The Effect of Citrate on the Rotation of the Molybdate Complexes of Malate, Citramalate and Isocitrate

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Extracts of liver and muscle convert citric acid into laevo-rotatory isocitric acid [Martius, 1938]. As the rotation of isocitric acid, like that of other α-hydroxy acids, is much increased by $(NH_4)_2MoO_4$*, the polarimeter can be used to measure low concentrations of isocitric acid in tissue extracts [Martius, 1938; Jacobsohn, 1940].

On applying this method to plant extracts we met with an unexpected complication. Aqueous extracts of rhubarb petioles showed a dextrorotation with molybdate, owing to the presence of *l*(−)malic acid. When the extracts were mixed with citrate the dextrorotation increased very considerably, e.g. from +1·75° to +3·25°. Investigation of this effect showed that the increase was not caused by an enzymic process; it proved to be due to an effect of citrate on the dextrorotation of the *l*(−)malate-molybdate complex. Under the test conditions $[\alpha]_D^{20°}$ was +716° for *l*(−)malic acid in the presence of $(NH_4)_2MoO_4$ and +1340° for *l*(−)malic acid in the presence of $(NH_4)_2MoO_4$ and citrate. Citrate was found also to change the rotation of the molybdate complexes of two substituted malic acids, viz. citramalic and isocitric acids.

The effect of citrate on the rotation of the malate-molybdate complex has already been observed by Auerbach & Krüger [1923] who realized that citrate interferes with the polarimetric determination of malic acid. Obviously citrate also interferes with the Martius polarimetric isocitric acid estimation. Isocitric acid is frequently accompanied in biological material by citric acid and many of the data calculated from polarimetric readings by previous authors therefore require revision.

EXPERIMENTAL

Materials. Samples of *l*(−)malic acid obtained from Messrs Schering-Kahlbaum and from Dr W. Fraenkel, Factory M 3, Treforest Estate, near Cardiff, were used, both giving identical results. Na-*l*(−)α-hydroxyglutarate was prepared according to Fischer & Moreschi [1912], citramalic acid according to Michael & Tissot [1892]; it was resolved with brucine according to Marckwald & Axelrod [1899]; the sample of *l*(+)lactic acid used was obtained from Messrs Schuchardt. Pure optically active isocitric acid was not available; a solution of the active acid was prepared by bacterial resolution of Na isocitrate synthesized according to Fittig & Miller [1889] and Wislicenus & Nassauer [1895].

Calculations. The specific rotation was calculated according to the formula $[\alpha]_D = 100/l \times c$. The values of *c* refer to the concentrations of the free organic acid (g. %), and not to the concentration of the molybdate complexes.

* This formula is used, for brevity's sake, to denote commercial ammonium molybdate.

RESULTS

Effect of citrate on the rotation of extracts of rhubarb petioles

Petioles of forced rhubarb were minced and then ground to a pulp in a mechanical mortar; half volume of H_2O was added during the grinding. Ten ml. pulp, 5 ml. *M* Na_3-citrate and 1 ml. 3 *M* acetate buffer of *p*H 4·0 were mixed and allowed to stand for 19 hr. at 18°, octanol serving as an antiseptic. Then 1·6 ml. glacial acetic acid and 14·4 ml. freshly prepared 29 % $(NH_4)_2MoO_4$ were added, the insoluble material filtered off and the filtrate cleared with a small quantity of charcoal.

A control tube contained water instead of the citrate solution. The polarimeter readings (2 dm. tube, Na-lamp) were: +3·25°; control, +1·75° (18°).

The results were independent of the period of incubation but approximately proportional to the quantity of pulp used. The effect of citrate could not therefore be attributed to the action of an enzyme.

Effect of citrate on the rotation of the molybdate complex of malic acid and other hydroxy acids

The rotation of *l*(−)malic acid (in the presence of $(NH_4)_2MoO_4$) is raised by citrate (Table 1) in the same proportion and in the same direction as is the rotation of the rhubarb extract, the increase being 85·5 % in the case of rhubarb extract and 87 % in the case of malic acid. The effect of citrate on the rotation of rhubarb extract can thus be explained as an effect on the rotation of malate (which is known to occur in rhubarb). The rotation of the molybdate complexes of lactic, tartaric and α-hydroxyglutaric acid are also changed by citrate but these effects are small as compared with the effect of the rotation of malic acid.

Citramalic acid (methyl malic acid) behaves similarly to malic acid. The rotations of the molybdate complexes of malic and citramalic acids are almost identical, both in the absence and in the presence of citrate (Table 1).

