluted or unfractionated extracts. Two precautions were necessary, especially when dealing with crude leaf extracts. First, succinate had to be added to the cuvettes before enzyme. If the enzyme was exposed to either of the dyes in the absence of succinate it was rapidly and apparently irreversibly inactivated. Secondly, with extracts containing chloroplasts it was necessary to avoid strong illumination of the cuvettes while the reagents were being mixed; otherwise the dichlorophenol-indophenol was reduced photochemically. When these precautions were taken, the amount of enzyme present was strictly proportional to the decrease in E up to rates of 0.12/min. Activity was not affected appreciably by halving or doubling the concentration of phosphate buffer, or by adding sucrose (0.3 m-mole) or small amounts of the extraction medium in which the tobacco leaves were disrupted.

Enzyme activity is expressed as μmoles of succinate oxidized/min./ml. of enzyme preparation added to the cuvette. This is calculated from the observed change in $E_{600\text{ m}\mu}$ by using the apparent molecular extinction coefficient of 20 500 found for the sample of 2,6-dichlorophenol-indophenol (at pH 7.6) with which most of the estimations were performed. The resulting values are only approximate as the purity of the dye is unknown.

In some experiments the succinate dehydrogenase was activated as described by Kearney (1957). Samples of the enzyme were incubated for 30 min. at 25° with succinate (0.12 m-mole) and NaH_2PO_4 (0.2 m-mole adjusted to pH 7.6 with KOH), before the other assay reagents were added. Blanks without succinate were then done on enzyme that had been incubated with phosphate buffer and water. Longer incubations (60 min.) did not increase the amount of activation; shorter ones (less than 16 min.) decreased it. No activation occurred when the mitochondrial preparations were treated with digitonin (20 mg./ml.) before the assay, or when 0.75 mM-calcium chloride (Singer & Lusty, 1960) was added to the assay medium.

Malate dehydrogenase. This was estimated spectrophotometrically by following the reduction of NAD. The experimental cuvette contained tris (0.3 m-mole, adjusted to pH 9 with HCl); sucrose (0.6 m-mole); sodium L-malate (0.1 m-mole); sodium cyanide (0.5 μmole); NAD (1.35 μmoles); leaf extract and water to 3 ml. The blank cuvette

contained the same reagents without NAD. The reaction was started by adding malate, and the increase in $E_{340\text{ m}\mu}$ was followed at room temperature for 2–5 min. on either the Unicam SP. 500 or the Optica SF4 spectrophotometer. The rate of reaction, expressed as μmoles of malate oxidized/min./ml. of enzyme preparation added to the cuvette, is calculated assuming the molecular extinction coefficient of NADH_2 to be 6220 (Horecker & Kornberg, 1948), so that an increase in $E_{340\text{ m}\mu}$ of 1.0 corresponds to the oxidation of 0.48 μmole of malate. Over the range of observed activities, the reaction rate was proportional to the amount of enzyme added. There was no reaction when either malate or enzyme was omitted.

Aconitate hydratase (isocitrate hydro-lyase, EC 4.2.1.3). This was measured spectrophotometrically, with isocitrate as substrate, by the method described by Bacon, Palmer & DeKock (1961). The increase in $E_{240\text{ m}\mu}$ was followed for 15 min. and was proportional to the amount of leaf extract added.

Succinate-oxidase system. This was measured manometrically as described by Pierpoint (1962).

Chemical estimations. Protein N was estimated by a micro-Kjeldahl method (Pierpoint, 1959), and chlorophyll by the method of Arnon (1949) with the values at $E_{652\text{ m}\mu}$ given by Bruinsma (1961).

RESULTS

Properties of leaf succinate dehydrogenase. A few of the properties of succinate dehydrogenase that are relevant to the assay conditions were determined with a mitochondrial preparation as a source of enzyme. The properties differ only slightly from those of a solubilized enzyme preparation from tobacco leaves measured under similar but not identical conditions (Hiatt, 1961). The Michaelis constant for succinate is 0.7–0.8 mM. The pH optimum (7.7) is 0.3 unit more alkaline than that of the soluble preparation, although the general shape of the two pH-activity curves are the same. The mitochondrial preparation is activated, generally about fourfold, by incubation with succinate. Finally, both preparations respond similarly to alterations in the concentration of the two dyes used in the estimations. The concentration of phenazine methosulphate used was apparently sufficient to saturate the enzyme; however, the activity may have been slightly underestimated by not extrapolating reaction rates to infinite dye concentration (Arrigoni & Singer, 1962).

