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EXTRACTION AND PURIFICATION OF PECTINS FROM ALCOHOL INSOLUBLE SOLIDS FROM RIPE AND UNRIPE APPLES

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ABSTRACT

Pectins are extracted from Alcohol Insoluble Solids of ripe and unripe apples and fractionated by ion exchange chromatography and gelfiltration. In the extracts mainly pectins with neutral sugar contents of 0·15, 0·24 and 0·53 mol neutral sugar residues/mole galacturonate residues are present. The pectin molecules contain rhamnose, arabinose, xylose, galactose, glucose and galacturonic acid residues. No mannose could be detected. The neutral sugar composition of the glycans bound to the galacturonan was found to be constant, except for the relative amount of galactose. During ripening the neutral sugar composition of the extractable pectin does not change.

INTRODUCTION

The chemical structure of pectin has been the subject of many scientific reports for more than 50 years. Elucidation of this structure is important because of the function of pectin in the cell wall as a 'lubricating' or 'cementing' agent (Rees & Wight, 1969), its role during ripening of fruits (Knee, 1978a, b), its role in food processing (Rombouts & Pilnik, 1978; van Buren, 1979) and its role as nutritional fibre.

The pectin molecule consists mainly of galacturonic acid, rhamnose, arabinose, xylose, galactose and glucose. In some pectins other sugars can be detected as minor constituents (e.g. in the 'RG 2' of Darvill *et al.*, 1978). The molecules have a galacturonan backbone, in which rhamnosyl residues are present (Aspinall & Fanshawe, 1961; Barret & Northcote, 1965; Aspinall *et al.*, 1967a, b; Foglietti & Percheron, 1968; Aspinall *et al.*, 1969; Aspinall *et al.*, 1970; Aspinall & Cottrell, 1970; Talmadge

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et al., 1973; Kato & Noguchi, 1976; Toman *et al.*, 1976; Pfister, 1977; Simson & Timell, 1978). The neutral sugar side chains are linked to the main chain via both galacturonic acid and rhamnose units. Covalent bonds between galactose and galacturonic acid and between xylose and galacturonic acid are often found (Bouveng, 1965; Aspinall *et al.*, 1967a, b; Stoddart *et al.*, 1967; Aspinall *et al.*, 1968; Foglietti & Percheron, 1968; Kikuchi & Sugimoto, 1976).

Covalent bonds of galactose and galacturonic acid and of xylose and galacturonic acid probably also occur in apple pectin (Barret & Northcote, 1965). The pseudo-aldobiuronic acid of galacturonic acid and arabinose (L-arabinofuranosyl-(1-3)-D-galacturonic acid) has also been detected in carnation root pectin and in lucerne pectin (Aspinall *et al.*, 1968; Foglietti & Percheron, 1968).

Methylation analysis reveals that rhamnose is present as chain unit, branch point unit and terminal unit (Talmadge *et al.*, 1973; Siddiqui & Wood, 1976; Simson & Timell, 1978).

Although the pectins of different species are structurally related (McNeil *et al.*, 1978), it cannot be said that the structure of pectin is independent of the species; an apiogalacturonan has been extracted from duck weed (*Lemna minor*) and a xylogalacturonan from mountain pine pollen (*Pinus mugo* var. Turra) (Mascaro & Kindel, 1977; Bouveng, 1965). The structure of pectin depends not only on the species, but also on the physiological state of the material studied (Gould *et al.*, 1965; Dalessandro & Northcote, 1977; Matheson & Saini, 1977).