Table 1. *Rotatory power of hydroxy acids in the presence of $(NH_4)_2MoO_4$ and other substances*

(2·5 ml. hydroxy acid solution; 2·5 ml. solution of the 'second substance'; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 18°; 1 dm. tube.)

Hydroxy acid (final conc.)	'Second substance' (final conc.)	α_D (°)	$[\alpha]_D$ (°)
l(−)Malic acid (0·0187 *M* = 0·25%)	—	+1·79	+ 716
	Na_3-citrate (0·25 *M*)	+3·35	+1340
l(+)Lactic acid (0·25 *M*)	—	−1·31	− 58
	Na_3-citrate (0·25 *M*)	−1·28	− 56
l(+)Tartaric acid (0·0334 *M*)	—	+3·30	+ 660
	Na_3-citrate (0·25 *M*)	+3·48	+ 696
l(−)α-Hydroxyglutaric acid (0·026 *M*)	—	−0·39	− 101
	Na_3-citrate (0·25 *M*)	−0·30	− 78
l(−)Citramalic acid (0·01 *M*)	—	−1·11	− 748
	Na_3-citrate (0·25 *M*)	−1·98	−1335
l(−)Malic acid (0·0187 *M*)	Na_2-racemate (0·25 *M*)	+1·21	+ 484
	dl-Na-lactate (0·25 *M*)	+1·31	+ 524
	dl-Na-β-hydroxybutyrate (0·25 *M*)	+1·73	+ 692
	Glycerol (0·6 *M*)	+1·44	+ 576
	Na_3-*trans*-aconitate (0·25 *M*)	+1·79	+ 716
	dl-Na_2-citramalate (0·25 *M*)	+1·84	+ 736

Effects of optically inactive hydroxy compounds other than citrate. Racemic acid, *dl*-lactic acid, *dl*-β-hydroxybutyric acid or glycerol all depress the rotation of the malate-molybdate complex, whilst *trans*-aconitic acid and *dl*-citramalic acid have no definite effect (Table 1).

Effect of citrate concentration. A concentration of citrate equivalent to that of malate already increases the rotation of malic acid considerably, but the maximum effect requires, under the test conditions, a citrate concentration of about 0·1 molar, i.e. about 5 times the concentration of malate. Very high concentrations of citrate (1 *M*) abolish the dextrorotation of the malate-molybdate complex, presumably by competitive reaction with the available molybdate (Table 2).

Table 2. *Effect of citrate concentration on the rotation of the malate-molybdate complex*

(2·5 ml. 1% *l*(−)malic acid solution—final malic acid conc.: 0·0187 *M*; 2·5 ml. Na_3-citrate solution diluted to different conc.; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 15°; 1 dm. tube.)

Citrate (final conc.) (*M*)	α_D (°)	$[\alpha]_D$ (°)
1·0	−0·08	—
0·5	+0·81	+ 324
0·25	+3·37	+1340
0·125	+3·36	+1335
0·0625	+3·16	+1260
0·03125	+2·82	+1130
0·01563	+2·48	+ 990
None	+1·79	+ 716

Effect of molybdate concentration. The rotation of the malate-molybdate complex does not significantly change when the concentration of $(NH_4)_2MoO_4$ varies between 3 and 13·5%. With citrate there is a diminished rotation when the molybdate concentration falls below 9%. Below this concentration the available molybdate is probably insufficient to 'saturate' both citrate and malate (Table 3).

Table 3. *Effect of $(NH_4)_2MoO_4$ concentration on the rotation of l(−)malic acid in the presence of citrate*

(2·5 ml. 1% *l*(−)malic acid; 0·5 ml. glacial acetic acid; 2·5 ml. 1 *M* Na_3-citrate or water; 4·5 ml. $(NH_4)_2MoO_4$ solution of varying strength; 15°; 1 dm. tube.)

$(NH_4)_2MoO_4$ (final conc.) (%)	α_D without citrate (°)	α_D with citrate (°)
13·5	+1·82	+3·50
9·0	+1·85	+3·37
6·0	+1·87	+0·96
3·0	+1·85	−0·08

Effect of the malic acid concentration. The specific rotation of the malate-molybdate-citrate complex is virtually constant when the malic acid concentration rises from 0·015 to 0·25% (Table 4).

Table 4. *Effect of malic acid concentration on the rotation of the malate-molybdate-citrate complex*

(2·5 ml. malic acid solution; 2·5 ml. Na_3-citrate 1 *M*; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 17°; 1 dm. tube.)

