DEHYDROGENASE ACTIVITY AND RESPIRATION: A QUANTITATIVE COMPARISON\textsuperscript{1,2}

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The evidence that the tricarboxylic acid cycle of Krebs plays an important part in plant metabolism is now very extensive (see the review of Burris (8)). It appears that all the necessary enzymes are present, at least in those few tissues which have been examined (12, 20, 21, 23, 25), and the oxidation of organic acids by in vitro preparations appears to proceed by way of the cycle (7, 12). However, the evidence as to whether the cycle functions as the main route of respiration in vivo is less convincing. In yeast, Krebs, Gurin and Eggleston (18) have shown that the cycle "cannot be the main mechanism of oxidation," but is rather a path for the synthesis of intermediary metabolites. Roberts et al (26) have recently come to a similar conclusion for Escherichia coli.

As to the in vivo role of the cycle in plants the evidence is still more incomplete, though there are several observations which are hard to reconcile with any central role for the cycle in the respiratory system. One of these is the relative insensitivity of respiration to malonate, found, for example, in freshly harvested oat coleoptiles (1, 4) and particularly in carrot roots (15). It is still more marked in Euglena; for Eichenberger, in unpublished experiments in this laboratory, has found that malonate does not inhibit the endogenous respiration even at pH 3.5, where it would be largely in the undissociated malonic acid form. Nevertheless, the oxidation of added succinate was powerfully inhibited by malonate under exactly the same conditions. Another difficulty is the failure of labeled organic acids to come into equilibrium with all of the cycle intermediates in tobacco leaves (29).

It seems therefore desirable to obtain further information as to the quantitative importance of the cycle in plant respiration.

If the activity of an enzyme in vitro is in excess of the rate of respiration of the tissue from which it was extracted, it may be concluded (with reservations) that this enzyme is non-limiting in respiration. The main reservation is that the conditions for the activity of the enzyme in vivo are no less favorable than those in vitro. Considering the degree of organization of the cell contents, this reservation does not seem to be an overriding objection to the formation of positive conclusions. However, if it is found that the enzyme activity in vitro is relatively less than the rate of respiration, the reverse conclusion would be open to serious doubts.

In comparing enzyme activities with rates of respiration, there arises immediately the problem of matching the conditions in the cytoplasm. With present knowledge it is obviously impossible to evaluate with precision even a single variable such as cytoplasmic (or mitochondrial) pH. However, a reasonable starting point is to compare respiration rate with the optimal rate of action of the extracted enzyme, invoking known or reasonable factors when available. For instance, the concentrations of coenzyme I and ATP where required in the experiments reported below are adjusted to $10^{-8}$ M. These may seem high, but the extraordinary activity of cofactors in intact mitochondria (16) and the upward revision of the estimates of coenzyme I and II concentrations in plants (2) lend support to this procedure. The role of other factors in establishing optimal rates was discussed in the previous paper (25).

While a comparison between respiration and the activities of individual enzymes of the cycle cannot be truly quantitative, moderately accurate assays together with the determination of optimal conditions for the enzymes involved are clearly needed. At least in etiolated tissues, these are available for alpha-ketoglutaric, succinic, and malic dehydrogenases (25).

As a basis for comparison, a measure is needed of the maximum level of respiration of the intact tissue (at the same temperature). Fortunately there have been many studies of the optimal conditions for plant respiration, so that the maximum rate of respiration of a tissue can be determined with fair certainty.

Having arrived at a figure for the rate of respiration, the next question is: how great must the activity of the cycle enzymes be in order to allow for their mediating the entire respiration? Since the cycle is composed of five dehydrogenations, it follows that in a respiratory system proceeding by it exclusively, each dehydrogenation would account for one fifth of the total oxygen uptake. However, one "turn" of the cycle results in the oxidation of one molecule of pyruvate,
with an RQ of 1.2, while the tissues here studied have a lower overall RQ; values as low as 0.8 were reported for the oat coleoptile (1), while pea stem internodes in auxin average 0.84 (10). Evidently, therefore, at least one other dehydrogenation outside the cycle must contribute to the respiration, and presumably this comprises the step between triose and pyruvate. Hence each dehydrogenation in the cycle only needs to proceed at one sixth of the total rate of oxygen consumption. In the comparisons between dehydrogenase activity and respiration which follow, the theoretical rate of substrate oxidation will therefore be considered as one sixth the oxygen uptake of the intact tissue.