In this paper the extraction and purification of pectins from Golden Delicious apple Alcohol Insoluble Solids (AIS) are described. The pectins were fractionated by ion exchange chromatography and gelpermeation chromatography and the amounts of the different neutral sugars in the pectin fractions were determined. Apples were chosen as the pectin source, not only because of the industrial use of apple marcs as a pectin source and the important contribution of apples to the diet of many people, but also because the apple is a climacteric fruit. Microscopic investigations show that after the climacteric, the middle lamella in apple fruit tissue has changed (Ben-Arie *et al.*, 1979). Pectin may be involved in this process as an important factor in fruit softening. We chose a sequential extraction with cold and hot acetate buffer, with oxalate/EDTA solutions and with dilute hydrochloric acid, realising that both the preparation of the AIS and the extractions have an impact on the chemical features of the pectins (Joslyn & Deuel, 1963). The temperature was kept below 70°C, in order to minimise the rate of hydrolysis of acid-labile glycosidic bonds. Ion exchange columns (i.e. DEAE-cellulose) have been successfully applied by various investigators to fractionate pectins (Kikuchi & Yokotsuka, 1972; Knee, 1973, 1978a, b; Berth *et al.*, 1977; Anger *et al.*, 1977). Ion exchange can be used to fractionate pectins according to their degree of esterification (van Deventer-Schriemer & Pilnik, 1976). However, it is likely that covalently linked neutral sugars and the molecular weight of the pectin also affect the elution profile (Anger *et al.*, 1977). Gelfiltration of pectin is also difficult to interpret because of the negative charge of the molecules; the molecular

weights (or rather the hydrodynamic volumes) found by calibration with dextrans must be considered as being too high as a result of the repulsion between sample and column material although this effect is probably reduced in the presence of buffers (Thibault, 1979).

METHODS

Preparation of AIS

Golden Delicious apples were obtained from 'de Boutenburg', an experimental apple orchard at Lienden, De Betuwe, The Netherlands. They were gathered on 12 October 1978, and stored in the open till 17 October 1978. Some of the apples were then allowed to ripen at 20°C for 4 weeks, the first week in an impermeable plastic bag. Both ripe and unripe apples were peeled, cored and soaked in a 0·2% solution of ascorbic acid to prevent browning before being mashed in a Kenwood Chef meat mincer. Immediately after mashing, portions of 1 kg were extracted three times with 2·5 litres 96% alcohol at 70°C. The AIS-preparation was air-dried overnight after solvent drying with acetone, ground in a hammer mill with a 10 μm sieve and stored at −40°C. The results of this procedure have been summarised in Table 1.

TABLE 1
Preparation of AIS from Apples

	Unripe	*Ripe*
Weight % of peels and cores	27·9	30·3
Weight % of pulp	72·1	69·7
Weight % of AIS compared with pulp	2·05	2·08
Anhydro-uronic acid content of AIS (mg/g)	279	284
Degree of esterification %	65	70

Extractions

Ten grams of AIS were extracted three times during 30 min (while stirred) with 300 ml 0·05 M Na-acetate buffer (pH = 5·2) at room temperature and the whole procedure was repeated at 70°C. The material was then further extracted with 0·05 M EDTA and 0·05 M NH_4-oxalate in 0·05 M Na-acetate buffer (again three times in 30 min at 70°C). After washing with water this extraction was followed by an extraction with dilute hydrochloric acid (three times, 30 min, 70°C, pH 2·5). The extracts were filtered and the pectins were precipitated with ethanol at 70% concentration. In the text these four extracts are referred to as the cold buffer, hot buffer, oxalate and acid extracts.

Ion Exchange

One-gram quantities of pectin were dissolved in 5 mM Na-phosphate buffer, pH = 5·1, and applied to a 30 x 1 cm column of DEAE-cellulose (Whatman DE 52). The pectins were eluted from the column (after washing thoroughly) with a linear gradient of 5–500 mM Na-phosphate buffers of pH = 5·1 (500 ml). Experiments were performed at room temperature.

Gelfiltration

Ten to fifty milligrams of pectin were dissolved in about 2 ml buffer and applied to a 80 x 2·5 cm column of Sephacryl S-300 (Pharmacia). The eluent was 0·1 M Na-phosphate, pH = 5·1. The flow rate was 0·3 ml/min, controlled by an LKB peristaltic pump. Experiments were performed at room temperature.

Analytical Methods

The anhydro-uronic acid (AUA, MW 176) content of pectin fractions was determined by an automated carbazole-sulphuric acid assay (van Deventer-Schriemer & Pilnik, 1976). The amount of AUA in the AIS preparations was determined according to Ahmed & Labavitch (1977) with meta-hydroxy-diphenyl. The neutral sugars were analysed gas chromatographically as their alditol-acetates (Darvill *et al.*, 1975). The methoxyl content was determined by gas chromatographic analysis of the methanol released on alkaline de-esterification (1 h at room temperature, 0·1 M KOH). Methanol was converted to methyl nitrite and determined according to Versteeg (1979).

