Micro-determination of *iso*Citric and *cis*-Aconitic Acids in Biological Material

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Tracer experiments of Wood, Werkman, Hemingway & Nier (1941) and of Evans & Slotin (1941) suggest that the order in which citrate, *iso*citrate and *cis*-aconitate arise from oxaloacetate and pyruvate in pigeon liver is:

$$\begin{array}{l}\text{oxaloacetate} \\ \text{+pyruvate}\end{array} \rightarrow \textit{cis}\text{-aconitate} \rightleftharpoons \textit{iso}\text{citrate}. \quad \begin{array}{c}\downarrow\uparrow \\ \text{citrate}\end{array}$$

and not, as had originally been suggested (see Krebs, 1943),

$$\begin{array}{l}\text{oxaloacetate} \\ \text{+pyruvate}\end{array} \rightarrow \text{citrate} \rightleftharpoons \textit{cis}\text{-aconitate} \rightleftharpoons \textit{iso}\text{citrate}.$$

In order to be able to examine the new hypothesis we have worked out methods for the micro-determination of *cis*-aconitic and *iso*citric acids, a preliminary account of which has been published in the Proceedings of the Biochemical Society (Krebs & Eggleston, 1943*b*). So far no specific micro-methods for the estimation of the two acids have been described. The polarimetric method (Martius, 1938) for the determination of *iso*citric acid, based on the high rotation of the molybdate complex of the acid, is unspecific and is therefore not generally applicable. Moreover, this method requires comparatively large quantities—about 25–50 mg.—if the determination is to be reasonably accurate. The determination of *iso*citric acid by isolation of its dimethylester lactone requires even larger quantities. The micro-determination of *cis*-aconitic acid (Johnson, 1939), depending on the manometric measurement of the hydrogen required for quantitative hydrogenation, is likewise unspecific and applicable only if other unsaturated compounds are absent.

The following principle permits the determination of a few milligrams of (−)*iso*citric and *cis*-aconitic acids in biological material provided that citric acid is not present in very much larger quantities. Three portions of neutral tissue extract or tissue suspension are measured. The first is incubated with 'aconitase' whereby *iso*citric and *cis*-aconitic acids are converted into citric acid, the latter being determined according to Pucher, Vickery & Leavenworth (1934). In the second portion citric acid is determined directly. From the third portion *cis*-aconitic acid is removed by conversion into the *trans*-form; it is then treated like the first sample. The difference in the citric acid content between the first and second samples indicates the sum of (−)*iso*citric and *cis*-aconitic acids in the sample; the difference between the second and third the amount of (−)*iso*citric acid; the difference between the first and third the amount of *cis*-aconitic acid.

Thus the determination of the two acids requires three determinations of citric acid. If only one of the acids is present (apart from citric acid) two citric acid determinations are required.

I. DETERMINATION OF CITRIC ACID

The method of Pucher, Sherman & Vickery (1936) for colorimetric citric acid determination in biological material (based on the conversion of citric acid into pentabromoacetone, which yields a yellow colour on addition of sulphide) is highly specific and is applicable to small quantities (0·2–1·0 mg.) of citric acid, but has one major disadvantage: its limits of error are considerable, amounting to about 10% when the method is applied to animal tissues. We have not been able to identify with certainty the steps where the errors arise; it is probable that they are connected with the relative instability of the colour-giving compound under the conditions of the estimation. This instability is shown by the following observations: 1 mg. pentabromoacetone was dissolved in 1 ml. 4% Na_2S, diluted with 5 ml. of water and 4 ml. of dioxan, and shaken for 15 min. with 50 ml. of various organic solvents. The colour of the aqueous solution was then compared with that of a standard solution prepared at the same time, but not treated with solvents. The recovery was as follows:

Solvent	Colour recovered (%)
Light petroleum (40–60°)	51
Toluene	58
Xylene	43
Ether	45
*cyclo*Hexane	47

Purification of light petroleum or toluene with concentrated H_2SO_4, alkaline $KMnO_4$ and bromine water did not improve the recovery. Gradual fading of the colour also occurred in the absence of the above solvents, the rate of fading being affected by substances in the solution; it is increased, for example, by ethanol. Within certain limits the loss of colour is in proportion to the concentration of pentabromoacetone, but at higher concentrations the loss is relatively smaller. This seems to be the reason why the amounts of citric acid to be determined by the colorimetric method of Pucher *et al.* (1936) should not exceed 1 mg.

Because of the relatively large margin of error of the colorimetric method we have, in the present

work, chiefly used the volumetric method of Pucher *et al.* (1934), with some modifications. This method is also based on the conversion of citric acid into pentabromoacetone, but the latter is determined volumetrically by titrating the bromide set free on treatment with sulphide. The main modifications concern the following points:

(1) The oxidation and bromination are carried out in more acid (3N) solution and $KMnO_4$ is gradually, instead of suddenly, added to the solution. This was found to increase the yield of pentabromoacetone to about 97%, whilst the original procedure yielded 88%. By increasing the acidity to 6N it is possible, as shown in Table 1, to raise the yield to the theoretical level of 100%, but at the same time the method becomes less specific (see later). For this reason we prefer to use an acidity of 3N.

Table 1. *Effect of acidity on the yield of pentabromoacetone from citric acid*

5 mg. citric acid; 'revised method' refers to the procedure described in the text, with the exception of the acid concentration which was varied as stated in the first column.

Normality of H_2SO_4	No. of exps.	Yield (%) Highest	Lowest	Mean
Method of Pucher *et al.* (1934)				
1·5	2	88·6	87·7	88·2
Revised method				
1·5	3	92·6	90·9	91·8
3	2	97·6	97·5	97·6
6	5	101·4	98·2	99·9
12	1	—	—	100·0

(2) For the extraction of pentabromoacetone from light petroleum a solution of 4% Na_2S in N-NaOH was used. This reagent extracts more rapidly than the original solution, which is of importance when the amount of citric acid exceeds 10 mg.

(3) The titration of the bromide is carried out with solutions of one-fifth of the strength used by Pucher *et al.* (1934). This increases the accuracy of the titration provided that the volume of the solution is kept low. Before the titration the volume is therefore reduced to about 7 ml. No loss of halide is incurred during the process if carried out as described later.

Procedure

Deproteinization. The solution to be examined is mixed with one-fifth of its volume of 20% metaphosphoric acid and filtered after 5 min. (For the keeping qualities of HPO_3 see Krebs, Smyth & Evans, 1940.)

Removal of further interfering substances (cf. Pucher *et al.* 1936). A measured portion of the filtrate containing 0·5–20 mg. citric acid, preferably 5–10 mg., is transferred to a 500 ml. conical flask, diluted with water to 80 ml., and, after addition of 6 ml. 20N-H_2SO_4, boiled until the volume is reduced to about 35 ml. This removes certain interfering substances such as β-ketonic acids (oxaloacetic, acetoacetic, acetonedicarboxylic acids), acetaldehyde and acetone, whilst no citric acid is lost. The solution is cooled to room temperature, an excess of bromine water (usually about 3 ml.) is added, and the mixture is allowed to stand for 10 min. If a precipitate is formed (as is commonly the case with plant material) the solution is allowed to stand for a further period of 20 min., with fresh additions of bromine water if the first portion of bromine is decolorized. The precipitate is filtered off and the filter is washed with a few ml. of 3N-H_2SO_4.

