THE ISOLATION OF L-CITRAMALIC ACID FROM THE PEEL OF THE APPLE FRUIT

by

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As briefly reported elsewhere[1], an acid differing in behaviour from any of the known fruit acids was detected during the examination by paper chromatography of extracts from the whole apples (excluding seeds) of mature Edward VII apples. Further work showed that this acid originated in the peel tissue and was, in all probability, either β-OH-glutaric acid or the isomeric citramalic acid, acids not, so far as the author is aware, previously reported in plant tissue. Elementary analysis, molecular weight determinations and silver salt analysis[2] established that the acid was a dibasic hydroxy acid of molecular formula $C_5H_8O_5$. Its melting point was in the neighbourhood of 95° C, and thus it could not be α-hydroxyglutaric acid (m.p. 72° C). Simultaneous oxidation with sulphuric acid and condensation with 2.4-dinitrophenylhydrazine gave a derivative which on the evidence of paper chromatograms and light absorption spectra appeared to be identical with the 2.4-dinitrophenylhydrazone prepared from synthetic acetone dicarboxylic acid. This agreed with the hypothesis that the acid was β-hydroxyglutaric acid. Paper chromatograms in which the acid was compared with synthetic β-hydroxyglutaric acid showed almost identical R_F values in two solvents but a noticeable difference in a third.

At this stage, larger and purer supplies (600 mg) of the acid were prepared. After several recrystallisations, the m.p. was found to be 109–111° C and when admixed with synthetic β-OH-glutaric acid (m.p. 95–96° C) a considerable depression of the m.p. was observed. Furthermore, preliminary polarimetric analysis showed the acid to be laevo rotatory; β-OH-glutaric acid contains no asymmetric carbon atom. It was clear, therefore, that the new acid could not be β-OH-glutaric acid. Other evidence had shown that it could not be α-OH-glutaric acid. The misleading evidence from the oxidation and reaction with 2.4-dinitrophenylhydrazine is presented and discussed in a subsequent paper.

Three other dicarboxylic hydroxy acids of molecular formula $C_5H_8O_5$ are known, namely citramalic acid (1-methyl malic) (I), 2-methyl malic acid (II) and itamalic acid (hydroxymethylsuccinic acid) (III). The last named acid does not occur in the free state (owing to lactone formation) and 2-methyl malic acid has no asymmetric carbon atom. The m.p. of citramalic acid is variously given as 95–110° C. This acid was synthesised and comparison with the acid from the apple peel established that this acid was, indeed, citramalic acid.

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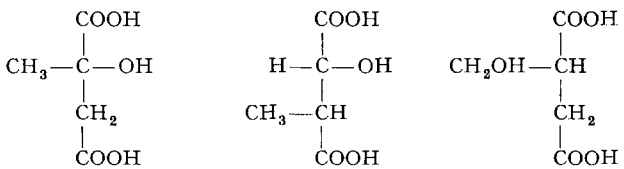

