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THE ESTIMATION OF DEHYDROGENASES IN PLANT TISSUE^{1, 2}

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Although a great deal of information has been amassed concerning dehydrogenases in animal tissues, there was for a long time little evidence that certain of these important enzymes even existed in plants. Malic and citric dehydrogenases were reported in 1929 in cucumber seeds (41), but it was not until 1939 that succinic dehydrogenase was found, first in pollen by Okunuki (28, 29) and then in certain other tissues (9, 11, 14). Nevertheless, the apparent absence or near-absence of succinic dehydrogenase in some tissues (2, 3, 6, 14, 42) as well as the occasional reports of the presence of individual enzymes (10, 23, 41, 42) seemed to indicate that the dehydrogenases, at least those of the 4 carbon and 6 carbon acids, were distributed only sporadically. It was during this period that the tricarboxylic acid cycle of Krebs (19), embodying many of these dehydrogenases, was becoming accepted as the main pathway of respiration in animal tissues. Respiration studies in plants (1, 5) pointed in the same direction.

In the last two years, the situation has greatly altered: the survey of Bhagvat and Hill (4), and the reports of Price and Thimann (32) on oat and pea seedlings and of Millerd (24) on potato tuber have shown that the earlier negative findings on succinic dehydrogenase were not justified, and have implied its general distribution in plants. Conn et al (8) have brought to light the enzyme causing simultaneous dehydrogenation and decarboxylation of malic acid and shown its wide distribution in plants; and most recently (20, 25, 26, 38), great progress has been made in identifying plant systems corresponding to the animal cyclophorase.

While the occurrence of the enzymes is thus no longer in doubt, their properties and their concentrations in plants remain little known. As to their properties, there is evidence that the methods of animal enzymology cannot always be employed unchanged in the study of plant dehydrogenases. For example, Latics (21) has discussed the special osmotic requirements of enzymatic particles from cauliflower, and our own studies (32) showed that the succinic dehydrogenase of oat coleoptiles is not only highly labile but

is inactive toward methylene blue and thionin, dyes widely used with the animal enzyme.

METHODS AND MATERIALS

PREPARATION OF ENZYMES: Since enzymes appear to alter in amount and type within a given tissue according to age, conditions of growth, and other factors, plant material capable of rigid standardization was selected: the apical 30 to 50 mm of 7-day-old etiolated pea stems (*Pisum sativum* var. Alaska) and the coleoptiles of 72-hour etiolated oat seedlings (*Avena sativa* var. Segerhavre). The conditions for growth were essentially those for studies of auxin-induced growth (39). The pea stem preparation served for many of the dehydrogenase experiments and for all of the oxidase manometric work. Except for the

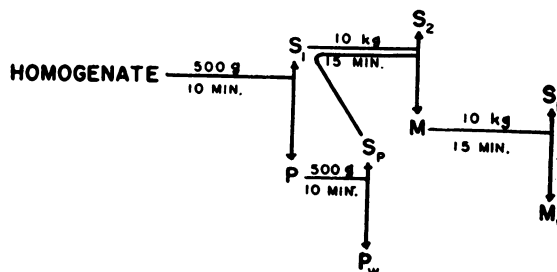


FIG. 1. Scheme of centrifugal fractionation. The inclusion of S_p in the first high-speed centrifugation is essential for quantitative recovery of M . The fraction used in subsequent experiments is M_w .

greater sensitivity to inactivation shown by the *Avena* particles, no qualitative differences were found between the enzymes from the 2 tissues.

Extraction and purification were conducted in a 3°C coldroom. Chilled tissue was ground in 1.5 to 10 times its weight of a solution 0.2 M in sucrose and 0.03 M in phosphate buffer of pH 7. A Potter-Elvehjem tissue grinder was employed for tissue weighing up to a gram, and a mortar and pestle for larger quantities. The macerate was then washed through a coarse sintered glass funnel to remove large cell debris.

Fractional centrifugation was accomplished by a modification of the widely accepted method of Hogeboom, Schneider, and Palade (17) as summarized in figure 1. A low-speed (500 to 1000 × g) sediment containing plastids, starch, and probably nuclei, was designated P ("plastid"); the washed plastid fraction

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was termed P_w . For quantitative recovery, it is essential to return the supernatant, S_p , from the plastid wash for further centrifugation (fig 1). The second, or mitochondrial,⁴ fraction, M , was almost completely precipitated below $8000 \times g$. From 8000 to $20,000 \times g$ very little was sedimented. Material remaining in the supernatant after centrifugation at $10,000 \times g$ (S_2 in figure 1) was not examined. Each sediment was washed with sucrose-phosphate mixture and resuspended by means of a small mechanical homogenizer. It was found beneficial to protect the mitochondria after the first wash by adding bovine serum albumin, 3 mg per ml solution.

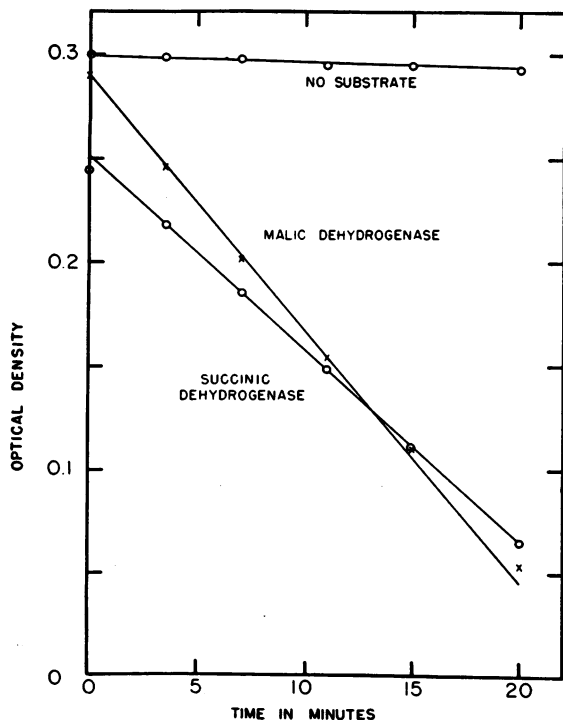


FIG. 2. Time course of dehydrogenation in the Thunberg tube. Malic and succinic dehydrogenases measured with particles (M_w) from 10 and 100 mg, respectively, fresh weight of oat coleoptiles.

Finally the sediments were diluted with sucrose-phosphate mixture to contain per ml the equivalent of either 100 mg fresh weight (FW) of tissue for the Thunberg dehydrogenase analyses or 1 to 3 gm fresh weight of tissue for the manometric oxidase determinations.

DEHYDROGENASE DETERMINATION: Although now used somewhat less than formerly, the Thunberg method has two advantages: it limits the number of enzymatic reactions under study, and it can yield quantitative results with very small amounts of plant tissue.

⁴ This solution was selected to give optimal oxidase activity, and not less than optimal dehydrogenase activity (see under Thunberg and manometric methods below).

The enzyme was added to Thunberg tubes chilled in cracked ice and evacuated by a mechanical pump with vigorous shaking. The tubes were then equilibrated 5 minutes in a $25^\circ C$ bath before the contents were mixed.

