Polarographic Estimation of Aconitates in Plant Materials

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A procedure was devised to determine *cis*- and *trans*-aconitate in plant materials through conventional polarography. The procedure minimizes both nonenzymatic aconitate isomerization and sample cleanup and manipulation. Variations in recovery of *cis*- and *trans*-aconitates added at known levels were within instrumental error. The *trans*-aconitate levels determined compared favorably with analyses by silica gel column chromatography, in tests with low-*cis*-aconitate plant materials. A discrepancy in high-*cis*-aconitate plants appeared to be due to enhanced isomerization rates in the chromatographic process. The procedure was applied to show variations in plant aconitate with time, species, plant part, and anatomical location.

Recent work has emphasized the general presence of *trans*-aconitate in plants (Burau and Stout, 1965; Stout *et al.*, 1967), and the wide range of concentrations found. Species of the family Graminaceae tend to accumulate *trans*-aconitate, whereas members of other families have generally had relatively low concentrations, with a few isolated cases of very high concentrations.

Aconitates are tricarboxylic acids existing in two stereoisomeric forms: The *cis*-form is considered to be a normal equilibrate in the isomerization between citric and iso-citric acid mediated in the tricarboxylic acid cycle by the Fe^{II}-requiring enzyme, aconitase (Conn and Stumpf, 1963). The *trans*-form is a competitive inhibitor of aconitase, causing accumulations of citrate and reductions in oxygen consumption rates in rat kidney cortex slices (Saffron and Prado, 1949). Blahuta and Mullen (1959) found that *trans*-aconitic acid completely inhibited the growth of *Pseudomonas aeruginosa*, but did not influence that of *Staphylococcus aureus*. Serum magnesium and phosphate were significantly reduced when Burt and Thomas (1961) fed heifer calves sodium citrate (also a tricarboxylic acid) as a dietary supplement at concentrations equivalent to 1% citric acid.

These considerations led to the suggestion that aconitates may be important in the metabolism and mineral nutrition of ruminants grazing on range forages. A need was felt for a method of quantitative determination of aconitates in plant materials that would be reasonably accurate, more rapid than chromatographic procedures (DeKock and Morrison, 1958), and more specific than spectrophotometric (Ventre *et al.*, 1946), selective extraction-titration (Balch *et al.*, 1946), or decarboxylation procedures (Ambler and Roberts, 1947). The procedure should also distinguish quantitatively between the *cis*- and *trans*-forms of aconitate.

Markman and Zinkova (1957) found that the half-wave reduction potentials of the stereoisomers of several unsaturated acids are sufficiently separated for their quantitative estimation by conventional polarography. These observations formed a basis for development of the following procedure for quantitative determination of *cis*- and *trans*-aconitate in the aboveground parts of higher plants.

RECOMMENDED PROCEDURE

Weigh 500 mg. of lyophilized ground plant material into a 125-ml. Erlenmeyer flask and add approximately 2.5 grams of activated charcoal (Darco G Elf or equivalent). Add 50.0 ml. of a buffered aqueous extracting solution that is 0.25*F* NH_3 and 0.25*F* NH_4Cl. Stopper the flask and agitate on a shaker for 30 minutes before filtering with suction through a cellulose-membrane filter of 0.2-μ porosity.

For polarography of total aconitate in the extract, take 5.0 ml. of the clear colorless extract, add 20.0 ml. of 0.2*F* HCl, mix, and deoxygenate with nitrogen gas. Measure the net diffusion current (i_d^{aq}) at −0.84 V. *vs.* a saturated calomel electrode (SCE) using the increment method (Meites, 1955, pp. 69–72).

For polarographic estimation of *cis*-aconitate, take 10.0 ml. of the extract and add 15.0 ml. of freshly prepared ethanol-water (70 to 30 v./v.), which is 0.6*F* in HCl. Again, mix the solution, deoxygenate, and polarograph, preferably at a slow voltage sweep rate (e.g., 0.05 V. per minute). Determine the net diffusion current for *cis*-aconitic acid (i_d^{Et}) at −0.72 V. *vs.* an SCE.

Since the reduced Ilkovic equation, $i_d = KC$, satisfactorily describes the polarographic behavior of these compounds, the algebraic relationships in the alcoholic (Et) and aqueous (aq) media are:

$$i_d^{Et} = K_{cis}^{Et} C_{cis}^{Et} \quad (1)$$

$$i_d^{aq} = K_{cis}^{aq} C_{cis}^{aq} + K_{trans}^{aq} C_{trans}^{aq} \quad (2)$$

in which i_d is the diffusion current, K is the polarographic constant, and C is the solution concentration of the aconitates (isomers distinguished by the subscripts). The values of K can be determined conveniently by polarographing standards, made up from pure organic acids, ranging from 0 to 3m*M* for *trans*-aconitic and from 0 to 0.6m*M* for *cis*-aconitic after final dilution in the polarographic solvent-extracting solution systems. When extracting solution was exposed to low-aconitate plant materials and charcoal and then spiked with known amounts of the aconitates, the K values were the same as when uncontaminated organic acid standards were used in the extracting solvent.