RESULTS AND DISCUSSION

Extractions

The mild extraction processes applied in these investigations release less than 50% of the uronic acid residues present in the apple AIS (Table 2). The pectins which remain in the AIS are probably tightly bound to other cell wall components, but the possibility cannot be ruled out that the preparation of the AIS (especially drying) may cause some of the pectin molecules to become insoluble. More severe conditions of extraction give higher yields, but the products obtained are certainly degraded. The only way to elucidate the structure of the non-extractable pectins is to release them with purified enzymes (Darvill *et al.*, 1978; Voragen *et al.*, 1979). After ripening more galacturonan can be extracted. The average sugar contents in both cases are about the same, but a marked difference exists in the distribution of the neutral sugars among the extracts; the oxalate extract of the AIS from ripe apples has a remarkably low neutral sugar content. The larger part of the sugar residues in the extracts is covalently linked to the galacturonan (Table 2); after ripening the amount of unbound neutral sugars (free glycan) increases. This increase is almost completely due to xylose and glucose (results not shown in the table); about equal amounts of xylose and

TABLE 2
Properties of Pectins Extracted from AIS from Unripe and Ripe Apples

	Anhydro-uronic acid (mg/g AIS)	*DE (%)*	*Bound glycan sugars[a] (mg/g AIS)*	*Free glycan sugars[b] (mg/g AIS)*
Unripe apples				
Cold buffer	29	76	3	1
Hot buffer	28	71	6	2
Oxalate	22	77	11	0
Acid	19	68	19	5
Total	98[c]		39	8
Ripe apples				
Cold buffer	30	80	5	3
Hot buffer	30	76	7	3
Oxalate	31	78	5	1
Acid	40	63	42	9
Total	131[d]		59	16

Conditions during the extractions described in the text.
[a] Inseparable from galacturonan by DEAE-cellulose ion exchange chromatography.
[b] Separable from galacturonan by DEAE-cellulose ion exchange chromatography.
[c] The total amount of anhydrogalacturonate material extracted represents 35% of the amount present in the AIS.
[d] The total amount of anhydrogalacturonate material extracted represents 46% of the amount present in the AIS.
DE = degree of esterification.

glucose are released, indicating that a xyloglucan is released from the cell wall material during ripening.

The overall degree of esterification is almost the same in ripe apples as in unripe apples. It is interesting to note that the pectin that cannot be extracted from the AIS by the extraction methods used in this study has a high degree of esterification. The pectin in the oxalate extracts also has a high degree of esterification.

Fractionations

The fractionation of pectin on DEAE-cellulose ion exchange chromatography was used in this study to establish the distribution of the neutral sugars among the pectin molecules. The pectins from the four extracts elute from the DEAE-cellulose columns in one tailing peak, which was collected in many fractions. About 10% of the pectin does not bind to the column and must be fractionated after partial cold alkali saponification. A small percentage must be eluted with 0·01 N NaOH. The pectin fractions from each extract were combined to obtain 10 'pools' with equal amounts of uronic acid residues, as shown in Fig. 1 for the cold buffer extract of ripe apple AIS. In this way 50 pools were obtained from the four extracts, including 10 pools for the pectin fraction that does not bind to the column and the pectin fraction that must be eluted with 0·01 N NaOH. For each of these 50 pools the neutral sugar content was deter-

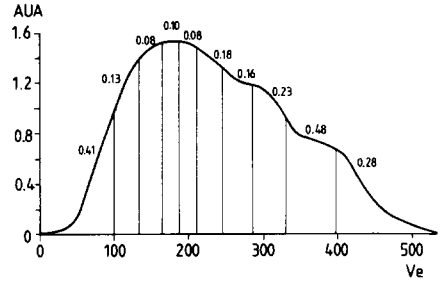

Fig. 1. Fractionation of the pectin from the cold buffer extract of ripe apple AIS on DEAE-cellulose. AUA, anhydro-uronic acid, mg/ml; Ve, elution volume, ml. The DEAE-cellulose column was eluted by a gradient of 5–500 mM Na-phosphate buffer pH = 5·1. The numbers in the figure indicate the neutral sugar residue content of the fractions, expressed as moles neutral sugar residues/mole of galacturonate residues.

