WATER-SOLUBLE CONSTITUENTS OF FRUIT

I. SOME OBSERVATIONS ON THE OCCURRENCE OF FREE GALACTURONIC ACID IN FRUIT

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Summary

Glucuronic acid showed no tendency to convert to the lactone when run on paper chromatograms in a variety of solvent mixtures. There were only slight differences between the R_F values of glucuronic and galacturonic acids in all the solvents used, including two mixtures for which large differences in R_F had been recorded.

By the use of paper chromatography, a free uronic acid was detected in several varieties of pears after ripening at 20°C, but not in green or tree-ripened pears. Similar results were obtained with freestone peaches. No free uronic acid was detected in apricots whether ripened on the tree or at 20°C.

Chemical tests applied to the free uronic acid in pears, after elution from paper chromatograms, indicated that it was galacturonic acid. In the sample which gave the strongest uronic acid spot, the concentration of free galacturonic acid was 350 μg/g fresh fruit.

I. Introduction

Derivatives of polygalacturonic acid are important cell-wall constituents of fruit but, until recently, there has been no evidence for the occurrence of free galacturonic acid among the soluble constituents of fruit.

Harris (1948) isolated galacturonic acid from rotten apples as the *p*-bromphenylhydrazine salt of its *p*-bromphenylhydrazone and, using a colorimetric method, found 1580 μg/ml of free uronic acid in juice from rotten apples and 13-54 μg/ml in juice from different varieties of sound apples. Harris suggested that the estimation of free uronic acid could be used to establish whether rotten apples had been used in the manufacture of juices and jellies. This work was continued by Winkler (1949, 1951, 1952) and Mills (1951, 1952) who found, for example, 1149 μg/g of free uronic acid in rotten pears and 40-52 μg/g in fresh strawberry juice. These authors used colorimetric methods to estimate the uronic acid in the total acids separated as the lead salts, or by using ion-exchange resins; consequently they did not obtain direct evidence that the free uronic acid in sound fruit was galacturonic acid.

This paper presents same evidence that the free uronic acid in sound ripe fruit is galacturonic acid and that its occurrence may depend on the method of ripening. The investigation is being continued with the aim of isolating the free uronic acid. The effect of different methods of ripening is outside the scope of the work and is not being further investigated.

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II. Methods

(*a*) *Fruit Extracts*

Samples of 6-20 (usually 12-20) sound fruits were used for the preparation of 80 per cent. alcoholic extracts. Cut tissue was dropped straight into a Waring Blendor containing alcohol cooled to − 20°C. The mixture was blended for 4 min, bringing the temperature to about 20°C, and then filtered or centrifuged. This procedure was used to minimize enzyme action in the fruit and chemical reactions in the extract. Judging only by paper chromatograms, the same results were obtained with ethanol at room temperature, or when the blended mixture was boiled for 10 min with, or without, calcium carbonate. The extracts were stored at − 20°C and gave apparently identical chromatograms over long periods of storage.

(*b*) *Paper Chromatography*

Spots were put on filter paper sheets (24 by 24 in.) with calibrated haemocytometer pipettes or an "Agla" microsyringe. Chromatograms were developed by descending flow in apparatus similar to that described by Woiwod (1949). The tanks were housed in an inside room where the variation in temperature was generally less than 1°C during each run; the annual temperature range was 17-28°C. The solvent was allowed to drip off the bottom edge of the paper (Jermyn and Isherwood 1949) which was serrated with pinking scissors. The papers were dried in a fume-cupboard at room temperature.

The solvents used in all experiments, except those described in Section III (*a*), were ethyl acetate-pyridine-water (2·5 : 1 : 3·5 v/v) (McFarren, Brand, and Rutkowski 1951), *n*-butanol-acetic acid-water (4 : 1 : 1 v/v), and phenol-water 4 : 1 w/v).

The spots were revealed by means of the following reagents:

(i) *Silver Nitrate-Sodium Hydroxide.*—The reagent of Trevelyan, Proctor, and Harrison (1950) was used and the papers were then fixed with sodium thiosulphate (10 per cent.) containing sodium metabisulphite (1·5 per cent.). To obtain maximum sensitivity it was necessary to use a freshly prepared solution of alcoholic sodium hydroxide for spraying.