Conversion into pentabromoacetone. To the solution are added 2 ml. 11·9% KBr and, drop by drop, enough 4·7% $KMnO_4$ to produce a permanent dark purple colour. During the addition of $KMnO_4$ the flask should be shaken vigorously. The solution is allowed to stand for 15 min. during which the excess of permanganate, as indicated by the purple colour, must be maintained. After the lapse of this period ferrous sulphate (20% $FeSO_4.7H_2O$ in 1% H_2SO_4) is added in 1–2 ml. quantities until the colour of the solution turns orange. Then an excess of 5 ml. ferrous sulphate is added which changes the colour to lemon. If more than 2 mg. of citric acid are present a precipitate of pentabromoacetone is usually visible.

Extraction of pentabromoacetone. The solution is transferred to a 350 ml. pear-shaped separating funnel freshly greased with 'rubber grease' and provided with a stem of at least 7 mm. internal diameter (narrower stems tend to retain fluid through capillary forces). The transfer is completed by rinsing into the separating funnel with several 5 ml. quantities of light petroleum (b.p. 40–60°). In all, 25 ml. petroleum are added and the mixture is shaken for 2 min. The aqueous layer is then drawn off and the petroleum layer (contaminated with traces of the aqueous layer) is collected in another vessel. The funnel is rinsed with 2 portions of 3–5 ml. petroleum which are added to the main extract. The aqueous layer is quantitatively washed into the funnel and shaken again with 25 ml. petroleum. The aqueous layer is drawn off and discarded. This second extract (with a trace of the aqueous layer) is combined with the first extract. The petroleum is then washed four times with 15 ml. water to remove halide and iron salts.

Extraction with sulphide. 5 ml. of 4% Na_2S in N-NaOH are measured into the funnel and shaken with the petroleum for 1 min. The aqueous phase is collected in a 200 ml. long-necked boiling flask. The shaking is repeated twice with 5 ml. of water and then again with 5 ml. alkaline sulphide and two 5 ml. portions of water. The presence of pentabromoacetone is indicated by a yellow colour of the aqueous phase. If the quantity of citric acid to be determined exceeds 20 mg. it may be necessary to make a third series of extractions with the sulphide

solution and water. Whether this step is needed is indicated by the intensity of the yellow colour of the second sulphide portion.

Dehalogenation of pentabromoacetone. To the combined sulphide solutions and water washings are added 1 ml. glacial acetic acid for every 5 ml. Na_2S used and a few grains of alundum or quartz. The solution is boiled to remove H_2S and to reduce the volume to about 7 ml. After cooling, 1·5 ml. $KMnO_4$ (4·7 %) are added; after about half a minute (during which the purple colour of the solution must be maintained, if necessary by further addition of $KMnO_4$) enough H_2O_2 (about 0·5 ml. '100 vol.') is added to decolorize the solution.

Titration. The solution is acidified with 2 ml. conc. HNO_3, and 1 ml. iron alum solution (100 g. $Fe(NH_4)(SO_4)_2.12H_2O$ dissolved in 100 ml. water) is added as indicator. When less than 2 mg. citric acid are expected to be present, 5 ml. 0·01042N-$AgNO_3$ are added and back-titrated with 0·01042N-KCNS. When more citric acid is present, larger quantities, or more concentrated solutions, of $AgNO_3$ must be used. 1 ml. 0·01042N-$AgNO_3$ is equivalent to 0·4 mg. anhydrous citric acid.

Discussion of special points

Preliminary ether extraction. Pucher, Wakeman & Vickery (1941) first dry plant material at 80°, acidify the powder with H_2SO_4 and extract the material with ether. It is unnecessary to carry out this procedure with animal tissues, since interfering substances are removed, without loss of citric acid, by deproteinization with metaphosphoric acid, boiling with acid, and treatment with bromine water. In our experience the ether extraction may as a rule be omitted also in the case of plant material.

Deproteinization. For testing the loss of citric acid in the course of deproteinization, a muscle suspension containing 20 g. minced muscle and 100 ml. phosphate buffer was heated to 58° for 1 hr., in order to inactivate aconitase. To 10 ml. muscle suspension were added 5 mg. citric acid (as citrate), a deproteinizing agent and water to make up to 50 ml. The recovery is shown in Table 2. The data show that recovery is complete when metaphosphoric acid is used.

Table 2. *Effect of various deproteinizing agents on the recovery of citric acid*

The concentrations stated refer to final concentrations. Citric acid was determined according to the revised method except that the concentration of H_2SO_4 was 6N during the oxidation of citric acid.

Deproteinizing agent (final concentration)	Recovery (%)
Trichloroacetic acid (3%)	90·1
Tungstic acid (0·03M)	85·5
Trichloroacetic acid (3%) + acetone (20%)	97·8
Metaphosphoric acid (5%)	100·7, 101·3, 98·2

Sources of error. The most frequent source of errors is the faulty manipulation of the petroleum extractions. Too much 'citric acid' is found if the water-washing of the petroleum is incomplete so that halide ions are left in the separating funnels, or if an accidental contamination with volatile halogen compounds (fuming HCl, $CHCl_3$, bromine vapour) occurs. Low values result from loss of petroleum extract during the washings. Special attention should therefore be paid to the careful handling of the extractions.

Recovery of citric acid. Tables 2 and 3 show that the recovery of varying quantities of citric acid from pure solutions and from tissue suspensions is satisfactory. The error does not exceed ±2·5%.

Table 3. *Recovery of citric acid from pure solutions*

Further data as in Table 2.

Citric acid		
Added (mg.)	Recovered (mg.)	Recovery (%)
0·25	0·244	97·6
1·00	0·986	98·6
2·00	2·00	100·0
4·00	4·06	101·5
10·00	10·20	102·0
20·00	20·45	102·25

Specificity of the citric acid determination. Whilst the method of Pucher *et al.* (1934) and the modification described in this paper possess a considerable degree of specificity, it

Table 4. *Specificity of the citric acid determination*

Unless stated otherwise the determination was carried out as described under 'Procedure'.

Substance	Tested (mg.)	'Citric acid' formed (mg.)	Modifications of the method
Group I			
Butyric acid	100	0·6	Solution not boiled before treatment with $KMnO_4$
Crotonic acid	100	7·8	
n-Valeric acid	100	0·4	
n-Caproic acid	100	0·5	
Group II			
Citraconic acid	100	0·4	
Mesaconic acid	100	0·6	
Citramalic acid	100	7·3	
Tricarballylic acid	100	1·6	
Group III			Conc. of H_2SO_4 before addition of $KMnO_4$
Aspartic acid	100	4·2	
Asparagine	100	1·7	
Tyrosine	100	3·8	
β-Hydroxybutyric acid	16·4	7·1	6N
	16·4	3·9	3N
	16·4	2·4	1·5N
	1·64	0·2	3N
	3·28	0·4	3N
	6·56	0·9	3N
	13·12	1·8	3N
Quinic acid	10	1·4	12N
	10	1·0	6N
	10	0·7	3N
	2	0·0	3N
	4	0·1	3N
	8	0·5	3N
	16	0·8	3N
	32	3·9	3N

must be borne in mind that biological material may contain substances other than citric acid which, under the conditions of the estimation, form either pentabromoacetone or other organic bromine compounds reacting like pentabromoacetone (i.e. passing into the petroleum phase and liberating Br ions with Na_2S).

None of the following substances when tested in quantities of 100 mg. yielded 'citric acid': succinic, fumaric, maleic, tartaric, malic, lactic, pyruvic, oxaloacetic, acetoacetic, α-hydroxyglutaric, α-ketoglutaric, acetic, malonic, glutaric, adipic, *iso*citric, *cis*- and *trans*-aconitic, acetone dicarboxylic, tiglic, meconic acids; glucose, glycerol, ethanol, ascorbic acid, glycine, alanine, glutamic acid, phenylalanine, coumarin and coumaric acid.

