12. *Ester and Lactone Linkages in Acidic Polysaccharides. Part I.*

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Simple carbohydrate esters and hexono- and urono-lactones with hydroxylamine under alkaline conditions yield hydroxamic acids which provide a basis for the estimation of these lactonised groups in natural products.

Uronolactones have been detected in dried specimens of various polyuronides.

HYDROXYLAMINE has been widely investigated as a means of detection and estimation of micro-amounts of carboxylic esters (Feigel, "Spot Tests," Elsevier, New York, 1939); these are very readily transformed into hydroxamic acids which give intensely coloured compounds with ferric salts. Hestrin (*J. Biol Chem.*, 1949, **180**, 249) used the method successfully for the quantitative assay of, notably, choline esters and Whittaker and Wijesundera (*Biochem J.*, 1951, **51**, 348) developed it for chromatographic separations. Its mechanism is considered to be analogous to that postulated for the formation of amides from esters by ammonia (Gordon, Miller, and Day, *J. Amer. Chem. Soc.*, 1948, **70**, 1946; 1949, **71**, 1245).

The reactions proceed equally readily with internal esters, *i.e.*, lactones, and acid anhydrides as in the assay of the penicillins (Baker, Dobson, and Martin, *Analyst*, 1950, **72**, 651; Albans and Baker, *ibid.*, p. 657).

It has now been shown that in the carbohydrate series 3 : 6-, 1 : 4-, and 1 : 5-lactones behave in the same way as simple esters and may be determined in the presence of the parent hydroxy-acid. The behaviour of uronic (3 : 6-)lactones is of particular interest because of the frequent occurrence of uronic acids in numerous plant and animal polysaccharides and the possibility of the existence of internal or external lactone bridges in these macro-molecules.

In a preliminary survey absorption spectra were observed for pure acethydroxamic acid reacting with ferric chloride at various acidities. A single absorption peak was found at 5050 Å, where a linear relation existed between extinction and concentration (0—10 μmoles). The colour was stable for 10 minutes and independent of the presence of excess of hydroxylamine. Whilst the intensity increased very slightly with increase in pH to a maximum value at pH 1·7 in the case of acethydroxamic acid (formed *in situ* from ethyl acetate and hydroxylamine and treated with ferric chloride under the same conditions), at 5050 Å it decreased when the pH value fell below 1·0; at lower pH's the intensity at lower wavelengths increased. Accordingly, a final pH value between 1·0 and 1·2 was adopted for later experiments. Otherwise, the results agreed closely with those obtained with preformed acethydroxamic acid. At pH 1·2 a linear relation was found between the extinction and the amount of ethyl acetate (0—10 μmoles). Acetamide reacted only slowly with hydroxylamine, being detectable, under the prescribed conditions, only in comparatively high concentration. Acetic anhydride reacted readily with two mols. of hydroxylamine. In most cases indications were found of a second absorption band at about 4100 Å but it was not possible to use it for quantitative work.

D-Glucurone gave absorption curves with a maximum at 4750 instead of 5050 Å. This was thought to be due to interference from the reducing group. D-Glucose had a maximum at about 4400 Å under similar conditions and correction of the D-glucurone absorption for this superimposed absorption caused maximum to shift from 4750 to 5050 Å. The absorption of D-glucurone was then similar to that of ethyl acetate, and involvement of the lactone ring was demonstrated by failure to detect more than slight absorption at 5050 Å with sodium D-glucuronate. In spite of this superimposed absorption, in the case of D-glucurone the absorption–concentration relation is linear at 5050 Å both before and after correction. Thus, while it is possible to estimate free carbohydrate lactones by this method, in general reducing sugars should be removed or glycosides formed from them. 1 : 4- and 1 : 5-Lactones typified by 2 : 3 : 5- and 2 : 3 : 4-trimethyl L-arabonolactone reacted readily with hydroxylamine, and their estimation could be carried out in the presence of glycosides.

Sugar esters could readily be estimated and as glycosides did not interfere it was possible to determine the total uronic acid content of a polysaccharide (by using the simple uronic ester glycoside as standard) if the material was first esterified; this was most readily accomplished with methanolic hydrogen chloride at 100°. The amount of ester determined in the resulting solutions and the acid values agreed closely with those obtained by titration.

In this way pneumococcus type III polysaccharide gave a value of 54·7% uronic acid components, a value of 50% (acid equivalent 350) being obtained by titration and structural studies (Reeves and Goebel, *J. Biol. Chem.*, 1941, **139**, 511). In the case of pneumococcus type II polysaccharide an acid equivalent of 972 (17·0% uronic acid residues) was found by the present method compared with 1010 determined by titration of identical material. Good agreement has been found in the case of other polysaccharides, *viz.*, hyaluronic acid, alginic acid, etc.