(ii) *Aniline Phosphate.*—Orthophosphoric acid (3 ml of 98 per cent.) was added to a solution of aniline (6 ml) in water-saturated butanol (600 ml) (cf. Bryson and Mitchell 1951). The sprayed chromatogram was heated for 5 min at 105-110°C. Uronic acids gave pinkish orange spots which appeared as dark spots in ultraviolet light.

(iii) *Naphthoresorcinol-Trichloracetic Acid.*—One volume of naphthoresorcinol (1 per cent. in ethanol) was mixed with 9 volumes of trichloracetic acid (10 per cent.) in water immediately before use. The sprayed chromatograms were heated for 8 min at 95-100°C. The mixture used by Partridge (1948) was less sensitive.

(*c*) *Elution of Compounds from Paper Chromatograms*

The spots of the required compound were located in the usual way (cf. Dent 1947, p. 244) by spraying marker strips, three strips being adequate for

a 24-in. chromatogram. The apparatus used for elution was similar to that described by Dent (1947), except that a short piece of filter paper was held between two microscope slides to act as a feeder strip. The strip to be eluted was touched against the wet end of the feeder strip and thus hung from it. Strips were sprayed to test whether the compound had been completely eluted; collection of 1 ml of eluate was found adequate. With one feeder strip, the collection of 1 ml took ½-2 hr, depending on the length of the strip being washed. The rate of elution was increased when desired by using two or three feeder strips superimposed.

III. Results

(*a*) *Paper Chromatography of Galacturonic and Glucuronic Acids*

(i) *Preparation of Glucuronic Acid Solution.*—A solution of sodium glucuronate was prepared by the electrometric titration of glucuronolactone to pH 8 with sodium hydroxide (Heald 1952; Hirsch 1952). The salt was converted to the free acid by shaking the solution with a cation-exchanger ("Zeokarb 225"). This solution of glucuronic acid contained no lactone (i.e. it gave no lactone spot on chromatograms run in butanol-acetic acid-water) immediately after preparation, and was completely stable at room temperature for at least several days.

(ii) *Formation of Glucuronolactone.*—Aqueous solutions (1 per cent.) of glucuronic and galacturonic acids were refluxed for 30 min and then compared on paper chromatograms run for 64 hr in butanol-acetic acid-water. Glucuronic acid was partially converted to the lactone (cf. Quick 1927; Hirsch 1952) as shown by the appearance on the chromatogram of a second spot which ran the same distance as authentic glucuronolactone. This spot was well separated from that due to glucuronic acid. Galacturonic acid was unchanged.

(iii) *Attempted Separation of the Uronic Acids on Paper.*—Glucuronic and galacturonic acids were not separated by butanol-acetic acid-water (4 : 1 : 1 v/v) or water-saturated *iso*butyric acid (cf. Partridge 1948), ethyl acetate-pyridine-water (2·5 : 1 : 3·5 v/v) (cf. Isherwood and Jermyn 1951), ethyl acetate-acetic acid-water (3 : 1 : 3 v/v) (cf. Rao, Beri, and Rao 1951), acetic acid-water (2·5 : 1 v/v), or ethanol-*iso*butyric acid-water (4 : 1 : 1 v/v). When run in amyl alcohol-pyridine-water (7 : 7 : 6 v/v) (Werner and Odin 1949; Masamune and Maki 1951) on acid-washed No. 1 Whatman paper at 22°C, there was a slight difference in the distance run but the separation was incomplete (e.g. distance from starting line after a 48-hr run, glucuronic acid 11·3, galacturonic 9·8 cm; after a 64-hr run, glucuronic 25·0, galacturonic 23·4 cm).

Both acids gave normal spots when run in water-saturated *iso*butyric acid or acetic acid-water on unwashed No. 1 or No. 4 Whatman paper; in all of the other solvent mixtures used, both acids gave elongated or double spots on unwashed No. 1 or No. 4 Whatman paper.