Malic acid (final conc.) (%)	α_D (°)	$[\alpha]_D$ (°)
0·25	+3·34	+1335
0·125	+1·76	+1407
0·0625	+0·90	+1440
0·03125	+0·45	+1440
0·01563	+0·23	+1470

Effect of temperature on the rotation of the malate-molybdate-citrate complex. At 6·5°, 17·5° and 37·5° we find for α_D +3·44°, +3·34° and +3·03° (under the conditions stated in Table 4).

Effect of citrate on the rotation of the citramalate-molybdate complex. The effect of citrate on the rotation of the citramalate-molybdate complex is similar in magnitude to the effect on the malate complex (Table 5).

Table 5. *Effect of citrate on the rotation of the citramalate-molybdate complex*

(3 ml. 0·445% (−)citramalic acid solution—final citramalic acid conc. 0·01 *M*; 2 ml. Na_3-citrate solutions of varying conc.; 0·5 ml. glacial acetic acid; 4·5 ml. 29% $(NH_4)_2MoO_4$; 22°; 1 dm. tube.)

Citrate (final conc.) (*M*)	α_D (°)	$[\alpha]_D$ (°)
0·2	−2·04	−1380
0·1	−2·00	−1350
0·05	−1·90	−1280
0·025	−1·73	−1170
0·0125	−1·55	−1040
0·00625	−1·41	− 452
0·00313	−1·27	− 856
None	−1·11	− 748

Experiments with isocitric acid

Since pure optically active isocitric acid was not available a solution of the active acid was prepared by bacterial resolution of synthetic Na_3-isocitrate. *Pseudomonas pyocyanea*, *Aerobacter aerogenes* or *Bact. prodigiosum* all resolve isocitrate [Martius, 1938]. The culture medium contained 1 g. KH_2PO_4, 1 g. Na_2HPO_4, 5 g. NH_4Cl, 0·2 g. $MgSO_4$, $7H_2O$, 0·05 g. $CaCl_2$ and 9·6 g. isocitric acid (as Na salt) per litre. 100 ml. medium were sterilized in Roux culture flasks of 1 l. capacity and inoculated with one of the above organisms. The flasks were laid out flat in order to facilitate aeration. When a heavy inoculum was used the maximum rotation was reached after 3 or 4 days' incubation at 37°, α_D of the medium being +1·69° (2 dm. tube; 5 ml. medium diluted with 0·5 ml. glacial acetic acid and 4·5 ml. 29% molybdate, filtered).

Like muscle tissue [Martius, 1938], the bacteria tested metabolize 50% of synthetic isocitrate. This is shown by the observation that a washed suspension of *Aerobacter aerogenes* (*p*H 6·8; 40°) absorbed 438 μl. extra O_2 on addition of 0·1 ml. 0·05 *M* citrate, and 226 μl. on addition of the same amount of synthetic isocitrate (calc. for complete oxidation: 504 μl.). The fact that less than the calculated amount of O_2 was absorbed was presumably due to the synthesis of cell material from the substrate [cf. Clifton, 1937].

For the following experiments 300 ml. medium were incubated for 3 days with *Aerobacter aerogenes*; the culture was then concentrated on the steam-bath to about 50 ml., and filtered after addition of charcoal. The effect of citrate on the rotation of the isocitrate solution in the presence of molybdate is shown in Tables 6 and 7. The increase caused by 0·1 *M* citrate varies between 40% and 120%.

Table 6. *Rotation of isocitrate solutions of varying concentrations in presence of citrate and molybdate*

(10 ml. isocitrate solution; 1·0 ml. glacial acetic acid; 9 ml. 29% $(NH_4)_2MoO_4$; 2 dm. tube; for the measurements recorded in the last two columns the isocitrate stock solution was diluted with Na_3-citrate solutions; the citrate concentration stated refers to the final concentration.)

Relative conc. of isocitrate (Data are no. of times stock solution was diluted)	α_D (°)		
	No citrate added	0·1 *M* citrate	0·2 *M* citrate
Undiluted	+9·64	—	—
2	+5·62	+7·86	+6·44
4	+2·72	+4·52	+3·96
8	+1·23	+2·47	+2·12
16	+0·58	+1·26	+1·12
32	+0·30	+0·66	—

Table 7. *Effect of citrate concentration on the rotation of the isocitrate-molybdate complex*

(Stock solution of isocitrate diluted with 3 vol. H_2O; 4 ml. diluted stock solution; 1 ml. citrate; 0·5 ml. glacial acetic acid; 4·5 ml. 29% molybdate; 20°; 1 dm. tube.)

