2. Whereas extracts of the wild type catalysed the reduction of diphosphopyridine nucleotide in the presence of α -oxoglutarate, thiamin pyrophosphate and coenzyme A, extracts of the mutant did not. Addition of mutant extract to that of the wild type did not affect the latter's ability to oxidize α -oxoglutarate; this shows that the inability of the mutant extract to effect this reaction was due not to the presence of an enzymic inhibitor, but to the lack of an enzymic component of the α -oxoglutarate-dehydrogenase system.

3. Extracts of the wild-type organism catalysed the anaerobic evolution of carbon dioxide from α -oxoglutarate in the presence of thiamin pyrophosphate and ferricyanide as electron acceptor. The rate of gas evolution was proportional to the amounts of extract added. Extracts of the mutant failed to effect this reaction.

4. Extracts of the wild-type organism in the presence of thiamin pyrophosphate catalysed the incorporation of isotope from [^{14}C]bicarbonate into α -oxoglutarate, by isotopic exchange. Extracts of the mutant did not catalyse this reaction.

5. Extracts of both types of organism contained succinic thiokinase, lipoic dehydrogenase and lipoic trans-succinylase: a new assay procedure for the last-named enzyme is reported.

6. These results show that the mutant lacked the enzymes catalysing the first step or first two steps in the overall oxidation of α -oxoglutarate. They also indicate that the formation of the succinic semialdehyde-thiamin pyrophosphate complex and carbon dioxide from α -oxoglutarate is effected by a discrete enzymic component of the α -oxoglutarate-dehydrogenase system.

7. Undialysed cell-free extracts of the mutant, in the absence of added electron donors, catalysed the anaerobic reduction of [^{14}C]malate to [^{14}C]succinate: this reflects the ability of the mutant to grow anaerobically even in the absence of succinate.

We thank Professor Sir Hans Krebs, F.R.S., for his interest and encouragement, Professor B. D. Davis for gifts of *E. coli* types W and 309-1R, and Miss Jennifer Twining for technical assistance. This work was supported by the Rockefeller Foundation, by the U.S. Public Health Service and by the Office of Scientific Research of the Air Research and Development Command, United States Air Force, through its European Office, under contract no. AF 61(052)-180.

REFERENCES

- Davis, B. D., Kornberg, H. L., Nagler, A., Miller, P. & Mingioli, E. (1959). *Fed. Proc.* **18**, 211.
 Dixon, G. H., Kornberg, H. L. & Lund, P. (1960). *Biochim. biophys. Acta*, **41**, 217.
 El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.
 Goldberg, M. & Sanadi, D. R. (1952). *J. Amer. chem. Soc.* **74**, 4972.
 Gunsalus, I. C. (1953). In *The Mechanism of Enzyme Action*, p. 545. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
 Gunsalus, I. C., Barton, L. S. & Gruber, W. (1956). *J. Amer. chem. Soc.* **78**, 1763.
 Hager, L. P. & Gunsalus, I. C. (1953). *J. Amer. chem. Soc.* **75**, 5767.
 Kaufman, S. (1955). In *Methods in Enzymology*, vol. 1, p. 714. Ed. by Colowick, S. P. and Kaplan, N. O. New York: Academic Press Inc.
 Kornberg, H. L., Davies, R. E. & Wood, D. R. (1952). *Biochem. J.* **51**, 351.
 Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
 Massey, V. (1958). *Biochim. biophys. Acta*, **30**, 205.
 Massey, V. (1960). *Biochim. biophys. Acta*, **38**, 447.
 Sanadi, D. R., Langley, M. & White, F. (1959). *J. biol. Chem.* **234**, 183.
 Schweet, R. S., Fuld, M., Cheslock, K. & Paul, M. H. (1953). In *Phosphorus Metabolism*, vol. 1, p. 246. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
 Searls, R. L. & Sanadi, D. R. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 697.
 Straub, F. B. (1939). *Biochem. J.* **33**, 787.