The procedure was applied to dried specimens of polysaccharides to test the possible occurrence of lactone linkages. A virtually ash-free specimen of pneumococcus type II polysaccharide gave no indication of the presence of such linkages even after extensive drying in a vacuum. Pneumococcus type III polysaccharide (ash content 2%), dried at room temperature, was found to contain a small but distinct number of uronic acid residues in the lactone form and this number increased when the polysaccharide was dried at 61°

in a vacuum (1 lactonised unit per 12 uronic acid residues). In the light of the structure proposed by Reeves and Goebel (*loc. cit.*) in which the glucuronic acid residues are linked through positions 1 and 3, the usual 3 : 6-lactone bridge cannot be present and the possibility of an intramolecular lactone being formed in any other way is remote. Probably therefore the lactone bridges in type III polysaccharides are intermolecular. According to Meyer, Fellig, and Fischer (*Helv. Chim. Acta*, 1951, **34**, 939) the glucuronic acid units of hyaluronic acid are also linked in the 1 : 3-positions and this is consistent with the present finding of only small amounts of uronolactone residues in isolated specimens. There is little evidence to support the conjecture of K. Meyer (*J. Biol. Chem.*, 1948, **176**, 993) that uronic anhydrides occur in the native material. It is not known whether the linkages are in fact those of lactones or of anhydrides and the lack of correlation between lactone content and viscosity suggests that if the linkages are intermolecular then they are of minor importance in cross-linkage between chains.

In the case of 1 : 4-linked acidic polysaccharides, *e.g.*, *Rhizobium radicicolum* or alginic acid, the hydroxyl group at $C_{(3)}$ is available for internal lactonisation. *Rhizobium* polysaccharide, dried at room temperature, was found to contain many lactonised residues. According to Schlücterer and Stacey (*J.*, 1945, 776), the material comprises D-glucose and D-glucuronic acid and thus it appears that in the dried material the uronic acid residues are to a large extent lactonised. Similarly, evidence was obtained that dry alginic acid exists chiefly in a lactonised condition. In this case, the extreme insolubility of the material caused difficulties. In general it appears that internal lactonisation (*i.e.*, 3 : 6-) may occur readily when the structure permits.

Experimental

Absorption Spectra of Acethydroxamic Acid–Iron Complex.—(i) *Effect of acid.* Aqueous solutions of acethydroxamic acid (Schroeter, *Ber.*, 1898, **31**, 2191; Jones and Oesper, *Amer. Chem. J.*, 1909, **42**, 518) (m. p. 87°; 10 μmole in 2 ml.) were diluted with water (0·9, 0·8, 0·7, 0·6 ml. severally) and treated with sodium hydroxide (3·5M; 1 ml.) followed immediately by hydrochloric acid (3·34M; 1·1, 1·2, 1·3, 1·4 ml.) and ferric chloride (0·37M in 0·1M-hydrochloric acid; 1 ml.). The final pH's of the solutions were 1·7, 1·2, 1·0, and 0·8 respectively. The absorption spectra were then immediately measured by means of a Beckman spectrophotometer (1-cm. cells). The compensating cell contained respectively, water (2·9, 2·8, 2·7, 2·6 ml.), hydrochloric acid (3·34M; 1·1, 1·2, 1·3, 1·4 ml.), sodium hydroxide (3·5M; 1 ml.), and ferric chloride (1 ml.). (The method used by Hestrin, *loc. cit.*, for preparing a compensating solution by adding the reagents in the reverse order to an aliquot of the solution under test was not found satisfactory.) Results were :

pH value	1·7	1·2	1·0	0·8
Log I/I_0 at 5050 Å	1·145	1·100	1·065	1·030

(ii) *Fading.* At pH 1·2 the extinction of the appropriate foregoing solution was determined at 5050 Å as a function of time :

Time (min.)	0	10	15	35	50	70
Log I/I_0	1·114	1·109	1·102	1·035	1·015	0·994

(iii) *Extinction–concentration relation.* Solutions of pure acethydroxamic acid (10, 7·5, 5·0, 2·5 μmole) in water (2·8 ml.) were treated as in (i) (final pH 1·22) and the extinction was measured within 10 minutes at 5050 Å. :

Acethydroxamic acid (μmole)	10·9	7·5	5·0	2·5
Log I/I_0	1·118	0·801	0·511	0·247

Ultra-violet Absorption of the Iron Complex obtained directly from Ethyl Acetate and Hydroxylamine.—(i) *Effect of acid.* Solutions of ethyl acetate (9 μmole) in 5% aqueous methanol (2 ml.) were diluted with water (1·1, 1·0, 0·9, 0·8 ml. severally) and mixed with a freshly prepared solution of hydroxylamine (2 ml.; from 2M-hydroxylamine hydrochloride and an equal volume of 3·5M-sodium hydroxide). After 4 minutes at room temperature (cf. Hestrin, *loc. cit.*) the solutions were treated with hydrochloric acid (3·34M; 0·9, 1·0, 1·1, 1·2 ml.) and ferric chloride (0·37M in 0·1M-hydrochloric acid; 1 ml.). The final pH values of the solutions were respectively

