116. *A New Method of End-group Assay for Laminarin and similarly constituted Polysaccharides.*

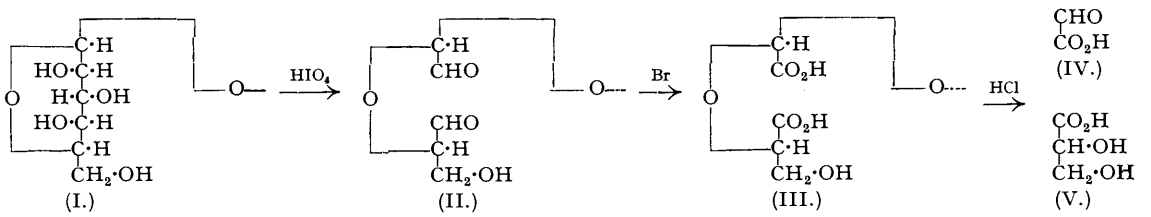
By VINCENT C. BARRY.

Periodic acid attacks only the non-aldehydic end-groups of laminarin, introducing into each of these two aldehydo-groups with loss of one carbon atom. Subsequent oxidation with bromine yields a polysaccharide whose end-group now contains two carboxyl groups. Estimation of the dicarboxylated end-group indicates a chain-length of 16 glucose units for laminarin.

IN previous work (Barry, *Sci. Proc. Roy. Dublin Soc.*, 1939, **22**, 59; 1941, **24**, 423), laminarin has been shown to consist for the most part of β-glucopyranose units linked from the aldehydic carbon atom of one glucose unit to carbon atom 3 of the adjacent unit. Regulated hydrolysis of the polysaccharide has been shown to yield,

among other products, a disaccharide, laminaribiose, 3-[β-*d*-glucosido]-*d*-glucose (*loc. cit.*). This 1 : 3 linkage of laminarin, as opposed to the 1 : 4 linkage of cellulose, has been confirmed by the behaviour of laminarin towards periodic acid. The consumption of periodic acid in the latter case is negligible in comparison with what takes place with starch or cellulose (Barry, Dillon, and McGettrick, this vol., p. 183). Nevertheless, a small but definite reduction of the periodic acid takes place and this has now been shown to be due to the oxidation of terminal non-aldehydic glucose units. As these are the only units in the polysaccharide molecule which present a :C(OH)·C(OH): group for attack, it was to be expected that each of these units would be modified as is α-methylglucoside by the periodic acid (Hudson and Jackson, *J. Amer. Chem. Soc.*, 1937, **59**, 2049).

On the assumption that the oxidation takes the same course in both cases, a terminal non-aldehydic glucose unit (I) undergoes cleavage of the carbon chain, as shown, with loss of carbon atom 2. The modified unit (II) now contains two aldehydo-groups, which on further oxidation with bromine are converted into carboxyl groups as shown in (III). The modified end-group can now be assayed directly by neutralisation or alternatively by conversion into the silver salt and estimation of the silver. The results from the two methods are in close agreement and the figures indicate that one terminal non-aldehydic unit is present for every 15 other units.



The oxidised laminarin with its modified end-unit (III) is designated as laminarinic acid. This product on hydrolysis should yield, in addition to glucose, a small quantity of glyoxylic acid (IV) and glyceric acid (V). The presence of glyoxylic acid was shown by the Adamkiewicz–Hopkins–Cole reaction in the hydrolysate obtained by the action of hot dilute hydrochloric acid on laminarinic acid. Also, a small quantity of oxalic acid was produced when laminarinic acid was hydrolysed and the neutralised hydrolysate oxidised subsequently with bromine.

Further confirmation was afforded by hydrolysing the dialdehydic laminarin (II) and treating the neutral hydrolysate with phenylhydrazine acetate. In the cold a small amount of glyoxalphenylosazone was obtained. No such result was obtained when unoxidised laminarin was treated in a similar way. The recognition of glyoxal in this case and of glyoxylic and oxalic acids above, confirms the mechanism of the oxidation as outlined. The dialdehydic laminarin showed no greater reactivity towards alkaline hypoiodite than did laminarin itself.

Oxidation of the laminarin need not proceed for more than 4 or 5 days with 0·25M-periodic acid in order to effect complete modification of the end-units to the dialdehydic form. In one experiment the oxidation was continued for 10 weeks without causing any alteration in acid value of the subsequently produced laminarinic acid. Longer standing with the reagent caused a partial oxidation of the aldehydo-groups to carboxyl. Similar behaviour was indicated by parallel experiments with starch and cellulose. Both of these polysaccharides on standing for 5 days with 0·25M-periodic acid gave products with neutralisation equivalents of about 145, indicating that about two-thirds of the aldehydo-groups introduced by the reagent were already further oxidised to carboxyl. When the untreated laminarin was subjected to the action of bromine of the same strength and under the same conditions as are applied in the conversion of the dialdehyde into laminarinic acid, the recovered laminarin had a definite neutralisation value and apparently a fixed amount of carboxyl had been introduced into the molecule. A second treatment with bromine did not increase the carboxyl content of the polysaccharide. It is probable that oxidation takes place of some of the terminal aldehyde units to modified carboxylated units. Allowance was made for this carboxyl content in calculating, from the alkali consumption, the percentage of dicarboxylated end-group in laminarinic acid. Some laminarinic acid obtained by oxidation of laminarin with bromine, then with periodic acid and finally with bromine again, contained no greater amount of carboxyl than when the first oxidation with bromine was omitted.