Biochem. J. (1961) **78**, 198

The Measurement of Aconitase Activity in the Leaves of Various Normal and Variegated Plants

By J. S. D. BACON, M. J. PALMER AND P. C. DEKOCK

Departments of Biochemistry and Plant Physiology, Macaulay Institute, Aberdeen

(Received 26 May 1960)

Aconitase, an enzyme widely distributed in living organisms, was first found in higher plants (kidney beans, cucumber seeds) by Martius (1939), and was later shown to occur in leaves of cabbage (Jacobsen & Soares, 1940) and of rhubarb (Morrison & Still, 1947).

Studies by Dickman & Cloutier (1951) and by Morrison (1954) have shown that during its purification from animal tissues the enzyme loses activity, which can be restored by the addition of ferrous iron together with cysteine, thioglycollate, ascorbate or glutathione. It has therefore been

assumed that *in vivo* the enzyme is associated with an iron compound which is lost or inactivated during the process of purification. Although no observations on this point have been made with plant aconitase, one of us has suggested (DeKock, 1954-55) that aconitase in plants might also be associated with iron, and hence that iron-deficient plants might contain less aconitase than normal ones.

We have developed a procedure, based on that of Racker (1950), for measuring aconitase activity in leaves. This paper describes its application to various species of plants, including some with variegated foliage. Its application to iron-deficient plants has been reported to the Biochemical Society (Bacon, DeKock & Palmer, 1959).

MATERIALS AND METHODS

Unless otherwise stated plants were grown in the gardens or greenhouses of this Institute. From September to April the temperature of the greenhouses was kept between 5° and 20°, and extra illumination was provided by groups of fluorescent discharge tubes from 5 a.m. until 9 p.m. each day. Leaves were picked shortly before use, and were conveyed to the laboratory in a dry beaker surrounded by crushed ice in a vacuum flask. The weighed samples were sliced with plastic scissors before being disintegrated in a suitable extraction medium (see below).

Substrates. Solutions of ($\pm$)-isocitrate were prepared by the hydrolysis of ($\pm$)-isocitric acid lactone (L. Light and Co. Ltd.) with 1.5N-NaOH at 100° for 30 min., and subsequent neutralization with 2N-HCl to pH 8.5. Citrate, malate and *cis*-aconitate solutions were made by dissolving citric acid, malic acid and *cis*-aconitic acid anhydride (prepared by the method of Malachowski & Maslowski, 1928) respectively in water and adjusting the pH to 8.5 with N-NaOH. Oxaloacetic acid was a commercial preparation (L. Light and Co. Ltd.) or was prepared from sodium diethyloxaloacetate (Eastman Kodak Ltd.) as described by Heidelberger (1953).

Estimations. Citrate was estimated by the method of Taylor (1953), and ascorbic acid by titration with 2,6-dichlorophenolindophenol (Roe, 1954) at a dye concentration of 0.1 mg./ml.

RESULTS

Measurement of aconitase activity

First attempts to measure aconitase activity directly in the spectrophotometer by the addition of leaf extract to the buffered citrate or isocitrate solutions described by Racker (1950) were not successful. Incubations were therefore made with larger proportions of leaf extract, and small samples were deproteinized before measurement of the absorption at 240 m μ . In this way it was found that for optimum activity the leaf enzyme required a higher pH and substrate concentrations about ten times as great. On the basis of these results the following procedure was then developed:

