The Isolation of Chlorogenic Acid from the Apple Fruit

By A. C. HULME

*Ditton Laboratory, Department of Scientific and Industrial Research,
East Malling, Maidstone, Kent*

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Chlorogenic acid, the depside formed by the condensation of caffeic and quinic acids, was first obtained in crystalline form by Gorter (1909) from green coffee beans, although considerably earlier Ludwig & Kromeyer (1854) had noted the presence in the seeds of the sunflower of a compound which was undoubtedly chlorogenic acid. The acid was first thought to be a glycoside of caffeic acid, but Gorter (1909) showed that the 'sugar' was, in fact, quinic acid and that the acidity was due to the free carboxyl group of this acid. Finally, Fischer (see Freudenberg, 1933) proved chlorogenic acid to have the following structure:

Gorter found the acid not only in coffee beans but also in coffee leaves, latex of *Castilleja elastica*, and the seeds of *Helianthus annuus*, *Kopsia flavida* and *Strychnos nux vomica*. On the basis of a colour reaction involving ferric chloride, he suggested that the acid was present in the plants of a large number of natural orders but, as Onslow (1931) has pointed out, Gorter's test will give a positive result with any *o*-dihydroxyphenolic compound. Oparin (1921, 1927) suggested that chlorogenic acid might act as a 'respiration-pigment' as defined by Palladin (1912) and claimed that it could bring about the oxidation of amino-acids and peptides especially in the presence of phosphates.

Recent interest in chlorogenic acid has centred on its function as a substrate for polyphenolase and it appears that the darkening of fruits on injury may be due to the oxidation of chlorogenic acid rather than to the oxidation of true tannins (Joslyn & Ponting, 1951). Cruess & Alsberg (1934) consider that a glucoside of caffeic acid and phenol obtained from olives is responsible for the browning of these fruits. Rudkin & Nelson (1947) showed that the addition of small quantities of chlorogenic acid, which they isolated from the sweet potato, in-

creased the oxygen uptake of, and also the carbon dioxide given off by, thin slices of the root of this plant.

The isolation of considerable quantities of quinic acid from the young fruits of Worcester Pearmain apples, and also its presence in pears and other fruits, suggested (Hulme, 1951) that one of several possible functions of the acid might be to combine with caffeic acid to form chlorogenic acid. The reactions of extracts of the young apples suggested that, in fact, they might well contain chlorogenic acid since they gave a positive Gorter test, slowly turned brown on the surface on being made alkaline, and gave paper chromatograms showing a spot with an intense ultraviolet fluorescence turning duck-egg green on exposure to the fumes of ammonia. Meanwhile, the presence of chlorogenic acid has been established in the vegetative parts of apple and pear trees and in the juice of mature pears (Bradfield, Flood, Hulme & Williams, 1952).

In experiments designed to isolate amino-acids from extracts of 50–100 kg. of the tissue of young apples, by absorption on large columns of cation-exchange resin followed by absorption of organic acids on anion-exchange resins, there was found to be present in the extracts a substance (or substances) which, when in contact with the anion-exchange resin or with the ammonia used to displace the amino-acids from the cation-exchange resin, was apparently transformed into a dark-coloured compound which clogged the columns. The apple extracts appeared to contain a considerable amount of this substance, and from its behaviour on the columns it seemed likely that absorption on the cation-exchange resin was not ionic but due to van der Waal's forces, and that the strongly basic nature of the anion-exchange resin (Dowex 2, R. W. Greif & Co. Ltd.) was modifying the substance to give the strongly pigmented compound. The substance behaved, in fact, like a soluble *o*-dihydroxyphenolic compound and its removal from the apple extracts was essential to a satisfactory separation of the amino-acids and organic acids.

For the removal of aromatic amino-acids, which behave in an anomalous manner on cation-exchange resins (Partridge, 1949), partially deactivated charcoal prepared by the method of Schramm & Primosigh (1943) has been used. It was found that

if the apple extracts were treated with sufficient of this charcoal to remove all colour, the extracts no longer darkened on being made alkaline. Furthermore, the substance originally responsible for the alkali-darkening could be recovered from the charcoal by displacement with the phenol-acetic acid reagent of Schramm & Primosigh (1943). As described below, considerable quantities of this substance, amounting to several grams, were isolated, purified and identified as chlorogenic acid.