On the other hand, a number of substances shown in Table 4 simulate citric acid. With reference to the practical significance, these substances are classed in this table in three groups. Group I comprises lower fatty acids. As these are volatile with steam they can be removed, if present, by boiling the acid solution until the characteristic odour has disappeared. The lost water must be replaced from time to time when the volume has fallen below 35 ml. Group II contains compounds which have not been reported to occur in biological material. As a rule, therefore, they need not be considered. Group III includes substances which occur widely in biological material: aspartic acid, asparagine, tyrosine, β-hydroxybutyric and quinic acids. The first two compounds yield small quantities of 'citric acid' and therefore interfere only when their concentration is at least 10 times that of citric acid. Tyrosine can be removed by adjustment of pH to about 5·0, and filtration. β-Hydroxybutyric acid, as far as is known, occurs in animal material only. Since 1 mg. simulates about 0·2 mg. citric acid, the citric acid determination is unreliable when significant amounts of β-hydroxybutyric acid are present.

Modified colorimetric procedure in presence of interfering substances. To overcome this difficulty we make use of the fact that the bromine compound arising from β-hydroxybutyrate (which is not pentabromoacetone) forms on treatment with sulphide a very much weaker yellow colour than pentabromoacetone. Hence β-hydroxybutyric acid interferes relatively little when the colorimetric method of Pucher *et al.* (1936) is used (as already noted by these authors). The procedure for this method follows that described above up to the stage of the washing of the light petroleum extract with water. The organic bromide is then extracted successively with 3, 2 and 1 ml. of alkaline Na_2S solution. The extracts are drained into a 10 ml. measuring cylinder containing 2 ml. dioxan and further dioxan is added to make the volume up to 10 ml. After filtration the colour is compared with a standard prepared from 1 mg. citric acid. The specimen examined should not contain more than 1 mg. citric acid. The colorimetric method should also be used where aspartic acid and asparagine are present in larger quantities.

Quinic acid is known to be a common constituent of plants; Gorter (1909) claims to have detected chlorogenic acid—a depside yielding quinic and caffeic acids on hydrolysis—in the leaves of 98 out of 230 species tested. As quinic acid forms citric acid on treatment with oxidizing reagents (Fischer & Dangschat, 1934) it is not surprising that, when treated with $KMnO_4$ and Br, it forms pentabromoacetone (which was isolated and identified by m.p., mixed m.p. and Br content). Fortunately small amounts of quinic acid—below 5 mg.—yield only negligible quantities of pentabromoacetone. The problem arising from the presence of larger quantities of quinic acid and its precursors has yet to be solved.

It will be noted that both β-hydroxybutyric and quinic acids yield more 'citric acid' the stronger the acidity during the bromination. For this reason a lower degree of acidity was adopted for the routine procedure although the yield of pentabromoacetone from citric acid under these conditions is only 97%. This deficit was corrected by multiplying the result by the factor 1·031.

It follows from Table 4 that caution is needed in interpreting the results of the pentabromoacetone methods. Small quantities of 'citric acid' can be simulated by other substances. However, for the purposes of this work, i.e. for the determinations of *iso*citric and *cis*-aconitic acids, the lack of complete specificity is, as a rule, unimportant as the method essentially depends on the additional citric acid arising on incubation with aconitase.

II. ACONITASE

Measurement of aconitase activity. The activity of various tissue preparations was ascertained by polarimetric determination of the *iso*citric acid formed from *cis*-aconitic acid: 2 ml. freshly prepared 0·5 M-solution of sodium *cis*-aconitate (156 mg. *cis*-aconitic anhydride dissolved with ice-cooling in 1·5 ml. cooled 2 N-NaOH and made up to 2 ml. with water), 0·5 ml. tissue suspension (1 part tissue suspended in 5 parts medium, preserved with octanol), 2·5 ml. 0·1 M-phosphate buffer pH 7·4, and 5 ml. of water were incubated at 40° for 30 min. To stop the reaction 1·25 ml. glacial acetic acid were added. 9 ml. of this mixture, cooled in ice (to retard the formation of coloured phosphomolybdate complexes interfering with polarimetric readings), were mixed with 2 ml. M-Na_3-citrate (see Krebs & Eggleston, 1943*a*) and 9 ml. cooled 30% NH_4-molybdate. The mixture was filtered after addition of about 0·5 g. charcoal. A freshly prepared suspension of pigeon muscle gave the following readings (2 dm. tube):

min.	$\alpha_D^{20°}$
10	−0·68
20	−1·30
30	−1·51
60	−2·32
120	−2·80

Under the conditions of the measurements a reading of one degree indicated the presence of about 16 mg. *iso*citric acid in the 10 ml. of the incubated mixture.

In most of the following experiments (which were carried out under the same conditions) only one reading, after 30 min. incubation, was taken to assess the relative activity of the enzyme. It should be pointed out that the above test conditions are not suitable for the study of the equilibrium between citric and *cis*-aconitic acids because of the instability of the enzyme.

Instability of aconitase in aqueous solution. In aqueous media aconitase proved to be remarkably unstable, as shown in Table 5. Aqueous solutions are therefore unsuitable for the prolonged storage of the enzyme. It is noteworthy that fumarase is much more stable under the same conditions. As shown in Table 6, the aconitase activity of an aqueous extract had fallen to almost nil after 5 days whilst the fumarase activity was unchanged. This

confirms Jacobsohn's conclusion that fumarase and aconitase are different chemical entities (Jacobsohn, Soares & Tapadinhas, 1940).

Table 5. *Aconitase activity of aqueous muscle suspensions*

1 part minced pigeon breast muscle + 5 parts medium; the suspensions were stored in the refrigerator.

Medium	α_D (after 30 min. incubation, degrees) 1 day storage	3 days' storage	7 days' storage
Water	−1·60	−1·30	−0·47
Water	−1·86	—	−0·01
0·1 M-phosphate buffer pH 6·8	−1·50	−0·78	−0·17
0·1 M-phosphate buffer pH 7·4	−1·49	−0·89	−0·61
Acetic acid 1%	—	—	−0·10
$NaHCO_3$ 1%	—	—	−0·43

Table 6. *Comparative stability of aconitase and fumarase in extracts from pigeon breast muscle*

The conditions of testing for fumarase activity were the same as those described for aconitase, except that fumarate was used as the substrate.

		α_D (degrees) Fumarate	*cis*-Aconitate
Fresh muscle extract	15 min.	+4·81	—
	30 min.	+7·79	−1·60
Same muscle extract stored 5 days in refrigerator	15 min.	+4·85	—
	30 min.	+7·82	−0·02

Effect of glycerol on the stability of aconitase. Treatment of freshly minced tissue with 5 vol. of ice-cold ethanol or acetone and rapid drying resulted in the loss of the major part of the original aconitase activity as did drying of muscle *in vacuo* over P_2O_5.

Addition of glycerol had a stabilizing effect on aconitase in tissue extracts, as shown in Table 7. However, the relatively large quantity of glycerol necessary for the stabilization was found to interfere with the citric acid determination, by reducing very large quantities of $KMnO_4$, and we therefore abandoned the use of glycerol extracts.

