1033. *Studies on Uronic Acid Materials. Part IV.*[1] *Aqueous Decarboxylation of Uronic Acids, and the Decarboxylation of Pectic Materials during Extraction.*

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Rate-constants for the decarboxylation of some uronic acids in de-ionised water have been determined. The extent of the decarboxylation of pectic materials when heated with (*a*) aqueous 70% ethanol, (*b*) water, (*c*) aqueous ammonium oxalate and (*d*) aqueous ammonium oxalate–oxalic acid has been investigated. The results are discussed with respect to the extraction procedures now used in structural studies; in particular, extraction with ammonium oxalate–oxalic acid solutions at 85—90° should be avoided.

Extraction is an all-important step in structural studies of polysaccharides: fractionation, degradation, chemical modification, and the creation of artefacts[2] are possible consequences of carelessly designed extraction schemes. In initial investigations, one can only use extraction conditions which are, on the basis of previous experience with some similar material, apparently the mildest available, consistent with extraction of the desired polysaccharide in reasonable yield. Poor yields may imply that only a non-representative fraction has been obtained.[3] Preliminary studies establishing an approximation to the composition of a new material may therefore be necessary before the optimum method of extracting a particular component can be devised;[4] even the cation composition can be important.[5] Often, however, as in starch chemistry (cf. ref. 6), less degraded products of higher molecular weight are obtained only after a continued series of studies has refined the method of extraction.

Studies assessing the degradation suffered by the product during extraction should be complementary to structural studies. All too often, structural studies are reported on materials claimed to have been extracted " by the mildest possible means " when, in fact, no experiments were carried out to establish the validity of this statement.

Some polysaccharides, *e.g.*, pectic materials, can at present only be extracted or subsequently purified by methods which degrade and/or chemically modify the material present in the plant. It is then even more important to assess the extent of (*a*) the degradation and (*b*) the modification, such as demethylation and decarboxylation; degradation and chemical modification may be quite independent effects for some polysaccharides. Much is known of the degradation of pectic materials, which can occur in cold alkaline solution,[7,8] buffered (pH 7) " Versene " solution, or, at elevated temperatures, in weakly

[1] Part III, Anderson and Garbutt, *Talanta*, 1961, **8**, 605.
[2] Anderson and King, *Talanta*, 1961, **8**, 497.
[3] Goring and Timell, *J. Phys. Chem.*, 1960, **64**, 1426.
[4] Anderson and King, *J.*, 1961, 2914.
[5] McCready and McComb, *Analyt. Chem.*, 1952, **24**, 1986.
[6] Killion and Foster, *J. Polymer Sci.*, 1960, **46**, 65.
[7] Vollmert, *Makromol. Chem.*, 1950, **5**, 110.
[8] Neukom and Deuel, *Z. schweiz. Forstv.*, 1960, **30**, 223.

acidic solution (cf. ref. 9). Vollmert [7] concluded that depolymerisation of pectic materials in alkali ceased when concurrent de-esterification became complete; recent work [10] has shown that only about one de-esterification in 80 leads to cleavage of a glycosidic linkage.

Less is known, however, of the possible modification suffered by pectic materials during extraction. Exhaustive extraction with hot 70% aqueous ethanol [11] and hot water usually precede the extraction of the pectic complex with hot oxalate solutions. This paper shows that some decarboxylation occurs during each of these stages.

In studies of the various methods of decarboxylating uronic acids,[12] refluxing with de-ionised water caused complete decarboxylation of galacturonic acid in about 80 hours in a vessel continuously swept with carbon dioxide-free nitrogen. Kinetic studies of the evolution of carbon dioxide from some uronic acids were then made; these gave the results in Table 1 (it is there assumed that 1 mole of carbon dioxide resulted from decarboxylation of 1 mole of uronic acid and that all the carbon dioxide evolved came from carboxyl groups; under the experimental conditions the amounts of carbon dioxide liberated in

TABLE 1.

Decarboxylation (%) of uronic acids in boiling conductivity-grade water.

Time (10^3 sec.)	Glucurone	Galact-uronic acid	Trigalact-uronic acid	Ca L-sorburonate	Alginic acid
10	5·6	7·8	12·8	35·0	3·7
20	11·1	15·1	24·2	50·9	7·3
30	16·6	21·9	33·6	60·2	11·0
40	21·8	28·3	41·4	66·4	14·6
60	31·5	39·8	54·2	73·3	21·7
80	40·4	50·0	64·1	78·3	28·5
100	48·7	59·2	72·2	87·4	35·1
150	66·3	78·2	87·2	90·2	50·7
200	79·4	91·4	96·0	96·4	65·6
250	88·4	97·8	99·4	98·3	79·4
Hence $10^6 k_1$ (sec.$^{-1}$) =	5·18	6·56	12·5	57·6	1·42

side reactions or from general degradation or decomposition would be expected to be very small; cf. ref. 1).