TABLE 1

OCCURRENCE OF A URONIC ACID IN FRUIT (DETECTED ON PAPER CHROMATOGRAMS RUN IN ETHYL ACETATE-PYRIDINE-WATER)

Sample*	Fruit	Variety	Season	Maturity†		Ripened at 20°C (days)	Uronic Acid‡
				Picked	Extracted		
1*a*	Pears	Winter Cole	1950	Green	Green	—	—
1*b*	Pears	Winter Cole	1950	Green	Ripe	9	++
2	Pears	Winter Cole	1950	Green	Ripe	10	++++
3*a*	Pears	Winter Cole	1951	Green	Green	—	—
3*b*	Pears	Winter Cole	1951	Green	Ripe	10	+
4*a*	Pears	Winter Cole	1951	Green	Green	—	—
4*b*	Pears	Winter Cole	1951	Green	Ripe	13	+++
5*a*	Pears	Winter Cole	1952	Green	Green	—	—
5*b*	Pears	Winter Cole	1952	Ripe	Ripe	—	—
5*c*	Pears	Winter Cole	1952	Ripe	Overripe	—	—
5*d*	Pears	Winter Cole	1952	Medium	Ripe	4	+
5*e*	Pears	Winter Cole	1952	Green	Ripe	9	++
6*a*	Pears	Packham's Triumph	1950	Green	Green	—	—
6*b*	Pears	Packham's Triumph	1950	Green	Ripe	10	++
7*a*	Pears	Packham's Triumph	1952	Green	Green	—	—
7*b*	Pears	Packham's Triumph	1952	Medium	Ripe	8	+
7*c*	Pears	Packham's Triumph	1952	Green	Ripe	13	+++
8*a*	Pears	Josephine	1950	Green	Green	—	—
8*b*	Pears	Josephine	1950	Green	Ripe	10	+
9	Peaches	Blackburn Elberta	1950	Ripe	Ripe	—	—
10	Peaches	Blackburn Elberta	1951	Green	Ripe	6-8	+++
11	Peaches	Blackburn Elberta	1952	Medium	Ripe	*c.* 4	+
12	Peaches	J. H. Hale	1950	Green	Ripe	*c.* 8	+
13	Peaches	Golden Queen	1950	Medium	Ripe	*c.* 4	—
14	Apricots	Trevatt	1950	Medium	Medium	1	—
15	Apricots	Trevatt	1951	Green	Ripe	4-5	—
16*a*	Apricots	Trevatt	1952	Green	Green	—	—
16*b*	Apricots	Trevatt	1952	Ripe	Ripe	—	—
16*c*	Apricots	Trevatt	1952	Green	Ripe	5	—
16*d*	Apricots	Trevatt	1952	Medium	Ripe	4	—
17*a*	Apples	Granny Smith	1951	Green	Green	—	—
17*b*	Apples	Granny Smith	1951	Green	Ripe	35	—

* Samples of fruit received are indicated by numbers. Letters indicate subsamples obtained by sorting or ripening the fruit received.

† "Green" fruit had been picked at commercial maturity but was not suitable for eating raw. "Ripe" fruit was eating-ripe. Sample 5 (200 lb of fruit) was cold-stored at −1°C for 4 months and when opened was sorted into green, medium, ripe, and overripe; total solids contents of samples extracted were 5*a*, 15·3; 5*b*, 20·8; 5*c*, 20·1; 5*d*, 18·4; 5*e*, 16·6; confirming that there had been a wide range of maturity at picking. Sample 7 was stored at 0°C and then sorted into green and medium; total solids contents of samples extracted were 7*a*, 17·2; 7*b*, 18·2; 7*c*, 17·5 per cent.

‡ The approximate range of uronic acid contents was 0·4-2 μg in the spot on the paper, corresponding with approximately 60-300 μg/g fresh fruit. The relative values were assessed visually on papers sprayed with silver nitrate-sodium hydroxide.