**METHODS AND MATERIALS**

The enzyme assays, employing both the Thunberg and the manometric techniques, were described in detail in a preceding communication (25). Unless otherwise stated, conditions were as given therein. The substrate concentrations were 0.02 M throughout. For malic dehydrogenase (Thunberg) DPN was normally 10^{-3} M. For malic and alpha-ketoglutaric "oxidases" DPN and ATP were both 10^{-3} M. For the Thunberg tests the redox dye was 10^{-3} M 2,6-dichlorophenol-indophenol; equivalent oxygen uptake is calculated from the rate of dye reduction. The addition of cytochrome c under the conditions employed was not required.

The tissues chosen for study were leafless coleoptiles of 3-day-old oat seedlings (*Avena sativa* var. "Segerhavre") and the third internodes of 7-day-old etiolated pea seedlings (*Pisum sativum* var. "Alaska"). The conditions of growth were those which have been standardized for auxin assays, and are described in detail elsewhere (6, 9).

The optimal conditions for respiration of coleoptiles as described by J. Bonner (5) are: 0.01 M KH_{2}PO_{4} (pH 4.5), 2% sucrose, 100 mg/l MnSO_{4}, 0.005 M pyruvate, and 3 mg/l indoleacetic acid. Pea stem internodes appear to be adequately provided with endogenous sugars, and are therefore relatively insensitive to added nutrients or cofactors other than auxin; nevertheless, identical solutions were employed, except that indoleacetic acid was 1 mg/l.

Respiration was measured with samples of up to 250 mg fresh weight (FW) of diced tissue. Under the conditions employed, the rates were not limited by oxygen tension. Oxygen uptake was linear for at least 1 hour. All measurements of both respiration and dehydrogenase activity were conducted at 25°C.

The following abbreviations are employed: FW, fresh weight of original tissue; M_{w}, washed mitochondrial fraction; Q_{O_{2}} (N), microliters O_{2} exchanged per hour per mg N; Q_{O_{2}} (FW), microliters O_{2} exchanged per hour per gram FW; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate.

**EXPERIMENTAL**

**Efficiency of Extraction:** The activity of separate respiratory enzymes can only be truly compared with the rate of respiration if three requirements are fulfilled: the extraction of the enzyme must be quantitative, the measurement of activity of the enzymes must be quantitative, and the level of respiration must be maximal.

The third of these requirements has been approximated by employing conditions which yield the highest rate of oxygen uptake. The rates obtained compare favorably with those in other published reports. The first and second requirements were considered in a previous paper (25) and are explored here in further detail.

The chilled and diced tissue was ground in a mortar at 3°C with 2 to 5 volumes of sucrose and phosphate solution, and extracted three times further with smaller amounts of solution. It was reported in the previous paper, and has been confirmed since, that the residue from coleoptiles prepared in this manner is essentially free from sucinic dehydrogenase activity. Pea stem sections, however, contain a higher ratio of vascular tissue to parenchyma, and therefore were not completely extracted. In one experiment summarized in table I, the oxygen uptake of the residue was compared with that of intact sections, a homogenate, and washed particles. It is seen that the residue has about one fifth the respiration of the original sections. It may be reasoned that the respiration of the residue must be due either to intact cells, corresponding to tissue sections; broken cells, corresponding to a homogenate; or trapped particles, corresponding to washed particles (M_{w}). Other experiments had shown that intact sections were not stimulated more than 20 to 25% by added p-phenylenediamine or succinate. An examination of table I suggests, therefore, that the respiration of the residue is due largely to unbroken cells. This is in partial support of the assumption of Millerd (22). Duplicate experiments indicated that the residue accounted for an average of roughly 20% of the respiration of the intact tissue. It may be assumed in consequence that the dehydrogenase activity of pea stem homogenates is approximately 80% of that available in the intact tissue.
TABLE II
COMPARISON OF DEHYDROGENASE ACTIVITY AND RESPIRATION *