It is clear, therefore, that under the conditions applying during these oxidations, a certain modification of a limited number of terminal aldehydic hexose units does take place. The extent of this modification, however, is fixed and if allowance is made for it, the proportion of terminal non-aldehydic units, after alteration by periodic acid and subsequent treatment with bromine, may be accurately measured.

The isolation of the oxidation products in these experiments is rendered particularly easy by the property of spontaneous precipitation possessed by laminarin. This property is not affected in any way by the modification taking place in the terminal units and the laminarin flakes out of solution during the oxidation in a form in which it is very sparingly soluble in cold water. No difference in the ordinary properties of the polysaccharide could be detected before and after the oxidations. The results obtained are shown below :

Neutralisation value*	2553	2474	2478	2642	2556	2554	2663	2424	2666; mean 2553
Mol. wt. from Ag salt†	2580	2421	2739	2730	2578	2637	2604	2613	— ; mean 2606

* Weight of laminarinic acid which neutralises 2000 c.c. of N-sodium hydroxide.
† Calculated from weight of silver laminarinate which contains 216 g. of silver.
Allowance is made in all cases for the carboxyl introduced into the aldehydic terminal groups by the bromine.

This chain-length of 16 units has not been confirmed so far by the Haworth–Hirst method of end-group assay. In only one of a number of experiments has fully methylated laminarin been found to give a detectable quantity of tetramethyl glucose on hydrolysis. In this case the proportion of end-group was calculated to be 1 in 73. It must be admitted, however, that 9 g. was the greatest weight of methylated material available in any of these experiments. When greater quantities of methylated polysaccharide have been accumulated, hydrolysis may be found to yield a greater proportion of tetramethyl glucose. This possibility is being investigated.

Whatever the results of these experiments, the obvious properties of laminarin appear to indicate a macromolecule of much less than 73 glucose units. As already described (Barry, *Sci. Proc. Roy. Dublin Soc.*, 1938, **21**, 56), laminarin deposited from aqueous solution is obtained in grains very sparingly soluble in cold water. On the other hand, it is precipitated from aqueous solution by alcohol as a white powder soluble in 65—70% alcohol and readily soluble in cold water. A concentrated aqueous solution may be obtained which is water-clear, non-viscid and easily filterable. It has since been shown that the addition of glycerol to the aqueous solution will postpone indefinitely the spontaneous precipitation of the polysaccharide. It appears, therefore, as has already been suggested (*loc. cit.*), that the aggregation of the laminarin in aqueous solution is a physical process and that the solubility of the polysaccharide is related directly to the size of the colloidal molecule. Evidence has been given in a previous paper which indicates a spiral chain of β-glucopyranose units for laminarin and the evidence here presented suggests that about 16 glucose units make up this chain. Such a molecule might be expected to have properties similar to those cited above. The evidence is, however, not sufficient to rule out the possibility of a looped-chain structure or some form of laminated formula for laminarin, such as has been postulated by Hirst and Young for rice starch (J., 1939, 1471). Estimates of the reducing power of the polysaccharide as measured by alkaline hypoiodite all showed that the terminal aldehydo-group does not react stoicheiometrically with iodine. This may be due to a modification of some of the terminal aldehydic units, such as the formation of a 1 : 6 non-reducing anhydro-ring, as has been suggested by Freudenberg in the case of other polysaccharides (*Angew. Chem.*, 1934, 675). With alkaline copper solutions the values obtained were conflicting and could not be reproduced.

This method of end-group assay would not be applicable as such to laminarin if a small proportion of the glucose units in the molecule were united by 1 : 4- or 1 : 6-glucosidic links, as each of these linkages would uncover a further point of attack to the periodic acid. In this event, however, the results would show that for each of these 1 : 4- or 1 : 6-linkages present, there is a chain of 15 units joined by 1 : 3-linkages. In the light of the view taken at present of the chain structure of polysaccharides, the presence of these other linkages does not seem very probable and it must also be taken into account that no trimethyl glucose other than 2 : 4 : 6-trimethyl glucopyranose has so far been detected among the hydrolysis products of fully methylated laminarin.

Experimental.

The laminarin was purified by six successive depositions from water (see Barry, *Sci. Proc. Roy. Dublin Soc.*, 1938, **21**, 615).