The tubes were constructed with shanks which fitted into a Klett-Summerson Colorimeter; readings of optical density were taken at 2- to 5-minute intervals for 20 minutes. Figure 2 is a plot of such readings against time. This method allows the use of such dilute suspensions that settling of the particles does not occur, yet light scattering is negligible. This direct determination of the linear rate of bleaching is very greatly preferable to the usual estimation by dye decolorization time.

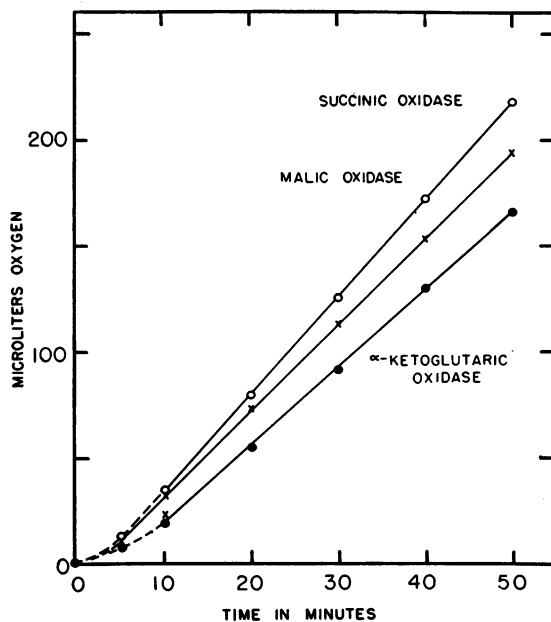


FIG. 3. Time course of oxidation in Warburg vessels. Oxidase activity measured with particles (M_w) from 3 grams fresh weight of pea stem internodes.

While this method determines the rate of hydrogen transfer, directly or via a flavoprotein, to the ultimate acceptor, to convert the rate to terms of oxygen uptake it is necessary only to know the molecular weight of the dye and its redox equivalent. In the case of 2,6-dichlorophenolindophenol, the molecular weight (of the sodium salt) is 290 and the equivalent is 2. Empirically it was found that 1.0 unit of optical density corresponds to the reduction of $0.53 \mu M$ of dye and is, therefore, equivalent to the utilization of $13 \mu l$ of oxygen.

The activities of the enzyme preparations described herein were of the order of $Q_{O_2}^N = 300$ for succinic dehydrogenase and = 1000 or higher for malic dehydrogenase.

The concentrations of components for succinic and malic dehydrogenase preparations are listed in the following protocol:

Component	Final Concentration
Succinate	0.025 M
Phosphate, pH 6.8	0.05 M
Dichlorophenolindophenol, 0.5 ml	0.0001 M
Bovine serum albumin	0.2 mg per ml
Washed particles (M_w)	50 mg FW per tube
Water	to 4.0 ml
Malate	0.025 M
Phosphate, pH 7.8	0.05 M
Coenzyme I	0.001 M
Sodium cyanide	0.01 M
Dichlorophenolindophenol, 0.5 ml	0.0001 M
Bovine serum albumin	0.2 mg per ml
Washed particles (M_w)	10 mg FW per tube
Water	to 4.0 ml

OXIDASE DETERMINATION: Oxygen uptake was measured in the Warburg vessel by conventional methods. The enzyme preparation was added to chilled vessels and equilibrated for 5 to 10 minutes in a 25°C constant temperature bath. Readings were plotted against time (fig 3) for an hour or more, and linear rates of O_2 uptake for 30 minutes were taken as a measure of enzyme activity; these rates were then converted to microliters O_2 uptake per hour. The rates averaged 300 to 600 $\mu l O_2/hr \times mg N$.

The final concentrations of components found optimal for the 3 oxidases are listed in the following protocol:

Component	Final Concentration
α -Ketoglutarate	0.02 M
Phosphate, pH 7.0	0.04 M
Magnesium sulfate	0.001 M
ATP	0.001 M
Sucrose	0.2 M
Bovine serum albumin	3 mg per ml
Washed particles (M_w)	1 to 3 gm FW per vessel
Water	to 1.5 to 3.0 ml
Succinate	0.02 M
Phosphate, pH 7.0	0.01 M
Sucrose	0.2 M
Bovine serum albumin	3 mg per ml
Washed particles (M_w)	1 to 3 gm FW per vessel
Water	to 1.5 to 3.0 ml
Malate	0.02 M
Phosphate, pH 7.15	0.005 M
Coenzyme I	0.001 M
ATP	0.001 M
Magnesium sulfate	0.001 M
Sucrose	0.2 M
Bovine serum albumin	3 mg per ml
Washed particles (M_w)	1 to 3 gm FW per vessel
Water	to 1.5 to 3.0 ml

MATERIALS: Glass-distilled water was used throughout. Alpha-ketoglutaric acid, m.p. 115 to 116°C, with the correct neutralization equivalent and free from succinate, was prepared by a modification of earlier methods (31). Phosphate buffers were made up from KH_2PO_4 and Na_2HPO_4 . Bovine serum albumin was purchased from Armour and Company. Cytochrome c, 65% coenzyme I (corrected to pure Co I through-

out), adenosine triphosphate, and Sequestrene (a thrice-recrystallized preparation of the disodium salt of ethylenediaminetetraacetic acid) were commercial samples.

Alpha-keto acids were determined by the method of Friedemann and Haugen (12). Succinate was determined with the ox heart succinoxidase assay according to Umbreit et al (43).

The following abbreviations will be used: ATP (adenosine triphosphate), BSA (bovine serum albumin), DIP (2,6-dichlorophenolindophenol), MB (methylene blue), and FW (fresh weight of original tissue).

EXPERIMENTAL RESULTS

THUNBERG METHOD: Succinic and malic dehydrogenases could be readily demonstrated by the method outlined, but no dehydrogenase could be detected for

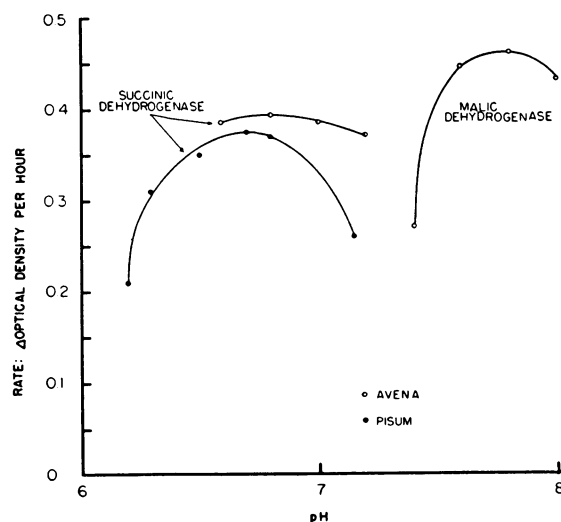


FIG. 4. Effect of pH on succinic and malic dehydrogenase activity.

pure α -ketoglutaric acid. Furthermore, the dye was not reduced by plant particles under the conditions described for the α -ketoglutaric dehydrogenase of pig heart (33). Commercial preparations of α -ketoglutaric acid contain appreciable amounts of succinate (31) which rapidly reduce the dye, and in addition some samples of the acid contain an orange impurity which decolorizes DIP non-enzymatically.