The rate constants (k_1) were calculated by Guggenheim's method.[13] (The results reported were obtained by using conductivity-grade water; traces of metal ions catalyse decarboxylation,[14] and results obtained by adding salts and other substances will be given elsewhere.)

Alginic acid was the most stable of the materials refluxed with water, yet 7% decarboxylation occurred in 5 hours (*i.e.*, the original uronic anhydride content was reduced from 97 to 90%). A total reflux period of 5 hours would not be excessive if exhaustive aqueous extraction were required; it is difficult to remove starch completely by aqueous extraction,[15] and the contamination of pectins by starch is well known.[16] The study of aqueous decarboxylation was therefore extended to a commercial apple pectin, pectic acids from *N. translucens* [17] and *C. australis*,[18] ammonium pectates from *N. translucens*,[17] and lucerne.[19] Reflux in aqueous solution for several hours caused significant decarboxylation, as shown by the percentages quoted in Table 2. The same Table shows also that some decarboxylation occurs on prolonged refluxing with 70% aqueous ethanol.

[9] Albersheim, Neukom, and Deuel, *Arch. Biochem. Biophys.*, 1960, **90**, 46.
[10] Launer and Tomimatsu, *J. Org. Chem.*, 1961, **26**, 541.
[11] Williams and Bevenue, *J. Assoc. Off. Agric. Chem.*, 1956, **39**, 901.
[12] Garbutt, Ph.D. Thesis, Univ. of Edinburgh, 1960.
[13] Guggenheim, *Phil. Mag.*, 1926, **2**, 538.
[14] Zweifel and Deuel, *Helv. Chim. Acta*, 1956, **39**, 662.
[15] Anderson and Greenwood, *J. Sci. Food Agric.*, 1955, **6**, 587.
[16] Bock, Baum, Döring, and Wardsack, *Ernährungsforsch.*, 1960, **5**, 539.
[17] Anderson and King, *Biochem. Biophys. Acta*, 1961, **52**, 441.
[18] Anderson and King, *Biochem. Biophys. Acta*, 1961, **52**, 449.
[19] Fanshawe, Ph.D. Thesis, Univ. of Edinburgh, 1960.

After exhaustive extraction with 70% ethanol and with hot water, pectic substances are usually extracted by repeated treatments at 80—90° with 0·5% aqueous ammonium oxalate.[11,19,20] The percentage decarboxylation caused by this reagent is shown in Table 2. In recent years, extraction with hot water containing 0·25% of oxalic acid and 0·25% of ammonium oxalate has been preferred for certain materials.[11,21] Our experiments have shown, however (see Table 2), that this extractant causes extensive decarboxylation. (Control determinations showed that the carbon dioxide evolved did not result from decomposition of the oxalate solution.)

The pectic acid and ammonium pectate samples studied had all been isolated by hot oxalate extraction of material which had been pretreated with 70% ethanol and with hot water, yet carbon dioxide was evolved from each sample on further treatment with

TABLE 2.

The apparent percentage of decarboxylation * of some pectic materials when treated with aqueous solutions used in extraction.

Samples ‡ decarboxylated	Extractant and temperature	Period of extraction † (hours) 2	4	5	10	12	20	24	32	40	50	60
1	De-ionised water, b. p.	2·7	4·1	—	—	5·0	—	6·1	—	—	—	—
2		1·8	2·6	—	—	5·1	—	7·6	—	—	—	—
3		0·9	1·6	—	—	4·2	—	9·4	—	—	—	—
4		1·4	3·2	—	—	6·9	—	10·9	—	—	—	—
2	70% aq. ethanol, b. p.	—	1·0	—	—	—	1·5	—	—	—	3·0	—
3		—	1·5	—	—	—	2·3	—	—	—	4·1	—
5		—	1·6	—	2·4	—	4·5	—	7·0	—	11·9	—
6		—	1·8	—	3·4	—	6·2	—	9·1	—	14·6	—
7		—	1·9	—	3·9	—	6·8	—	9·6	—	15·2	—
1	0·25% aq. ammonium oxalate + 0·25% aq. oxalic acid 85—90°	—	4·9	—	—	—	—	28·0	34·2	—	—	—
2		5·9	7·9	—	—	—	—	31·0	36·0	—	—	—
3		2·7	3·8	—	5·9	—	—	14·4	—	24·0	28·0	—
4		2·2	6·0	—	—	—	—	32·0	38·4	—	—	—
5		—	—	4·6	7·5	—	14·3	—	—	26·6	—	36·2
6		—	—	5·0	8·4	—	15·9	—	—	29·2	—	40·2
7		—	—	5·8	10·1	—	19·4	—	—	36·1	—	48·8
1	0·5% aq. ammonium oxalate, 85—90°	1·0	—	—	4·8	5·7	—	11·5	—	—	—	—
2		5·0	6·6	—	—	10·0	12·0	—	—	—	—	—
3		1·4	—	—	—	4·3	—	5·9	—	—	—	—
4		1·6	2·6	—	—	6·1	—	9·6	—	—	—	—

* Values shown are the average of duplicate determinations made on each material under the stated conditions; agreement in the duplicate determinations was within ±10%.