I II III

EXPERIMENTAL

Several bushels of mature, gas-stored Edward VII apples were rapidly peeled with a mechanical peeler, the peel being dropped, as it left the apples, into a petroleum ether-"dry ice" mixture contained in large vacuum-jacketed containers. By this procedure the peel was frozen hard within a few seconds after leaving the fruit and the tissue showed no sign of darkening. The peel was rapidly removed from the freezing mixture, the petroleum ether drained off, and the peel placed in cooled tins which were then placed in a room at $-20°$ C to await further treatment. Some days later the frozen peel (about 3.5 kg) was ground (at $-10°$ C) and plunged, 500 g at a time, into boiling ethanol the final concentration of which (allowing for the water-content of the tissue) was ~80%. The tissue "mush" was filtered into large Soxhlet thimbles which were placed in the extraction vessel of a large Soxhlet-type extractor and extracted under reduced pressure with 80–85% ethanol until the liquid leaving the thimbles was colourless (48 hour). Three and a half kilograms of peel tissue was extracted in one loading of the extractor. The extract was evaporated under reduced pressure at 45° C until all the alcohol had been removed. A novel feature of the all glass extractor and the evaporator was that the large glass condensers used were cooled by a circulating stream of glycol cooled to ~1° C in a refrigeration machine. This, together with the use of a ballast type* vacuum pump, practically eliminated loss of solvent during the extraction and greatly reduced the evaporation time when removing the alcohol. The evaporate (volume = 1 litre) was filtered with suction through a pad of asbestos and the pad washed with warm water. The final 1.5 litres of filtrate and washings was a clear bright red liquid. It was shaken with 40 g of acid-treated charcoal[3] in a mechanical shaker for 18 hours. The charcoal was filtered off, washed and the combined filtrate and washings, which was a very pale yellow clear solution, contained 10 g of acid calculated as malic acid. Cations and amino acids were removed by passing this solution after dilution to 4.5 litre down a large column of Zeokarb 215 and the organic acids (18 g calculated as malic acid) collected on a column of Deacidite E using the procedure previously described[4]. The Deacidite column was washed free of sugars using ~8 litres of distilled water. Strongly basic columns such as Dowex 2, although leading to a better fractionation of the organic acids cannot be used at this stage because such columns break down sugars to lactic and other acids (PHILLIPS AND POLLARD[5], HULME[2]). The acids were displaced from the Deacidite column with 0.1 *N* HCl at a flow-rate of 75 ml/hour. Seventy five fractions of ~35 ml each were collected using a mechanical fraction collector.

Hydrochloric acid first appeared in fraction 66. Fractions 1–64 were bulked and the solution (~2300 ml) contained 15 g of acid expressed as malic acid. Fractions 65–75 were bulked and evaporated to a small volume; paper chromatograms indicated the

* Supplied by Messrs. W. Edwards & Co., Ltd.

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presence of malic acid, citric acid, a trace of 'G' acid (see later) and HCl in high concentration. This combined fraction was discarded.

The bulked fraction 1–64 was evacuated to remove CO_2 and then passed down a two-tier column of Dowex 2 resin (top column, 60 cm × 2.5 cm; bottom column, 14 cm × 1 cm) taking precautions to prevent the resin coming into contact with CO_2. The acids absorbed on the column were displaced by 0.1 *N* HCl at a rate of ~40 ml per hour. Ninety-one fractions of ~16 ml each were collected. Paper chromatographic analysis of these fractions, using *n*-butanol-formic acid-water (40:10:50 v/v) as the solvent system, run with marker spots of the appropriate acids (where known) indicated the presence of the following acids:

Fractions 1–17. Quinic acid only (HULME[4]).

Fractions 18 and 19. Quinic acid; an unidentified acid (A) having an R_F value rather lower than that of citric acid; an unidentified acid (B) having an R_F value rather higher than that of malic acid.

Fractions 20 and 21. Quinic acid; two unidentified acids (C and D) having very low R_F values (below quinic acid).

Fractions 22–65. Trace of quinic acid; malic acid and an acid (G) having an R_F value higher than that of acid B (above) both these acids were in high concentration.

Fractions 66–83. Malic acid in high concentration and acid G at a lower concentration than in fractions 22–65.

Fractions 84–85. Malic acid, citric acid and a trace of G together with a small amount of the displacing HCl.

The remaining fractions contained only HCl.

