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Determination by a Kinetic Method of the Nearest-Neighbour Frequencies in a Fragment of Alginic Acid

BJØRN LARSEN, OLAV SMIDSRØD, ARNE HAUG and TERENCE PAINTER

Norwegian Institute of Seaweed Research, N.T.H., Trondheim, Norway

An acid-soluble fragment (*A*) of alginate, having a number-average degree of polymerisation (P_n) of 20, and containing residues of mannuronic acid (M) and guluronic acid (G) in the ratio of 1.2:1, was partially hydrolysed at 100° and pH 2.8. Under these conditions, hydrolysis was characterised by two first-order rate-constants (k_M and k_G), the ratio (k_M/k_G) of these being about 4.3.

At degrees of scission (α) near 0.5, the yields of the mono- and tri-saccharides were lower, and those of the di- and tetra-saccharides higher, than the yields calculated from the Kuhn equation on the assumption of random cleavage. On the other hand, two essentially homopolymeric fragments of alginate, composed mainly of mannuronic acid residues (fragment *B*) and guluronic acid residues (fragment *C*), respectively, gave yields of mono- and oligo-saccharides closely agreeing with those predicted by the Kuhn equation.

An expanded equation, permitting calculation of the yields of mono- and oligo-saccharides in terms of the k_M/k_G ratio, α, and the nearest-neighbour frequencies, was derived and used to analyse the data obtained with fragment *A*. The results indicated that contiguous residues of guluronic acid were not present in *A*, and that the average length of the groups of contiguous mannuronic acid residues was 1.2 units.

It was concluded that about 90 % of *A* consisted of sequences of alternating mannuronic and guluronic acid residues, and that the average length of these was at least 10 monomer units.

The identification of oligosaccharides, liberated by partial acid hydrolysis, provides information about the kinds of glycosidic linkages present in polysaccharides, but the yields of easily identifiable fragments are usually much too low to give a quantitative picture of the structure of the whole polysaccharide chain.[1-4]

Freudenberg and his co-workers [5,6] were the first to use kinetic methods to study the structure of a complete polysaccharide molecule. The rates of hydrolysis of cellulose and of oligomers derived from it were measured, and with the help of Kuhn's equations,[7] it was shown that all the internal glucosidic

linkages in cellulose were hydrolysed at the same rate, and were therefore most probably identical. It was also recognised [6,7] that measurement of the yields of various oligomers for a given degree of scission could be used to obtain the same information.

In this laboratory, it has been shown that alginate is a block copolymer, containing long sequences of contiguous mannuronic acid residues, similar sequences of guluronic acid residues, and also long heteropolymeric sequences composed of both monomers. The heteropolymeric parts are readily split out from the molecule as acid-soluble material (fragment *A*) during mild acid hydrolysis, whereas the homopolymeric parts remain insoluble, and are isolated from the residue as two discrete fragments (*B* and *C*), containing mainly mannuronic acid residues and mainly guluronic acid residues, respectively.[8–10]

Attempts to determine the arrangement of the two types of uronic-acid residue in *A* have so far shown that the diuronides obtained from it (in about 20 % yield) by further acid hydrolysis consist largely of the two heteropolymeric ones, M—G and G—M (Refs. 8, 9, 11). It thus appeared that fragment *A* was rich in alternating mannuronic and guluronic acid sequences, but how much of it was so constituted could not be determined without first arriving at an understanding of the kinetics of acid hydrolysis of the material.

Such an understanding was recently reached, with the demonstration that, over a wide range of pH values, the rate of hydrolysis of *A* could be expressed, with reasonable accuracy, in terms of two first-order rate-constants, which were associated, respectively, with the two types of monomeric unit.[11] At low pH, the two rate-constants were very similar in magnitude, giving rise to approximately random hydrolysis, whereas between pH 2 and pH 4 they were widely different. This lack of randomness in the hydrolysis in weakly acidic solution, which was caused by direct participation of the carboxyl groups in the hydrolytic mechanism (intramolecular catalysis),[11] was expected to influence the yields of oligomeric intermediates in a manner dependent upon the arrangement of mannuronic and guluronic acid residues along the chain.

The present work therefore consists in an extension of the methods of Kuhn and Freudenberg to cover this form of non-random depolymerisation, and the use of the expanded formulae, with the help of the digital computer, to determine the structure of fragment *A*.

THEORY

Effect of molecular weight and molecular-weight distribution upon the yields of oligomers from finite chains. Fragment *A* had a number-average degree of polymerisation (P_n) of 19 ± 1, and, as could be expected from the method of its isolation, it was polydisperse with respect to molecular weight. It is therefore necessary to ascertain whether P_n and the chain-length distribution have to be taken into account in deriving a formula for the yields of oligosaccharides from *A*. It is helpful first to consider the influence of these quantities in random depolymerisation.

For random cleavage of a polymer whose molecules are all N units in length, the yield (Y_n) of n-mer is given [12] by

$$Y_n = (nw/N)(1-w)^{n-1}[2+(N-n-1)w] \quad (1)$$

where w is the fraction of all the original inter-unit linkages cleaved.