EXPERIMENTAL

Several thousand immature fruits of average weight 13.6 g. were gathered from Worcester Pearmain apple trees and placed in galvanized-iron tanks through which was passed a stream of humidified air at 15°. After 7 days the fruits were free of starch (the removal of starch was required for work not reported here on the ethanol-insoluble material of the fruit). After rapidly halving the fruits (at 1°) and removing the embryonic seeds they were frozen at -20°. When frozen, the fruit was peeled at -10° and the pulp tissue ground to a fine powder, also at -10°. Approximately 7 kg. of this powder were plunged, in batches of 500 g., into boiling 95% ethanol (the final ethanol concentration was ~80%). The combined batches (liquid and tissue) were poured into large Soxhlet thimbles. All the thimbles were placed in a Soxhlet type extractor and extracted under reduced pressure with 80-85% ethanol until the liquid leaving the thimbles was colourless (48 hr.). All the ethanol extracts were combined (approximately 14 l.) and the ethanol removed below 45°. The residual liquid (2 l.) was filtered through a pad of asbestos to remove green, waxy material and the reddish brown, slightly opalescent filtrate was treated with a few ml. CHCl_3 and stored at 1°. Nine similar extractions of 7 kg. of pulp tissue were carried out, approximately 2 l. of filtered extract being obtained from each. Each 2 l. batch was shaken for several hours with 80-90 g. of acid-treated charcoal (Schramm & Primosigh, 1943; Partridge, 1949). The charcoal was filtered off and washed until free of sugar. The various charcoal filter cakes were stirred into a solution of 20% (v/v) acetic acid solution containing phenol (5%, w/v) (Partridge, 1949)—about 8 l. in all being used—and stored at 1° for several days. Subsequent operations were carried out on batches of approx. 1 l. of the well stirred charcoal suspension. Each batch was filtered, the charcoal washed with a little dilute acetic acid and the filtrate and washings shaken with 1 l. of ethyl ether in 100 ml. lots in a separating funnel to remove the phenol. The acetic acid in the phenol-free residual liquid, which was orange coloured, was removed by repeated evaporation and dilution with water. The final evaporate, about 500 ml., had a pH of between 3.2 and 3.3. It was rapidly neutralized (pH 6.8) by the addition of solid Na_2CO_3 ; during this stage it was impossible to prevent some darkening of the liquid. Neutral lead acetate (5 ml., 0.5 M) was added and the small grey precipitate which formed was discarded since it contained a large amount of impurity in relation to its chlorogenic acid content. The deep-red supernatant liquid was treated with excess neutral lead acetate; the precipitate formed was dissolved in 5% acetic acid and reprecipitated at pH 8.5; this precipitate was suspended in water, the lead carefully removed with H_2SO_4 , the lead-free filtrate brought

to pH 2.0 and extracted with ethyl acetate (1300 ml. in 100 ml. lots in a separating funnel, 'first extract', and then in a liquid-liquid extractor, 'second extract'), etc. following the procedure used by Rudkin & Nelson (1947) to isolate chlorogenic acid from sweet potato. Before extracting chlorogenic acid in the undissociated form with ethyl acetate, Rudkin & Nelson first made their extracts 2 M in KH_2PO_4 and then acidified them to pH 2. This addition of phosphate was found, in the present work, to cause the formation of orange-coloured, insoluble agglomerates during the extraction with ethyl acetate, and the crude chlorogenic acid obtained proved difficult to purify. The addition of phosphate was, therefore, discontinued after treatment of the first litre of charcoal eluate.

It was found advantageous to carry out all evaporations under reduced pressure in a stream of N_2 .

The crude preparations (hereafter called 'crude chlorogenic acid preparations'), obtained by precipitation from the concentrated ethyl acetate extracts by the addition of excess light petroleum (b.p. 60-80°) followed by centrifugation, were dried *in vacuo* after treatment with a few drops of benzene. Unlike Rudkin & Nelson's crude preparations from the sweet potato they were coloured pink, the depth of colour increasing during drying. From the evidence presented below (under 'Examination and characterization of chlorogenic acid preparations from the apple'), and from microchemical tests, it appeared that these crude preparations contained, in addition to chlorogenic acid, various catechins. Attempts were made to purify the preparations by the counter-current distribution method as used by Rudkin & Nelson, but it was clear that the contaminants were not of the type of component A (closely related to chlorogenic acid) of these workers. Preliminary experiments using silica-gel chromatography also failed to effect a separation from all the coloured impurities. Since the catechins do not crystallize readily from water and, as Kirby, Knowles & White (1951) have shown, exhibit mutual solubilities, it was decided to try to crystallize the chlorogenic acid from water at 1° in presence of a little HCl (Freudenberg, 1933, mentions that mineral acids reduce the solubility of chlorogenic acid). This method proved immediately successful and after recrystallizing several times, washing the crystals each time with ice-cold water, and drying *in vacuo* over P_2O_5 a white crystalline compound was obtained which, on examination as described below, proved to be chlorogenic acid. To prevent the compound becoming slightly tinged with pink during drying it was necessary to place the wet crystals immediately in the desiccator (at 1°) and fill with N_2 before connecting to the vacuum pump.