Glass-distilled water was found to be the best extracting medium. The addition of sucrose beyond 0.4 M, or phosphate beyond 0.05 M, led to decreased activity; inert protein (100 mg/l BSA) produced no effect. In 0.01 M phosphate, of pH 5.5 to 7.5, identical succinic dehydrogenase activities were recovered; at pH 5 and pH 8 the recovery was depressed.

When particles obtained by fractional centrifugation (M_w of figure 1) were maintained at 3° in the following solutions for 60 minutes, no loss could be detected: phosphate up to 0.05 M, sucrose up to 0.6 M, and BSA at 100 mg/l. This shows that the

60 minutes required for preparation entails no loss in activity (36).

The pH optimum of succinic dehydrogenase was found to be 6.8 (fig 4) which agrees with that of the enzyme from potato tuber (24). The pH optimum was found to be essentially independent of the dye concentration.

The pH optimum for malic dehydrogenase is 7.8 (fig 4) which falls on a plateau previously reported for this enzyme by Berger and Avery (3), although the pH-dependence appears to be sharper than was reported by these workers.

The absence of succinic dehydrogenase in the *Avena* coleoptile (3) was probably due to the choice of redox dye, as mentioned earlier (32). The oat and pea enzymes not only fail to decolorize methylene blue and thionin, but are strongly inhibited by one of them (MB) as shown in table I.

Malic dehydrogenase reduces methylene blue, as Berger and Avery (3) also found, but oxidation is much more rapid with the indophenol dye (fig 5a). Even DIP is toxic at moderate concentrations; at 10^{-3} M dilution there is no enzyme activity, but at 10^{-4} M dilution the rate of reduction is not critically dependent on DIP concentration.

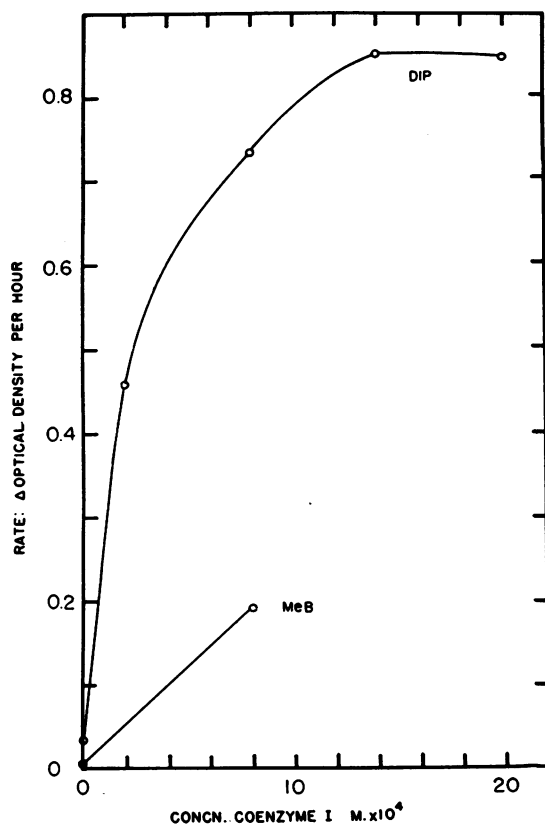


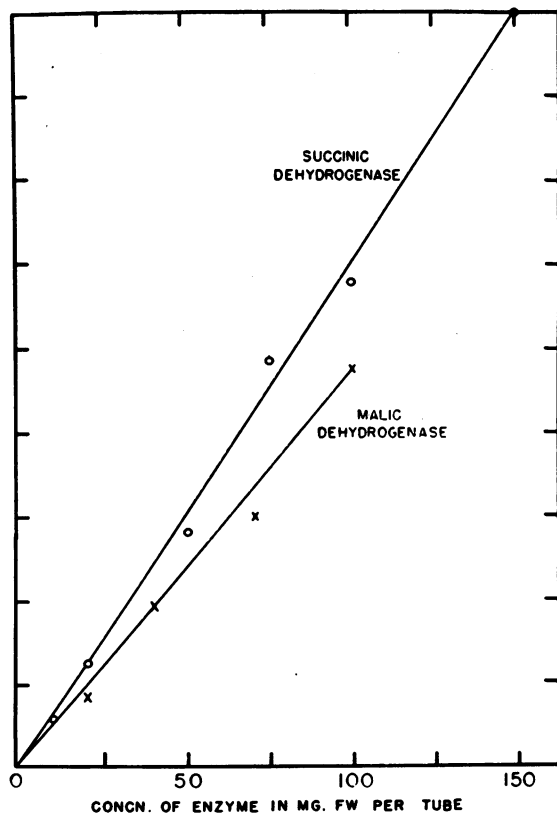
FIG. 5a. Saturation of malic dehydrogenase with coenzyme I. With DIP as redox dye, coenzyme I is non-limiting at 1.4×10^{-5} M. With MB as dye the reaction is strongly inhibited.

FIG. 5b. Proportionality between enzyme velocity and enzyme concentration. In practice the Thunberg method is found to be quantitative over a 20-fold range of activity.

TABLE I
EFFECT OF METHYLENE BLUE (MB) AND DICHLOROPHENOLINDOPHENOL (DIP) ON DEHYDROGENASE ACTIVITY. ENZYME ACTIVITY (V) EXPRESSED AS DECREASE IN OPTICAL DENSITY PER HOUR

SUBSTRATE	MB	DIP	V	% DIP CONTROL
	<i>Molar</i>	<i>Molar</i>		
Succinate	0	14^{-4}	0.200	..
"	2×10^{-5}	10^{-4}	0.192	96
"	1×10^{-5}	10^{-4}	0.180	90
"	5×10^{-5}	10^{-4}	0.122	61
"	1×10^{-4}	0	0	0
Malate	1×10^{-4}	0	0.192	..
"	0	10^{-4}	0.734	..
α -Ketoglutarate	1×10^{-4}	0	0	..
"	0	10^{-4}	0	..

The Lineweaver and Burk (22) double reciprocal plot of dehydrogenase activity against substrate concentration departs slightly from the linear with succinic dehydrogenase (fig 6). Depending on the substrate concentration, the calculated K_s varies from



0.00023 M to 0.00059 M, subject to the reservations pointed out by Slater and Bonner (37). These K_s values indicate a greater enzyme-succinate affinity than any previously reported, save that for *Neurospora* (36). The enzyme from potato tuber (24) has a K_s of 0.019 M.

Malic dehydrogenase, as already shown (3), manifests an almost absolute requirement for coenzyme I (fig 5a); the enzyme is saturated at somewhat more than 10^{-3} M. Such data as can be found (35) suggest that this value is a little higher than for other coenzyme-requiring dehydrogenases.