(*b*) *Detection of a Uronic Acid in Fruit Extracts*

Crude 80 per cent. alcoholic extracts consistently gave compact spots on paper chromatograms. The uronic acid was recognized by its R_F values and its reactions with the three reagents described (Section II (*b*)). Other ions reacted only with the silver nitrate reagent and did not interfere with the recognition of the uronic acid spots (cf. Plate 1). The spots considered to be the uronic acid were not affected in any way by the removal of cations with a cation-exchange resin ("Zeokarb 225" or "Dowex 50"), but disappeared after the removal of anions with an anion-exchange resin ("Amberlite 1R-4B"). The results obtained with the various extracts are set out below:

(i) *Two-dimensional Chromatograms.*—Alcoholic extracts (approx. 60 μl, equivalent to 12·4 mg fruit) of ripe apricots, peaches, pears (3 lots), and apples were applied to No. 1 Whatman filter paper. The chromatograms were run for 48 hr in phenol-water and then 64 hr in butanol-acetic acid-water and were compared with chromatograms of known sugars run under the same conditions. The peaches and two lots of pears had a uronic acid spot corresponding in position to galacturonic or glucuronic acid. There was no spot in the area where glucuronolactone would lie.

(ii) *Chromatograms Run in Phenol-Water.*—Alcoholic extracts (approx. 30 μl, equivalent to 6·2 mg fruit) of green pears, ripe pears, ripe peaches, ripe apricots, green apples, and ripe apples (8, 7, 4, 3, 2, and 1 samples respectively) were applied to No. 1 Whatman paper. The chromatograms were run for 48 hr. Uronic acid spots, corresponding with galacturonic or glucuronic acid, were detected in ripe pears and ripe peaches.

(iii) *Chromatograms Run in Ethyl Acetate-Pyridine-Water.*—As before, approximately 30 μl of each extract was applied to No. 1 Whatman paper. The chromatograms were run for 16 or 24 hr. The results obtained are shown in Table 1 and, for samples 3, 4, and 7, in Plate 1, Figures 1 and 2. Known amounts of galacturonic acid were added to a pear extract which apparently contained no uronic acid, and the chromatograms were sprayed with silver nitrate-sodium hydroxide; 0·2 μg was barely detectable, 0·4-2 μg covered the range given by the fruit extracts.

(*c*) *Tests to Determine the Identity of the Uronic Acid in Pears*

(i) *Collection of Uronic Acid.*—The 80 per cent. alcoholic extract of sample 2 (Table 1) was concentrated under reduced pressure. Spots of the concentrate (equivalent to 31 mg fresh fruit) were applied to No. 3 MM Whatman paper. Chromatograms were run in ethyl acetate-pyridine-water and marker strips were sprayed. The uronic acid was eluted from appropriate strips cut from the unsprayed sections of the papers.

(ii) *Attempt to Form a Lactone.*—Aqueous solutions (1 per cent.) of this uronic acid which had been refluxed for 30 min did not give a lactone spot on paper chromatograms (cf. Section III (*a*) (ii)).

(iii) *Ehrlich Basic Lead Acetate Test.*—This test (Ehrlich 1932) was applied to eluted uronic acid (600 μg) dissolved in water (0·05 ml). When a freshly prepared saturated solution of basic lead acetate (2 drops) was added a white precipitate formed which became orange after heating at 100°C. The solution evaporated, leaving an orange residue. A similar quantity of authentic galacturonic acid gave the same colour and glucuronic acid gave a pale yellow. An eluate from a blank paper run in ethyl acetate-pyridine-water gave no colour.

(iv) *Dische Cysteine Colour Reaction.*—Authentic galacturonic acid gave the characteristic green colour described by Dische (1948), but the same sample, run on paper in ethyl acetate-pyridine-water and then eluted, failed to give the green colour. The reaction also failed when an eluate from a blank paper, which had been irrigated with ethyl acetate-pyridine-water, was added to a solution of galacturonic acid.