Distribution of succinate dehydrogenase among leaf fractions. The succinate dehydrogenase of leaf extracts is completely sedimented by centrifuging at 10 000g for 30 min. Most of the activity (60–80%) is recovered in the mitochondrial fraction, where its specific activity ($\mu\text{moles/min./mg.}$ of protein nitrogen) is six to seven times that of the original extract (Table 1). The remainder is in the chloroplast fraction with a specific activity similar to that in the leaf extract.

Table 1. *Distribution of succinate dehydrogenase and protein nitrogen in fractions of tobacco leaves*

Tobacco leaves (20 g.) were disrupted in 100 ml. of extraction medium, and the resulting suspension was filtered through muslin and then fractionated by centrifuging at 1000*g* for 7 min. and 10 000*g* for 30 min. The washed chloroplast and mitochondrial preparations were suspended in washing medium. Samples taken for succinate-dehydrogenase estimations were activated by preincubation with succinate. The total succinate-dehydrogenase activity of each fraction (μ moles/min./total fraction) was obtained by multiplying the specific activity of the enzyme by the protein-nitrogen content of the fraction.

	Protein N		Succinate dehydrogenase		
	(mg.)	(% of total)	(μ moles/min./total fraction)	(% of total)	(μ mole/min./mg. of protein N)
Initial leaf extract	36.5	100	1.56	100	0.043
Chloroplast fraction	9.3	25	0.31	20	0.034
Mitochondrial fraction	4.2	11.5	1.21	78	0.290
Supernatant fraction	22	60	0	0	0
Percentage recovered in the three fractions	—	96.5	—	98	—

Table 2. *Distribution of succinate dehydrogenase and chlorophyll in sucrose gradients*

The chloroplast and mitochondrial preparations were made from different batches of tobacco leaves, layered on sucrose gradients and centrifuged. The contents of the centrifuge tubes were then divided into nine fractions (the first being taken from the top and the ninth from the bottom), and succinate dehydrogenase and chlorophyll were measured in samples of each. Before the enzyme measurements, all samples were activated by incubation with succinate.

Fraction no.	Chlorophyll (μ g./ml.)		Succinate dehydrogenase (μ m-moles/min./ml.)	
	Mito-chondria	Chloro-plasts	Mito-chondria	Chloro-plasts
1	126	56	11.4	0.97
2	160	62	23.2	0.97
3	87	44	35.0	2.46
4	28	49	20.0	3.75
5	9	60	4.0	0.97
6	3	140	0	0
7	3	390	0	0
8	0	295	0	0
9	0	17	0	—

The estimations on which the values of Table 1 are based were made after the enzymes had been activated by incubation with succinate. When activation was omitted the recovery of activity among the fractions of the leaf extract was less (50–60 %) because the mitochondrial fraction apparently contained less enzyme. Activation apparently affected the enzyme in this fraction more than that in the chloroplast fraction or the extract.

The succinate dehydrogenase of the chloroplast fraction and the mitochondrial fraction behaved in the same way when these preparations were centrifuged in sucrose gradients. Most activity was recovered in the second, third and fourth fractions

taken from the gradients, with the highest concentration usually in the third (Table 2). The distribution of activity was the same when the fractions were activated by incubation with succinate. No activity was detected in those fractions previously (Pierpoint, 1962) shown to contain relatively intact chloroplasts. Recovery of activity when either chloroplast or mitochondrial preparations were centrifuged in the sucrose gradients averaged about 70 %.