1·5, 1·2, 1·0, 0·8. Compensating solutions were prepared similarly with 5% aqueous methanol. The resulting solutions were shaken under reduced pressure (15 mm.) for 1 minute to remove dissolved gases, and the extinction was measured as above:

pH	1·5	1·2	1·0	0·9
Log I/I_0	0·843	0·923	0·984	0·952

(ii) *Extinction–concentration relation.* Solutions of ethyl acetate (10·0, 8·1, 7·2, 6·4, 4·5, 4·0, 2·7, 2·4, 1·0 μmole) in 5% aqueous methanol (2 ml.) were treated as above (final pH 1·2), results being:

Ethyl acetate (μmole)	10·0	8·1	7·2	6·4	4·5	4·0	2·7	2·4	1·0
Log I/I_0	1·030	0·855	0·750	0·655	0·460	0·405	0·280	0·250	0·097

Ultra-violet Absorption of Iron Complexes from Various Acetyl Derivatives and Hydroxylamine.—Aqueous solutions (5% aqueous methanol for water-insoluble substances), treated as described in the foregoing paragraph, gave:

Acetic anhydride (μmole)	9·0	7·0	4·0	3·0	1·0
$E_{max.}$ (5050 Å)	1·795	1·393	0·803	0·635	0·215
Acetylcholine chloride (in 0·001N-sodium acetate; cf. Hestrin, *loc. cit.*) (μmole)	5·5	4·1	2·7	1·4	
$E_{max.}$ (5000) Å	0·850	0·645	0·445	0·230	
Acetamide (μmole)	165·0	82·5	50·0	33·3	
$E_{max.}$ (5000 Å)	0·500	0·250	0·160	0·110	

Ultra-violet Absorption of the Iron Complex from D-*Glucurone and Hydroxylamine.*—Aqueous solutions, treated as described above, gave the following results:

μmole	9·2	8·3	7·4	6·9	6·4	5·5	4·6	2·3
Glucurone, E_{4750}	0·982	0·822	0·750	0·710	0·630	0·549	0·475	0·235
,, E_{5050}	0·920	0·780	0·705	0·665	0·585	0·510	0·425	0·205
Glucose, E_{5050}	0·170	0·135	0·125	—	0·105	0·095	—	0·030
Glucurone, E_{5050}, "corr."	0·750	0·645	0·580	—	0·480	0·415	—	0·175

Ultra-violet Absorption of Iron Complexes from Carbohydrate Esters and Lactones with Hydroxylamine.—The following results were similarly obtained:

Dimethyl mucate (μmole)	8·0	6·0	4·0	2·0	1·0
$E_{max.}$ (5050 Å)	1·165	0·900	0·580	0·295	0·198
Methyl methyl-D-galacturonoside (μmole)	10·5	8·4	6·3	2·1	1·05
$E_{max.}$ (5050 Å)	0·850	0·735	0·550	0·205	0·100
*iso*Propylidene D-glucurone (μmole)	9·3	8·36	4·18	1·0	
$E_{max.}$ (5000 Å)	0·950	0·840	0·422	0·104	
2 : 3 : 5-Trimethyl L-arabonolactone (μmole)	10·6	5·3	1·0		
$E_{max.}$ (5050 Å)	1·07	0·489	0·094		
2 : 3 : 4-Trimethyl L-arabonolactone (μmole)	9·73	4·86	2·43	1·0	
$E_{max.}$ (5050 Å)	0·761	0·369	0·179	0·078	
D-Glucono-1 : 4-lactone (μmole)	8·0	6·0	4·0	2·0	1·0
$E_{max.}$ (5050 Å)	0·670	0·570	0·425	0·225	0·075

Total Carboxylic Acid Content of Polysaccharides.—(i) Pneumococcus type III polysaccharide was precipitated by ethanol from an acidic solution. After reprecipitation from aqueous solution it was dialysed in a Cellophane container against distilled water, then was isolated by being freeze-dried. It contained no alkoxyl groups (Zeisel). 1·40 Mg. were heated at 100° with methanolic hydrogen chloride (1%; 0·1 ml.). After 30 minutes the material was added to 1·9 ml. of water and the concentration of ester was measured as described for ethyl acetate. E at 5050 Å = 0·392, equiv. to 4·43 μmole. Total uronic acid content = 55·6; 53·8% in duplicate tests. Authentic D-glucurone, esterified and estimated in this way, gave 98% recovery.