(*d*) *Quantitative Determination of Galacturonic Acid in Pears*

Nineteen spots (each 15 μl ≡ 3·1 mg fruit) of an alcoholic extract of sample 2 (Table 1) were put on a sheet of No. 1 Whatman paper. The chromatogram was run in ethyl acetate-pyridine-water, three marker strips were sprayed, and duplicate strips, each containing eight uronic acid spots, were eluted. The eluates were evaporated and the galacturonic acid estimated by the Dische carbazole reaction, using the proportions of sulphuric acid and water given in the modified method (Dische 1950) but heating at 100°C for 20 min (Dische 1947). The sulphuric acid used was heated strongly with ammonium sulphate to eliminate oxides of nitrogen which gave high blank values. Very good agreement was obtained between the duplicates, the average being 320 μg/g. The estimation was repeated using a concentrated extract of sample 2, each spot being the equivalent of 15·5 mg fruit. The mean of good duplicates was 380 μg/g.

IV. Discussion

(*a*) *Paper Chromatography of Glucuronic and Galacturonic Acids*

Using an aqueous solution of glucuronic acid containing no trace of the lactone, it was found that the acid was stable at room temperature for at least several days, and was only partially converted to glucuronolactone by refluxing for 30 min (Section III (*a*) (i) and (ii)) (cf. Quick 1927; Hirsch 1952). Glucuronic acid was also stable when run on paper chromatograms in a variety of solvent mixtures, as shown by the absence of streaking and of the lactone spot.

Partridge (1948) first described the behaviour of glucuronic acid and its lactone on paper chromatograms, using a mixture of the acid and lactone obtained by the hydrolysis of menthol-glucuronide according to the method of Quick (1927). It is clear (Partridge 1948, p. 243) that it was this mixture which was applied to the paper chromatograms, and which gave two spots corresponding respectively with glucuronic acid and glucuronolactone. However, also on p. 243 (Partridge 1948) it is stated that "glucuronic acid and galacturonic acid

may be differentiated by the formation of the glucurone spot by the former," and on p. 248, in the discussion, it is said that "in acidic solvents solutions of glucuronic acid give rise to two well separated spots, one of them due to the lactone." It appears that there has been some misunderstanding due, firstly, to starting with a mixture of glucuronic acid and its lactone and secondly, to the formation of elongated spots by glucuronic acid which sometimes had "a marked forward trail." Both galacturonic and glucuronic acids give double or elongated spots in a number of solvent mixtures (Partridge 1948; Section III (*a*) (iii) above), and it has now been shown (Anet, Ash, and Reynolds, unpublished results) that the effect is due to certain of the cations in the paper.

Glucuronic acid ran slightly further than galacturonic acid in amyl alcohol-pyridine-water, but the acids ran the same distance in all of the other solvents tried, including ethyl acetate-acetic acid-water (Section III (*a*) (iii)). Whatman No. 1 and No. 4 papers were used in this work and the results for amyl alcohol-pyridine-water agreed with those obtained by Werner and Odin (1949) on Munktell OB paper; on the other hand Masamune and Maki (1952), using the same solvent mixture and Toyo No. 2 paper, recorded a good separation of these acids (R_F values 0·00 and 0·13). Rao, Beri, and Rao (1951) recorded R_F values of 0·17 and 0·30 for glucuronic and galacturonic acids on circular chromatograms run with ethyl acetate-acetic acid-water at 35°C, whereas in the present work these acids ran the same distance on descending chromatograms run at 20°C.

It appears therefore that galacturonic and glucuronic acids cannot at present be distinguished on paper chromatograms, and consequently it becomes necessary to elute the uronic acid from the paper and apply tests to the eluted acid as described in Section III (*c*).

(*b*) *Occurrence of Galacturonic Acid in Fruit*

A free uronic acid was detected by means of paper chromatography in several varieties of pears when the fruit was ripened at 20°C (Section III (*b*); Table 1; and Plate 1, Fig. 1); it was never found in the fruit as picked, although it may have been present in quantities not detectable under the conditions used. Where there was a variation in maturity at picking, the amount of uronic acid was related to the time of ripening (Table 1; Plate 1, Fig. 2).

A free uronic acid was also present in freestone peaches which had been ripened at 20°C, but was not detected in tree-ripened peaches (Table 1, samples 9-12). The free uronic acid was not detected in ripened clingstone peaches, nor in green or ripened apricots or apples (Table 1, samples 13-17*b*).