This distribution of succinate dehydrogenase among the sucrose fractions corresponds closely to that of the succinate-oxidase system (Table 3), and, within the limits of the estimations, the ratio of the two activities in each fraction is constant. Although the two activities were measured under different conditions, it is possible to make a very rough comparison of their rates under similar ones. From the known variation of succinate-dehydrogenase activity with pH, and assuming Q_{10} 1.6, the same as for the solubilized yeast enzyme (Singer, Thimot, Massey & Kearney, 1956), the succinate-dehydrogenase activity of fraction 3 (Table 3) corresponds to about 0.37 μ mole of succinate oxidized/ml./hr. under the condition of the succinate-oxidase assay. This would probably have been increased fourfold, to 1.5 μ moles of succinate oxidized, had the extract been activated with succinate. The succinate-oxidase activity of this fraction is 1.6 μ moles of succinate oxidized/ml./hr., assuming that all the oxygen uptake is associated with the single-step conversion of succinate into fumarate. There is therefore probably enough succinate dehydrogenase in this fraction to account for its likely role in the succinate-oxidase system.

Properties of tobacco-leaf malate dehydrogenase. In the assay conditions used, the enzyme was saturated with respect to malate (Michaelis constant approx. 4.5 mM), but not to NAD. Increasing the concentration of NAD 2.6-fold gave a 60 %

increase in reaction rate. The pH-activity curve was rather sharp with a well-defined optimum at 8.8. The reaction rate was decreased (16–20 %) by adding small amounts of sodium chloride (0.05M), and by increasing the concentration of tris from 0.10 to 0.13M. Conversely it was stimulated about 10 % when the concentration of tris was decreased from 0.1M to 66 mM. The large stimulation of a plant malate dehydrogenase by low concentrations of salts (Hiatt & Evans, 1960) was observed in an assay medium of low ionic strength.

The above properties were observed with a chloroplast preparation as the source of enzyme. Similar results were found when the enzyme present in other leaf fractions was used.

Distribution of malate dehydrogenase among leaf fractions. Over half (55–70 %) of the malate dehydrogenase of the leaf extracts was recovered in the supernatant fraction (Table 4), from which it could not be sedimented by additional centrifuging

at 25 000g for 2 hr. The rest of the activity was spread through the two sedimented fractions and their washings. The chloroplast fraction contained small amounts (5–7 %) and the mitochondrial fraction rather more (7–19 %). When the washings of the chloroplast and mitochondrial fractions were collected separately they had appreciable quantities of enzyme of high specific activity (Table 4). In another experiment two successive washes of a mitochondrial preparation removed 48 % and 11.5 % of its total malate dehydrogenase; they had specific activities that were 3.5 and 2 times that of the residual mitochondria, or 5 and 2.8 times that of the supernatant fraction. It is very unlikely that the enzyme in the washings was derived from contaminating supernatant fluid, and much more probable that it was eluted from the sedimentable particles. When either mitochondrial or chloroplast preparations were centrifuged in a sucrose gradient, enzyme activity was most concentrated in the top fractions, but was present in nearly all the others as well (Table 5). There was no apparent correlation between its distribution and that of intact chloroplasts, chloroplast fragments, or with the succinate-oxidizing system, even when twice-washed mitochondrial preparations were used. The activity is more diffuse than that observed with glycollate oxidase (Pierpoint, 1962), an enzyme not apparently attached to any sedimentable particles, and suggests that the malate dehydrogenase is eluted from components of both the chloroplast and mitochondrial fractions during sedimentation in the sucrose gradients.

The average recovery of malate-dehydrogenase activity after centrifuging in a sucrose gradient was 96 %.

Distribution of aconitate hydratase among leaf fractions. This enzyme has not been studied in the same detail as the two dehydrogenases. In two experiments 84 and 73 % of the recovered activity was in the supernatant fraction, and the rest in the mitochondria. But medium in which the mitochondrial fraction had been washed had almost as

Table 3. *Distribution of succinate dehydrogenase, the succinate-oxidase system and chlorophyll in a sucrose gradient*

An unwashed mitochondrial preparation was centrifuged in a sucrose gradient, and the contents of the tubes were divided into eight fractions (the first fraction being taken from the top of the tube and the eighth from the bottom). Chlorophyll, succinate dehydrogenase and the succinate-oxidase system were measured in samples from each fraction.