When a dropping mercury electrode (DME) that gave a diffusion current constant of $I = 1.884$ (Meites, 1955, p. 47) was used in the acidic aqueous system and held at zero V. *vs.* an SCE, K_{trans}^{aq} was 6.47, K_{cis}^{aq} was 6.83, and K_{cis}^{Et} was 2.57 μa/m*M*.

It was appropriate to calculate C_{cis}^{Et} first; then, C_{cis}^{aq} is half that value by dilution and can be substituted in Equation 2 for calculating C_{trans}^{aq}. These values can then be used in calculating aconitate concentrations in the plant sample.

Sample Preparation. The severed plant or plant part is placed in a polyethylene-film bag and quick-frozen in an insulated container cooled with solid CO_2. The samples are kept frozen before and during lyophilization. Each

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Table I. Recovery by Polarographic Method of *cis*- and *trans*-Aconitic Acid Added to 500-Mg. Samples of *Poa pratensis*, μmole

trans-Aconitic Acid		*cis*-Aconitic Acid	
Added	Recovered	Added	Recovered
11.5	11.9	2.6	2.5
23.0	24.3	5.1	4.8
46.0	45.8	10.2	10.0
92.0	91.3	15.3	15.6
130.0	134.3		

sample is ground in a rotary-knife mill to pass a 40-mesh screen. A subsample of 15 to 20 grams is then powdered in an oscillating carborundum ball-mill.

RESULTS AND DISCUSSION

Extraction and Polarography. Extraction is performed at pH 9.5 by an NH_3–NH_4^+ buffer because this system results in neither positive nor negative adsorption of aconitate by the plant material or the charcoal. At neutral or slightly alkaline pH's, extraction can be complete, but only by successive extractions, involving extra manipulations and volume control. At pH values less than 4, in contrast, the charcoal strongly adsorbed both aconitate stereoisomers.

Charcoal treatment of the extract is necessary because polarography of untreated extracts results in shifts of half-wave potentials to more negative voltages, distortions of the diffusion plateaus, and decreases in the diffusion currents. The exact nature of this interference was not determined.

The data in Table I suggest that aconitates in solution are recovered quantitatively from a system composed of low-aconitate plant material, charcoal, and extracting buffer. Since aconitate recoveries were calculated from K values determined for aconitates in pure solutions, it is also apparent that soluble plant components which are not adsorbed by the charcoal do not affect the diffusion coefficient of the aconitates or the electrochemical reduction mechanisms.

The pH and buffer capacity of the final solution affect both the half-wave potential ($E_{1/2}$) and the i_d.

In strongly buffered aqueous solutions, the i_d for *trans*-aconitate decreased linearly as pH increased from 0.5 to 2.0. The decrease was 4.5% per pH unit. In these same solutions, $E_{1/2}$ shifted to more negative potentials at the rate of 86.6 mV./pH. As Elving and Teitelbaum (1949) pointed out for fumaric and maleic acids, which have a polarographic behavior similar to that of the aconitates, a low buffer capacity in the polarographic solution may result in spurious $E_{1/2}$ shifts, the appearance of double-waves, and decreases in the i_d of the primary wave resulting from local H^+ consumption in the reduction process at the mercury droplet surface. The above recommended procedure utilizes the high buffer capacity of HCl and keeps pH constant between samples.

Interferences. Removing undetermined interferences with charcoal has already been discussed. Incomplete removal of such materials is first reflected in the slope of the aconitate diffusion plateau, making it steeper than in the standards.

Unsaturated polycarboxylic acids, with carboxyl groups attached to both carbons of the double bond, will also interfere, for their halfwave potentials are close to those of the aconitates in acid media. Positive interference will most likely be from fumaric acid, but can also be caused by significant concentrations of alpha-keto acids, such as pyruvic or alpha-ketoglutaric. Of the transition metal ions, only Zn^{II} will cause any serious interference. The polarographic solution concentrations of Zn^{II} which might result from plant extracts will ordinarily be insignificant, although it has been found desirable to redistill certain lots of HCl to reduce Zn contamination from this source.

Analytical Procedure Comparison. Leaf samples of eight grasses selected arbitrarily to give a wide range of aconitate concentrations were collected in the field and analyzed for *cis*- and *trans*-aconitate by the above polarographic procedure using a conventional 3-electrode polarograph. The samples were also analyzed by the silica-gel column-chromatographic procedure of DeKock and Morrison (1958). Chromatographic fractions were successively titrated under a nitrogen atmosphere by an automatic titrimeter using a glass-SCE couple.