Examination and characterization of chlorogenic acid preparations from the apple

The author is greatly indebted to Dr J. M. Nelson of Columbia University for an authentic sample of chlorogenic acid which was compared with the material from the apple in the following procedures.

Filter-paper chromatography. Chromatograms were prepared on Whatman no. 2 'chromatographic' paper. The solvent system used was Campbell, Work & Mellanby's (1951) modification of Partridge's (1948) original 4:5:1 butanol/water/acetic acid mixture. Although the former authors inferred that

the upper layer of this mixture as prepared by them had the same composition as the upper layer of Partridge's system, it was found that phenolic bodies such as chlorogenic acid gave considerably elongated spots with the Partridge mixture, whereas they gave almost round spots with the Campbell, Work & Mellanby mixture (48 hr. allowed for esterification before use with both solvent systems). Paper chromatography showed that at least six substances giving an orange coloration when sprayed with the diazotized *p*-nitroaniline reagent of Bradfield, Penney & Wright (1947) (modified for use as a spray by the addition of sodium acetate, Bradfield (1948)) were present in the neutral lead acetate precipitate, and five of these continued to be present up to and including the crude light petroleum precipitate. From chromatographic evidence two of these compounds appeared to be epicatechin and ($\pm$)-catechin. No compounds reacting to the diazotized *p*-nitroaniline spray travelled faster than ($\pm$)-catechin. This rules out the presence of the epicatechin gallates which have considerably higher R_F values than ($\pm$)-catechin (Bradfield & Bate-Smith, 1950). Filter-paper chromatograms of the recrystallized compound showed one spot only, fluorescing blue in ultraviolet light, changing to duck-egg green when exposed to the fumes of ammonia and giving a positive reaction when sprayed with Bradfield's modified diazo reagent (see above), or with Roberts's (1951) modification for spraying of Hoepfner's (1932) nitrite test, or with Partridge's (1948) ammoniacal silver nitrate reagent. Nelson's sample (see above) of chlorogenic acid, when run on chromatograms alongside the present preparation, gave identical results and an identical R_F value (0.57).

Chemical methods. The crystalline compound had m.p. 207–209° not depressed by admixture with authentic chlorogenic acid (m.p. 207–209° corr.). (Found: C, 54.1; H, 5.4. Calc. for $C_{16}H_{18}O_9$: C, 54.24; H, 5.12%.) A sample was hydrolysed for 15 min. at 20° with potassium hydroxide (13%, w/v) and neutralized (pH 4) with sulphuric acid (10%, w/v) (cf. Freudenberg, 1933). The product contained chlorogenic acid, quinic acid and caffeic acid (chromatographic comparison with authentic specimens).

Absorption spectra. The absorption spectrum in ultraviolet light of the crude light petroleum precipitate in ethanol showed very high absorption below 240 $m\mu$. and gave peaks at 283 $m\mu$. and approx. 328 $m\mu$., the former being considerably more pronounced than the latter. Bradfield & Penney (1948) showed that the maxima for the catechins, gallocatechins and catechin gallates (in ethanol) all occur between 271 and 280 $m\mu$. In addition, all their catechins showed strong absorption below 240 $m\mu$. These facts provide support for the

suggestion arising from the paper-chromatographic data that the crude chlorogenic acid preparations were contaminated with a series of catechins.

The recrystallized chlorogenic acid preparations gave an ultraviolet absorption spectrum identical with that given by Nelson's sample of the acid (Fig. 1). The molar extinction coefficient at the peak wavelength of 328–300 $m\mu$. ($\epsilon_{\max}$) was 19700.

Fig. 1. Absorption spectra of chlorogenic acid obtained from the apple (Hulme), —; and from the sweet potato (Nelson), - - -.

DISCUSSION

These results demonstrate the presence of chlorogenic acid in the young fruits of the Worcester Pearmain apple. The 70 kg. of pulp tissue yielded approx. 19 g. of 'crude chlorogenic acid preparation' in the combined first ethyl acetate extracts and approx. 3.5 g. in the combined second ethyl acetate extracts. The light absorption measurements showed that the first ethyl acetate extracts were richer in chlorogenic acid in relation to 'catechins' than the second ethyl acetate extracts, so that some form of counter-current extraction should, in future work, greatly facilitate the separation of chlorogenic acid and catechin.

