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TRANS-ACONITATE IN PLANT TISSUES

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Abstract—Measurements of ^{14}C (from acetate-^{14}C) in *cis*- and *trans*-aconitates show that 95 per cent of the total aconitate is present in the *trans* form in corn tissues. *Trans*-aconitate-1,5,6-^{14}C was metabolized by corn and pea root sections but not by carrot discs. It is suggested that aconitate in corn tissues is out of equilibrium with other TCA cycle acids because it accumulates preponderantly in the *trans* form in the tissue.

INTRODUCTION

ACONITIC acid has been found in a variety of plant tissues.[1] In some of these, aconitic is, in fact, the major acid component but in others only trace amounts occur. Although the *cis* form is biologically active,[2,3] the *trans* form has been isolated apparently free of the *cis* form from such different plants as tomato,[4] a moss[5] and sugar cane.[6] Beath[7] crystallized aconitate melting at 172–173° from several plants. He pointed out that the melting points reported for aconitic acid isolated from plant tissues were usually in the range between 165° and 191°, i.e. between the melting points of the *cis* form (125°)[8] and the *trans* form (194–195°).[8] This indicated that a mixture of the two forms was present.

The *trans* form of aconitate is the most stable and many authors assume that the *cis* form has been converted to the *trans* form during extraction. When heat is used during the extraction such a conversion is probable since various authors[8,9] have shown that a large *cis* to *trans* conversion can occur under such conditions. Conversion of *cis*- to *trans*-aconitate has even been shown to occur at room temperature when the acid was eluted from silica gel.[10] Krebs and Eggleston[9] have also reported conversion of *trans*- to *cis*-aconitate. These data do not rule out the possibility suggested by Martius[2] that the aconitic acid in tissues may already be in the *trans* form in storage pools.

In previous work[11] in which corn root tissues were provided with acetate-1-^{14}C it was found that the pattern of ^{14}C incorporation into aconitate was different from that into citrate. It was also found that aconitate accounted for a greater proportion of the total acid present in corn tissues as they matured and was the major acid in the older tissues.

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[11] D. H. MACLENNAN, H. BEEVERS and J. L. HARLEY, *Biochem. J.* In press.

This work was aimed at determining why aconitate was not in equilibrium with citrate in the tissue and what factors affected its accumulation.

RESULTS

The different behavior of aconitate and citrate could be explained if aconitate were formed by reactions other than those of the TCA cycle. "Aconitic Hydrase" which forms citrate exclusively from *cis*-aconitate was looked for in corn root extracts using the protein fractionation procedures described by Neilson.[12,13] Isocitrate and citrate were both converted to aconitate by mitochondria and soluble protein extracts. The relative aconitase activities with isocitrate and citrate as substrates were 4·4:1 in each fraction. All of the aconitase activity was recovered in the protein which precipitated from the soluble extracts when the ammonium sulfate level was increased from 35 per cent to 60 per cent of saturation and the ratio of activity towards isocitrate and citrate remained at 4·4:1. There is thus no evidence for a protein similar to that found by Neilson[12,13] in *Aspergillus.*

TABLE 1. SPECIFIC ACTIVITIES OF ACONITIC ACIDS ISOLATED FROM CORN ROOTS PROVIDED WITH ACETATE-1-^{14}C

	cis-Aconitic	*trans*-Aconitic
^{14}C in acid (c.p.m.)	120	7820
Acid present* (microequivalents)	23·1	25
Specific activity c.p.m./microequivalent	5·2	313

* Unlabeled *cis*-aconitate was added to extraction medium.

To determine whether aconitate in corn tissue existed in the *cis* or *trans* form, intact roots were allowed to take up acetate-1-^{14}C for 30 min to label the aconitate in the tissue.[11] The material was then ground in 0·5 N H_2SO_4 containing an excess of unlabeled *cis*-aconitate. *Cis*- and *trans*-aconitate were then separated. The unlabeled aconitate accounted for most of the titratable acidity and after the initial *cis*/*trans* separation, 25 per cent of the aconitate acidity was in the *trans* form while 75 per cent was still in the *cis* form.