Fraction no.	Chlorophyll (μg./ml.)	Uptake of O ₂ by succinate-oxidase system (μm-moles/ml./min.)	Succinate dehydrogenase (μm-moles/ml./min.)
1	190	4.6	2.2
2	255	8.4	2.9
3	138	13.6	5.5
4	63	9.8	3.4
5	38	4.9	0.8
6	21	1.6	0.6
7	7	0	0
8	7	0	0

Table 4. *Distribution of malate dehydrogenase in tobacco-leaf fractions*

The leaves (17 g.) were disrupted in 95 ml. of extraction medium, and the fractions separated as usual. Both chloroplast and mitochondrial fractions were washed in wash medium (20 ml. and 6 ml. respectively) and the washings were kept separately.

Fraction	Protein N		Malate dehydrogenase		
	(mg.)	(% of total)	(μmoles/min./total fraction)	(% of total)	(μmoles/min./mg. of protein N)
Initial extract	28.5	100	78	100	2.74
Chloroplast fraction	7.2	25	5.1	7	0.71
Chloroplast washings	1.9	6.7	8.5	11	4.5
Mitochondrial fraction	2.0	7	5.3	7	2.65
Mitochondrial washings	0.34	1.2	3.9	5	11.5
Supernatant fraction	14.8	53	46	60	3.1

Table 5. *Distribution of malate dehydrogenase and chlorophyll in sucrose gradients*

Mitochondrial and chloroplast preparations were made from separate batches (17 g.) of tobacco leaves, and after the chloroplast preparations had been washed twice, they were centrifuged in sucrose gradients. The contents of the centrifuge tubes were divided into eight fractions (the first being taken from the tops of the tubes) and samples taken for chlorophyll and malate-dehydrogenase estimations.

Fraction no.	Chlorophyll ($\mu\text{g./ml.}$)		Malate dehydrogenase ($\mu\text{mole/min./ml.}$)	
	Mito-chondria	Chloro-plasts	Mito-chondria	Chloro-plasts
1	164	38	0.81	0.13
2	193	35	0.72	0.12
3	70	35	0.28	0.11
4	23	59	0.27	0.09
5	7	134	0.15	0.06
6	1	245	0.10	0.04
7	0	154	0.02	0.03
8	0	50	—	0.02

much activity as the washed mitochondrial fraction, suggesting that some of this enzyme, too, was leached out of the particles.

DISCUSSION

The distribution of succinate dehydrogenase among fractions of leaf extracts, and among fractions of sucrose density gradients, closely resembles that of the succinate-oxidase system, as well as that of fumarate hydratase and cytochrome oxidase (Pierpoint, 1962). There can be no doubt that the major and probably only location of succinate dehydrogenase in these extracts is the mitochondria. The observation by Sissakian & Chamova (Sissakian, 1958) that chloroplasts contain some activity is probably because their preparations were contaminated with mitochondria. The concentrated leaf extracts and the centrifuging procedure usually used by Sissakian and his colleagues make contamination likely (see Mosolova & Sissakian, 1961; Pierpoint, 1962), and Leech & Ellis (1961) demonstrated that it is difficult to remove contaminating mitochondria by the usual technique of washing. The immature chloroplasts of *Avena* coleoptiles reduced neotetrazolium chloride in the presence of succinate (Sorokin & Thimann, 1960) and may, unlike tobacco-leaf chloroplasts, contain succinate dehydrogenase. But an alternative explanation, more in keeping with the present work, is that the reduction results from the conversion of succinate into malate and its subsequent oxidation by chloroplast malate dehydrogenase.

The distribution of malate dehydrogenase is

more complex. The high specific activity of medium in which both mitochondrial and chloroplast fractions were washed, as well as the presence of activity in those sucrose-gradient fractions containing chloroplasts or mitochondria, make it likely that the enzyme is derived from both these particles and suggest that it was located there in the cell. Possibly all the enzyme in the plant cell is so located, and the 60% or so that is recovered in the supernatant fraction is leached from the organelles during preparation. It may be relevant here that Davies (1961) showed only two chromatographically distinct forms of the enzyme in extracts of pea epicotyls and cauliflowers. However, there could well be a third, 'soluble', form of malate dehydrogenase that is not derived from any particles. Such a malate dehydrogenase occurs in animal tissues, and has a chemical composition distinct from that of the mitochondrial enzyme (Siegel & England, 1962).