Table 7. *Stabilizing effect of glycerol on aconitase*

Medium	α_D after 30 min. (degrees) 2 days' storage	5 days' storage	15 days' storage	36 days' storage
Water	−1·86	−0·20	0	0
100% glycerol	—	−1·49	−0·73	−0·69
50% glycerol (aqueous)	−1·91	−1·60	−0·23	−0·06

Stability of aconitase in frozen tissue. Freezing minced pigeon breast muscle in the freezing chamber of a refrigerator, as recommended by Cohen (1940) for the preservation of succinic dehydrogenase, stabilizes aconitase sufficiently to allow the use of the stored material over a period of several weeks. Under the test conditions the following activities were measured:

After 3 days' storage: $\alpha_D^{20°}$ −1·55°.
After 6 days' storage: $\alpha_D^{20°}$ −0·87°.
After 25 days' storage: $\alpha_D^{20°}$ −0·64°.

The procedure for the determination of *iso*citric acid, as described later in this paper, requires an activity equivalent to a rotation of at least −0·50° under the above conditions; the preservation of the enzymes by freezing is therefore satisfactory. Pigeon breast muscle can be replaced by mammalian heart muscle whose aconitase activity is only slightly lower than that of pigeon breast muscle.

Inhibition of aconitase. The effects on aconitase of a number of substances which might occur in material to be tested for *iso*citric acid were examined under standard conditions. As shown in Table 8,

Table 8. *Effect of various substances on the activity of aconitase.*

Standard conditions, frozen minced pigeon breast muscle.

Substance added	α_D (degrees) Control	With added substance
$CaCl_2$ (0·01 M)	−1·52	−1·57
$BaCl_2$ (0·01 M)	−1·52	−1·57
$MgCl_2$ (0·01 M)	−1·52	−1·63
Na-oxalate (0·1 M)	−1·16	−1·48
$CuSO_4$ (10^{-4} M)	−1·06	0
$CuSO_4$ (10^{-5} M)	−1·06	−0·74
HCN (2×10^{-3} M)	−0·89	−0·06
HCN (2×10^{-4} M)	−1·57	−1·29
Na_2S ($1·2 \times 10^{-3}$ M)	−0·89	−0·22
NaCl 2%	−1·47	−0·51
NaCl 4%	−1·47	−0·23
NaCl 8%	−1·47	−0·23
$HgCl_2$ (5×10^{-4} M)	−1·89	−0·02
$HgCl_2$ (5×10^{-5} M)	−1·89	−1·17
NaF (2×10^{-2} M)	−1·54	−1·46
Na-benzoate (4×10^{-2} M)	−1·17	−1·13
Na-iodoacetate (10^{-2} M)	−1·54	−1·36
Na-pyrophosphate ($7·5 \times 10^{-3}$ M)	−1·54	−0·60
Na-cacodylate (10^{-2} M)	−1·72	−1·50
Alloxan (10^{-2} M)	−2·32	−0·50

the chlorides of Ca, Ba and Mg (0·01 M) had no effect. Oxalate somewhat accelerated the reaction, an effect which is so far unexplained. Cu ions, $HgCl_2$, HCN and Na_2S in relatively low concentrations inhibit the enzyme. No significant inhibition was found with various animal and plant tissue extracts, but it is evidently necessary to examine the activity of aconitase in media containing untested substances before the enzyme is used.

III. PROCEDURE FOR THE DETERMINATION OF THE SUM OF (−)*iso*CITRIC AND *cis*-ACONITIC ACIDS

The material containing suitable quantities (0·5–20 mg.) of the acids to be determined should be neutral and must not contain substances which precipitate protein or otherwise inhibit aconitase.

30 ml. of the neutral solution or suspension, or smaller quantities diluted to 30 ml., are mixed in a 50 ml. graduated stoppered measuring cylinder with 5 ml. phosphate buffer (pH 7·4; 0·1M) and 5 ml. 'aconitase' (1 g. frozen pigeon breast muscle or mammalian heart muscle suspended in 5 ml. water). The mixture is placed in a water-bath at 40° for 60 min. and occasionally shaken. In a second 30 ml. sample of the material aconitase is replaced by 5 ml. water; it is otherwise treated in the same way. At the end of the incubation 20% metaphosphoric acid is added to make the volume up to 50 ml. The precipitate is filtered off into a graduated cylinder, the volume of the filtrate is measured and citric acid is determined as described in § I.

If 30 ml. of the solution or suspension do not contain enough of the acids to be determined, larger quantities are taken and the amounts of buffer, aconitase and metaphosphoric acid are increased in proportion.

Lacto-*iso*citric acid which may be present in dried material derived from certain plants does not react with aconitase (see later). If the lactone is to be included in the determination, it must be hydrolyzed before treatment with aconitase as described in § V.

Calculation. Let a = the initial amount of citric acid in the sample (as determined in the control sample not treated with aconitase); b = the amount of citric acid in the sample after incubation with aconitase; x = the sum of (−)*iso*citric and *cis*-aconitic acids in the sample. The equilibrium mixture of the three tricarboxylic acids contains (approximately) 89·5% citric acid, 6·2% *iso*citric acid and 4·3% aconitic acid (Krebs & Eggleston, 1943*a**). The concentration of *iso*citric acid is thus 6·93% and that of *cis*-aconitic acid 4·81% of the concentration of citric acid when the equilibrium is established. The final concentration of *iso*citric acid in the sample, after treatment with aconitase, is therefore 0·0693b, that of *cis*-aconitic acid 0·0481b. The sum of the initial amounts of the three acids equals the sum of the final amounts:

$$a + x = b + 0{\cdot}0693b + 0{\cdot}0481b,$$

or

$$x = 1{\cdot}117b - a. \qquad (1)$$

a, b and x in (1) refer to molecular equivalents, but not to weights. If a and b are expressed in mg., x is the sum of (−)*iso*citric and *cis*-aconitic acids expressed in mg. of *iso*citric (or citric) acid.

Accuracy of the determination of the sum of (−)*iso-citric and* cis*-aconitic acids.* The accuracy of the method largely depends on the accuracy with which the difference between a and b can be measured. The larger the amounts of citric acid relative to the amounts of *iso*citric and *cis*-aconitic acids in the sample, the greater are the errors in the determination of the difference. If the concentration of citric acid is 10 times above that of the other two acids the method becomes unreliable. If, on the other hand, the other acids prevail, their determination may be as accurate as the citric acid determination, viz. ±3%.

The accuracy would also be affected by errors in the figures for the composition of the equilibrium mixture of the three acids in the presence of aconitase. The data so far available (see Krebs & Eggleston, 1943*a*; Martius & Leonhardt, 1943) are only approximate, and the error in figure for *iso*citric acid may even be of the order of 15%; for the published values given for the specific rotation of the molybdate complex of *iso*citric acid—on which calculations of the *iso*citrate concentration have been based—differ considerably; Martius (1938) found −413°, Pucher (1942) −363°. The error in the figure for *cis*-aconitic acid is probably no more than 5%. However, the calculation according to the formula (1) does not directly depend on the accuracy of these data, but on the accuracy of the figure for the concentration of citric acid in the equilibrium mixture. Errors of 15% for *iso*citric acid and 5% for *cis*-aconitic acid, even if they were additive, would cause an error of only 1·1% in the value of citric acid (on the highly probable assumption that the equilibrium mixture contains no other acids). The factor 1·117 used in formula (1) may therefore be taken to be accurate within 1·1%.

Recovery of pure iso*citric and* cis*-aconitic acids*

*Preparation of synthetic lacto-*iso*citric acid.* To test the method synthetic preparations of the two acids were used. Fittig & Miller (1889) prepared *iso*citric acid and lacto-*iso*citric acid from trichloromethylparaconic acid by converting the latter first into Ba-*iso*citrate and decomposing the latter with H_2SO_4. According to Nelson (1930) the over-all yield is 80%. We simplified the procedure by omitting the isolation of the Ba salt.