It seemed unlikely that acid 'G', the one under consideration here, could be completely separated from malic acid by means of Dowex 2. Small scale experiments showed that this acid was more soluble in methyl *iso*butyl ketone (MIK) than malic acid and that the two acids could be separated by partition chromatography on silica gel using this solvent. Buffering of the silica gel with phosphate at pH 2.9[6] improved the separation. Combined fractions (22–65) from the Dowex column were considered as the most suitable fraction from which to separate G acid. This fraction was evaporated to dryness in vacuo and the residue repeatedly extracted, with mechanical shaking, by MIK (all the methyl *iso*butyl ketone used was redistilled since the commercial solvent gives, on evaporation, a small oily deposit). The extracts were combined and filtered and 200 ml of solution was obtained. The unextracted residue was shown to consist entirely of malic acid. The extract contained 7.5 g of acid calculated as malic acid. It was carefully poured, 20 ml at a time, onto a silica gel column containing 115 g (dry weight) of the gel buffered with a phosphate buffer of pH 2.9. The column was prepared by pouring the gel, made into a slurry with MIK saturated with the phosphate buffer, into a glass tube (3.5 cm diameter and 50 cm long) half filled with buffered MIK; it was washed through with 1 l of buffer-saturated MIK the final washings being allowed to sink to the level of the top of the gel before the acid solution was added. When all this acid solution had passed into the gel, a reservoir of buffer-saturated MIK was placed on the top of the column and liquid was allowed to flow through the column at a rate of ~50 ml per hour. Fractions varying between 25 and 50 ml each were collected on a mechanical fraction collector. As the chromatogram was "developed" two broad bands appeared as areas more transparent than the rest of the gel. These bands were well

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separated from one another and the effluent was discarded until the lower band was about 5 cm from the bottom of the column. Subsequently small aliquots (0.1–1 ml depending on the concentration of acid) were taken from each fraction and titrated against 0.02 *N* baryta using an indicator composed of 40 mg of thymol blue, 3 ml of 0.1 ml NaOH and 200 ml water. The progress of the acids leaving the column was followed by means of these titrations; this is shown diagrammatically in Fig. 1.

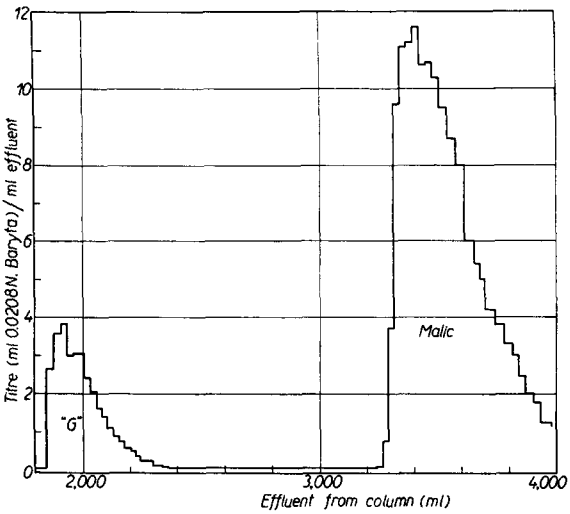

Fig. 1. Chromatogram on buffered silica gel of "G" acid (citramalic) and malic acid run in methyl *iso*-butyl ketone.

Acid commenced to leave the column when ~1850 ml of solvent had been passed down. This acid (G) had been completely eluted from the column after ~2400 ml of solvent had run from the column. This was checked by means of paper chromatograms. Fractionation was continued until most of the malic acid was eluted (~4000 ml effluent—see Fig. 1) to obtain an estimate of the relative amounts of the two acids present in the extract originally placed on the silica gel column. The effluent containing G acid only (*i.e.* that between 1850 ml and 2400 ml) was evaporated to a small volume and placed in a small crystallising dish at — 20° C overnight. On scratching the sides of the dish with a glass rod crystals appeared and formed a solid mass in the dish. The crystals were taken up in water and some of the water removed by evaporation *in vacuo* at 45° C. The resultant rather viscous solution was placed "*in vacuo*" in a desiccator over P_2O_5 at 1° C. After several days crystals appeared. The crystals were filtered off and washed with ether at 10° C. Finally the crystals were dried *in vacuo* over P_2O_5 at room temperature.