Chemical tests on some uronic acid eluted from paper chromatograms of pear extracts (Section III (*c*)) indicated that it was galacturonic acid. This was subsequently confirmed by other methods for peaches (Anet and Reynolds 1953) and pears (Anet and Reynolds, unpublished results).

The concentration of galacturonic acid in the sample which gave the strongest uronic acid spot (sample 2, Table 1) was 350 μg/g fresh fruit. The smallest amount of galacturonic acid which could be detected on paper chro-

matograms lay between 0·2 and 0·4 μg, corresponding with 30-60 μg/g fresh fruit. Most of the values obtained by earlier workers (Harris 1948; Winkler 1951, 1952; Mills 1952; cf. Section I above) were less than 60 μg/g.

Two significant points emerge from the present work. Firstly, a number of samples of pears and peaches ripened in a constant-temperature room at 20°C contained much higher concentrations of free galacturonic acid than any previously recorded. Secondly, where there was an appreciable variation in maturity at picking, the amount of free galacturonic acid present after ripening was greater in fruit which was originally less mature, and consequently required a longer time at 20°C to ripen.

The most probable mode of formation of free galacturonic acid in fruit would be by the enzymic degradation of pectin, which would require the presence of both pectin esterase and polygalacturonase (Lineweaver and Jansen 1951). Both of these enzymes can be produced by microorganisms, and pectin esterase is widely distributed in higher plants (Lineweaver and Jansen 1951; Kertesz 1951). However, Kertesz (1951) stated that, except in germinating barley, it was doubtful whether polygalacturonase occurred in higher plants. Since then Joslyn, Mist, and Lambert (1952), Weurman (1952), and Roelofsen (1953) have published the results of experiments which indicated the presence of polygalacturonase in apple juice, pears, and tomatoes respectively. Weurman (1952) also found that the pears studied (var. Doyenné Boussoc) contained a substance which acted as an inhibitor of polygalacturonase activity, and suggested that the occurrence of an inhibitor explained the failure of earlier attempts to establish the presence of polygalacturonase in fruits.

It therefore appears possible that the increased production of free galacturonic acid in fruit ripened at 20°C, now described, was due to a disturbance in the relationship between an inhibitor and an enzyme. On the other hand, pectic enzymes of fungal origin, if present, would become active when the fruit was transferred to 20°C. Most of the pear samples were ripened and extracted after the fruit had been stored for 4 or 5 months at −1° or 0°C and this long storage period would increase the possibility of contamination by fungal enzymes. However, two pear samples were ripened and extracted after only 1 or 2 months storage, and none of the peach samples was cold-stored for more than a few days. Furthermore, there was no visible evidence of infection in the fruit used. It therefore seems unlikely that the results were due to the activity of fungal enzymes.

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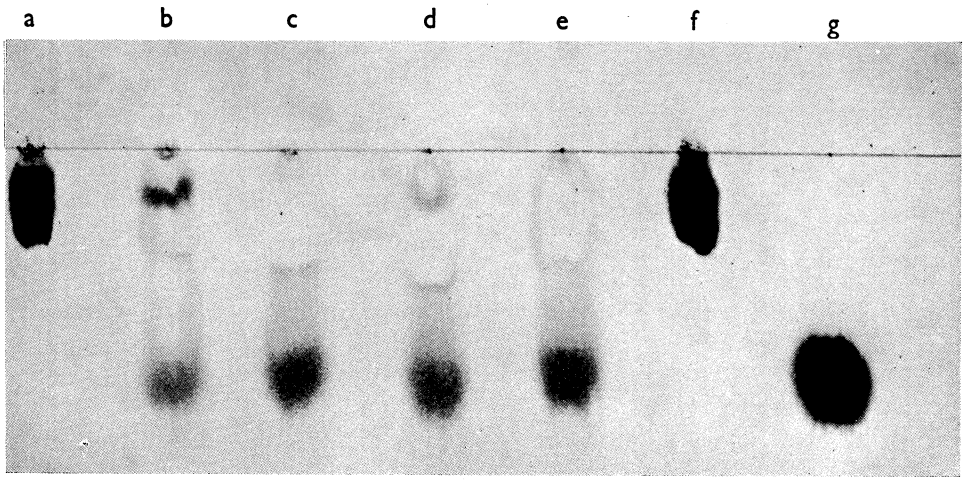