If all the ^{14}C in aconitate had been in the *cis* form at the time of extraction and if all of the added unlabeled aconitate had been in the *cis* form, then later *cis* to *trans* conversions would have resulted in *cis*- and *trans*-aconitates of equal specific activity. Alternately, if all of the added aconitate were in the *cis* form and all of the aconitate-^{14}C were in the *trans* form, the *cis*-aconitate isolated from the tissue would be unlabeled (if no *trans* to *cis* conversion occurred) while the *trans*-aconitate would have all of the ^{14}C regardless of how great the *cis* to *trans* conversion. In Table 1 are given the specific activities of the *cis*- and *trans*-aconitates isolated from the tissue. The *trans*-aconitate had a specific activity 62 times greater than that of the *cis*-aconitate. The *cis*-aconitate-^{14}C was diluted with unlabeled *cis*-aconitate but the *trans*-aconitate-^{14}C was also diluted by an unlabeled *trans*-aconitate impurity in the commercial *cis*-aconitate. This impurity amounted to 25 per cent of the

[12] N. E. NEILSON, *Biochim. Biophys. Acta*, **17**, 139 (1955).
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total aconitate acidity (see Methods). The ^{14}C in *cis*-aconitate was, therefore, diluted 3 times more than the ^{14}C in the *trans* acid. To determine the total ^{14}C in the *cis* and *trans* forms at the time the tissues were ground the specific activity values must be corrected as follows:

$$\frac{\text{total } ^{14}C \text{ in } \textit{trans}\text{-aconitate}}{1} \Big/ \frac{\text{total } ^{14}C \text{ in } \textit{cis}\text{-aconitate}}{3} = 62$$

or

$$\frac{\text{total } ^{14}C \text{ in } \textit{trans}\text{-aconitate}}{\text{total } ^{14}C \text{ in } \textit{cis}\text{-aconitate}} = 21.$$

By converting total ^{14}C in each acid to percentages it can be determined that at the time of grinding 95·2 per cent of the ^{14}C was in the *trans* acid while 4·8 per cent of the ^{14}C was in the *cis* acid. These values change very little if the *trans* dilution is considered to have occurred at the time of grinding or subsequent to the grinding procedure.

TABLE 2. BREAKDOWN OF *trans*-ACONITATE BY VARIOUS PLANT TISSUES

	Tissue		
	Corn root	Pea root	Carrot root
Total ^{14}C in added acid (c.p.m.)	75834	75834	75834
^{14}C in fractions (c.p.m.)			
External solution	56298	52813	43065
CO_2	5327	3239	31
Organic acids	6500	9672	23530
Acidic amino acids	988	52	0
Neutral and basic amino acids	754	0	0
Sugars	0	0	0
^{14}C recovered (c.p.m.)	69867	65776	66626
Per cent recovery	92	87	88

Since *trans*-aconitate is found in the tissue, it seemed likely that a mechanism for its further metabolism would exist. The aconitase enzyme isolated from corn tissue which was found to be active toward citric, isocitric and *cis*-aconitic acids was not active towards *trans*-aconitic acid.

When labeled *trans*-aconitate was fed to corn, pea and carrot root tissues, it was readily metabolized by corn and pea tissues but not by carrot tissue. The results of this experiment are presented in Table 2. Approximately one third of the added ^{14}C was absorbed by each tissue. In the corn tissue, ^{14}C was recovered in CO_2, organic acids, and amino acids showing that it was extensively metabolized. The $^{14}CO_2$ evolved accounted for 7 per cent of the ^{14}C added, but for 27 per cent of the ^{14}C taken up. Further separation of the organic, and acidic amino acid fraction showed that ^{14}C was present in succinate, malate, citrate, glutamate, and aspartate as well as aconitate.

In the pea tissue, $^{14}CO_2$ evolved accounted for 4·3 per cent of the ^{14}C added and 14 per cent of the ^{14}C taken up. Slight labeling was found in the acidic amino acids. In the carrot tissue ^{14}C was recovered only in the organic acid fraction. This fraction was not separated but it is very probable that all of the ^{14}C was in aconitate. In previous work,[11] aconitate could not be detected in carrot.

A variety of techniques were tried in an effort to demonstrate the utilization of *trans*-aconitate in mitochondrial and soluble preparations from corn roots. These were unsuccessful.

DISCUSSION

In this work it has been shown that about 95 per cent of the aconitate accumulated in corn roots from acetate during a 30-min period exists in the *trans* form in the tissue. In this form it is no longer a substrate for the aconitase enzyme and so cannot directly enter the TCA cycle. This may be a factor in the extensive accumulation of aconitate in older corn root tissue and probably explains why, in experiments with acetate-^{14}C, the ^{14}C content of aconitate continued to increase at times when the ^{14}C content of other TCA cycle acids was falling.[11] Since the *cis* form of aconitate is unstable[8,9] and especially under acid conditions such as exist in at least parts of plants cells[14] the conversion of *cis*- to *trans*-aconitate may well be spontaneous. An enzymic conversion cannot be ruled out, however.