| EXPT. NO. | TISSUE | RESP. OBS. | MALIC DEHYDROGENASE
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SUCCINIC DEHYDROGENASE</td>
</tr>
<tr>
<td>1</td>
<td>Avena</td>
<td>571</td>
<td>95  133</td>
</tr>
<tr>
<td>2</td>
<td>Avena</td>
<td>566</td>
<td>93  146</td>
</tr>
<tr>
<td>3</td>
<td>Pisum</td>
<td>370.5</td>
<td>62  68.5</td>
</tr>
<tr>
<td>4</td>
<td>Pisum</td>
<td>373</td>
<td>62  70.5</td>
</tr>
</tbody>
</table>

* Dehydrogenases determined by rate of reduction of 2,6-dichlorophenolindophenol, by homogenates prepared as in text. Rates given as equivalent oxygen uptake.
** Pisum dehydrogenase values multiplied by 1.25 to allow for incomplete extraction.

SUCINIC AND MALIC DEHYDROGENASE AND RESPIRATION: The Thunberg method, as adapted to the determination in plants of the dehydrogenases for succinate and malate, is very sensitive and fairly accurate, according to a number of criteria. Since the dilution employed is considerable (10 to 50 mg FW per 4 ml of test solution), it is possible to assay homogenates of substrate-rich tissues without encountering significant blanks.

The results of several experiments, comparing the dehydrogenase activities and the rates of respiration of tissue aliquots, are summarized in table II. The results, expressed in microliters of oxygen uptake per hr per gm FW, show that the succinic dehydrogenase activity of the oat coleoptile can consume oxygen at a rate equal to about one quarter of the rate of respiration. For the pea stem it corresponds to about one fifth the rate. The maximal malic dehydrogenase activity, with coenzyme I nearly saturating, corresponds to about twice the entire rate of respiration. Since the decarboxylating "malic enzyme" requires coenzyme II, its activity is not included in this measurement.

Thus, the succinic dehydrogenase activity is slightly more than theoretical for operation of the Cycle, while malic dehydrogenase has many times more activity than is required.

ALPHA-KETGLUTARIC, SUCCINIC, AND MALIC "OXIDASES": Dehydrogenase activity can also be measured by means of oxygen uptake, whereby the dehydrogenase is linked, through the endogenous cytochrome system, to oxygen ("oxidase"). In the case of plant a-ketoglutaric dehydrogenase, this is the only means thus far developed for its detection. As discussed earlier (25), the enzyme specificity is lower with oxygen than with dye reduction since the initial rate of oxygen uptake corresponds to secondary oxidations of the reaction product. It is possible, nonetheless, to estimate, by means of empirically determined factors, the approximate rate of dehydrogenation of the organic acid from the measured oxygen uptake.

In the simplest case, the entire oxygen uptake would be due to a single-step oxidation. This is true for the initial stage of malate oxidation, in which direct analysis shows that a mole of malate disappears per atom of oxygen used. With succinate, the corresponding factor was found to be 0.9. With a-ketoglutarate, however, the factor was only 0.5 (24) which means that half the oxygen uptake is due to oxidations beyond succinate. Hence the organic acid oxidized may be approximated by multiplying the Qo2 values by 1.0, 0.9 and 0.5 for malate, succinate and a-ketoglutarate, respectively. In order to allow for incomplete extraction, each factor must be further multiplied by 1.25 when comparing the enzyme activity to the rate of respiration in pea stems.