0.5 g. of leaf was disintegrated in an all-glass Potter-Elvehjem homogenizer with 2.5 ml. of extraction medium previously cooled in ice. The extraction medium contained 0.5M-sucrose, 0.2M-2-amino-2-hydroxymethylpropane-1:3-diol(tris)-HCl buffer (pH 8.5) and 5 mM-ethylenediaminetetra-acetic acid (EDTA). The suspension was passed through muslin into a cooled tube and centrifuged at about 1300 *g* for 5 min., during which time the temperature rose to about 15°. The supernatant fluid was decanted into a cooled tube and kept on ice. Two 1 cm. cells of the spectrophotometer (Unicam SP. 500) were set up, one containing sodium ($\pm$)-isocitrate, and both a sucrose solution buffered with tris-HCl at pH 8.0. After addition of the leaf extract (0.025-0.10 ml.) the final concentrations were: 0.05M-($\pm$)-isocitrate, 0.5M-sucrose and 0.1M-tris, in a total volume of 3 ml. The isocitrate solution itself contributed to the mixture an extinction of 0.4-0.8 at 240 m μ depending upon the batch of lactone used. Addition of leaf extract to both cells increased the extinction considerably, so that with some extracts more than 0.05 ml. made the instrument so insensitive that readings were unreliable. Usually, however, it was possible to measure an increase of extinction (240 m μ) of between 0.05 and 0.50 unit during a 30 min. period after the addition of the extract: in some extreme cases the change was as great as 1.0 unit with 0.05 ml. of extract (*Sambucus*). The temperature was controlled at 25° in the constant-temperature cell holder supplied with the Unicam SP. 500. The increase was almost always linear with time, and from it the μ moles of aconitate formed were calculated from an extinction value given by Racker (1950) of 0.71 for 0.2 mM-*cis*-aconitate. Aconitase activities are expressed as μ moles of *cis*-aconitate formed/g. fresh wt. of leaf/hr. (Table 2).

On the assumption that all the measured extinction increment was the result of aconitase activity, the following factors were examined for their influence on the extractability of the enzyme. (The species on which a particular test was performed is given in parentheses.) Sampling error, which is great when only 0.5 g. of leaf is taken, was largely overcome by using opposite leaflets, or half-leaflets from the same leaf (tomato, broad bean) or opposite sides of simple leaves (mustard). Extracts prepared in this way showed good agreement of activities, often better than $\pm 5\%$.

Method of disintegration. The use of a large (21 cm. diam.) mortar and pestle, cooled overnight in the refrigerator, with or without sand, gave extracts of similar activity to those prepared in the homogenizer. Extension of treatment beyond the time needed to disintegrate all large particles tended, if anything, to decrease the activity.

Extraction medium. Sucrose was originally included with the intention of preserving the mitochondria, but later experiments showed that practically all the aconitase activity was present in the supernatant after centrifuging at 25 000–40 000 g for 15 min.

Tris buffer was preferred to phosphate because in preliminary experiments the latter seemed to depress the activity (cf. Pierpoint, 1959), but later tests with improved technique showed no appreciable difference. Sodium EDTA has a strong absorption at 240 m μ and contributes an extinction of about 0.02 when 0.05 ml. of extract is added to a final volume of 3.0 ml. of buffered substrate. It was retained in the extraction medium as a safeguard against heavy-metal contamination. It delayed the darkening of some leaf extracts (e.g. broad bean) but did not prevent a slow increase in absorption at 240 m μ . Its omission did not seem to affect the aconitase activity (cabbage, *Abutilon*). Addition of citrate to the medium (Pierpoint, 1959) increased the activity by 5–10% (*Abutilon*).

pH and molarity of buffer. The pH of the final extract was usually somewhat lower (at most 0.5 unit) than that of the extraction medium, in which the buffer concentration was 0.2M. Experiments with cabbage and *Abutilon*, and more extensive experiments with tomato leaves, showed that much the same activity was measured after extraction with media of pH between 8.0 and 9.0, but that distinctly lower values (50–75%) were found with media of pH 7.0–7.5.

Centrifuging at 1300 g. This removes cell debris, chloroplast material, starch granules, etc. When it was omitted (*Abutilon*) the extract appeared darker and more opaque, but did not absorb much more at 240 m μ : the aconitase activity was somewhat higher (about 10%).

Storage of the low-speed supernatant. Even at 0°, extracts of some leaves lost activity fairly rapidly. Thus a tomato-leaf extract lost one-quarter of its

activity in 7 hr. at 0°, one-half in 6 hr. at 25° and more than two-thirds in 4.5 hr. at 40°. Extracts from cabbage and mustard leaves were much more stable. The activity lost in this way could not be restored by treatment with ferrous ammonium sulphate and cysteine at 0° (Morrison, 1954).

Composition of the test medium. The activity had a broad optimum between pH 7.5 and 8.5 (cabbage, broad bean). The omission of sucrose or replacement of the tris-HCl by phosphate did not affect the activity. Activity was not a maximum at the concentration of isocitrate used; no precise measurements of K_m were made, but it was estimated to lie between 0.5 and 1.0×10^{-2} M ($\pm$) isocitrate (broad bean, parsley, *Abutilon*).