Approximately 3 g. of pure chlorogenic acid were obtained from the 19 g. of crude chlorogenic acid preparation from the first ethyl acetate extracts; practically none crystallized from the crude preparation from the second ethyl acetate extracts. Some of the acid undoubtedly remained uncrystallized from the catechin-rich supernatant liquids, as was confirmed by an examination of these liquids by filter-paper chromatography. Since catechin gallates, the only catechin compounds having a high molecular extinction coefficient between 270 and 280 $m\mu$. (Bradfield *et al.* 1947; Bradfield & Penney, 1948) appear to be absent from

the crude chlorogenic preparations (see p. 338) it was possible, from an examination of the ultraviolet absorption spectra of these crude preparations, to conclude that at least twice as much chlorogenic acid was present in the 70 kg. of apple pulp tissue as was isolated in a crystalline form, i.e. 6–7 g. in all or approximately 10 mg./100 g. of pulp tissue. The same pulp tissue contains 800 mg./100 g. of quinic acid (Hulme, 1952) and, in spite of the disparity in these quantities, it is tempting to conclude that the two acids are metabolically related in the fruit. It is possible that one role of quinic acid is, by combining with the relatively insoluble caffeic acid, to render the *o*-dihydroxyphenolic properties of this latter acid more readily available in the form of the soluble chlorogenic acid. Chlorogenic acid would then be analogous to the glycosides of the insoluble flavone and flavonol pigments, the quinic acid residue in the former playing the same 'solubilizing' role as the sugar residues in the latter. Glycosides of caffeic acid are not commonly known to occur; the

caffeic acid-phenol glycoside of olives of Cruess & Alsberg mentioned earlier appears to be the only one so far reported.

SUMMARY

1. The occurrence and possible function of chlorogenic acid in plants is discussed.
2. A method is described whereby the acid can be isolated free of catechins from the young fruits of Worcester Pearmain apples.
3. An estimation is made of the amount of chlorogenic acid present in these young apples.
4. Its possible relationship to quinic acid, also present in the fruits, is discussed.

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REFERENCES

- Bradfield, A. E. (1948). Private communication.
 Bradfield, A. E. & Bate-Smith, E. C. (1950). *Biochim. biophys. Acta*, **4**, 441.
 Bradfield, A. E., Flood, A. E., Hulme, A. C. & Williams, A. H. (1952). *Nature, Lond.*, **170**, 168.
 Bradfield, A. E. & Penney, M. (1948). *J. chem. Soc.* p. 2249.
 Bradfield, A. E., Penney, M. & Wright, W. B. (1947). *J. chem. Soc.* p. 32.
 Campbell, P. N., Work, E. & Mellanby, E. (1951). *Biochem. J.* **48**, 106.
 Cruess, W. V. & Alsberg, C. L. (1934). *J. Amer. chem. Soc.* **56**, 2115.
 Freudenberg, K. (1933). *Tannin, Cellulose, Lignin*. Berlin: J. Springer.
 Gorter, K. (1909). *Arch. Pharm., Berl.*, **247**, 184, 436.
 Hoepsner, W. (1932). *Chem. Z.* **86**, 991.
 Hulme, A. C. (1951). *J. exp. Bot.* **2**, 298.
 Hulme, A. C. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 317.
 Joslyn, M. A. & Ponting, J. D. (1951). *Advanc. Food Res.* **3**, 1.
 Kirby, K. S., Knowles, E. & White, T. (1951). *J. Soc. Leath. Tr. Chem.* **35**, 338.
 Ludwig, E. & Kromeyer, O. (1854). *Arch. Pharm., Berl.*, **99**, 285.
 Onslow, M. W. (1931). *Principles of Plant Biochemistry*. Cambridge University Press.
 Oparin, A. (1921). *Biochem. Z.* **124**, 90.
 Oparin, A. (1927). *Biochem. Z.* **182**, 155.
 Palladin, W. (1912). *Z. GarPhysiol.* **1**, 91.
 Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
 Partridge, S. M. (1949). *Biochem. J.* **44**, 521.
 Roberts, E. A. H. (1951). Private communication.
 Rudkin, G. O. & Nelson, J. M. (1947). *J. Amer. chem. Soc.* **69**, 1470.
 Schramm, G. & Primosigh, J. (1943). *Ber. dtsch. chem. Ges.* **76**, 373.

Separation and Estimation of Blood Keto Acids by Paper Chromatography

By M. F. S. EL HAWARY AND R. H. S. THOMPSON

Department of Chemical Pathology, Guy's Hospital Medical School, London

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Owing to the interest attaching to the level of pyruvic acid in the blood, and its variations under physiological and pathological conditions, a number of methods have been devised for its estimation. Based on the earlier work of Dakin & Dudley (1913), the 'hydrazone' method was introduced by Barrenscheen & Dreguss (1931) and Case (1932). Its modifications by Peters & Thompson (1934), Lu (1939a), Bueding & Wortis (1940) and others,

afforded a more specific method for the estimation of total keto acids than the 'bisulphite-binding' method of Clift & Cook (1932a). However, even the later precautions introduced by Friedemann & Haugen (1943), while improving still further the specificity of the method for the estimation of pyruvic acid, did not completely exclude interference from other keto acids present in blood or other biological materials.