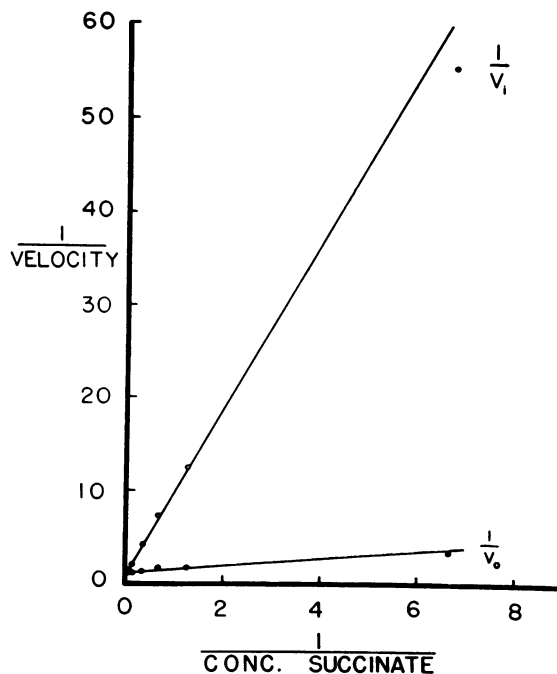


FIG. 6. Competitive inhibition of succinic dehydrogenase by malonate. Lower curve, succinate alone; upper curve, in presence of 5×10^{-4} M malonate. From this and similar Lineweaver-Burk plots of enzyme velocity as a function of substrate concentration, the K_s for succinic dehydrogenase of oat coleoptiles is found to range from 0.00023 M to 0.00059 M. The K_i for malonate is 0.0019 M.

With respect to phosphate concentration in the reaction mixture, the rates of succinic and malic dehydrogenases are unaffected from 0.01 to 0.1 M. At lower concentrations malic dehydrogenase activity falls rapidly, an effect which may be due to impaired buffering, since the substrate offers no effective buffering at pH 7.8. Succinic dehydrogenase remains constant at phosphate concentrations as low as 0.001 M, as has been reported by several workers for the corresponding enzyme from animal tissues (7). It must be borne in mind that mitochondria contain phosphate (15).

Similarly, no benefit is derived from the addition of sucrose to the reaction medium. In fact, malic

dehydrogenase appears to be inhibited by sucrose above 0.4 M, but this might be due to the increased viscosity affecting the efficiency of evacuation.

The lack of any real effect of these 2 non-specific agents, namely phosphate and increased osmotic pressure, on the dehydrogenase activity of plant particles constitutes an important difference between the requirements of enzymatic dehydrogenation when measured directly and when coupled through the cytochrome system to O_2 (see below).

As noted above, it was found unnecessary to add protective agents to maintain full activity during short-term storage at 3°C. During the reaction in the Thunberg tube, however, the particles are diluted and raised to 25°C. This may be the reason for an observation, confirmed many times, that the dehydrogenase activity of washed particles is low unless stabilized by one of a number of non-specific agents incorporated into the reaction mixture: gelatin, ovalbumin, BSA,

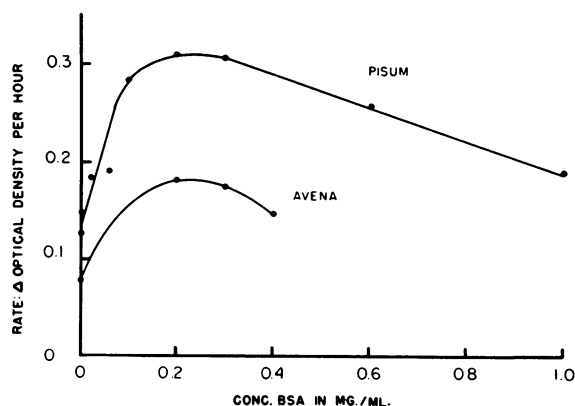


FIG. 7. Effect of protein on succinic dehydrogenase activity. Bovine serum albumin can be replaced by a number of proteins and, to some extent, by Sequestrene.

a globulin prepared from germinating pea endosperm, asparagine, or the chelating agent, Sequestrene. The latter duplicates the effect of BSA on pea stem particles at a much lower concentration (0.03 mg/ml sequestrene \sim 0.3 mg/ml BSA), but is less effective on the more sensitive particles from oat coleoptiles.

BSA, since it is commercially available in a crystalline form, was selected as a standard. Figure 7 shows a plot of activity as a function of protein concentration; 0.2 mg/ml appears optimal. The decrease in rate above the optimum is due to foaming during evacuation, which decreases the efficiency of air removal.

ACCURACY AND SPECIFICITY: One of the questions to be considered in assessing the accuracy of an enzyme determination is: to what extent has the enzyme deteriorated prior to or during the time required for estimation? The answer is that, excluding changes caused by cell disruption, there is no deterioration of these two dehydrogenases between maceration and reaction, and their activity is unaffected by considerable variation in the extracting medium.

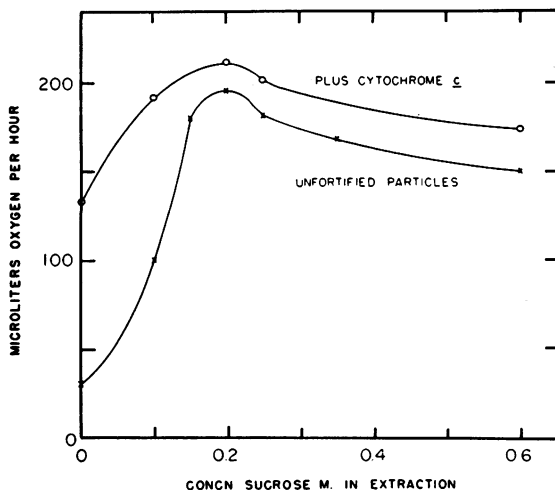


FIG. 8. Effect of sucrose in the extraction medium on the final succinic oxidase activity and its response to cytochrome c. Final reaction mixtures made up to identical sucrose concentrations. Final concentration of cytochrome c: 10^{-4} M.

A valid method must measure activity as proportional to enzyme concentration. This is found to be the case over a wide range with succinic and malic dehydrogenases (fig 5b).

Since the identical particles were used for the study of these two dehydrogenase activities, it is conceivable that each substrate molecule is oxidized more than one step; so that, e.g., the rate of dye decolorization with succinate as substrate was due not only to the dehydrogenation of succinate but of malate as well. However, succinic dehydrogenase was found insensitive to coenzyme I up to a concentration of 2×10^{-4} M, while malic dehydrogenase requires it; hence, with this factor withheld the subsequent steps are prevented. With malic dehydrogenase the subsequent steps are similarly prevented in the usual way by introducing 0.01 M HCN to trap the reaction product.

In the past the effect of malonate has been used for the specific identification of succinic dehydrogenase. Thus, concentrations up to 0.02 M have no effect on malic dehydrogenase; in one experiment the velocity was 0.474 in the absence of malonate and 0.476 in 0.02 M malonate. These concentrations strongly inhibit succinic dehydrogenase (fig 6). The K_I for malonate inhibition of succinate dehydrogenase appears to be 0.0019 M, which represents an affinity some 0.1 that of the substrate for the enzyme. This is remarkable even allowing the interpretation for varying values of K_M/K_I offered by Thorn (40).