With plants there is a further restriction on the manometric method, namely that the tissue homogenates are too dilute to give accurate estimations; as

TABLE III
COMPARISON OF "OXIDASE" ACTIVITY OF WASHED MITOCHONDRIAL SUSPENSIONS WITH RESPIRATION OF PEA STEM INTERNODES *

<table>
<thead>
<tr>
<th></th>
<th>a-KETGLUTARIC OXIDASE</th>
<th>SUCCINIC OXIDASE</th>
<th>MALIC OXIDASE</th>
<th>Resp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qo2 (FW)</td>
<td>Qo2 (N)</td>
<td>Qo2 (FW)</td>
<td>Qo2 (N)</td>
<td>Qo2 (FW)</td>
</tr>
<tr>
<td>85.0</td>
<td>102</td>
<td>120</td>
<td>421</td>
<td></td>
</tr>
<tr>
<td>44.5</td>
<td>295</td>
<td>78</td>
<td>271</td>
<td></td>
</tr>
<tr>
<td>49.5</td>
<td>317</td>
<td>106</td>
<td>55†</td>
<td></td>
</tr>
<tr>
<td>Ave.</td>
<td>60</td>
<td>103</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>Factor **</td>
<td>0.625</td>
<td>1.125</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>Qo2 (ave.) × factor †</td>
<td>37</td>
<td>116</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>% theoretical</td>
<td>67</td>
<td>210</td>
<td>229</td>
<td></td>
</tr>
</tbody>
</table>

* Separate rows refer to averaged values from separate experiments.
** Equivalents organic acid
Equivalents Qo2 × 1.25. For derivation see text.
† Rate of organic acid disappearance expressed as equivalent oxygen uptake.
†† Theoretical ⋆ † respiration.
a result, mitochondrial or other particulate suspensions, which can be concentrated from a large amount of tissue, must be employed. But unfortunately, many enzymes are only partially included in these fractions (11, 25, 28); and hence the values will in many cases be low. In addition, the manometric method is insufficiently sensitive for small amounts of plant tissue; in the case of the oat coleoptile, for example, more than 100 ten-mm sections are desirable for a single vessel.

These limitations must be borne in mind in making comparisons of the respiration and "oxidase" activity of pea stem tissue, as summarized in table III. Nevertheless, it is evident that the oxygen consumption of the mitochondrial fraction agrees moderately well with the theoretical levels required for the cycle. Only α-ketoglutarate is oxidized at a rate lower than theoretical, its value being one-third too low. Since the total oxygen uptake with malate is greater than with α-ketoglutarate, a limiting element not common to the two "oxidases" is indicated. The addition of a crude coenzyme A preparation did not stimulate oxygen uptake. The dehydrogenase itself appears therefore to be limiting in the electron transfer chain and the assay is correspondingly validated. However, in view of the considerable reservations attendant to this method, it might be predicted that a direct assay measuring α-ketoglutaric dehydrogenase of the whole homogenate would show activity equivalent to or exceeding theoretical.

**TABLE IV**

**COMPARISON OF DEHYDROGENASE AND "OXIDASE" ACTIVITY***  

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>DEHYDROGENASE</th>
<th>&quot;OXIDASE&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>232</td>
<td>342</td>
</tr>
<tr>
<td></td>
<td>294</td>
<td>395</td>
</tr>
<tr>
<td>Malate + 10⁻⁴ M DPN</td>
<td>...</td>
<td>332</td>
</tr>
<tr>
<td>Malate + 10⁻³ M DPN</td>
<td>995</td>
<td>445</td>
</tr>
<tr>
<td></td>
<td>1075</td>
<td>483</td>
</tr>
</tbody>
</table>

*Single preparation of washed mitochondria in Thunberg and manometric tests; except for particle dilution and terminal oxidant, identical conditions throughout: 0.02 M substrate and 0.01 M phosphate, pH 7. Oxygen uptake calculated as Qo₂ (N). The sets of data are from two separate experiments.

Indeed, considering the 30-fold difference in enzyme concentration as well as the difference in the hydrogen acceptors, the correlation is quite good.