Pure trichloromethylparaconic acid (24·7 g. 0·1 g.-mol.) (m.p. 97°; Cl content: found, 43·4%; calc. 43·0%) was dissolved in 305 ml. 2N-NaOH (A.R.) (slight excess over 0·6 g.-mol.). The solution was kept for 20 min. at 60°. Under these conditions the reaction

$$C_6H_5O_4Cl_3 + 6NaOH = Na_3C_6H_5O_7 + 3NaCl + 3H_2O$$

proceeds quantitatively as judged by the appearance of chloride ions and the disappearance of alkali. Then 32 ml. 10N-HCl (A.R.) were added (slight excess over 0·3 g.-mol.) and the solution was evaporated on a steam-bath until it was almost dry. The dish was then completely dried in a vacuum desiccator over P_2O_5 and NaOH. This required 1–2 days, and the material usually became crystalline in the desiccator. The perfectly dry mixture of crystals of lacto-*iso*citric acid and NaCl was treated with several 50 ml. portions of hot ethylacetate to dissolve the lacto-*iso*citric acid. The combined extracts were filtered and evaporated to about 80 ml. Crystals of lacto-*iso*citric acid readily

* Martius & Leonhardt (1943) found independently and with partly different methods 89·2% citric acid; 7·7% *iso*citric acid; 3·1% *cis*-aconitic acid.

appeared on cooling and the crop was much increased by gradual addition of chloroform; it was recrystallized from ethylacetate, m.p. 163°. Yield 11·5 g. (60% of calc.).

Preparation of cis-*aconitic acid.* *cis*-Aconitic anhydride, m.p. 78°, prepared according to Malachowski & Maslowski (1928), contained an impurity even after repeated recrystallization from benzene, as indicated by the yellow colour arising on addition of alkali to the aqueous solution and by incomplete yields of citric acid on treatment with aconitase (Table 9). A pure sample of the acid was prepared as follows: 3·12 g. (0·02 g.-mol.) *cis*-aconitic anhydride (m.p. 77°) were dissolved in about 25 ml. warm ethyl acetate which had been saturated with water at room temperature. Chloroform was added until the solution became turbid. The acid crystallized in white needles, m.p. 130° (5° higher than reported by Malachowski & Maslowski, 1928). Analysis (Drs Weiler and Strauss, Oxford): found: C, 40·97; H, 3·22%. Calc. C, 41·38; H, 3·47%. Alkaline solutions of the acid are colourless. The crystals are stable for at least 3 months at room temperature with exposure to air.

Recovery. Table 9 shows that recovery from pure solution is satisfactory, the error of a single analysis being no more than 4%.

Table 9. *Recovery of* iso*citric acid and* cis*-aconitic, acid from pure solutions*

Substrate added (mg.)		*iso*Citric or *cis*-aconitic acids: Expected* (mg.)	Found† (mg.)	Recovery (%)
*r-iso*Citric acid lactone‡	10·9	6·08	6·26	103
	11·35	6·35	6·28	99
	19·2	9·60	9·76	102
cis-Aconitic anhydride§	7·80	8·70	8·30	95
	7·80	8·70	8·03	92
	3·90	4·35	4·16	96
	3·90	4·35	4·02	92
cis-Aconitic acid	9·45	9·45	9·28	98
	8·7	8·7	8·63	99
	8·7	8·7	8·35	96
	8·7	8·7	8·50	98

* In the case of synthetic *iso*citric acid the expected yield is 50% of the total acid (Martius, 1938).

† A correction was made allowing for a yield of 97% of citric acid.

‡ Lacto-*iso*citric acid was hydrolyzed for 20 min. at 40° with 0·01 N-NaOH.

§ Probably impure; see text.

IV. SOME REACTIONS OF *cis*-ACONITIC AND *iso*CITRIC ACIDS

As a preliminary to work on the separate determination of the two acids it was necessary to study: (*a*) the stability of *cis*-aconitic acid and the interconversion of the *cis* and *trans* acids; (*b*) the interconversion of *iso*citric and lacto-*iso*citric acids; (*c*) the behaviour of lacto-*iso*citric acid towards enzymes.

Known quantities of the acids were exposed to varying conditions and their concentration was measured by the method described in the preceding section. The method permits the determination of *cis*-aconitic acid in the presence of *trans*-aconitic acid, and of *iso*citric acid in the presence of its lactone, for *trans*-aconitic acid and lacto-*iso*citric acid do not react in the presence of aconitase to form citric acid.

Stability of cis*-aconitic acid in aqueous solutions.* Few details are known about the stability of *cis*-aconitic acid and its salts. Malachowski & Maslowski (1928) state that in aqueous solution *cis*-aconitic acid is completely converted into the *trans* form in 5 min. at 100° and that at 60° the major part of the acid is transformed in 10 min. Our data (Table 10)

Table 10. *Stability of* cis*-aconitic acid and its salts in aqueous solutions*

Medium containing 8·7 mg. *cis*-aconitic acid	Temp.	Time	*cis*-Aconitic acid found (mg.)	*cis*-Aconitic acid removed, as % of added acid
5 ml. water	100°	10 min.	7·66	11·9
1 ml. water	40°	3 days	1·14	86·7
5 ml. 0·1 N-HCl	100°	1 hr.	0·92	89·3
,,	100°	2 hr.	0·79	90·8
5 ml. 0·5 N-HCl	100°	10 min.	2·16	74·9
10 ml. N-HCl	40°	2 days	1·34	84·5
5 ml. water; neutralized with Na_2CO_3	40°	24 hr.	8·08	5·9
,,	40°	5 days	7·53	13·4
,,	100°	2 hr.	7·27	16·4
10 ml. M-Na_2CO_3	40°	2 days	6·80	20·9
5 ml. 0·5 M-Na_2CO_3	100°	10 min.	6·85	20·2
5 ml. 0·1 N-NaOH	100°	1 hr.	1·83	78·7
5 ml. N-NaOH	100°	10 min.	1·25	85·5

roughly confirm these statements, but they show that the neutral solutions of the Na salt are relatively stable. After 24 hr. at 20° or 39° less than 4% disappeared. In 5 days at 39° about 10% disappeared. Even at 100° the change is slow: after 2 hr. 12% were lost. In acid or alkaline solutions *cis*-aconitic acid is less stable. In 0·1 N-HCl about 90% and in 0·1 N-NaOH about 80% disappeared at 100° within 1 hr. Under none of the test conditions, it will be noted, was the conversion into the *trans* form complete.

Conversion of trans*-aconitic acid into* cis*-aconitic acid.* A specimen of 'aconitic acid' obtained from British Drug Houses Ltd., m.p. 191°, was used. It contained less than 0·1% citric acid. A freshly prepared aqueous solution was free from *cis*-aconitic acid. Heating aqueous solutions of the acid and its salts to 100° produced considerable quantities of *cis*-aconitic acid, as shown in Table 11, the yields exceeding under some conditions 30%. The extent of the conversion depends much on the H^+ concentration. Virtually no *cis*-aconitic acid is formed in neutral solutions, but the conversion takes place on both sides of the neutral point.