Intracellular location of enzyme

It was thought that if the aconitase were associated with a particulate fraction it might be separated from the crude extracts by high-speed centrifuging, but tests on various species showed that this was not feasible. Centrifuging at 40 000 g in a refrigerated centrifuge for periods up to 1 hr. yielded a pellet which usually contained only 5–15% of the total activity (Table 1). The activity in the pellet was clearly more than could be accounted for by the supernatant fluid it contained, but it also seemed to be less stable, and the point was not investigated further. Simultaneous estimation of fumarase in the cabbage extract showed that a rather greater proportion of the total (10–15%) was associated with the pellet.

Evidence for the presence of aconitase

The increase in extinction at 240 m μ in the presence of isocitrate is only presumptive evidence for the formation of *cis*-aconitate. We therefore tested other aspects of the reactions catalysed by aconitase: the formation of *cis*-aconitate from citrate, the disappearance of added *cis*-aconitate and the formation of citrate from either isocitrate

Table 1. *Distribution of aconitase activity after high-speed centrifuging of leaf extracts*

Crude extracts were centrifuged for 15 min. at 25 000–40 000 g in a refrigerated centrifuge. Each sediment was resuspended in the original volume of extraction medium, and 0.05 ml. samples were taken for each assay.

Species	pH of extraction medium	Aconitase activity (μ moles/g. fresh wt./hr.)			Percentage in sediment [100 b/(a + b)]
		Whole extract	Supernatant (a)	Sediment (b)	
<i>Vicia faba</i>	8.5	43	40	6	13
	8.5	30	23	5	19
	7.5	45	38	5	12
	7.5	55	43	3	7
<i>Brassica oleracea</i>	8.5	—	21	1	5
	8.5	—	21	1	5
	8.5	—	24	1	4
<i>Sinapis alba</i>	8.5	—	24	5	17

or *cis*-aconitate. With citrate as substrate there was also an increase in extinction at 240 m μ , but this was always less than that observed with the standard concentration of *isocitrate*, even when high concentrations of citrate were used. A series of comparisons was made with extracts of broad-bean leaves having different activities in the standard test (figures in parentheses), at the concentrations of the two substrates [10 mM-($\pm$)-*isocitrate*; 30 mM-citrate] selected by Racker (1950), and the following ratios of activities were found: 1.6 (55), 1.5 (41), 2.0 (74) and 1.7 (82). With 0.25 mM-*cis*-aconitate as substrate there was a fall in extinction comparable with the increase with 10 mM-($\pm$)-*isocitrate*; for three broad-bean extracts with a wide range of activity the ratios of the activities towards *cis*-aconitate and *isocitrate* were 0.68, 1.0 and 1.1. Estimations of citrate showed that it was formed under the assay conditions at a rate comparable with the rate of conversion of *isocitrate* and *cis*-aconitate. Dr R. I. Morrison kindly examined the products of an incubation of leaf extract with *isocitrate* by partition chromatography on silica gel (DeKock & Morrison, 1958), and showed that both citrate and *cis*-aconitate were formed. The agent or agents responsible for all these changes were destroyed by heating the extract, or even, with some extracts (e.g. tomato), by leaving them overnight at room temperature.

Effect of inhibitors of aconitase. Potassium cyanide at concentrations from 0.4 to 50 mM had an inhibitory effect on extracts of various species (broad bean, cabbage, tomato, mustard, *Heracleum*, *Hosta*, *Sambucus*). In these experiments neutral potassium cyanide solutions were added to the

extracts at 0° to give the concentration stated, and 0.05 ml. samples were withdrawn at varying intervals (up to 2 hr.) for tests in the spectrophotometer. In very few cases was the activity completely inhibited, and in the majority the degree of inhibition varied, even from extract to extract of the same species, as might be expected with crude extracts. Extracts treated with 1–10 mM-cyanide for 30 min. at 0° lost 50–75% of their activity. The increase in extinction during the enzyme assays was always linear, suggesting that no reversal of inhibition took place when the extract was diluted. The inhibitory effects of $\alpha\alpha'$ -dipyridyl and *o*-phenanthroline were slow to develop (cf. Dickman & Cloutier, 1951). In these cases inhibition was indicated by a quicker loss of activity in the presence of the inhibitor than in its absence when the extract was kept at 0° for periods of 2–6 hr. (*Abutilon*).