Oxidation of Laminarin with Bromine.—A solution of laminarin (2 g.) in hot water (100 c.c.) was cooled, bromine (3 c.c.) added, and the mixture shaken from time to time in a stoppered flask. After standing for 1 hour, the laminarin began to be deposited and the mixture was kept in the dark for 5 days. The excess of bromine was now removed by aeration, and the separated laminarin collected (centrifuge), washed with water, hot alcohol, and ether, and dried over sulphuric acid in a vacuum desiccator to a white ash-free powder (moisture, 6·3%). 1·0 G. (absolutely dry) of the product neutralised 11·85 c.c. of N/100-sodium hydroxide. In subsequent calculations of neutralisation values, this volume of N/100-alkali is always subtracted from the actual alkali required for neutralisation.

Oxidation of Laminarin (i) with Periodic Acid.—A solution of laminarin (13 g.) in hot water (90 c.c.) was cooled, 0·44M-periodic acid (100 c.c.) added, and the mixture kept in a stoppered flask. The laminarin began to settle in flakes almost immediately. 0·25 C.c. of this mixture immediately after making up required, after addition of excess of potassium iodide and acidification, 4·37 c.c. of N/10-sodium thiosulphate. This titre dropped after 8 days to 3·88 c.c. The liquid was now decanted, and the oxidised laminarin washed in the centrifuge with water until the washings were free from periodic acid. It was then washed with hot alcohol and ether and dried first over sulphuric acid in a vacuum desiccator and then to constant weight in a high vacuum at 90°. 1·0 G. required 15·00 c.c. of N/100-sodium hydroxide, indicating that a small fraction of the aldehydo-groups had been oxidised to carboxyl by the periodic acid itself.

Further Oxidation (ii) of this Product with Bromine.—The oxidised laminarin (10·7 g.) was dissolved in warm water (200 c.c.), bromine (5 c.c.) added to the cooled solution, and the mixture shaken intermittently for a few hours and then kept in the dark (7 days). The twice oxidised laminarin (laminarinic acid), recovered as described above, was indistinguishable in appearance from laminarin. 1·0 G. of the dry laminarinic acid neutralised, on boiling, 9·04 c.c. of N/10-sodium hydroxide (phenolphthalein) or after correction 7·86 c.c., *i.e.*, 2000 c.c. of N-sodium hydroxide would neutralise 2545 g.

Silver Laminarinate.—To the neutral solution from above, excess of silver nitrate solution was added, and the silver laminarinate precipitated by the addition of absolute alcohol (4 vols.) as a white flocculent material which turned brown readily on exposure to light. It was separated on the centrifuge, washed a few times with absolute alcohol and then with ether, and dried over sulphuric acid in a vacuum desiccator to a pale yellow powder (moisture content, 7·84%). 0·4624 G. (absolutely dry) gave on ignition 0·0380 g. of silver. A similar allowance (see above) being made for the carboxyl introduced into the terminal aldehydic unit by the bromine oxidation, this gives a molecular weight of 2578.

Oxidation with Periodic Acid of Laminarin which has been already oxidised with Bromine.—The material (1 g.) was dissolved in hot water (10 c.c.), and the cooled solution treated with 0·44M-periodic acid (10 c.c.). The titre (N/10-sodium thiosulphate) was reduced in 8 days from 4·00 c.c. to 3·82 c.c. The oxidised laminarin was recovered as before; 0·5 g. neutralised 17·00 c.c. of N/100-sodium hydroxide.

Conversion of this Product into Laminarinic Acid.—0·4 G. was dissolved in warm water (10 c.c.), bromine (0·4 c.c.) added, and the mixture well shaken. The laminarinic acid was recovered as already described. 0·2 G. (dry) neutralised

18·00 c.c. of N/100-sodium hydroxide. With the usual correction (see above) this gives for 2000 c.c. of N-sodium hydroxide a weight of 2560 g.

Glucose and Glyoxylic Acid in the Hydrolysate.—Laminarinic acid (0·5 g.) was heated with 5% hydrochloric acid (10 c.c.) on the boiling water-bath for 4 hours, the liquid neutralised with ammonia, and a little of it treated with phenylhydrazine acetate in the usual way. Glucosazone was formed in quantity. A further quantity of the neutral hydrolysate on treatment with casein and concentrated sulphuric acid gave a deep blue-purple ring between the two liquids. The colour spread in an hour through the solution.

Oxalic Acid in the Oxidised Hydrolysate.—About 1 g. of the laminarinic acid was hydrolysed as described above, and bromine (0·8 c.c.) added to the neutralised hydrolysate. After shaking, the mixture was kept for a week. The liquid was again treated with excess of ammonia and then with calcium chloride solution, yielding a small amount of precipitate, insoluble in dilute acetic acid, soluble in dilute hydrochloric acid.

Glyoxal in the Hydrolysate from Periodic Acid-oxidised Laminarin.—About 1 g. of the laminarin after oxidation with periodic acid was hydrolysed by boiling for ½ hour with 5% sulphuric acid (50 c.c.). The liquid was neutralised with barium carbonate; on addition of phenylhydrazine acetate to the filtrate a colourless crystalline precipitate separated in small quantity. This was recrystallised from aqueous alcohol, giving colourless jagged spear-shaped plates identical with glyoxalphenylosazone.

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UNIVERSITY COLLEGE, GALWAY. [*Received, May 18th,* 1942].