When aqueous solutions of *trans*-aconitic acid are dried at 100° and kept at this temperature for some time much *cis*-aconitic acid is formed under certain conditions as shown

Table 11. *Conversion of* trans-*aconitic acid into* cis-*aconitic acid in aqueous solutions*

trans-Aconitic acid added (mg.)	Medium	Temp.	Time (min.)	*cis*-Aconitic acid formed* (mg.)	*cis*-Aconitic acid formed, as % of added acid
69·6	5 ml. water	100°	30	8·46	12·1
69·6	5 ml. water; neutralized with $NaHCO_3$	100°	30	0·14	0·2
348	5 ml. 0·1 N-HCl	100°	30	39·3	11·3
348	,,	100°	60	38·8	11·1
34·8	,,	100°	60	11·8	33·9
17·4	,,	100°	60	2·88	16·5
8·7	,,	100°	60	1·89	21·7
348	5 ml. N-HCl	100°	30	39·5	11·5
69·6	0·8 ml. N-$NaHCO_3$ + 2 ml. water	100°	300	16·3	23·4
69·6	0·4 ml. N-$NaHCO_3$ + 2 ml. water	100°	300	20·5	29·5
69·6	5 ml. N-NaOH	100°	30	7·33	10·8
69·6	0·8 ml. N-$NaHCO_3$ + 2 ml. water	140°†	120	10·4	14·9
69·6	0·4 ml. N-$NaHCO_3$ + 2 ml. water	140°†	120	2·82	4·1

* Controls showed that no citric acid was formed.

† Heated in autoclave, pressure 40 lb./sq. in.

in Table 12. The neutral salt and the free acid formed no *cis*-isomeride, the monosodium salt 31·6%, the disodium salt 12·1%. No *cis*-aconitic acid was formed when the solution of the Na and Na_2 salts were dried *in vacuo* at room temperature. These observations are relevant when plant material is prepared for analysis (§ VI).

Table 12. *Conversion of* trans-*aconitic acid into* cis-*aconitic acid on evaporation of aqueous solutions*

Treatment of 69·6 mg. *trans*-aconitic acid dissolved in 2 ml. water	*cis*-Aconitic acid formed (mg.)	*cis*-Aconitic acid formed, as % of added acid
Dried at 100°; kept dry at 100°, 2 hr.	0·2	0·3
+0·4 ml. M-$NaHCO_3$ (to form mono-Na salt); dried at 100°; kept dry at 100°, 2 hr.	22·0	31·6
+0·8 ml. M-$NaHCO_3$ (to form di-Na salt); dried at 100°; kept dry at 100°, 2 hr.	8·45	12·1
+0·4 ml. M-$NaHCO_3$ (mono-Na salt); dried in vacuum desiccator, 24 hr., room temp.	0·1	0·14
+0·8 ml. M-$NaHCO_3$ (di-Na salt); dried in vacuum desiccator, 24 hr., room temp.	0	0
+1 2 ml. M-$NaHCO_3$ (to form tri-Na salt); kept dry at 100°, 2 hr.	0·1	0·14

The ready conversion of the *trans* into the *cis* form may be responsible for the fact that the m.p. of pure *trans*-aconitic acid shows considerable variations. Malachowski & Maslowski (1928) remark that specimens melting at 182·5 and 194·5° both gave correct analytical values.

Hydrolysis of iso*citric acid lactone.* When 0·2 ml. *iso*citric acid lactone was added to 3 ml. 0·02 M-$NaHCO_3$, saturated with 5% CO_2 in O_2, 127 μl. CO_2 were liberated (measured manometrically). The evolution of CO_2 was completed after 5 min. at 40°. During the following 60 min. no measurable quantities of CO_2 were evolved. On the assumption that the lactone is not hydrolyzed, 125 μl. CO_2 were calculated. Thus at 40° and pH 7·3 the rate of hydrolysis of the lactone is insignificant.

The rate of hydrolysis by alkali under other conditions was measured in the following way: a known weight of lactone (about 20 mg.) was dissolved in 0·01 N-$NaHCO_3$ or NaOH. The solutions were kept for varying periods at different temperatures and at the end of the period 3 ml. were pipetted into a conical Warburg vessel provided with a side-arm containing 0·3 ml. 10% H_2SO_4. The gas space of the vessel was filled with a gas mixture containing 5% CO_2. The CO_2 combined with the excess of alkali in the solution and the bicarbonate content of the solution was measured manometrically in the usual way. The difference in the amount of bicarbonate between the control containing no *iso*citric acid and the *iso*citrate solution indicates the amounts of acidic groups. The method permits the measurement of the degree of the hydrolysis of the lactone on 1–2 mg. material with an accuracy of about 2%.

In bicarbonate solution (pH between 7 and 8) 18% of the lactone was hydrolyzed in 30 min. at 100°. In dilute NaOH (initial concentration 0·01 N and the excess NaOH after complete hydrolysis being about 0·003 N) the hydrolysis was complete within 10 min. at 100°. At 40° the following rate was observed, when 34·8 mg. lacto-*iso*citric acid were dissolved in 20 ml. 0·04 N-NaOH (required for neutralization of *iso*citric acid, 20 ml. 0·03 N; thus excess of alkali after neutralization was 0·01 N). After 10 min. 91·2%, after 30 min. 98·5%, after 90 min. 99·2% were split. To effect hydrolysis it is therefore sufficient to add an excess of 0·01 N-NaOH and to keep the solution for 30 min. at 40°.

The lactone is also readily hydrolyzed, though not to completion, on heating in acid solution; 12·65 mg. synthetic lactone were dissolved in 10 ml. 0·5 N-HCl and heated for 30 min. at 100°. After cooling, the solution was neutralized with M-$NaHCO_3$. The *iso*citric acid determination yielded 5·36 mg. (calc. for complete hydrolysis, 6·37 mg.).

*Behaviour of lacto-*iso*citric acid towards enzymes.* As already reported by Martius (1938) and confirmed by Table 8, 50% of synthetic *iso*citric acid reacts with aconitase. Lacto-*iso*citrate forms no citric acid when incubated with muscle or liver suspensions at pH 7·4. These tissues thus do not contain enzymes capable of hydrolyzing the lactone.

V. SEPARATE DETERMINATION OF (−)*iso*-CITRIC ACID AND *cis*-ACONITIC ACID

The separate determination of these two acids met with unexpected difficulties when the following principle was applied. In a measured portion of the material the sum of the acids was measured. A second portion was acidified and evaporated to dryness. As shown in Table 13, *cis*-aconitic acid is quantitatively removed from pure solutions by this treatment and it was expected that thus the remaining *iso*citric acid could be determined separately; the difference between the results of the first and second analyses would then be the amount of *cis*-aconitic acid in the sample. However, whilst it is possible to remove *cis*-aconitic acid from pure solutions, we find that its conversion into the *trans* form is not quantitative when certain other substances are present; among these are citric acid, *iso*citric acid and plant tissues. Under the conditions tested, between 4 and 16% of the added *cis*-aconitic acid remained unchanged. We have not yet found a method which removes *cis*-aconitic acid quantitatively without affecting the concentration of *iso*citric acid, and as yet the following procedure is the best available for the separate determination of the two acids. The method gives correct values for the sum of the acids, or for the individual acids when present alone. When both are present in about equal quantities the values for *iso*citric acid are between 10 and 20% too high, those for *cis*-aconitic between 10 and 20% too low.

Procedure. In the first two measured portions the sum of the two acids is analyzed as already described. A further portion, in a porcelain evaporating dish (about 8 cm. diameter), is acidified with 1 ml. conc. HCl. The dish is heated on a steam-bath for 30 min. after the evaporation of the water. After cooling, the material is dissolved or suspended in 10–15 ml. water, quantitatively transferred to a 50 ml. stoppered measuring cylinder and made alkaline to phenol red with 2N-NaOH. A further excess of 1 ml. 2N-NaOH is added to hydrolyze the *iso*citric lactone. Incubation at 40° completes the hydrolysis within 30 min. The next step is the adjustment of the pH to 7·2–7·4 (orange to phenol red) with M-H_3PO_4. The material is diluted to 35 ml. and is then ready, without further addition of buffer, for treatment with aconitase as described in § III.