The *trans* form of aconitate, when supplied externally, was shown to be extensively metabolized by corn roots. The fact that *trans*-aconitate accumulates in corn tissues argues more for its compartmentation away from metabolic sites than for its inability to be further metabolized. It is not known at present what the intermediate reactions are which enable *trans*-aconitate to reenter the metabolic sequence in those tissues in which it can be utilized but some metabolism would be expected if the *trans* form gives rise to the *cis* form.[9]

EXPERIMENTAL

Cis- and *trans*-aconitate were separated on a 1 × 11 cm Dowex-1 (formate) column using a linear gradient elution technique.[15] When 23·5 N formic acid was used in the acid reservoir and 3-ml fractions were collected, *trans*-aconitate was eluted in fractions 77–87 and *cis*-aconitate was eluted in fractions 123–132. Ordinarily 8 N formic acid was used and *trans*-aconitate was eluted in fractions 145–155. *Cis*-aconitate was not eluted under these conditions. *Cis*- and *trans*-aconitate were also separated on silica gel columns using the protocol of Bulen *et al.*[4] Columns 1 cm in diameter were packed with silica gel and developed, under pressure, with 20% butanol in chloroform equilibrated with 0·5 N H_2SO_4. Three milliliter fractions were collected and *trans*-aconitate was eluted in fractions 11–19 and *cis*-aconitate in fractions 40–50.

Trans-aconitate-1,5,6-^{14}C was prepared by providing acetate-1-^{14}C to 2-day-old corn roots. Tissues were killed after 4 hr and extracted in boiling ethanol. Water soluble materials were fractionated as described previously.[11] The organic acid fraction was taken to dryness, dissolved in 0·5 ml 0·5 N H_2SO_4 and separated on a silica gel column. The *trans*-aconitate peak was collected, and again separated on a Dowex-1 (formate) column using 8 N formic acid in the acid reservoir. The acid recovered had a specific activity of 38,000 c.p.m. per

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[15] D. T. CANVIN and H. BEEVERS, *J. Biol. Chem.* **236**, 988 (1961).

micromole. Two micromoles were fed to 2 g samples of excised root tissues of corn, carrot and peas using the apparatus described previously.[11]

To label aconitate in corn roots for specific activity determinations, four, 2-day-old corn seedlings were placed in a beaker with their roots immersed in a solution of acetate-1-^{14}C for 30 min. Roots were removed and immediately ground in 0·7 ml 0·5 N H_2SO_4 containing 30 mg *cis*-aconitate (A grade obtained from Calbiochem). One half milliliter of this solution was separated on a silica gel column. The *cis* and *trans* peaks were collected separately, placed on separate Dowex-1 (formate) columns and fractionated using 8 N formic acid in the acid reservoir. Acids eluted in the *trans* area from each Dowex column were titrated and counted to obtain specific activity as described previously.[11] When commercial *cis*-aconitate was separated on silica columns, a *trans* component equal to 25 per cent of the total acid was obtained. This was probably already in the *trans* form before the acid was placed on the column.[10] The *cis* peak collected from the silica column was further converted to the *trans* acid during the manipulations subsequent to its elution from the silica column and the specific activity of this later formed *trans* acid was measured and taken to be the same as the specific activity of the *cis* form eluted from the silica gel column.

Mitochondria from corn roots were prepared and assayed for oxidative activity by the method of Lund *et al.*[16] Assay of the supernatant from the mitochondrial preparation was carried out with the same reaction mixture as that used for mitochondria. "Aconitase" was prepared by grinding root tissues in 0·1 M phosphate buffer at pH 7·6. The suspension was centrifuged at 15,000 × *g* for 30 min. The supernatant solution was fractionated with ammonium sulfate and that fraction precipitating between 40% and 60% ammonium sulfate saturation was redissolved in phosphate buffer and used as the enzyme preparation. The reaction mixture consisted of 0·3 ml 0·5 M KH_2PO_4 pH 7·6, 0·1 ml acid (3 μmoles), 0·1 ml enzyme, 2·5 ml H_2O. The assay was based on aconitate absorption at 240 millimicrons as described by Racker.[17]

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