Aconitase activities in the leaves of various plants

In view of the above findings it seemed probable that the procedure adopted would permit a comparison of the relative aconitase activities of leaves. The activities measured for fully expanded leaves of plants grown in soil are given in Table 2. In view of the effects of leaf age and position, one should not attach too much importance to the mean values given.

Particularly high values were found for two variegated plants, *Sambucus nigra variegata* (see Table 4) and *Abutilon striatum thompsonii*. The succulents tested all gave low or zero values by the standard procedure; the low absorption of these extracts made it possible sometimes to test as much as 0.25 ml. Previous vacuum-infiltration

Table 2. *Aconitase activities of extracts of green leaves of various species*

The mature leaves of plants grown in soil in the open or in the greenhouse were tested as described in the text. The plants are divided into three groups: crops of economic importance, garden plants and succulents.

Species	Common name	No. of extracts tested	Aconitase activity (μ moles/g. fresh wt./hr.)	
			Range	Average
<i>Allium cepa</i>	Onion	1	—	55
<i>Avena sativa</i>	Oats	1	—	35
<i>Brassica oleracea</i>	Cabbage	5	32–60	47
<i>Lycopersicum esculentum</i>	Tomato	28	21–86	46
<i>Sinapis alba</i>	Mustard	8	15–34	27
<i>Vicia faba</i>	Broad bean	16	15–55	33
<i>Abutilon striatum</i>	—	18	49–164	92
<i>Corydalis lutea</i>	Yellow fumitory	2	80–118	99
<i>Digitalis purpurea</i>	Foxglove	1	—	49
<i>Heracleum giganteum</i>	—	3	71–84	80
<i>Petroselinum hortense</i>	Parsley	3	82–134	115
<i>Tropaeolum majus</i>	Nasturtium	1	—	65
<i>Bryophyllum crenatum</i>	—	1	—	12
<i>Crassula perforata</i>	—	1	—	0
<i>Rochea coccinea</i>	—	1	—	0
<i>Sedum prealtum</i>	—	5	—	0

with 1% ammonia solution (Coles & Waygood, 1957) did not alter the zero values given by *Sedum*. Although the addition of extracts of this species to test solutions containing, for example, *Abutilon* extract caused a drop in activity, this did not seem to be proportional to the volume of extract added and never exceeded one-third of the original activity; some inhibition was shown by a boiled *Sedum* extract.

Differences in activity among leaves of the same plant

Measurements made while a leaf in a particular position on the plant was expanding showed that (per unit of fresh weight) the aconitase activity fell in broad bean and rose in tomato (Table 3); it changed little in mustard. There was an influence of leaf position. We have the most complete information about this in mustard plants, where leaves 7, 8 and 9 may have twice the activity of the earlier ones (1-3). Some analyses of broad-bean plants grown in solution culture showed a tendency for the top leaves (7 and 8), even when fully expanded, to have two to three times the activity of the lowest (1), and the total activity per leaf to be as much as five times as great in leaves 6-8 as in the lowest.

Leaves of variegated plants

In view of the connexion between iron status and chlorophyll formation some analyses were made of green and chlorophyll-deficient areas of leaf taken from variegated plants (cf. DeKock & Morrison, 1958). Although large differences were found there was no consistent trend (Table 4). A single albino plant of broad bean showed a

higher leaf aconitase than the normal plant on which it was grafted.