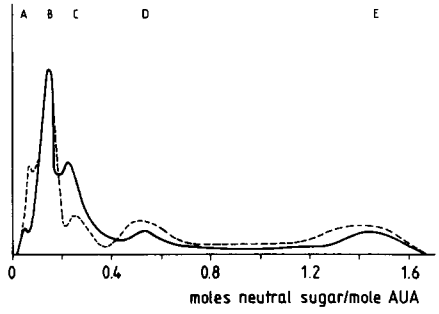

Fig. 2. Neutral sugar distribution curves of pectins extracted from ripe and unripe apple AIS. Construction described in the text. Abscissa: neutral sugar content as moles neutral sugar per mole anhydrogalacturonic acid. The total area represents 100% of the AUA present in the four extracts. —— Unripe apples; – – – ripe apples.

mined and the neutral sugar residues were analysed. The neutral sugar content varied from 0·04 to 1·7 mol neutral sugar residues per mole of galacturonic acid residues. However, the composition of the neutral sugars appeared to be constant, except for the relative amount of galactose. The galactose/arabinose ratio decreases with increasing neutral sugar content. In each of the four extracts the same neutral sugar composition was found. No mannose could be detected. Table 3 gives the neutral sugar composition of the fractions of Fig. 1, expressed as moles of neutral sugar residues per mole of galacturonic acid residues.

TABLE 3
Neutral Sugar Composition of the Pectin Pools of Fig. 1 Expressed as Moles Neutral Sugar Residues/Mole of Galacturonate Residues

Fraction number	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Rhamnose	0·024	0·005	0·004	0·004	0·005	0·007	0·008	0·010	0·026	0·018
Arabinose	0·231	0·060	0·035	0·042	0·035	0·098	0·091	0·128	0·296	0·158
Xylose	0·018	0·004	0·003	0·003	0·003	0·004	0·006	0·009	0·021	0·013
Galactose	0·120	0·058	0·034	0·053	0·032	0·059	0·064	0·077	0·104	0·079
Glucose	0·024	0·005	0·003	0·005	0·003	0·010	0·010	0·009	0·029	0·013
Total	0·42	0·13	0·08	0·10	0·08	0·18	0·18	0·23	0·48	0·28

More information about the distribution of the neutral sugars among the pectin molecules was obtained by constructing neutral sugar distribution curves. These curves were constructed as follows. The 50 pools from ripe apple pectin extracts and the 50

pools from unripe apple pectin extracts were arranged in ascending order of neutral sugar content. From these data two cumulative neutral sugar distribution curves were constructed. Numerical differentiation of these two curves results in the neutral sugar distribution curves of Fig. 2. This figure suggests that in pectin a discontinuous, rather than a continuous, distribution of the neutral sugars is present. The cold buffer extracts contain mainly pectins indicated in Fig. 2 as A, B and C. The cold buffer extract of ripe apple AIS also contains some pectin of type E. The hot buffer extracts contain B, C and D pectins while the oxalate extracts consist of pectins of types A, B, C and D. In the acid extracts C, D and E are the dominating types. The pectins of types A and E elute from the DEAE-cellulose column at lower ionic strength than the other types. Types C and D elute at the end of the gradient. Chromatography on DEAE-Sephadex A 25 gives the same results as those obtained on DEAE-cellulose. Table 4 shows (with the exception of galactose) the fairly constant composition of the neutral sugars. The values for the galactose/arabinose, xylose/arabinose ratios, etc., are average values for pectin fractions of about the same neutral sugar content from different extracts. Pectins of the same type (A, B, C, D or E) but from different extracts have the same composition.