Citrate (final conc.) (*M*)	α_D (°)
0·1	+1·89
0·05	+1·77
0·025	+1·58
0·0125	+1·41
0·00625	+1·27
0·003125	+1·11
None	+1·04

Equilibrium between citric, isocitric and cis-aconitic acids

Martius [1938] calculated from polarimetric readings that the equilibrium mixture of the three tricarboxylic acids in the presence of aconitase contains 9·4% isocitric acid. The calculation was based on the assumption that the specific rotation of isocitric acid in the presence of molybdate is −428°. Since this value applies to pure isocitric acid solutions it is no doubt too low for the conditions of Martius's experiments, where about 0·09 *M* citrate was present.

The data presented in Tables 6 and 7 permit an approximate estimate of the specific rotation of isocitric acid in the presence of citrate. Under the conditions of Martius's measurements the increase brought about by citrate is about 82%; this would bring $[\alpha]_D$ to −780°. Using this figure for the recalculation of Martius's data we obtain a value of 5·15% isocitric acid in the equilibrium mixture.

A number of further measurements of the final rotation of solutions in which the enzymic equilibrium between the three tricarboxylic acids was established, are shown in Table 8. The solutions

contained 0·2 *M* Na citrate, varying quantities of buffer, minced tissue and a drop of octanol. The mixture was incubated at 38°. For the determination of the rotation 10 ml. were acidified with 1 ml. glacial acetic acid, ice-cooled, and mixed with 9 ml. 29 % $(NH_4)_2MoO_4$. The solution was filtered after adding a small quantity of charcoal, and rapidly examined in 2 dm. tubes. Ice-cooling was necessary in order to prevent the development of the blue colour of the reduced phosphomolybdate complex. Under the conditions of the measurements the concentration of citrate was about 0·09 *M*.

Table 8. *Equilibrium at 38° between citrate, isocitrate and* cis*-aconitate in the presence of aconitase*

(Unless otherwise stated the tissue concentration in the enzyme preparation was 3·0 g. of fresh tissue in 50 ml.; the buffer consisted of phosphate except in the one instance indicated, in which $NaHCO_3/CO_2$ was used.)

Buffer		Period of incubation	$\alpha_D^{18°}$ (° for 2 dm.)			*cis*-Aconitic acid
Final conc. *M*	*p*H	hr.	Without citrate	With citrate	Difference	%*
		(*a*) Enzyme from guinea-pig liver				
0·030	7·4	18†	+0·06	−1·74	−1·80	4·08
0·025	6·8	17	+0·13	−1·70	−1·83	3·52
0·025	7·1	17	+0·12	−1·68	−1·80	—
0·025	6·8	17	+0·11	−1·79	−1·90	4·19
0·025	6·8	17	+0·12	−1·72	−1·84	5·04
0·050	6·8	17	+0·02	−1·73	−1·75	4·26
0·060‡	6·4‡	24	−0·14	−1·81	−1·67	—
0·025	6·8	17	+0·13	−1·77	−1·90	4·73
0·025	6·8	17	+0·16	−1·72	−1·88	4·22
		(*b*) Enzyme from guinea-pig muscle				
0·010	6·8	41	−0·06	−1·82	−1·76	4·33
		(*c*) Enzyme from pigeon breast muscle				
0·025	6·8	22	−0·01	−1·77	−1·76	4·22

* The figures in this column are

$$\frac{\text{molecules of } cis\text{-aconitic acid found}}{\text{molecules of citric acid added}} \times 100.$$

† Tissue conc. = 1·5 g./50 ml.
‡ Buffer = $NaHCO_3/CO_2$.

The rotations observed were a little higher than those reported by Martius [1938] and by Jacobsohn, Soares & Tapadinhas [1940]. The average of six measurements of α_D under the same conditions (*p*H 6·8; 0·025 *M* phosphate) is −1·85°. The deviations from this mean are considerably greater than the experimental error (which is ±0·04°). They may be connected with the rotation of the control. As the crude tissue extracts used in our experiments contained cathepsin and amylase, optically active substances may have been formed during the incubation from protein or carbohydrates. It is possible that the high concentration of citrate affects these enzymes and this would invalidate the control experiments. More accurate estimations of the rotation caused by isocitric acid would require the use of a purified aconitase free from other enzymes and their substrates.

On the basis of the assumption that $[\alpha]_D$ for the experimental conditions is −780°, the rotation of −1·85° indicates that 6·2 % of the citric acid was converted into isocitric acid. The average proportion of aconitic acid (determined according to Johnson [1939]) was 4·3 %. This leaves 89·5 % for citric acid. Determinations of citric acid according to Pucher, Sherman & Vickery [1936] and Kometiani [1931] agreed with this calculation within the limits of the error of the methods, which proved to be about ±10 %.