Measurement of fumarase activity

The same spectrophotometric method may be used to estimate fumarase (Racker, 1950), if isocitrate is replaced by malate. DL-Malate was not found to be suitable for this purpose, presumably because of the inhibitory action of the D-isomer. With L-malate, activities were measured in a number of leaf extracts. Yellow fumitory (*Corydalis lutea*), a plant which accumulates fumaric acid, and *Meconopsis cambrica*, a related plant, showed higher activities (60-80 μ moles/g. fresh wt./hr.) than mustard and broad bean, for which values lay in the range 15-30. The fumarase activity seemed to be more stable than the aconitase. In *Corydalis* the activity at pH 8.5 was higher than at 8.0, but higher pH values were not tested. There seemed to be no connexion between fumarase and aconitase activities; thus *Coleus*, which yields little or no aconitase, has as much fumarase as *Abutilon*. Extracts of the yellow portions of leaves of *Sambucus*, which had an aconitase activity (111) nearly twice that of extracts of green portions (67), had rather less fumarase (37 against 51).

DISCUSSION

The main purpose of this work was to establish a reliable method for estimating aconitase activity extractable from plants of differing iron status. The method introduced by Racker (1950), although not acceptable for some purposes (cf. Herr, Sumner & Yesair, 1956), has proved very con-

Table 3. *Aconitase activity of extracts from young and older leaves*

Mature leaves were compared with leaves just unfolding or expanding on the same plant.

Species	Aconitase activities (μ moles/g. fresh wt./hr.)			
	Mature leaves		Younger leaves	
	Range	Average	Range	Average
<i>Lycopersicum esculentum</i>	35-80 (9)	55	27-43 (6)	33
<i>Vicia faba</i>	22-55 (6)	35	71-131 (6)	96

Table 4. *Aconitase activities of extracts of the leaves of variegated plants*

Some of the leaves of the *Bougainvillea* were devoid of chlorophyll and were compared with wholly green leaves. In all other cases the yellow or green areas were cut from the variegated leaves.

Species	Aconitase activities (μ moles/g. fresh wt./hr.)	
	Green tissue	Yellow tissue
<i>Bougainvillea glabra</i>	63, 67	21, 43, 47, 23
<i>Coleus blumei</i>	13, 6, 3	3, 0, 0
<i>Hosta lancifolia</i>	102, 96	116, 135
<i>Remonium variegata</i>	0	14
<i>Sambucus nigra variegata</i>	152, 55, 105, 90, 55, 64, 68 (average: 84)	185, 102, 84, 102, 105, 105 (average: 114)
<i>Sidalcea rosea</i>	35	54

venient and could if necessary be modified to measure the enzyme activity in even smaller samples of leaf than the 0.5 g. chosen here. We have not attempted to apply it to leaves too tough or fibrous to be disintegrated in a glass homogenizer; most of our results therefore refer to young and quickly growing leaves and for this reason are probably above the average that would be found for plant tissues generally.

On the other hand, the enzyme is rather labile in crude extracts [Whatley (1951-52) found none in extracts of acetone-dried powders], and so our estimates of activity must represent less than the total enzyme originally present in the leaf. However, the losses suffered in this way probably never exceeded 20-25% and the results may form a sufficient basis for the comparison of activities in leaves of various kinds.

In some plants the values obtained were considerably above the average, but suspicion attaches to some of them. For example, variegated *Abutilon* is known always to be virus-infected, and the variegated *Sambucus nigra* may also be. With higher activities towards isocitrate the ratio of the activity with *cis*-aconitate to that with isocitrate tended to be lower; this might be a sign that some enzyme other than aconitase was acting. All leaves with high aconitase activities had high ascorbic acid contents (200-400 mg./100 g. fresh wt.), but the converse was not true. The possibility cannot be altogether excluded that ascorbic acid, which has a high absorption in the ultraviolet, may sometimes interfere with the measurement of aconitase activity. The very low values for the succulents might be a reflexion of much slower growth, or they may be due to the presence of an inhibitor.

With the data accumulated it is difficult to generalize about the changes in aconitase activity as the leaf unfolds and matures. More information about these changes in *Sinapis alba* will be reported separately.