MANOMETRIC METHODS: Extraction: Unlike the Thunberg tube method, the respirometer requires very specific extraction solutions; in particular, the sucrose concentration is extremely critical. Figure 8 shows that only about 15% of the optimum oxidase activity was recovered when the tissue was ground in distilled water. The optimum sucrose concentration is about

0.2 M. The lower oxidase activity on either side of 0.2 M appears to be partly related to the availability of endogenous cytochrome c, since the addition of cytochrome c to the reaction mixture at a concentration of 10^{-4} M leads to more nearly uniform rates (fig 8).

The presence of phosphate during extraction exerts a beneficial effect on all 3 oxidase activities. A broad optimum from 0.02 to 0.05 M was found (fig 9).

The addition of protein or Sequestrene to the extracting medium is of little consequence, since abundant soluble pea proteins are present in the homogenate. During further purification, however, a definite effect was shown by these agents. In one experiment succinic oxidase activity was determined after 2 washings of the particles. When BSA at 3 mg/ml or Sequestrene at 1 mg/ml was added to the washing mixture, the succinic oxidase activity was 20% and 9%, respectively, greater than the control.

Reaction: There is a further effect of added protein on the linearity of reaction rate (fig 10). A constant rate of O_2 uptake for up to 90 minutes has been recorded in the presence of protein; Sequestrene did not substitute satisfactorily.

The optimal concentration of sucrose for the reaction itself was found to be identical with that for the extraction solution: 0.2 M.

It is not clear whether phosphate is required for O_2 uptake. Although no attempt was made to free the preparation rigorously from phosphate, an absolute requirement with succinic and malic oxidases could not be detected. However, added phosphate does increase the rate (7).

The pH optima are close to that of 7.15 reported

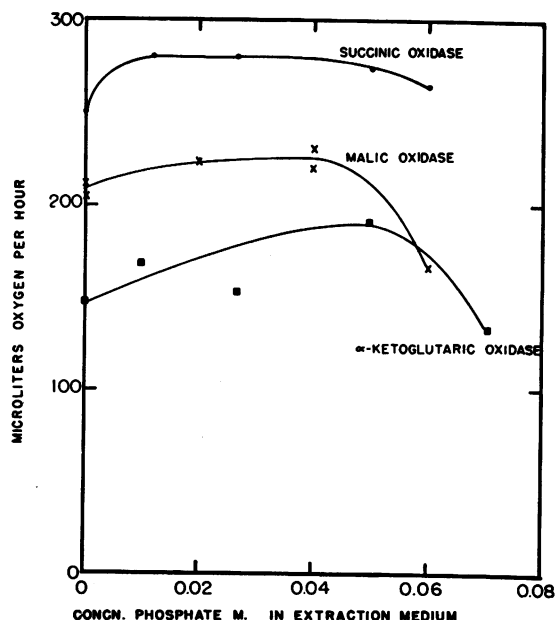


FIG. 9. Effect of phosphate in the extraction medium on the final oxidase activity. With each substrate, concentrations of phosphate in reaction mixture identical.

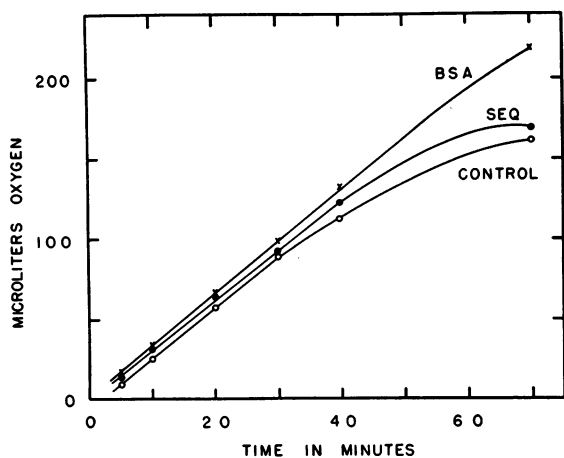


FIG. 10. Effect of protein and Sequestrene on linearity of succinic oxidase activity with time. Thrice-washed particles centrifuged without added protein. BSA or Sequestrene, both 1 mg/ml, added in reaction mixture.

for potato cytochrome oxidase (24), although slight differences are seen amongst the three substrates (fig 11a). The optimum for malic oxidase evidently differs considerably from that for malic dehydrogenase (fig 4).

The phosphate optima for the action of the three oxidases vary from 0.005 M with malic oxidase to 0.04 M with α -ketoglutaric oxidase (fig 11b). In 5 of 6 experiments with succinic oxidase there was a definite phosphate optimum at 0.01 M. This is not

simply an osmotic effect since the same value was obtained at several different sucrose and substrate concentrations.

Maximal rates of O_2 uptake were uniformly obtained at about 0.02 M substrate for the 3 oxidases.

Coenzyme I stimulates both α -ketoglutaric oxidase and malic oxidase (table II). With α -ketoglutaric oxidase this promotion is duplicated by ATP. With malic oxidase, in the presence of added coenzyme I, ATP does affect the initial rate of O_2 uptake slightly (table II). The absence of stimulation by ATP alone disagrees with the conclusions of Laties (20). However, added ATP does maintain the linearity of the reaction; in its absence the rate falls off rapidly after 40 minutes, whereas with 3×10^{-3} M ATP, it remains constant for at least 75 minutes. These and other experiments lend themselves to the interpretation that ATP exerts a sparing action on endogenous and added coenzyme I.

Magnesium ion is very clearly a cofactor for α -ketoglutaric oxidase (table III). At 10^{-3} M both O_2 uptake and the disappearance of the substrate are doubled. Malic oxidase is also promoted by magnesium, but to a lesser extent.

The effect of these cofactors on succinic oxidase will be treated in the following section.

Accuracy and specificity: The accuracy of a manometric determination may be judged from the same criteria as were listed for the dehydrogenase-dye system. The linearity of reaction rate has been shown in figure 3. The particles alone do not show exact proportionality between activity and enzyme concentration, but if BSA is added the proportionality becomes clearly linear over a moderate range (fig 12).

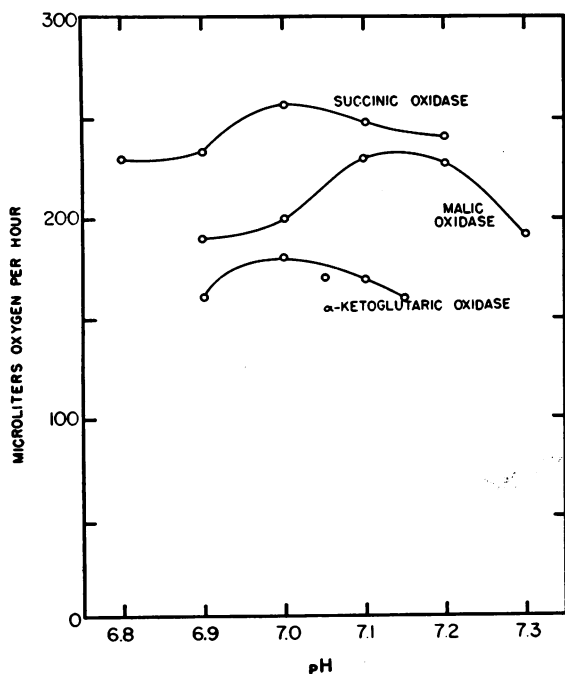


FIG. 11a. Effect of pH on oxidase activity.