In the case of malic dehydrogenase, agreement is obtained only at low coenzyme concentration. In manometric experiments (20, 25) there is considerable activity in the absence of added coenzyme, but additions of it do not lead to as high values as with the dye as acceptor. It would appear, therefore, that a step between flavoprotein and oxygen is limiting.

**DISCUSSION**

Some years ago the comparison of rates of respiration and enzyme activity excited great interest (see the review of Krebs (17)), but in the animal tissues studied, it is extremely difficult to estimate the maximum level of respiration of an intact organ or tissue. The problem with plants is much more straightforward, since tissues are easily excised with a minimum of damage and are presumably less subject to a complex array of hormones. Nonetheless, few such studies have been undertaken (13, 14, 22); only one of these, that of Millerd (22), purports to deal with a comparison of the Krebs cycle enzymes and respiration, as does the present study.

In Millerd's report the oxygen uptake of a mitochondrial preparation oxidizing succinate (45 μl/hr × gm FW) is considered to account for the entire respiration of the intact tissue (150 μl/hr × gm FW), by assuming only 50% efficiency of extraction and 50% loss during centrifugation. These assumed losses were based on visual observation and do not correspond to our data on extraction, given above, nor to our data on recovery from centrifugation (25), although these were obtained with very similar tissue.

However, Millerd assumed that all of the oxygen uptake with succinate represented complete oxidation through the cycle. This was not proven, and indeed, the oxygen uptake in the presence of succinate, both of plant particles (25) and of muscle homogenates (table 3 of ref. 19) is due almost entirely to the oxidation of succinate itself and not of other cycle intermediates. As a result, the theoretical requirement for succinic oxidase is much lower than it would be for cyclic oxidation. Considerably larger respiratory rates and enzyme activities were found in the present paper (tables III and IV) and one may conclude that the plant tissues studied can oxidize succinate at a rate greater than one-sixth of the oxygen uptake of the intact tissue. With a tissue QO of 1.0 or less the succinic dehydrogenase activity thus equals or exceeds the theoretical level for Krebs cycle respiration.

A similar argument can be made for malic dehydrogenase. The total malic dehydrogenase, and even that fraction of it associated with the mitochondria which can be coupled with the oxidase, appears sufficient for the purpose. In view of the apparent excess of malic dehydrogenase, it may be suggested that the 75% of this enzyme appearing in the soluble fraction (25) has a separate function. "Malic enzyme" may presumably be ruled out, since it is specific for coenzyme II.
α-Ketoglutaric acid oxidation appears to have the most complex requirements of those tested; it is unlikely therefore that truly optimal conditions have been established. In fact considerable variability in the \( Q_{o_2} \) (N) values has been observed, many single values appearing to be higher than those reported in table III. It is possible, therefore, that the activity found in these experiments is on the low side and that the true α-ketoglutaric dehydrogenase of the particulate fraction is sufficient for the operation of the cycle. However, it must be recalled that α-ketoglutaric acid was one of the least active acids in promoting growth of isolated coleoptiles (see (6) table 2). This behavior certainly suggests that its oxidizing enzyme might be a limiting factor.

Although it is quite possible that the enzyme activities in vivo may be higher than we have been able to demonstrate in vitro, there are several considerations that suggest that the in vivo activities are not maximal. When more than one substrate is added to a particulate preparation, the rate of oxidation is not additive, indicating competition for pathways of electron transport. Furthermore, there is mutual inhibition among the cycle intermediates. With malate oxidation these effects are very pronounced.

Of the two other dehydrogenases of the cycle, little of a quantitative nature can be said. Iso-citric dehydrogenase appears to be fairly active in plant extracts, but pyruvate oxidation has been found so far to go only slowly (12, 21, 22).

From the present study it can be concluded that of the five dehydrogenases required for respiration through the Krebs cycle, two are present in adequate amounts and a third one may perhaps be. This is affirmative evidence, within its limitations, for the cycle as the main route of respiration in the plant.