The values for *cis*-aconitic acid are in accordance with those previously reported from this laboratory by Johnson [1939]. Johnson calculated the isocitric acid concentration of the equilibrium mixture by deducting the sum of the aconitic and citric acid recovered from the amount of citric acid added. In the light of more recent experience with the citric acid determination according to Pucher *et al.* [1936] [see also Dickens, 1941], it is probable that Johnson's recovery of citrate was somewhat incomplete. We find that the yield is frequently low when permanganate is added rapidly to the citrate solution and we therefore now add the reagent gradually, as recommended by Reichard [1934]. This precaution was not observed in Johnson's experiments; his citrate values therefore were probably too low, and the calculated isocitrate values too high.

Change of *p*H from 6·8 to 7·4, or the substitution of phosphate buffer by bicarbonate buffer, does not appreciably affect the equilibrium (Table 8). Confirming Jacobsohn's [1940] observation we find that addition of $MgCl_2$ (0·12 *M*) reduces the concentration of isocitric acid in the equilibrium mixture from 6·2 to 2·6 % (assuming $[\alpha]_D$ to be −780°). Under the same conditions (*p*H 6·8; 0·025 *M* phosphate buffer) the concentration of *cis*-aconitic acid fell from 4·2 to 1·6 %. $MgCl_2$ thus shifts the equilibrium in favour of citrate, presumably because of the formation of a Mg-citrate complex [see Hastings, McLean, Eichelberger, Hall & da Costa, 1934; Nordbö, 1938] which results in a lowering of the concentration of 'free' citrate in the system.

Polarimetric determination of malic and isocitric acids

To overcome the difficulty arising from the interference by citrate with the rotation of the molybdate complexes, we add citrate to the solution in quantities sufficient to bring the citrate concentration to about 0·1 *M*; 4 ml. of the solution to be examined (which should be neutral) are mixed with 1 ml. *M* Na_3-citrate, 0·5 ml. acetic acid and 4·5 ml. 29 % $(NH_4)_2MoO_4$. The data recorded in Tables 4 and 6 show that the rotations of malic and isocitric acids under these conditions are approximately proportional to the hydroxy acid concentration. This procedure has the further advantage of increasing the sensitivity of the polarimetric method.

SUMMARY

1. Citrate increases the rotation of the molybdate complexes of malic acid (as already observed by Auerbach & Krüger [1923]), of citramalic acid and of isocitric acid. The increase can amount to more than 100%. The magnitude of the effect under varying conditions is investigated.

2. The effect of citrate must be taken into account when the above acids are determined polarimetrically by the molybdate method. Isocitric acid concentrations calculated from polarimetric readings by previous investigators who were unaware of the citrate effect require revision.

3. Preliminary experiments show that the equilibrium mixture of isocitrate, *cis*-aconitate and citrate in the presence of liver or muscle aconitase contains 6·2% isocitrate, 4·3% *cis*-aconitate and 89·5% citrate (38°; *p*H 6·8; 0·025*M* phosphate buffer). The effect of *p*H is small between 6·8 and 7·4. $MgCl_2$ shifts the equilibrium in favour of citrate.

4. A modified polarimetric method is suggested for the determination of malic and isocitric acids, applicable to solutions containing citrate.

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Aerobic Oxidation of Aromatic Hydrocarbons in the Presence of Ascorbic Acid

THE REACTION WITH ANTHRACENE AND 3:4-BENZPYRENE

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It has long been known that oxidation of the aromatic hydrocarbons plays a fundamental part in their elimination from the animal body, but isolation of the products excreted has contributed little to knowledge of the initial introduction of oxygen into the molecule. This problem has assumed even greater importance recently in connexion with the fate of carcinogenic hydrocarbons in the animal body. Many such hydrocarbons are now known and, in most cases, introduction of oxygen into the molecule leads to considerable or complete loss of carcinogenic activity. Thus in these carcinogens oxidation is equivalent to 'detoxication' from the point of view of cancer induction.

It is well known that the feeding of naphthalene to rabbits leads to the production of cataract. The crystalline lens normally contains much ascorbic acid. Simultaneously with the development of opacity in the lens during cataract formation the ascorbic acid content falls to a very low value. It has also been reported that the administration of ascorbic acid to animals on a naphthalene diet inhibits cataract production. It must be admitted that the whole question of the relation of ascorbic acid to cataract production by naphthalene is still undecided, and there is no general agreement on the facts. However, these experiments suggested to Jorissen [1937] the desirability of testing the