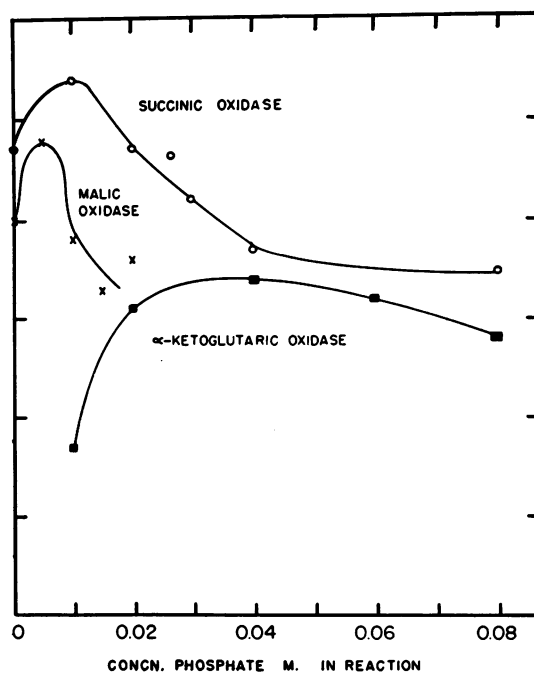


FIG. 11b. Effect of phosphate in reaction mixture on oxidase activity. All substrates 0.02 M.

TABLE II

EFFECT OF COENZYME I AND ATP ON RATES OF OXYGEN UPTAKE WITH MALATE AND KETOGLUTARATE. ENZYME RATES CALCULATED OVER LINEAR PORTION OF TIME CURVE

SUBSTRATE	CONC.	CONC.	- $\mu\text{L O}_2$ ¹	STIMU- LATION
	COENZYME I	ATP		
	M	M	per hr	%
Malate	0	0	108	...
"	0.0001	0	155	43
"	0.0004	0	183	69
"	0.001	0	227	110
Malate	0	0	124	...
"	0	0.001	105	-15
"	0	0.003	86	-33
"	0.001	0	141.5	...
"	0.001	0.0003	189	34
"	0.001	0.001	173	22
"	0.001	0.003	169	19
α -Ketoglutarate	0	0	76	...
"	0.0001	0	128.5	69
"	0.0004	0	141.5	86
"	0.001	0	161	112
α -Ketoglutarate	0	0	76	...
"	0.0004	0.0003	151	99
"	0	0.001	153	101
"	0	0.003	160	111
"	0.0004	0.001	157.5	107

Because the oxidation of any one substrate may proceed by concatenary reactions through the Krebs cycle, the rate of O_2 uptake measured with a complete system like the particles studied here may not be a measure of any single enzyme reaction. On this account the specificity of the enzymes can only be demonstrated by direct analysis for the disappearance of substrate or formation of the product.

With α -ketoglutarate the succinic acid produced is generally oxidized further by succinic oxidase. The second step can be prevented by addition of malonate

TABLE III

EFFECT OF MAGNESIUM ON OXYGEN UPTAKE AND ON THE FORMATION OR DISAPPEARANCE OF KETO ACIDS

SUBSTRATE	CONC.	- $\mu\text{L O}_2$	STIMU- LATION	$\Delta \mu\text{L}$ α -KETO- ACID	STIMU- LATION
	Mg^{++}				
	M	per hr	%		%
α -Ketoglu- tarate	0	101	..	- 45	...
"	0.0002	200	99	- 96	113
"	0.0005	189	88	- 110	145
"	0.0015	181	79	- 107	140
Malate	0	143	..	+ 27	...
"	0.0005	174	21
"	0.001	182	27	+ 34 *	26
"	0.004	165	15
Malate	0	166
"	0.001	220	32

* α -Keto acid identified, by absorption spectrum of dinitrophenylhydrazon, as oxalacetate or pyruvate.

(27), but it has been found with the present preparations that any concentration of malonate sufficient to repress the second step results in a considerable inhibition of α -ketoglutarate oxidation. The minimum concentration of malonate needed to reduce the O_2 consumption to the theoretical value, i.e., 0.5 mole of O_2 per mole of α -ketoglutarate consumed, is 0.003 M. At this concentration, however, α -ketoglutarate disappearance is reduced by 25 % (31). Over a large number of experiments it has been found that in the absence of malonate the rate of substrate disappearance with this enzyme corresponds to 0.4 to 0.6 times the rate of oxygen uptake. This ratio is essentially independent of Mg^{++} or ATP concentrations. It indicates that in the normal course of events α -ketoglutarate is oxidized smoothly to the stage of fumarate. The relative constancy of this ratio means that O_2 uptake

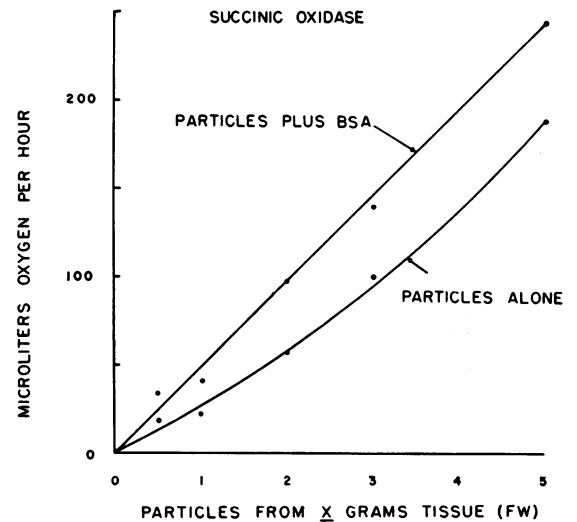


FIG. 12. Proportionality of succinic oxidase activity and enzyme concentration. Linear relation found only when protein added; effect exaggerated in this case with thrice-washed particles.

measurements can be used to give a rough estimate of the actual enzyme rate.

Succinic dehydrogenase is perhaps the one dehydrogenase that has never been shown to require a coenzyme or cofactor. Data with the Thunberg method (above) confirm this conclusion. It is surprising, then, to find reports (20, 25) that the succinic oxidase of plant particles responds strongly to ATP and Mg^{++} . These findings, however, were confirmed in our own experience. With pea stem particles the effect of ATP alone is slight and variable. In most cases the stimulation by either ATP or Mg^{++} alone was of the order of 10 %, but together they produced a promotion of more than 30 % (table IV). Larger promotions were obtained from tissue that could be regarded as suboptimal, such as after storage in the cold room. By employing such tissue, it was possible to analyze directly for the site of stimulation. The data of table V, obtained from plants stored 24 hours

TABLE IV
EFFECT OF ATP AND Mg^{++} ON OXYGEN UPTAKE WITH
SUCCINIC OXIDASE

CONC. Mg^{++}	CONC. ATP	$-\Delta \mu L O_2$	STIMULATION
<i>M</i>	<i>M</i>	<i>per hr</i>	<i>%</i>
0	0	204	..
0	0.001	206	1
0	0	246	..
0	0.001	272	10
0	0	250	..
0.0015	0	272	9
0	0.001	282	13
0.0015	0.001	342	37

at 3°C, indicates that ATP promotes only the oxidation of malate and further intermediates, and does not affect succinic oxidation per se. On the other hand, Mg^{++} clearly affects the oxidation of succinate itself. With ATP and Mg^{++} together succinate disappearance is not further increased, but O_2 consumption is, and a considerable pool of oxalacetate is formed. From the standpoint of succinic oxidase estimation, it is sufficient to observe that in the absence of cofactors, disappearance of succinate accounts for nearly 90% of the observed O_2 uptake.