**Summary**

The maximum activities of succinic and malic dehydrogenase, and of succinic, malic and α-ketoglutaric "oxidases" have been determined as accurately as possible in Avena coleoptiles and Pisum stem sections. These activities have then been compared with the observed maximum rates of respiration of the intact tissues, in order to determine whether the Krebs cycle is the main route of respiration or not. The limitations within which such comparisons are valid have been discussed. It is concluded that the amounts of succinic and malic dehydrogenase are sufficient to allow for all of the tissue respiration to proceed through them; in the case of α-ketoglutaric acid the amount of enzyme appears a little short of the theoretical. In the absence of information on the other dehydrogenations of the cycle, the evidence for the Krebs cycle as a main respiratory pathway remains incomplete.

**Literature Cited**


Studies of the CO₂ content of the air have been published over the last 200 years. Stepanova (20) gives 229 abstracts in her 1952 review, without including several extensive studies published in botanical journals or any work in which CO₂ measurements were incidental to studies of photosynthesis, soil respiration, etc. Although DeSaussure (7) showed diurnal variations in the CO₂ content of field air as early as 1816, and correctly interpreted them as due principally to photosynthesis and respiration, one 1952 paper is listed as an endeavor to clarify the disputed point of whether the CO₂ content of the air is constant or variable.

The point is of more than academic interest, for Böning (3), Chapman and Loomis (5), Decker (6) and Thomas and Hill (21) have shown that photosynthesis varies directly with the CO₂ concentration at levels present in the field. Normal CO₂ is generally considered to be 0.03 volume percent, which is 300 ppm or 0.594 mg/liter of air at sea level and 0° C. Glueckauf (9) believes, however, that the average concentration is increasing with the utilization of oil and coal, and is now above 330 ppm. Balloon and rocket flights have shown that the volume percentage of CO₂ is essentially uniform to heights of at least 70 km or 42 miles (9). A value of 0.029 ± 0.002 % at 72,000 ft was reported by the Explorer II ascent (17). Atmospheric pressure at this altitude is about 5 % of that at sea level and the quantity of CO₂ per unit space is correspondingly low, but the volume percentage is unchanged (23).

Verduin and Loomis (24) reported reductions in the CO₂ content of field air during rapid photosynthesis in Iowa averaging 25 % of the "normal" concentration. Similar changes have been reported recently by other workers (4, 11, 15, 16, 22, 25). Chapman (4) found less fluctuation in the CO₂ content of the air in a dryland field at Alliance, in western Nebraska, than had been reported for Ames, Iowa. He assumed that the lower soil organic matter, moisture and temperature reduced the build-up of free CO₂ at night, and that the generally more sparse vegetation resulted in less utilization during the day. Results in Palestine (16) were similar to those in Iowa, but analyses at Milano, Italy (22) showed a considerable increase in average CO₂ content of the air in the summer. The daytime drop during the summer was of the same magnitude as that in Iowa.

Verduin and Loomis (24) noted that the low daytime CO₂ at ground level was not increased appreciably by winds of moderate velocity, thus raising the question of stratification and limited mixing of surface air with higher levels. In the summer of 1952 the opportunity arose of using the Iowa State College television tower for continuous sampling of air at a moderate height in comparison with analyses from an adjoining corn field.

Methods

A modification of Heinicke’s method (10) described by Chapman and Loomis (5) was used. Five lines of 3/16 inch I.D. copper tubing were connected to CO₂ absorption towers. These lines sampled the air at: (a) 1 m above the ground and about 20 m inside the border of a large corn field; (b) 1 m in the TV tower area, a grass plot about 40 by 60 m completely surrounded by corn; (c) 10 m above the ground on the tower; (d) 30 m on the tower, and (e) 152 m at the base of the transmitting antenna on the tower. Manometers and thermometers were inserted in the air lines just ahead of the flow meters to provide data for correcting apparent air volumes. Temperature differences were small; pressure drops due to line resistance varied between about 10 and 50 cm of water.