Brummond & Burris (1954) suggested that in green tissues (lupin) the enzyme was associated with a particulate fraction, and Davies (1952) showed the presence of aconitase (and fumarase) in preparations of mitochondria from pea hypocotyls. Estermann, Conn & McLaren (1959) have found it also in mitochondria from lupin cotyledons. Our own experiments do not support the idea that the enzyme is associated in any special way either with the chloroplasts or with the fractions sedimenting at 25 000 g. Preparations of 'mitochondria' made and tested by the procedure of Pierpoint (1959) had succinoxidase activities of the same order as (or sometimes less than) those made with our own medium modified by the addition of 0.02M-citrate and adjustment of the pH to 7.8. However, they

still carried only about 4% of the total aconitase of the extracts.

The aconitase from plant material has never been purified, but despite the earlier claims of Jacobsohn and his collaborators (cf. Jacobsohn, Soares & Tapadinhas, 1940) it seems likely that, as in animal tissues, a single enzyme catalyses the interconversions of citrate, *cis*-aconitate and isocitrate. There is also some evidence from the work of James (1956), and the present experiments, that the plant enzyme is sensitive to inhibitors such as $\alpha\alpha'$ -dipyridyl, which might be considered to indicate that iron is involved in its action; no effect of ferrous iron has been recorded.

If one takes a figure of 25 μ moles of citrate transformed/g. fresh wt./hr. as an average value for the leaf-aconitase activity one may calculate that this corresponds to a citric acid cycle Q_{O_2} of about 10, whereas the Q_{O_2} of most leaves falls between 1 and 2 (Spector, 1956). A weakness in any such calculation is the difficulty of knowing the effective citrate concentration *in vivo*, and speculation is not very profitable. Nevertheless, the wide range of aconitase activity in the species tested suggests that in some leaves at least the enzyme cannot be a limiting factor in respiration. The changes in activity which take place as leaves mature may reflect changes in the proportion of the metabolism devoted to the synthesis of cell structures, rather than changes in the nature of the respiratory pathway, but the latter cannot be excluded.

SUMMARY

1. The spectrophotometric method of Racker (1950) for the assay of aconitase has been adapted and applied to extracts of the leaves of a variety of plants. Reasons are given for considering that the method provides a measure of the aconitase originally present in the intact leaf.

2. Results of tests on various plants are given in detail, including some obtained with the separated green and yellow parts of variegated leaves.

3. A few assays of fumarase by the same method are reported.

4. Some quantitative aspects of the values obtained are discussed.

This work was done with the technical assistance of Miss D. M. Stewart. We are grateful to Mr W. G. Mackenzie, Curator of the Chelsea Physic Garden, for gifts of several succulent plants.

REFERENCES

- Bacon, J. S. D., DeKock, P. C. & Palmer, M. J. (1959). *Biochem. J.* **73**, 41 f.
Brummond, D. O. & Burris, R. H. (1954). *J. biol. Chem.* **209**, 755.
Coles, C. H. & Waygood, E. R. (1957). *Canad. J. Bot.* **35**, 25.

- Davies, D. D. (1952). *J. exp. Bot.* **4**, 173.
- DeKock, P. C. (1954-55). *Rep. Macaulay Inst. Soil Res.* p. 37.
- DeKock, P. C. & Morrison, R. I. (1958). *Biochem. J.* **70**, 266, 272.
- Dickman, S. R. & Cloutier, A. A. (1951). *J. biol. Chem.* **188**, 379.
- Estermann, E. F., Conn, E. E. & McLaren (1959). *Arch. Biochem. Biophys.* **85**, 103.
- Heidelberger, C. (1953). In *Biochemical Preparations*, vol. 3, p. 59. Ed. by Snell, E. E. London: Chapman and Hall Ltd.
- Herr, E. B., jun., Sumner, J. B. & Yesair, D. W. (1956). *Arch. Biochem. Biophys.* **62**, 29.
- Jacobsohn, K. P. & Soares, M. (1940). *C.R. Soc. Biol., Paris*, **133**, 112.
- Jacobsohn, K. P., Soares, M. & Tapadinhas, J. (1940). *Bull. Soc. Chim. biol., Paris*, **22**, 48.
- James, W. O. (1956). *New Phytol.* **55**, 269.
- Malachowski, R. & Maslowski, M. (1928). *Ber. dtsh. chem. Ges.* **61**, 2521.
- Martius, C. (1939). *Hoppe-Seyl. Z.* **257**, 29.
- Morrison, J. F. (1954). *Biochem. J.* **58**, 685.
- Morrison, J. F. & Still, J. L. (1947). *Aust. J. Sci.* **9**, 150.
- Pierpoint, W. S. (1959). *Biochem. J.* **71**, 518.
- Racker, E. (1950). *Biochim. biophys. Acta*, **4**, 211.
- Roe, J. H. (1954). *Meth. biochem. Anal.* **1**, 118.
- Spector, W. S. (1956). *Handbook of Biological Data*, p. 265. Philadelphia; W. B. Saunders Co. Ltd.
- Taylor, T. G. (1953). *Biochem. J.* **54**, 48.
- Whatley, F. R. (1951-52). *New Phytol.* **50**, 258.