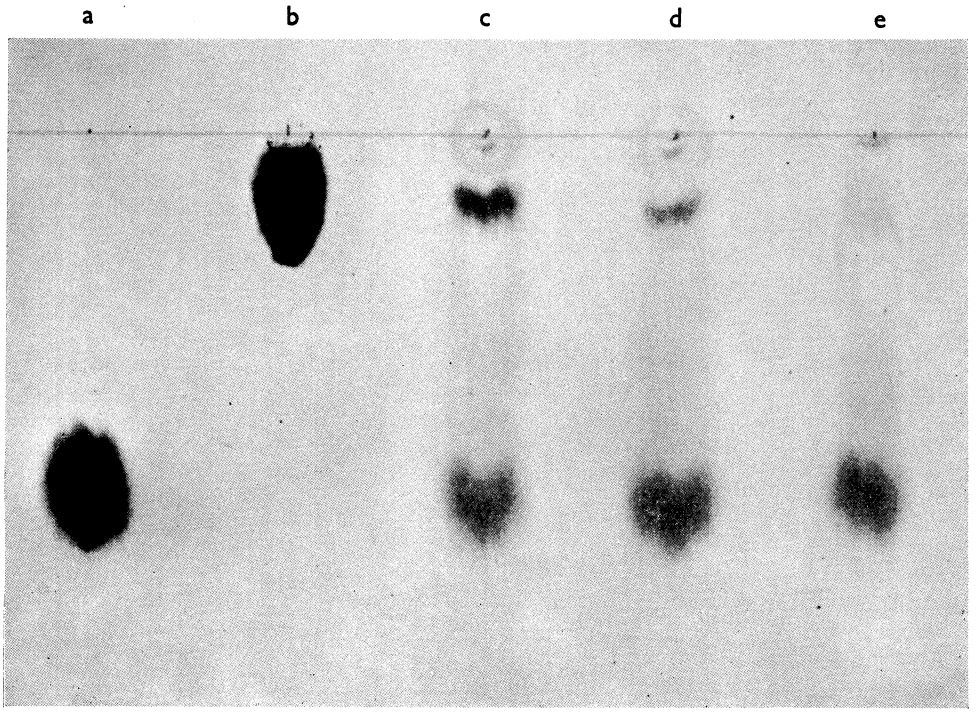

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EXPLANATION OF PLATE 1

Fig. 1.—Portion of paper chromatogram on No. 1 Whatman paper (unwashed) run 24 hr in ethyl acetate-pyridine-water and treated with silver nitrate-sodium hydroxide showing starting line and
(*a*) D-Galacturonic acid;
(*b*) Galacturonic acid and inositol in Winter Cole pears (1951), from Batlow, N.S.W., ripened 13 days at 20°C (Table 1, 4*b*);
(*c*) Inositol in same lot of pears before ripening (Table 1, 4*a*);
(*d*) Galacturonic acid and inositol in Winter Cole pears (1951) from Orange, N.S.W., ripened 10 days at 20°C (Table 1, 3*b*);
(*e*) Inositol in same lot of pears before ripening (Table 1, 3*a*);
(*f*) D-Galacturonic acid;
(*g*) Meso-inositol.

Fig. 2.—Portion of paper chromatogram on No. 1 Whatman paper (unwashed) run 24 hr in ethyl acetate-pyridine-water and treated with silver nitrate-sodium hydroxide showing starting line and
(*a*) Meso-inositol;
(*b*) D-Galacturonic acid;
(*c*) Galacturonic acid and inositol in Packham's Triumph pears (1952), from N.S.W., ripened from green to ripe in 13 days at 20°C (Table 1, 7*c*);
(*d*) Galacturonic acid and inositol in same lot of pears ripened from medium to ripe in 8 days at 20°C (Table 1, 7*b*);
(*e*) Inositol in same lot of pears before ripening (Table 1, 7*a*).