TABLE 4
Neutral Sugar Composition of Pectins Extracted from Ripe and Unripe Apples (AIS)

	A[a]	*B*	*C*	*D*	*E*	*On average*
Ripe apples						
Rhamnose	0·15	0·07	0·08	0·07	0·09	0·09
Xylose	0·09	0·10	0·09	0·08	0·08	0·09
Galactose	1·42	0·80	0·69	0·38	0·29	
Glucose	0·17	0·13	0·12	0·12	0·03	0·10
Sugar content (moles/mole gal. A)	0·08	0·15	0·24	0·54	1·42	
Unripe apples						
Rhamnose	0·08	0·05	0·07	0·06	0·07	0·07
Xylose	0·07	0·07	0·07	0·08	0·09	0·07
Galactose	1·90	0·83	0·60	0·21	0·21	
Glucose	0·08	0·09	0·08	0·11	0·07	0·08
Sugar content (moles/mol gal. A)	0·04	0·14	0·24	0·53	1·48	

[a] A–E refer to Fig. 2. The neutral sugar composition is expressed as moles sugar per mole arabinose. The values are averages of those for pectins with about the same neutral sugar content in the four extracts. Mannose was absent in all cases.

It was realised that because of the interference of the degree of esterification, the molecular weight and the neutral sugar content in the fractionation on DEAE-cellulose columns, it is necessary to produce more evidence to support the postulated neutral sugar distribution. The existence of the different types of pectin molecules is evident

from gelfiltration and from rechromatography on DEAE-cellulose. Examples of rechromatography and gelfiltration are given in Fig. 3 and Fig. 4. In fractions of low neutral sugar content a high molecular weight coincides with a relatively high amount of neutral sugars; in fractions of higher neutral sugar content the situation is more complicated. The pectin with a high neutral sugar content in the acid extracts (type E) has a lower molecular weight than the other pectins extracted.

The presence of the pectin molecules of types A-D, and especially the presence of pectin molecules with a very high neutral sugar content (type E), is in agreement with the conception of pectin as consisting of 'smooth' and 'hairy' regions. Much of the evidence produced (Barret & Northcote, 1965; Bhattacharjee & Timell, 1965; Zitko & Bishop, 1965; Stoddart *et al.*, 1967; Siddiqui & Wood, 1976; Berth *et al.*, 1977; Pfister, 1977; Knee, 1978a,b; Shibuya & Iwasaki, 1978; McNeil *et al.*, 1978) shows that pectin contains regions of 'homogalacturonan' and regions rich in neutral sugars,

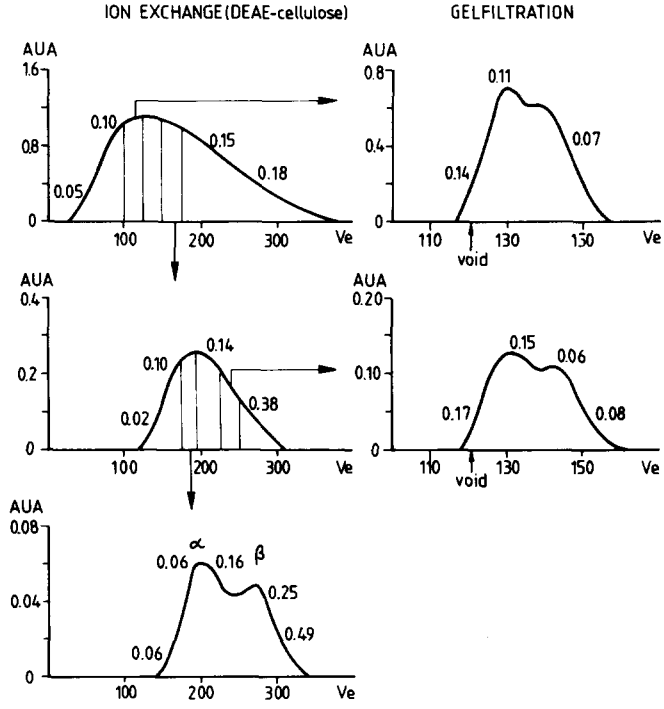

Fig. 3. Purification and fractionation of pectin from the oxalate extract of AIS from ripe apples. Ion exchange (left-hand side figures) and gelfiltration (right-hand side figures) as described in the text. AUA, anhydro-uronic acid, mg/ml; Ve, elution volume, ml. The numbers in the figures indicate the neutral sugar residue content of the fractions, expressed as moles neutral sugar residues/mole of galacturonate residues. The neutral sugar composition of the fractions is similar to the compositions given in Table 4, except for fraction α (Table 5). The arrows indicate fractions that have been rechromatographed on ion exchange or on gelfiltration columns.