The data (Table II) show reasonable agreement in results between the two methods, except that the concentrations of *cis*-aconitic acid detected in *Echinochloa* and *Hordeum* were substantial by polarography but not by chromatography. The values for *trans*-aconitic acid in the same grasses were lower by polarography than by chromatography. The probable reason for this discrepancy is that the acidic character of the mobile and immobile chromatographic phases causes significant conversion of the labile *cis*- to the more stable *trans*-configuration of the aconitic acid. Such conversion was observed on a similar chromatographic system by Coic *et al.* (1961). Although the final polarographic solutions are also acidic, it is not likely that analytically detectable errors will occur if polarography is performed within 2 hours of acidification. This is substantiated by data of Ambler and Roberts (1948) from pH 1.0 systems held at 25° C., from which a first-order specific reaction-rate constant of 0.314 day^{-1} can be calculated. From this value one would expect a 2% conversion of *cis*- to *trans*-aconitic acid in 1.5 hours. The data in Table I also support this contention. Data of Ambler and Roberts (1948) also suggest that nonenzymatic

Table II. Representative Plant Species, Leaf Contents of Nonvolatile Organic Acids by Silica-gel Column Chromatography, and of *cis*- and *trans*-Aconitic Acids by Polarography, μmole/Gram (Dry Weight)

	Chromatography					Polarography	
Species	Fumaric	Succinic	Malic	Citric + iso-citric	*Trans*-aconitic	*Cis*-aconitic	*Trans*-aconitic
Echinochloa crusgalli	tr	tr	4	6	290	61	252
Maize	5	5	40	24	199	1	203
Sorghum sudanense	3	8	37	11	83	2	88
Hordeum leporinum	5	8	30	18	86	24	71
Phalaris tuberosa	4	18	12	7	58	6	59
Sorghum vulgare	3	tr	26	107	33	1	35
Poa pratensis	5	8	68	22	2	1	3
Ehrharta calycina	tr	tr	18	32	tr	tr	3

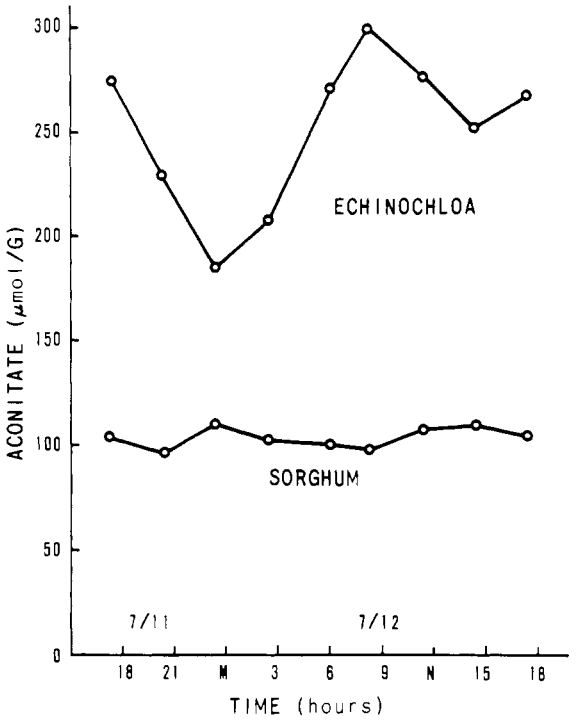

Figure 1. Aconitate concentration in upper leaves of field-sampled grasses

Table III. *trans*-Aconitate Concentration in Mature Sweet-corn Plant Separates, μmole/Gram

	Leaf	Stem
Upper	96	66
Middle	55	32
Lower	38	3

cis-trans conversion during extraction will be negligible in the pH 9.5 buffer.

With the above qualifications, it is clear that aconitate in these grasses was predominantly *trans*-aconitate, supporting contentions of Martius (1937) and MacLennan and Beevers (1964). The proportion of *cis*- to *trans*-aconitate in these grasses is not constant, however, but varies from 0.5%, in maize, to 25%, in *Hordeum*.

Application of Polarographic Method. Since the polarographic procedure is considerably faster than the chromatographic procedure, more samples can be examined and more-detailed questions asked. In July, samples of mature upper leaves of *Echinochloa* and *Sorghum vulgare* were collected every 3 hours for a 24-hour period. Total aconitate in these samples (Figure 1) shows that the two species differed distinctly in behavior. *Sorghum* aconitate concentrations were essentially invariant with time, whereas *Echinochloa* concentrations exhibited a substantial diurnal change. One-week-old sweet-corn seedlings grown in the greenhouse also showed diurnal changes, involving 2 maxima and 2 minima.

Aconitate content may be strongly influenced also by anatomical position or physiological age of the plant part, as shown in Table III by analyses of plant separates from a dough-stage sweet-corn plant grown in the greenhouse and harvested at 1100 hours. The younger tissues tend to exhibit distinctly higher aconitate concentrations. It is not likely that shading of lower leaves contributed to the gradient in this isolated plant. Results have been similar with other grasses.

These results raise questions similar to those of MacLennan and Beevers concerning mechanisms of metabolism and rate and direction of translocation of *trans*-aconitate in plants. They also suggest that the *trans*-aconitate burden of pasture grass forage may be a function of the time of day as well as of the anatomical position of the plant part removed in grazing.

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