Calculation. Let a = the initial amount of citric acid; b = the amount of citric acid after treatment with aconitase in the sample containing *cis*-aconitic acid; b' = the final amount of citric acid after treatment with aconitase in the sample from which *cis*-aconitic acid has been removed; y = the amount of (−)*iso*citric acid in the sample; z = the amount of *cis*-aconitic acid in the sample. Then

$$y + z = 1{\cdot}117b - a \text{ (as previously shown);}$$
$$y = 1{\cdot}117b' - a;$$
$$z = 1{\cdot}117\,(b - b').$$

If a, b and b' are expressed in mg. (instead of mM.) the last equation becomes

$$z = 1{\cdot}102\,(b - b').$$

Recovery. Applying the foregoing procedure to pure mixtures of (−)*iso*citric and *cis*-aconitic acids, we found the following recovery:

Added (mg.)			Recovered (mg.)		
(−)*iso*-Citric acid*	*cis*-Aconitic acid	Sum	Sum	*iso*-Citric acid	*cis*-Aconitic acid†
5·39	4·01	9·40	9·48	6·05	3·43
4·80	5·72	10·52	10·52	5·58	4·94

* Added as *r-iso*citric acid.
† Expressed in mg. (equimolar) citric acid.

Whilst recovery of the sum of the two acids was satisfactory the yield of *iso*citric acid was too high and that of *cis*-aconitic acid too low. In other words, part of the *cis*-aconitic acid appeared as *iso*citric acid. This was not due to a formation of *iso*citric (or citric) acid from aconitic acid during the treatment with hot HCl and dry heat, but to the incomplete conversion of the *cis* into the *trans* form.

Further data showing the effect of various substances on the conversion of *cis*- into *trans*-aconitic acid are recorded in Table 13. Only when no other substances are present is *cis*-aconitic acid quantitatively removed on evaporation of the acid solution.

Table 13. *Removal of* cis*-aconitic acid in the presence of other substances by treatment with* HCl *and evaporation to dryness*

For details of procedure see text

cis-Aconitic acid added (expressed in mg. of equimol. citric acid)	Other substances added	'*iso*Citric acid' found (mg.)	*cis*-Aconitic acid not converted into *trans* form (as mg. citric acid)	*cis*-Aconitic acid appearing as '*iso*citric acid' (as % of added *cis*-aconitic acid)
6·25	None	0	0	0
5·60	None	0	0	0
6·25	5·50 mg. (−)*iso*citric acid	6·01	0·51	8·1
6·35 *	6·10 mg. citric acid	0·37	0·37	5·8
6·35 *	6·10 mg. citric acid	0·33	0·33	5·2
6·33	5·55 mg. (−)*iso*citric acid	6·30	0·75	11·9
6·33	5·85 mg. (−)*iso*citric acid	6·42	0·57	9·0
12·66	5·85 mg. (−)*iso*citric acid	6·48	0·63	3·5
6·57	400 mg. dried foxglove leaves containing 3·50 mg. citric acid and 7·57 mg. *iso*citric acid	8·83	0·96	14·6
6·30	400 mg. dried leaves of *Delphinium* containing 2·35 mg. citric acid	1·02	1·02	16·2

* Duplicates.

As yet the available evidence does not indicate that *cis*-aconitic acid occurs in biological material except in very small quantities as an accompaniment of citric acid in animal tissues (and under artificial experimental conditions). In many cases, therefore, *iso*citric acid will be present alone, so that no error will result from incomplete removal of *cis*-aconitic acid.

Specificity of the method. The specificity of the determination of *iso*citric and *cis*-aconitic acids depends on the specificity of the aconitase preparation used. The two tricarboxylic acids are the only substances at present known to form citric acid under the conditions of the test. Although fresh muscle can under certain conditions form citrate when oxaloacetate is available, this does not apply to dilute suspensions of frozen muscle as used in the above procedure. There is therefore good reason to assume that the specificity of the method is very high, if not absolute. Thus if incubation with aconitase increases the amounts of citrate this increase is in all probability due to *iso*citrate or *cis*-aconitate. If, however, citric acid is present in the material, an error can arise from the fact that the specificity of the determination of citric acid is not absolute. The calculation of the amounts of *iso*citric and *cis*-aconitic acid is based on the assumption that addition of aconitase removes 10·5% of the preformed citric acid. Any 'pseudo' citric acid would not be attacked by aconitase and therefore simulates *iso*citric or *cis*-aconitic acid in an amount equivalent to 10·5% of the 'pseudo' citric acid.

It follows that if the citric acid determination shows no increase after incubation or with aconitase such values of '*iso*citric' or '*cis*-aconitic' acids as may be arrived at by applying formula (1) are somewhat uncertain.

VI. PREPARATION OF BIOLOGICAL MATERIAL

Extracts from animal tissues should be neutralized and, as already pointed out, must not contain substances which inhibit aconitase. Animal tissues should be minced or otherwise broken up, but it is not necessary to remove the tissue particles or proteins before the addition of aconitase. The methods as described can therefore be applied to suspensions of animal tissues without modification.

Since plants may contain *trans*-aconitic acid—it has been isolated from many species*—special care is required to avoid the artificial formation of *cis*-aconitic acid from the *trans* form. Drying of plant material at high temperatures, as practised for general analytical purposes by Pucher *et al.* (1941), is evidently unsatisfactory (see Tables 11 and 12). On the other hand, drying and pulverizing plant tissue is advantageous because it disrupts the tissues and facilitates the extraction of the cell contents. We dry the material—5–10 g. wet weight—in a vacuum desiccator over P_2O_5; the dried tissue is either ground in a mortar, or, in the case of fibrous material, chopped up with scissors. Portions of suitable size varying between 50 and 500 mg. according to the tricarboxylic acid content are used for the analysis. They are suspended in water and then treated in the same way as suspensions of animal tissues.

An ether extract prepared according to Pucher *et al.* (1941) may be used instead of a suspension of dried material.

VII. SOME APPLICATIONS OF THE METHOD

*iso*Citric acid in plants. Application of the method confirms Pucher & Vickery's (1942) discovery that the so-called 'Crassulacean malic acid' is (−)*iso*-citric acid. Table 14 shows that the leaves of the seven species examined contained between 3·8 and 12·7% *iso*citric acid (calculated on a dry-weight basis). *cis*-Aconitic acid was not present in measurable quantities.

No appreciable amount of *iso*citric or *cis*-aconitic acid was found in the leaves of the following species: *Epilobium angustifolium* L., *Saxifraga umbrosa* L., *Cucumis sativa* L., *Geranium pratense* L., *Geranium*

* It is not impossible that *trans*-aconitic acid isolated from plants arises from the *cis*-acid during the process of isolation. This question is under investigation.

Table 14. *iso*Citric acid in the leaves of Crassulaceae

The leaves were collected between 6 and 7 a.m. during 19 June and 3 July 1943 with the exception of those of *Sedum praealtum* which were collected on 5 May 1944.