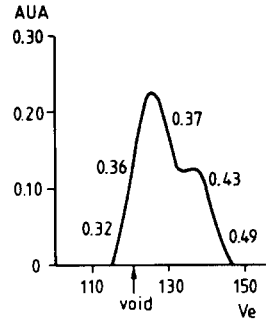

Fig. 4. Gelfiltration of a DEAE-cellulose purified pectin fraction from the acid extract of AIS from unripe apples. AUA, anhydro-uronic acid, mg/ml; Ve, elution volume, ml. The numbers in the figure indicate the neutral sugar residue content of the fractions, expressed as moles neutral sugar residues/mole of galacturonate residues.

'smooth' and 'hairy' regions. Our experiments do not support the suggestion of Aspinall *et al.* (1970) that pectin is a 'chemically homogeneous polydisperse system'; a polydisperse system implies a continuous distribution of the neutral sugar content.

In the cell wall model of Keegstra *et al.* (1973) chains of xyloglucan are linked to arabinogalactans which are, in turn, linked to the galacturonan backbone. Indeed, oligosaccharides consisting of galactose and xylose have been isolated (Aspinall *et al.*, 1967a, b; Kikuchi & Sugimoto, 1976). However, some experiments suggest that not only arabinogalactans but also xylans or xyloglucans are linked to the galacturonan backbone; the pseudo-aldobiuronic acid of xylose and galacturonic acid has repeatedly been isolated (see Introduction). Some of our pectin fractions have relatively high amounts of xylose and glucose (e.g. fraction α, Table 5). This might indicate the presence of separate xyloglucan side chains. However, this variation is only present in pectins of type A. This can be explained by assuming that pectins of type A are pectins with degraded hairy regions. After ripening more pectin of this type is present. The neutral sugar contents of the pectins of types B, C and D have ratios of 1 : 1·7 : 3·7 (Table 4). It might well be that these pectins correspond with molecules with 1, 2 and 3 or 4 hairy regions. Pectin E has 10 times the neutral sugar content as pectin B.

TABLE 5
Average Neutral Sugar Composition of Peaks α and β of Fig. 3, Expressed as Moles Neutral Sugar Residues/Mole of Arabinose Residues

	α	β	A^a
Rhamnose	0·28	0·11	0·15
Xylose	0·23	0·07	0·09
Galactose	1·04	0·40	1·42
Glucose	0·25	0·07	0·17

[a] Average neutral sugar composition of the pectins of type A (Table 4).

Pectins of type E occur dominantly in the acid extracts; they have a low molecular weight compared to the pectins of the other types and are probably the products of degradation.

Ripening

During ripening the neutral sugar content of the extracted pectins, the composition of the neutral sugar side chains and the overall degree of esterification do not change (Tables 1, 2 and 4). More pectin can be extracted after ripening (Table 2). Many authors found an increase in the amount of easily extractable pectin during ripening (e.g. Knee, 1973 and 1978a; Seipp, 1978). The softening of apple fruits, however, starts before the increase of water-soluble pectin (Doesburg, 1957). Some authors found differences between ripening on and off the tree, in respect to ease of pectin extraction (Esau *et al.*, 1962; Knee, 1973). This leads to the conclusion that the softening is not necessarily caused by pectin solubilisation.

Some degradation of the pectin occurs during ripening; as a result more pectin of type A and type E is present (Fig. 2) and the average apparent molecular weight of the acid extractable pectin has decreased (gelfiltration experiments). The latter can be explained by the increased amount of type E pectin in the acid extract as pectin molecules of type E are relatively small. The increased amount of type A pectin is in agreement with the results of Knee (1978a) who found that the neutral sugar content of DEAE-cellulose purified soluble pectin from apples decreased during ripening. As mentioned before, more xylose and glucose residues that are not bound to galacturonan appear in the buffer extracts. We have also extracted pectin from unripe apple AIS after treatment with partial purified cellulase (Voragen *et al.*, 1979). Cellulase treatment appears to have the same effect as ripening; the neutral sugar distribution curve looks like the curve for ripe apples, more pectin can be extracted and more unbound xylose and glucose residues are released from the AIS. The release of xylose and glucose residues (xyloglucan?) may indicate that the softening of fruits during ripening and, similarly, the processes of elongation growth (Albersheim, 1974) are due to events in the deeper parts of the cell wall.

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