Other experiments bear out the conclusion of table V, that the particles oxidize succinate to fumarate very much more rapidly than to subsequent stages. The R.Q. of succinate oxidation over a 1-hour period was found to be 0.23, which is in accord with a small contribution coming from secondary reactions. That of malate over the same period, on the other hand, was 1.08. In addition, the accumulation of α -keto acid shown in table III is restricted to less than one quarter of the observed O_2 uptake. Both these findings therefore indicate that malate is subject to several secondary oxidations.

It may be said in conclusion that the Thunberg method allows quantitative estimations of succinic and malic dehydrogenases while the manometric method yields less precise, but nonetheless practical assays for succinic and α -ketoglutaric oxidases in these particles.

INTRACELLULAR LOCALIZATION: All of the studies

TABLE V
EFFECT OF ATP AND Mg^{++} ON OXYGEN UPTAKE AND
SUCCINATE DISAPPEARANCE WITH PARTICLES FROM PEA
STEMS STORED 24 HOURS AT 3°C

CONC. ATP	CONC. Mg^{++}	$-\Delta \mu L O_2$	STIMULATION	$-\Delta \mu L$ SUCCINATE	STIMULATION	$\Delta \mu L$ OXALACETATE
<i>M</i>	<i>M</i>	<i>per hr</i>	<i>%</i>	<i>per hr</i>	<i>%</i>	<i>per hr</i>
0	0	212	..	188	..	0
0	0	208	..	184	..	0
0.001	0	296	40	186	..	2
0	0.001	333	58	252	35	0
0.001	0.001	380	81	256	38	45

TABLE VI

INTRACELLULAR FRACTIONATION OF SUCCINIC AND MALIC DEHYDROGENASES. CENTRIFUGATION PROCEDURE ACCORDING TO FIGURE 1. ENZYME ACTIVITY EXPRESSED AS CHANGE IN OPTICAL DENSITY PER HOUR PER GRAM FRESH WEIGHT OF TISSUE

FRACTION	SUCCINIC DEHYDROGENASE		MALIC DEHYDROGENASE	
	v/GM	% OF TOTAL ACTIVITY OF HOMOGENATE	v/GM	% OF TOTAL ACTIVITY OF HOMOGENATE
Homogenate	5.24	100	55.2	100
P_w	0.32	6	3.9	7
S_2	0.56	10.5	41.8	76
M_w	3.88	74	12.5	23
Total	...	90.5%	...	106%

described thus far were conducted on the particulate fraction, M_w , corresponding to the mitochondria of animal tissue. Many dehydrogenases, however, are known to be present in other centrifugal fractions.

In table VI the activities of succinic and malic dehydrogenases in the pea stem fractions are compiled. It is seen that succinic dehydrogenase is almost quantitatively associated with the mitochondria, a finding in agreement with the studies on liver tissue (17). Malic dehydrogenase is present both in particles and in solution. In neither case was there any significant activity in the "plastid" fraction.

The inherent difficulties in studying intracellular localization with the intact cytochrome system are compounded in the case of plant enzymes by their considerable dilution with the vacuolar sap. Nonetheless, a fractionation of succinic oxidase followed manometrically results in complete agreement with the dehydrogenase analysis (table VII).

DISCUSSION

THE ENZYME ASSAY: The modified Thunberg method employed here has many of the qualifications needed for quantitative assay: high sensitivity, specificity, continuous readings, proportionality with time and enzyme concentration, and in the case of malic dehydrogenase, practicality of saturation with coenzyme. The choice of 2,6-dichlorophenolindophenol as a redox dye is essential for plant succinic dehydrogen-

TABLE VII

INTRACELLULAR FRACTIONATION OF SUCCINIC OXIDASE. RATES (v) CORRECTED FOR BLANKS AND EXPRESSED IN $\mu L O_2$ PER HOUR PER GRAM FRESH WEIGHT OF TISSUE

FRACTION	v	% OF TOTAL ACTIVITY OF HOMOGENATE
Homogenate	97.5	100
P_w	1.2	1.2
M_w	103	105
M_w plus S_2	79	81
M_w plus P_w	102.5	105

ase and is advantageous for malic dehydrogenase. Our experience suggests that the procedures outlined will prove suitable for these enzymes from most plants with only slight alterations.

The neotetrazolium method has been adapted (13) to a demonstration of succinic dehydrogenase in the oat seedling, but the enzyme rate with this oxidant, as calculated from the published data, is no more than 3% of that with the indophenol dye in the Thunberg method.

The manometric method is not as easy to develop into a quantitative estimation. This is partly due to the properties of the particles. Thus, in Stafford's investigations (38) succinic oxidase, obtained by the centrifugation of homogenates made in water or 0.1 M phosphate, was weak and unstable, the rate decreasing rapidly after 30 minutes of reaction. Less than 60% of the activity of the original homogenate was recovered in unwashed particles.

The requirement of non-specific protein to maintain stability is not without precedent. Although in the present case no relation was found between phosphate and protein requirements, the suggestions of Keilin and Hartree (18) as to alteration in colloidal properties is clearly applicable. More recently, Sanadi and Littlefield (34) found that BSA promoted the activity of the most purified preparations of liver α -ketoglutaric dehydrogenase.

Another difficulty with the manometric determinations is imposed by the different osmotic and salt requirements of the particles from different plants. The sucrose concentration optimal for pea stem particles (0.2 M) compares closely to the optimum for liver particles (16), but with cauliflower 0.5 M sucrose is necessary (21) and with mung bean (25) the optimum differs between extraction and reaction. The absolute rates of oxidation by mung bean particles suggest that considerable inactivation may have proceeded even under the best conditions described. Although our measurements were conducted at 25°C rather than 30°C, the pea stem particles show Q_{O_2} per mg nitrogen from 300 to 600, or over twice those of the mung bean enzymes for all three substrates.

When a preparation is made under conditions leading to maximum oxidase activity, its activity in the Thunberg tube is not lower than maximal. Hence a direct comparison can be made using the factor of 13 μ l O_2 per unit of optical density. Thus in one experiment, the $Q_{O_2}^N$ for succinate was 395 manometrically and 294 in the Thunberg tube. This agreement is better than that obtained in animal preparations using methylene blue as carrier (37).