Characterisation of the acid, G

a. *By chemical methods.* The recrystallised acid had a melting point of 110–110.5° C. Elementary analysis (Weiler and Strauss, Oxford) gave C, 40.7; H, 5.5; calculated for $C_5H_8O_5$: C, 40.6; H, 5.4%. The silver salt contained 58.8% Ag; calculated for $C_5H_6O_5Ag_2$, 59.7%. The M.Wt. (Rast) was 145; calculated for $(C_5H_8O_5)$, 148. On determination of the "OH" groups by heating with acetic anhydride (SIGGIA, 1949) the substance blackened but gave 9.2% "OH"; calculated for $C_5H_7O_4OH$, 11.5% OH. $[\alpha]_{20}^{D}$ in a micro polarimeter tube was — 30°. $[\alpha]_{25}^{D}$ of D-citramalic acid is given in the literature as + 37°.

Heating (oxidation) with sulphuric acid followed by coupling with 2.4-dinitrophenylhydrazine gave a crystalline hydrazone soluble in alkali indicating the presence in the oxidation mixture of a keto acid (see HULME[7]).

Comparison of 'G' acid with β-OH-glutaric acid and DL-*citramalic acid.*

β-OH-glutaric acid was prepared by reduction of acetone dicarboxylic acid (pre-

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pared by the method of v. PECHMANN[8]) with sodium amalgam (v. PECHMANN AND JENISCH[9]). The m.p. of this acid, after recrystallisation from water, was 95–96° C. On admixture of 'G' acid with the synthetic β-OH-glutaric acid a m.p. of ~50° C was obtained.

DL-citramalic acid was prepared from acetoacetic ester by the method of MICHAEL AND TISSOT[11]. It had a melting point of 112° C. When mixed with 'G' acid the mixed melting point was 104° C. Since the m.p.'s of the DL- and L-acids are given variously in the literature as between 95 and 119° C, this mixed melting point may be considered as supporting the hypothesis that the two acids are identical. The recrystallised *p*-bromophenacyl derivatives were found to have m.p.'s as follows: 'G' acid (L-citramalic acid), 113–113.5° C: synthetic DL-citramalic acid, 137–137.5° C, and synthetic β-hydroxyglutaric acid, 166° C.

b. *By means of paper chromatography.* Paper chromatograms were prepared in the three solvent systems butanol-formic acid-water, propanol-ammonia-water and benzyl alcohol-*tert*-butanol-*iso*propanol-formic acid-water (STARK, GOODBAN AND OWENS[10])