Species	Dry leaf analyzed (mg.)	Citric acid found (mg.)	*iso*Citric acid found (mg.)	Percentage content (calculated for dry wt.) Citric acid	*iso*Citric acid
Bryophyllum calycinum Salisb.	50	1·94	5·46	3·88	10·92
Sempervivum tectorum L.	200	4·60	14·20	2·30	7·10
Sempervivum montanum L.	100	2·29	12·73	2·29	12·73
Sedum album L.	200	2·76	14·80	13·8	7·40
Sedum acre L.	80	1·27	3·01	1·57	3·76
Sedum praealtum Sol.	100	2·29	10·80	2·29	10·80
Escheveria sp.	50	0·465	2·42	0·93	4·84

sanguineum L., *Pelargonium* sp., *Solanum tuberosum* L. ('Duke of York'), *Rubus fruticosus* L., *Helianthus annuus* L., *Lysimachia nummularia* L., *Vicia faba* L. (broad bean).

Considerable quantities of *iso*citric acid (1–3 % of the dry weight) were found in the leaves of the foxglove (*Digitalis purpurea* L.). A sample of leaves collected from first-year plants in June gave the following results:

Before treatment with aconitase:	3·18 mg.	Citric acid in 400 mg. dry leaves
After treatment with aconitase:	9·88 mg.	
After removal of *cis*-aconitic acid:		
Before treatment with aconitase:	3·20 mg.	
After treatment with aconitase:	9·71 mg.	
Thus Citric acid content:	0·80 %	
*iso*Citric acid content:	1·93 %	
cis-Aconitic acid content:	Negligible	

Diurnal changes in Bryophyllum calycinum *and* Sedum praealtum. It has long been known that the concentration of titratable acid of the leaves of *Bryophyllum* and other Crassulaceae increases during darkness and this has been attributed mainly to changes in the malic acid concentration (see Bennet-Clark, 1933). Since the term 'malic acid' in this context was usually meant to include 'Crassulacean malic acid' it seemed of interest to re-examine the nature of the acid changes.

Tables 15 and 16 confirm (see Bennet-Clark, 1933) that the diurnal changes chiefly, though not exclusively, affect malic acid. In *Bryophyllum* 83 % of the changes in concentration of organic acid is due to malic acid, 8 % to *iso*citric acid and 9 % to citric acid; in *Sedum* 79 % is due to malic acid, 9 % to *iso*citric acid and 12 % to citric acid. It will be noted that the changes in organic acid concentration considerably exceed the changes in titratable acidity. As already pointed out by Bennet-Clark this indicates that part of the organic acid in the leaves is ionized.

SUMMARY

1. A modification of the volumetric citric acid determination of Pucher *et al.* (1934) is described and its specificity is examined. β-Hydroxybutyric and quinic acids are the most common substances in biological material which simulate citric acid.

Table 15. *Diurnal changes in the composition of the leaves of* Bryophyllum calycinum

'Light' refers to leaves collected at 3 p.m. on a sunny day (23 June 1943); 'dark' refers to leaves collected from the same plant after it had been kept in complete darkness at 23° for 16 hr.

	100 mg. dry* leaves contain				
	Light		Dark		Changes caused by transfer to darkness
	(mg.)	(μ-equiv.)	(mg.)	(μ-equiv.)	
Total titratable acidity	—	96	—	205	+109 μ-equiv.
*iso*Citric acid	10·2	159	10·9	171	+ 12 μ-equiv.
Citric acid	3·0	48	3·9	61	+ 13 μ-equiv.
Malic acid	4·5	67	12·8	191	+124 μ-equiv.
Succinic and fumaric acids	Trace	—	Trace	—	—
Sum of *iso*citric, citric, malic acids	—	274	—	423	+149 μ-equiv.
Total carbohydrates†	36·0	—	29·8	—	− 6·2 mg.

* The dry weight was 6·98 % of the wet weight.
† Reducing sugar after hydrolysis, expressed as glucose; according to Bennet-Clark (1933) a proportion of the carbohydrate is probably sedoheptose.

Table 16. *Diurnal changes in the composition of the leaves of* Sedum praealtum

'Light' refers to leaves collected at 2.15 p.m., 5 May 1944, after direct exposure to bright sunlight throughout the day; 'dark' refers to leaves collected from the same plant after it had been kept in complete darkness for 18 hr. at 25°.

	100 mg. dry* leaves contain				
	Light		Dark		Changes caused by transfer to darkness
	(mg.)	(μ-equiv.)	(mg.)	(μ-equiv.)	
Total titratable acidity	—	22	—	162	+140 μ-equiv.
*iso*Citric acid	12·5	195	13·7	214	+ 19 μ-equiv.
Citric acid	0·9	14	2·4	38	+ 24 μ-equiv.
Malic acid	5·3	79	15·9	238	+159 μ-equiv.
Sum of *iso*citric, citric, malic acids	—	288	—	490	+202 μ-equiv.
Total carbohydrate†	18·2	—	9·6	—	− 8·6 mg.
'Glucose'‡	8·4	—	2·2	—	− 6·2 mg.

* The dry weight was 4·84 % of the wet weight. † Reducing sugar after acid hydrolysis.
‡ Reducing sugar (Hagedorn-Jensen) expressed as glucose (the sugar is largely sedoheptose (Bennet-Clark, 1933)).

When β-hydroxybutyric acid is present the colorimetric method of Pucher *et al.* (1936) is preferable; the acid does not interfere with this method as long as its concentration is not more than 10 times higher than that of citric acid.

2. For the determination of (−)*iso*citric acid and *cis*-aconitic acids the material is divided into three equal portions. The first is treated with aconitase, and the citric acid formed from *iso*citric acid and *cis*-aconitic acid under the influence of this enzyme is determined. In the second, citric acid is determined directly. From the third portion *cis*-aconitic acid is removed by conversion into the *trans* form before it is treated in the same way as the first sample. The difference in the citric acid content between the first and second portions indicates the sum of (−)*iso*citric and aconitic acids, the difference between the second and third the amount of (−)*iso*citric acid, the difference between the first and third the amount of *cis*-aconitic acid.

3. An aconitase preparation suitable for analytical work is described and its properties are examined. Unlike fumarase, aconitase is unstable in aqueous tissue extracts. The enzyme is fairly stable in frozen tissue; it is inhibited by Cu ions, $HgCl_2$, HCN and H_2S.

4. Methods for the preparation of *r*-lacto-*iso*citric and of *cis*-aconitic acids are described.

5. The stability of *cis*-aconitic acid and the interconversion of *cis*- and *trans*-aconitic acids is investigated, the measurements being based on the fact that aconitase reacts with the *cis* form only. *cis*-Aconitic acid was found to be much more stable than data in the literature suggest; e.g. in neutral solution only 4% disappeared in 24 hr. at 20°; at 100° 12% was lost in 2 hr. *trans*-Aconitic acid is converted into the *cis* form in acid or alkaline solution at 100°; the yields may exceed 30%.

6. Pucher & Vickery's (1942) discovery that the 'Crassulacean malic acid' is (−)*iso*citric acid is confirmed. Leaves of *Bryophyllum*, *Sedum*, *Sempervivum* and *Escheveria* were found to contain between 3·8 and 12·8% (−)*iso*citric acid (calculated for dry weight).

7. The diurnal changes in titratable acidity in leaves of *Bryophyllum calycinum* and *Sedum praealtum* were found to be chiefly due to changes in the malic acid concentration.

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Human-milk Fat

2. COMPONENT GLYCERIDES

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The component acids of human-milk fat were shown in the preceding part of this series (Hilditch & Meara, 1944) to differ in several respects from those of cow-milk fat. Although palmitic, stearic and myristic acids are present in proportions similar to those in which they occur in cow-milk fats, the lower saturated acids are quite different, comprising only lauric and small proportions of decanoic acid. Again, in the unsaturated series, although oleic acid forms 30–37% (by wt.) of the total fatty acids, it is