Biochem. J. (1961) **78**, 204

The Maltophosphorylase of Beer Lactobacilli

By B. J. B. WOOD AND C. RAINBOW

Department of Applied Biochemistry, University of Birmingham*

(Received 13 July 1960)

The observation that certain beer lactobacilli grow better on maltose than on glucose (Moore & Rainbow, 1955) indicates that these bacteria metabolize maltose by a process with initial steps that do not involve the intervention of maltase. Since then, two other beer lactobacilli 'preferring' maltose for growth have been found by us. One of these (L1), which grew rapidly and well only on maltose, of many carbohydrate substrates tested, was selected for further study as being likely to possess a restricted system of carbohydrate-metabolizing enzymes, and therefore to be uncomplicated with respect to the means by which the initial steps of maltose breakdown take place.

EXPERIMENTAL

Materials

β -Maltose. This was the monohydrate (T. Kerfoot and Co. Ltd., Vale of Bardsley, Lincs.): it was chromatographically almost free from other reducing sugars.

α -D-Glucose 1-phosphate (dipotassium salt). This was synthesized by a modification of Hanes' (1940) enzymic method: it was crystalline material containing less than 0.05% of inorganic P.

β -D-Glucose 1-phosphate. This was synthesized chemically as the barium salt from α -acetobromoglucose by the method of Reithel (1945) and converted into the dipotassium salt by ion exchange as described below. We are also indebted to Dr W. Z. Hassid, University of California, for a gift of a derivative of authentic β -glucose 1-phosphate,

which was converted into the dipotassium salt for infrared examination.

Enzymes. Glucose oxidase and catalase (ex liver, 20 mg./ml.) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Yeast autolysate. This was prepared by incubating a suspension of pressed yeast in an equal weight of water overnight at 45°. After separating the supernatant by centrifuging, it was autoclaved at 15 lb./in.² for 15 min. with 1% of filter-aid and then filtered.

Organisms. These were the strains of lactobacilli designated L3, L4, L5 and L6 by Moore & Rainbow (1955) and also strains L1 and L2. L3 and L4 are strains of *Lactobacillus brevis*, and we are grateful to Dr M. Elizabeth Sharpe for carrying out a confirmatory identification of L4. L5 and L6 are also probably strains of this species, but L1 and L2 are unidentified lactobacilli isolated from brewer's top fermentation pitching yeast by Dr D. Kulka of this Department. Stock cultures of all strains were carried as stabs in malt agar.

Media. All strains were grown on the following medium (composition/l.): yeast autolysate, 100 ml.; casein acid hydrolysate, 10 g.; β -maltose monohydrate, 20 g.; KH_2PO_4 (A.R.), 0.5 g.; K_2HPO_4 (A.R.), 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (A.R.), 0.1 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (A.R.), 5 mg.; FeCl_3 (A.R.), 1 mg.; KOH soln. to pH 5.0. After dispensing in suitable vessels, the medium was sterilized by autoclaving at 15 lb./in.², momentarily (for volumes less than 50 ml.) or for 15 min. (for volumes exceeding 50 ml.).

Cultural methods

Batches of cells were usually grown in two portions, each of 750 ml., contained in 1 l. conical flasks. Each portion was inoculated with 5 ml. of a culture obtained by transferring from a stock stab to a 10 ml. portion of medium in

* Now Department of Biochemistry.