The malate-dehydrogenase activity of these tobacco-leaf extracts is about 50 times as great as that of the succinate dehydrogenase. This is shown by a comparison of the activities of the similar extracts described in Tables 4 and 1. Even though most of the malate-dehydrogenase activity is not attached to sedimentable particles, the mitochondrial preparations are about ten times as active in dehydrogenating malate as succinate. However, a rough estimate of the relative activity of the two mitochondrial dehydrogenases in the manometric conditions in which the oxidation of succinate and malate was measured (Pierpoint, 1960*a*) suggests that they are about equal. As mitochondrial preparations oxidize succinate and malate to the same extent (Pierpoint, 1960*a*), and the succinate dehydrogenase is just sufficient to account for the oxidation of succinate, it follows that the malate dehydrogenase is approximately sufficient to account for its expected role in the malate-oxidase system of these preparations.

The situation apparently differs from that previously reported for fumarate hydratase (Pierpoint, 1960*b*). There is at least 15 times more than would be required to account for the rates of oxygen uptake observed in the presence of fumarate.

The few experiments done with aconitate hydratase confirm the conclusion of Bacon *et al.* (1961) that little of this enzyme in leaves is recovered in the mitochondrial or chloroplast fractions. These experiments suggest, however, that some at least of the 'soluble' enzyme originated in the mitochondria. This may also have been the origin of the large amounts of fumarate hydratase in the supernatant fraction of cabbage leaves (Bacon *et al.* 1961), as in tobacco-leaf extracts the enzyme is predominantly recovered with the mitochondria (Pierpoint, 1960*b*; 1962).

SUMMARY

1. The activities of succinate dehydrogenase, malate dehydrogenase and aconitate hydratase were measured on fractions of tobacco-leaf extracts prepared by differential centrifuging. The dehydrogenases were also measured in fractions produced when the chloroplast and mitochondrial preparations were resolved by sucrose gradient centrifuging into chloroplasts, chloroplast fragments and mitochondria.

2. All the succinate dehydrogenase of the extracts behaved as if it were attached to mitochondria. It sedimented along with the succinate-oxidase system, and the succinate-dehydrogenase activity was sufficient to account for its expected role in this oxidase system.

3. Most of the malate dehydrogenase was recovered in the supernatant fraction. The small proportions (5–19%) present in the mitochondrial and chloroplast preparations were reduced by washing. More enzyme appeared to be washed out of the chloroplasts and mitochondria as they sedimented in sucrose gradients.

4. Most of the aconitate-hydratase activity of leaf extracts was recovered in the supernatant fraction. The rest occurred in the mitochondrial fraction and could be partially removed by washing.

5. It is suggested that some of the malate dehydrogenase that was recovered in the supernatant fraction originated in chloroplasts and mitochondria, and that some of the aconitate hydratase originated in mitochondria.

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Phosphoinositides

5. THE INOSITOL LIPIDS OF OX BRAIN*

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(Received 28 January 1963)

An enzyme from liver that hydrolyses inositol phospholipids was described by Kemp, Hübscher & Hawthorne (1961*b*). During studies of its action on the brain 'diphosphoinositide' fraction prepared by the method of Folch (1949), phosphate esters were observed which were more difficult to remove

from ion-exchange resins than the expected inositol diphosphates (Kemp, Hawthorne, Sammons & Ellis, 1961*a*). Further investigation showed that they were inositol triphosphates (Ellis, Kemp & Hawthorne, 1960), confirming results from other Laboratories (Grado & Ballou, 1960; Dittmer & Dawson, 1960).

A more complete study of the hydrolysis of the Folch 'diphosphoinositide' fraction is now presented, from which it is concluded that ox brain

* Part 4: Hawthorne (1961).

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