Alpha-ketoglutaric oxidase must be estimated under a further limitation, specificity. Approximately 2 atoms of O_2 are absorbed per molecule of keto-acid disappearing even at the beginning. This corresponds to a conversion of α -ketoglutarate to an acid of the oxidation level of fumarate. In the presence of malonate, only 1 atom of O_2 is taken up per molecule of substrate, and the reaction presumably stops at succinate (27). As pointed out above and discussed in detail elsewhere (31), however, it is not possible to

suppress the second oxidation without also inhibiting the first. Hence, the assay cannot be conducted with a high degree of accuracy manometrically.

This curious phenomenon of α -ketoglutarate oxidation linked to succinate oxidation is not simply due to the presence of the 2 enzymes on the same particles. With succinate as substrate malate does not begin to be oxidized appreciably until after nearly all of the succinate has disappeared. It would appear, therefore, that the carbon skeleton of α -ketoglutarate remains attached to the particle until after the second oxidation has occurred. Plaut and Plaut (30) have reported similar, but more striking behavior by heart mitochondria; pyruvate is oxidized vigorously via citrate, but citrate itself is not attacked. They reason that the pyruvate condensation and subsequent reactions occur in the mitochondria, but without release of the carbon skeleton until a higher oxidation level is reached.

The above phenomena indicate that manometric assays of complex enzyme systems cannot be interpreted without analysis of the reaction products. There remains, further, the probability that different combinations of linked oxidations may occur with particles from varied sources.

PHYSICAL NATURE OF THE PARTICLES: Some of the results reported here bear on the nature and identity of the enzymatic particles. On the basis of staining with Janus Green, and size, Millerd et al (26) identified as mitochondria the fraction centrifuged at 10,000 \times g from mung bean hypocotyls. Stafford (38), on the other hand, in a more intensive study of several fractions from germinating pea seedlings, regarded the morphological identification of the particles as unsatisfactory. She writes, "There appears to be a complete series of particles . . . ranging from plastid sizes of 6 to 7 microns down to 0.1 micron or even below." The largest particles are included in her series because a considerable fraction of the cytochrome oxidase and succinoxidase was found therein. This may perhaps have been due to some agglomeration of mitochondria brought about by carrying out the fractionation in water alone. In our experiments (table VII), the entire succinic oxidase activity was recovered in M_w , a fraction which so far as could be seen under the phase microscope does not include units of plastid size and whose homogeneity is directly comparable to liver mitochondria.

Thus, regardless of what future investigations may reveal regarding the identity of the enzymatic particles, the operational analogy between plant particles and animal mitochondria seems clear.

Lastly, some of the above data bear on the statement (25) that the rates of O_2 uptake by particles *in vitro* are sufficient to account for the respiration of the intact tissue. The actual rate of O_2 uptake with Millerd's best particulate preparation was 45 μ l O_2 per hour per gram fresh weight of tissue. The respiration of this tissue when intact was 150 μ l per hour. Two factors were employed to equate these values. Firstly, it was stated that only 50% of the cells appeared to be ruptured during grinding. With our

material, we find, however, a second grinding and extraction of the tissues produces only a small increase in yield. Also when coleoptile tissue had been extracted 3 times, the residue was found to be devoid of succinic dehydrogenase. Secondly, it is claimed that approximately half the mitochondria are lost during the centrifugation process. Table VII shows, however, that with our procedure, which differs from Miller's only in washing of the plastid fraction, the succinic oxidase activity is quantitatively recovered in M_w . It is believed therefore that the employment of these factors is quite unjustified. This question clearly deserves more detailed consideration, which will be given it elsewhere.

SUMMARY

This paper is concerned with the optimum conditions for the extraction of succinic and malic dehydrogenases as well as of succinic, malic, and α -ketoglutaric oxidases, and with the quantitative assay of these enzymes in plant tissue.

Particles containing the enzymes were prepared by fractional centrifugation from *Avena* coleoptiles and from the apical internodes of etiolated *Pisum* seedlings.

For the dehydrogenases (determined by dye reduction), water was the best extracting medium, but enzyme activity was increased in the presence of a protein preparation, or asparagine, or, to some extent, Sequestrene, during the dehydrogenation; crystalline bovine serum albumin was found very effective. Malic dehydrogenase requires coenzyme I, but succinic dehydrogenase does not; neither enzyme responds to the addition of sucrose or phosphate. As hydrogen acceptor, 2,6-dichlorophenolindophenol was much superior to methylene blue, which is toxic. Succinic dehydrogenase is present only in the particles, but malic dehydrogenase is present also in soluble form, the soluble enzyme accounting for three quarters of the activity of the homogenate.

For the oxidases (determined by O_2 uptake), sucrose (0.2 M) with phosphate (0.02 to 0.05 M) is the optimum extraction medium, and the addition of protein increases activity only of purified preparations. The influence of sucrose is probably exerted on the availability of cytochrome *c* in the particles. The activity of all 3 enzymes is promoted by magnesium. Details of pH and phosphate optima for the oxidase reactions are given.

Malic and α -ketoglutaric oxidases require coenzyme I; in the latter case, ATP can be substituted, but with malic oxidase ATP has only a slight effect, which is ascribed to its sparing action on coenzyme I. With succinic oxidase an increased O_2 uptake in presence of ATP is shown to be due to the promotion of oxidation processes beyond the stage of fumarate. In the absence of cofactors the disappearance of succinate accounts for nearly 90% of the observed O_2 uptake.

The limitations to the quantitative recovery of malic and α -ketoglutaric oxidases are discussed, and it is shown that malonate cannot be used to isolate the latter. The dehydrogenases and succinic oxidase

are recovered essentially quantitatively by the methods used. Problems involved in comparing the enzyme contents with the respiration of the intact tissue are discussed.

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SEED VITALITY AND OTHER COTTON CHARACTERS AS AFFECTED BY THE AGE OF SEED ¹

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The longevity of a large number of crop or weed seeds has been studied by several workers. Darlington (10, 11) and Toole and Brown (26) reported the results of two experiments, one of which was started by Beal in 1879, and the other by Duvel in 1902: some seed, buried in the soil, kept their vitality for 70 or 60 years respectively. According to other evidence (1, 5), two kinds of seed survived after 149 or 158 years. The life span of seeds has been reviewed by Crocker (9).

In the case of crop plants, seed vitality as affected by age (or various storage conditions) was investigated by Sifton (18), Sonavne (23, 24), Stevens (25), Kiesselbach (13), Robertson and Lute (15), Robertson et al (16, 17), Barton and Garman (4), and others. Alfalfa seed may be viable after 20 years, and

the same applies to oats or barley. Wheat also was viable for 15 to 20 years, whereas rye, maize, soybean, timothy, etc. are likely to lose their germinative value within much shorter intervals of time. The life span of crop seeds is greatly affected by the amount of moisture during the period of storage, no seed surviving long when the atmospheric moisture approaches saturation. There is certainly no truth in the belief that in Egypt, wheat seeds have survived from the times of the Pharaohs (14).

Regarding cotton, there is evidence that seeds may still be viable (at the rate of 6%), after being in storage for 25 years under dry conditions; under humid conditions (up to 11% moisture in the seed), they deteriorated rapidly after one to five years according to conditions (19, 20, 21). In the case of higher amounts of moisture, only a few months, weeks, or even days are sufficient for the loss of via-

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