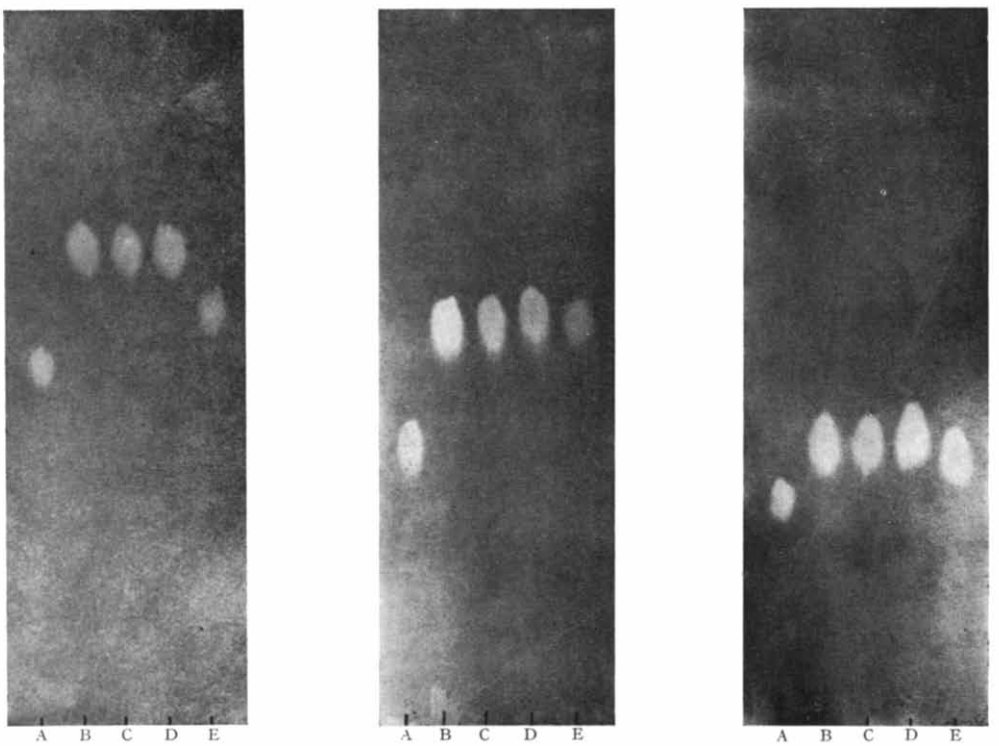

Fig. 2. Chromatograms in 3 solvent systems showing the identity of citramalic acid, isolated from apple peel, and synthetic citramalic acid, and comparing the rate of travel of the acid with that of malic acid and β-hydroxy-glutaric acid.

A. Malic acid
B. Citramalic acid from apple peel
C. as B plus synthetic citramalic acid
D. Synthetic citramalic acid
E. Synthetic β-hydroxy-glutaric acid

Solvent systems
1. Butanol-formic acid-water (4:1:5 v/v)
2. Benzyl alcohol-*tert* butanol-*iso*propanol-formic acid-water (24:8:8:8:1 v/v)
3. Propanol-NH_4OH-water (6:3:1 v/v).

Papers sprayed with 0.08% bromocresol green in 95% ethanol.

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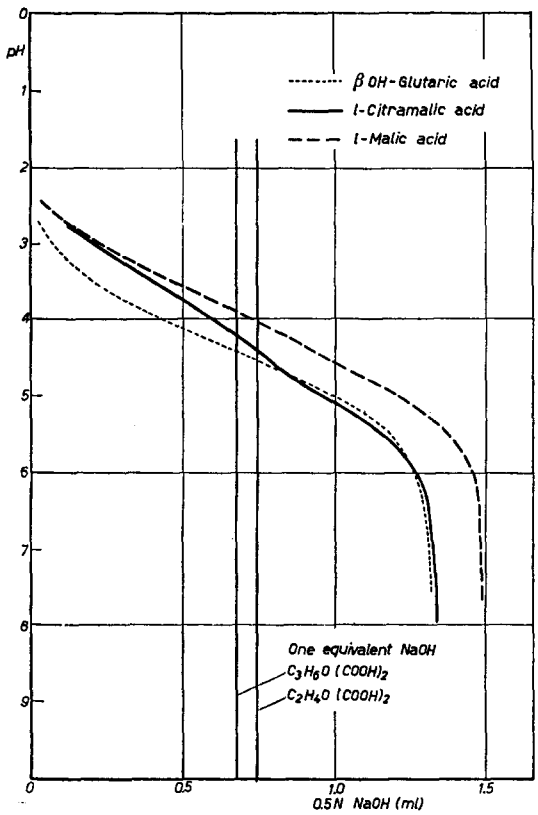

Fig. 3. Titration curves of citramalic acid (from the apple and synthetic), malic acid (from the apple) and synthetic β-hydroxyglutaric acid.

under carefully controlled conditions (see HULME[4]). 'G' acid was compared with synthetic DL-citramalic acid and β-hydroxyglutaric acid and the resulting chromatograms are shown in Fig. 2. The results provide strong evidence for the identity of 'G' and citramalic acid.

c. *Infra-red absorption spectra.* A comparison of the infra-red absorption spectra of β-OH-glutaric acid and 'G' acid over the wavelength range 3110 and 3680 CM^{-1} showed a large divergence in the region of the peak attributable to the OH group of the β-OH-glutaric acid, and confirmed the non-identity of 'G' acid and β-hydroxyglutaric acid.

The *titration curves* of malic acid, citramalic acid and β-OH-glutaric acid are shown in Fig. 3. The curves for the last two acids have not, so far as can be ascertained, been previously published. The shift produced by the introduction of a methyl group into the malic acid molecule is interesting. pK_1 for citramalic acid is considerably lower than for β-OH-glutaric acid, but the pK_2 values are identical for the two acids. β-OH-glutaric acid has a symmetrically oriented molecule.