(ii) Pneumococcus type II polysaccharide (1·69 mg.), was treated as above. Extinction at 5050 Å = 0·160, equiv. to 1·80 μmole. Total uronic acid content = 18·7%.

(iii) Hyaluronic acid (N, 2·96; ash, 2·61%) (2·53 mg.) isolated by Kaye's method (*Nature*, 1950, **166**, 478) from umbilical cord and deproteinised by the Sevag technique, was heated at

100° for 1 hour with methanolic hydrogen chloride (0·2 ml.; 1%). The ester content of the resulting solution was determined as before. Extinction at 5050 Å = 550, equiv. to 6·3 μmole. Total carboxylic acid content = 42·7% (cf. 44·9%, Kaye and Stacey, *Biochem. J.*, 1951, **48**, 249).

(iv) Alginic acid (2·07 mg.), reprecipitated in the acidic state and dried at room temperature with ethanol and ether, was heated at 105° for 2 hours with methanolic hydrogen chloride (0·3 ml.; 1%). The ester concentration of the contents was determined by the foregoing method. Extinction at 5050 Å = 0·990, equiv. to 11·2 μmole. Total uronic acid content 94·2% (cf. 100% required by structure of Hirst, Jones, and Jones, *J.*, 1939, 1880).

Lactone Linkages in Dried Acidic Polysaccharides.—(i) *Rhizobium radicicolum* polysaccharide (0·5 g.; $[\alpha]_D^{22}$ −18° in H_2O) was reprecipitated at pH 3 from acetic acid solution by gradual addition of ethanol (5 volumes), and the product was dried by being washed with absolute ethanol (twice), then with ether, and finally in a vacuum over phosphoric oxide at room temperature. The dried material had an ash content of 2·2% and contained no alkoxy-groups (Zeisel). The dried polysaccharide (3·00 mg.) was dissolved in distilled water (2 ml.), and the content of lactonised groups estimated by the foregoing method (*iso*propylidene glucurone as standard). Extinction at 5050 Å = 0·560, equiv. to 5·50 μmole, *i.e.*, 31·2% of the initial polysaccharide is composed of lactonised uronic acid groups. The structure proposed by Schlücterer and Stacey (*loc. cit.*) requires a uronic acid content of 33%; thus most of the uronic acid residues are in the lactone form.

(ii) Pneumococcus type III polysaccharide reprecipitated at pH 3 by ethanol (5 volumes) was dried with absolute ethanol and ether and finally in a vacuum at room temperature. 1·10 Mg. were dissolved in water (2 ml.), and the lactone residues were determined (*iso*propylidene glucurone as standard). Extinction at 5050 Å = 0·013, equiv. to 0·125 μmole, *i.e.*, 2·0% of the initial polysaccharide is composed of uronic acid groups or 4·0% of the total uronic acid groups is lactonised.

The amount of lactone was measured again after a specimen of the same material (1·1 mg.) had been dried at 61° in a vacuum over phosphoric oxide for 1 hour. The extinction value was 0·025, *i.e.*, corresponded to 8·0% lactonisation of the uronic acid residues. This value did not increase when specimens were dried at 61° for longer periods.

(iii) (*a*) A sample of the hyaluronic acid described above and having $\eta_{rel.}$ (at 1 g./l.) of 1·55 for a 0·03% solution was examined for lactone residues. 18·98 Mg. were dissolved in distilled water (2 ml.). The extinction value of the resulting hydroxamic acid–iron complex at 5050 Å was 0·085, equiv. to 1·1 μmole of uronolactone residues, *i.e.*, 2·0% of the acidic residues of the original material in the lactone form (" corrected " glucurone as standard).

(*b*) A sample of hyaluronic acid (pyridine method; precipitate 1) was prepared from umbilical cord by Hadidian and Pirie's procedure (*Biochem. J.*, 1948, **42**, 260). The material contained N, 3·4% (corrected for ash), and ash, 15·2%, and had $\eta_{rel.}$ (at 1 g./l.) of 2·8 for a 0·1% solution and 2·4 for a 0·03% solution. 18·90 Mg. of the polysaccharide, dried at 61° in a vacuum for 1 hour, were dissolved in water (2 ml.) and the amount of lactone was estimated. *E* at 5050 Å = 0·050, equiv. to 1·1% of the uronic acid residues in the lactone form (" corrected " glucurone as standard).

(*c*) Hyaluronic acid (3·26% N, ash, 8·50%; 21·8 mg.), isolated by extraction of umbilical cord with 1% phenol (Kaye, unpublished results) and dried at 61° for 30 minutes in a vacuum, was dissolved in water (2 ml.) and estimated as above. *E* at 5050 Å = 0·180, equiv. to 2·35 μmole of glucurone, *i.e.*, 3·55% of the uronic acid in the lactonised form (" corrected " glucurone as a standard).

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