† In presence of nitrogen.

‡ Origin of samples: 1, ammonium pectate from N. *translucens*; [17] 2, pectic acid from *C. australis*; [18] 3, commercial apple pectin; 4, ammonium pectate from lucerne; [19] 5, alginic acid; [23] 6, glucurone; 7, galacturonic acid.

these extractants. In view of the experimental conditions, it seems reasonable to postulate that this carbon dioxide arose from decarboxylation, although there may be a small contribution from general decomposition or degradation. Clearly, the samples must therefore have undergone decarboxylation to at least a similar extent during their original extraction from the plant material. The present results indicate that the customary extraction solutions cause decarboxylation of pectic materials to the following extent: (*a*) ~0·2% per hr. in refluxing 70% aqueous ethanol; (*b*) 0·4—0·6% per hr. in refluxing hot water; (*c*) 0·3—0·9% per hr. on extraction at 85—90° with 0·5% aqueous ammonium oxalate; (*d*) 1·4% per hr. on extraction with water containing 0·25% each of ammonium oxalate and oxalic acid at 85—90° for three laboratory-prepared specimens and 0·6—0·7% per hr. for commercial apple pectin. Contact with each hot extractant for 4 hours in the

[20] Aspinall and Cañas-Rodriguez, *J.*, 1958, 4020; Kertesz, " The Pectic Substances," Interscience Publ. Inc., New York, 1951, pp. 103—104; Adams and Castagne, *Canad. J. Chem.*, 1949, **27**, *B*, 924.

[21] Bishop, *Canad. J. Chem.*, 1955, **33**, 1521.

presence of nitrogen will cause a total of 3—6% decarboxylation if 0·5% aqueous ammonium oxalate is used; the total may reach 8% if the ammonium oxalate–oxalic acid treatment is employed. Increasing the extraction times increases the extent of the decarboxylation.

It is of interest that, in both the oxalate extractants, the commercial apple pectin (MeO = 5·1%) was more stable than the laboratory-prepared pectic acids, which contained no methoxyl. The closely related behaviour of the products from lucerne, *N. translucens*, and *C. australis* is interesting since the absence of methoxyl groups in the lucerne product was a consequence of de-esterification during isolation;[19] the products from *Nitella* and *Chara* came from algae whose cells contained no methoxyl.[17,18]

Previous workers[9,10] have established that the alkali-sensitivity of the 1,4-bonds in pectic materials depends on the presence of ester groups at position 6, the de-esterified product being practically stable to further degradation.[7,10] A second effect can now apparently be distinguished; de-esterified or initially non-esterified pectic materials are less stable to chemical modification than are esterified products.

Clearly, contact with any hot extractant must be as brief as possible if significant decarboxylation of pectic materials is to be avoided: in particular, hot oxalate–oxalic acid solutions cause considerable decarboxylation. If use of hot extraction solutions is unavoidable, the extent of the chemical modification suffered by the pectic material should be assessed, before structural studies, by decarboxylation experiments on the extracted material.

Experimental and Results

Origin of Samples.—(*a*) D(+)-Galacturonic acid monohydrate (Roche Biochemicals Ltd.) (Found: C, 34·8; H, 5·7. $C_6H_{12}O_8$ requires C, 34·0; H, 5·7%) had 96% purity [decarboxylation[22] in 19% (w/w) hydrochloric acid for 2½ hr.]; paper chromatography showed a trace of galactose. (*b*) D-Glucurone (Roche Biochemicals Ltd.) (Found: C, 40·8, 40·7; H, 4·6, 4·6. $C_6H_8O_6$ requires C, 40·9; H, 4·6%) had 97·2% purity. (*c*) Alginic acid was the sample prepared by cold extraction by Chanda *et al.*,[23] which had 97·4% purity. (*d*) Trigalacturonic acid and calcium 5-oxo-D-gluconate (calcium L-sorburonate) were kindly provided by Dr. W. W. Reid.[24] The former was free from mono- and di-galacturonic acid but contained a small quantity of a polygalacturonic acid (detected by chromatography); it had 95·0% purity. The 5-oxo-D-gluconate was 93% pure. (*e*) Commercial apple pectin (B.D.H. Ltd., 240 grade) had uronic anhydride[22] 58%, OMe[25] 5·1%. (*f*) Ammonium pectate from *Nitella translucens*[17] had no methoxyl content,[25] uronic anhydride[22] 51%. (*g*) Ammonium pectate from lucerne[19] had no methoxyl and 51% of uronic anhydride. (*i*) Pectic acid from *Chara australis*[18] had no methoxyl and 52% of uronic anhydride.