DISCUSSION

The data presented proves conclusively that 'G' acid isolated from apple peel is L-citramalic acid. From the viewpoint of paper chromatography, it is interesting that the addition of 'CH_3' to the molecule of malic acid to give citramalic acid or β-OH-glutaric acid produces an identical shift in the R_F value in one acid solvent (benzyl alcohol-*tert* butanol-*iso*propanol-formic acid-water) although the orientation of the two molecules are quite different, whereas another acid solvent (*n*-butanol-formic acid-water) shows up the difference in the molecule structure of the two isomeric dicarboxylic hydroxy acids.

From the data shown in Fig. 1, a determination of the malic acid remaining in fractions (22–65) see page 38—and in fractions (66–83) (the small amount of malic acid in fractions (84–85) could not be determined because of the large amount of HCl present) an approximate estimation of the relative amounts of citramalic and malic acids in the peel was obtained. On this basis the 3.5 kg of peel tissue contained ~11.5 g of malic acid and ~1.5 g of citramalic acid. What proportion of these acids were originally

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in the free or the combined state (as salts) cannot be determined. From a titration of combined fractions (1–17)—see page 38—which contained only quinic acid, ~2.5 g of this acid were also present.

It is difficult at this stage to suggest the part played by citramalic acid in the metabolism of the peel of apples (it appears to be absent from the pulp tissue of either young or mature fruits). There is as yet no critical evidence for or against the existence of a tricarboxylic (Krebs) cycle in apple tissue. It has been suggested that a seven-carbon atom carboxylic acid might be an intermediate in this cycle and that this acid might be oxalo-citramalic acid (erroneously called oxalo-citraconic acid), although present opinion inclines to the view that "active acetate" reacting with oxalo-acetate eliminates the need for such a hypothetical seven-carbon acid (see BALDWIN[12]). Oxalo-citramalic acid, if taking part in a Krebs cycle, would be formed by condensation of enol-α-keto-glutaric acid. Like other keto acids of similar structure, oxalo-citramalic would be unstable and, if present in the tissue, would be expected to break down during isolation procedures to yield citramalic acid.

Further discussion of the possible function of citramalic acid in the peel will be deferred to a later paper dealing with the oxidation of the acid.

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SUMMARY

1. A method is described whereby a "new" organic acid is isolated from the peel of mature fruits of the Edward VII apple.

2. The characterisation of the acid is described and evidence advanced for the view that the acid is L-citramalic acid rather than the isomeric β-hydroxyglutaric acid.

3. An estimation is made of the amount of citramalic acid present in the peel in relation to the amount of malic acid also present.

4. The possible position of citramalic acid in the metabolism of the peel-tissue is discussed.

RÉSUMÉ

1. L'auteur décrit une méthode qui lui a permis d'isoler du péricarpe des pommes mûres de la variété Edward VII un acide organique "nouveau".

2. Cet acide, qui a été caractérisé, est plus probablement l'acide L-citramalique qu'un isomère de l'acide β-hydroxy-glutarique.

3. La quantité d'acide citramalique présente dans le péricarpe a été déterminée et comparée à la quantité d'acide malique qui s'y trouve également.

4. Le rôle de l'acide citramalique dans le métabolisme du péricarpe est discuté.

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ZUSAMMENFASSUNG

1. Es wird eine Methode beschrieben, die zur Isolierung einer "neuen" organischen Säure aus den Schalen von reifen Edward VII-Äpfeln führte.

2. Die Charakterisierung der Säure wurde gegeben und die Anschauung vorgebracht, dass die Säure eher L-Citraapfelsäure wie die isomere β-Oxyglutarsäure ist.

3. Es wurde eine Bestimmung der in der Schale vorhandenen Citraapfelsäuremenge im Verhältnis zu der ebenfalls vorhandenen Apfelsäure durchgeführt.

4. Die mögliche Stellung der Citraapfelsäure im Stoffwechsel des Schalengewebes wird besprochen.

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