Apparatus.—Anderson's decarboxylation apparatus and reaction conditions[22] were used. A reaction flask, having a thermometer-pocket sealed into the flask-wall, was used in experiments where temperatures other than reflux temperature were used. Kinetic measurements were facilitated by fitting a two-way stop-cock after T_2 (see ref. 22) so that the gas stream could be switched from one absorption trap to a second identical trap. A matched pair of traps was used; in these, the sintered discs had closely matching porosity so that identical back pressures, and hence constant reflux temperatures, were maintained. The nitrogen flow-rate was stabilised by a capillary-buffer system, needle valves, and a Rotameter.

Decarboxylation of Uronic Acids in Water.—Conductivity-grade water from a laboratory de-ioniser was refluxed in the decarboxylation apparatus, then allowed to cool in an atmosphere of carbon dioxide-free nitrogen. The weighed sample of uronic acid was added to the reaction flask. The carbon dioxide evolved was determined at intervals during the refluxing; the results are shown in Table 1. Control experiments confirmed that carbon dioxide was not evolved when water alone was refluxed. The decarboxylation of uronic acids in acid solution has been shown to be of the first order;[12] on the assumption that this applies also to aqueous

[22] Anderson, *Talanta*, 1959, **2**, 73.
[23] Chanda, Hirst, Percival, and Ross, *J.*, 1952, 1833.
[24] Brooks and Reid, *Chem. and Ind.*, 1955, 325, 360; Jones and Reid, *Canad. J. Chem.*, 1955, **33**, 1682.
[25] Anderson and Duncan, *Talanta*, 1961, **8**, 241.

solutions, the rate constants shown in Table 1 were calculated by Guggenheim's method,[13] which does not require a knowledge of the precise purity of the substrates.

Decarboxylation of Pectic Materials in Hot Water.—Samples (110—190 mg.) of commercial apple pectin, ammonium pectates from *N. translucens* and lucerne, and pectic acid from *C. australis* were refluxed with conductivity-grade water; the carbon dioxide evolved was determined at intervals up to 24 hr. from the start of refluxing. It has been shown [1] that 98% of the carbon dioxide liberated when uronic acid materials are refluxed in acid solution comes from the carboxyl groups. On the assumption that this will also hold for aqueous solutions, yields of carbon dioxide were expressed as percentage decarboxylation. The results from the hot-water experiments are shown in Table 2. Each result is the average of duplicate runs made with the substance named; agreement to within $\pm 10\%$ was always obtained.

Decarboxylation in Aqueous 70% *Ethanol.*—Samples (120—200 mg.) of galacturonic acid, glucurone, alginic acid, commercial apple pectin, and pectic acid from *C. australis* were refluxed with 70% aqueous ethanol, and the carbon dioxide liberated was determined at intervals up to 50 hr. from the start of refluxing. A very small amount of carbon dioxide was evolved when 70% aqueous ethanol was refluxed alone ($\geqslant 5\%$ of that evolved when substrates were present) and the yields of carbon dioxide obtained were corrected for this. On the assumption that the corrected yield of carbon dioxide came from carboxyl groups, the results are shown in Table 2.

Decarboxylation in 0·25% *Aqueous Ammonium Oxalate* + 0·25% *Oxalic Acid Solution at* 85—90°.—Samples (100—150 mg.) of galacturonic acid, glucurone, alginic acid, commercial pectin, pectic acid from *C. australis*, and ammonium pectates from lucerne and *N. translucens* were treated with water containing 0·25% each of ammonium oxalate and oxalic acid, maintained at 85—90°. The carbon dioxide liberated was determined at intervals up to 60 hr. The yields of carbon dioxide were corrected for the very small amounts of carbon dioxide detected when the hot oxalate solution alone was maintained at 90°. The percentages shown in Table 2 were obtained from calculations based on the corrected yields of carbon dioxide.

Decarboxylation with Aqueous 0·5% *Ammonium Oxalate at* 85—90°.—Samples (100—150 mg.) of commercial apple pectin, ammonium pectate from lucerne and *N. translucens*, and pectic acid from *C. australis* were treated at 85—90° with 0·5% aqueous ammonium oxalate. The carbon dioxide evolved was determined at intervals up to 24 hr. from the start of refluxing. Very small amounts of carbon dioxide were detected when the ammonium oxalate solution alone was kept at 90°. The percentages shown in Table 2 were calculated from the corrected yields of carbon dioxide.

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