On the other hand, a polymer formed by random scission of an infinitely long chain has a chain-length distribution defined by the Kuhn equation:[7]

$$F^N = Ns^2(1-s)^{N-1} \quad (2)$$

where F^N is the weight-fraction of all chains N units in length, and $s=(1/P_n)$. The quantity s can of course also be regarded as a degree of scission, but for the present purpose, it is necessary to retain it as a symbol different from w. During further random depolymerisation of this polymer, the yield (Y_n^N) of n-mer originating by further cleavage of all chains of length N units, is given by combining eqns. (1) and (2):

$$Y_n^N = nws^2(1-w)^{n-1}(1-s)^{N-1}[2+(N-n-1)w]$$

The yield of n-mer originating by further cleavage of all chains containing more than n units is then

$$\sum_{N=n+1}^{\infty} Y_n^N, \text{ which simplifies to } nw(1-w)^{n-1}(1-s)^n[2s+w(1-s)]$$

Now the amount of n-mer present in the original polymer is $ns^2(1-s)^{n-1}$, and to obtain the amount remaining after further degradation, this must be multiplied by the probability, $(1-w)^{n-1}$, that none of the $(n-1)$ linkages had thereby been cleaved. Addition of this product to the amount of n-mer obtained by degradation of longer chains then gives:

$$Y_n = n(1-w)^{n-1}(1-s)^{n-1}[s+(1-s)w]^2 \quad (3)$$

Table 1. Theoretical yields of mono- and oligo-mers from different polymers randomly degraded to a degree of scission α of 0.5.

Description of polymer	P_n	Equation	Yield (%) of Monomer	Dimer	Trimer	Tetramer
Monodisperse	20	1	27.50	26.25	18.75	11.88
Infinite	∞	2	25.00	25.00	18.75	12.50
Polydisperse	20	3	27.56	26.18	18.65	11.81

In Table 1, eqns. (1) and (3), respectively, have been used to calculate the yields of various n-mers from two polymers, one of them monodisperse with respect to molecular weight, and the other having a chain-length distribution obeying the Kuhn equation. Both polymers have $P_n=20$; this condition was imposed by setting $N=20$ in eqn. (1) and $1/s=20$ in eqn. (3). In both cases,

the degree of scission (w) is 0.5. For comparison, the corresponding yields obtained by degradation of an infinitely long chain to the same degree of scission are included in the table; these were calculated by putting $s=0.5$ and $N=n$ in eqn. (2). The results show that, whereas the degree of polymerisation (P_n) of the starting material has a significant effect upon the yields of mono- and oligo-mers, the effect of the molecular-weight distribution is negligibly small for the chosen values of P_n and n.

It is instructive now to choose a quantity, α, such that $(1-\alpha)=(1-w)(1-s)$. Substitution of this into eqn. (3) reduces it to a form identical with the Kuhn eqn. (2). It is readily recognised that α is simply the degree of scission, calculated on the assumption that the starting material was of infinite molecular weight. Otherwise stated, α is the reciprocal of the number-average degree of polymerisation of the reaction mixture.

It can be concluded that, regardless of the molecular-weight distribution, the Kuhn equation can be applied to the random depolymerisation of fairly short chains, provided that α, rather than w, is regarded as the degree of scission. The corresponding proposition, that the equations to be derived for non-random depolymerisation of infinite chains are, subject to similar conditions, applicable to fragment A, is accepted here without undertaking a rigorous proof.

Non-random depolymerisation of a binary linear heteropolysaccharide of infinite molecular weight. Consistency in notation and nomenclature with earlier papers [13–15] is maintained, and reference is made to them for definitions. The copolymer is considered to be composed of monomer units A and B, the mole fractions of which are F^A and F^B, respectively. Its degradation is described by two first-order rate-constants, k_A and k_B, where these represent the rates of exposure of units of A and B, respectively, as end-groups. These end-groups must both be of the same kind, that is, they must either be both reducing end-groups or both non-reducing end-groups. Degradation leads at any time (t) to degrees of scission α_A and α_B, where these represent the respective fractions of the total A-units and B-units existing as the specified kind of end-group. It then follows that:

$$\alpha_A = 1-\exp(-k_A t) \text{ and } \alpha_B = 1-\exp(-k_B t) \quad (4)$$

Also, the fraction (α) of the total monomer units exposed as the specified kind of end-group is given by:

$$\alpha = \alpha_A F^A + \alpha_B F^B \quad (5)$$

As a first approximation, it is assumed initially that the distribution of monomer units along the chains is fully described by two nearest-neighbour frequencies,[15] p(AA) and p(BB). To facilitate reference to earlier work, these quantities are expressed in terms of two constants, a and b, such that $p(AA)=a/(1+a)$ and $p(BB)=b/(1+b)$.

The convention is adopted of scanning the chains in such a direction that the rate of cleavage of a given linkage is determined by the identity of the preceding monomer unit in the chain. Thus, if the ith unit in the chain is an A, the rate of cleavage of the ith linkage is k_A.

The liberation of homopolymeric fragments is considered first. The probability that, in a particular chain, the $(i+1)$th to the $(i+n)$th units inclusive are all A's is $P_i^{A}[a/(1+a)]^n+P_i^{B}[1/(1+b)]\ [a/(1+a)]^{n-1}$, where P_i^{A} and P_i^{B} are the probabilities that the ith unit is an A or a B, respectively. Imposing the condition that the ith and $(i+n)$th linkages must be cleaved, while the intermediate $(n-1)$ linkages are not, it is found that the probability of the n A's being split out as an n-mer is:

$$P_i^{A}[a/(1+a)]^n\alpha_A^2(1-\alpha_A)^{n-1}+P_i^{B}[1/(1+b)]\ [a/(1+a)]^{n-1}\alpha_A\alpha_B(1-\alpha_A)^{n-1}$$

If the chain is N units in length, the fraction (Y_n^{A}) of the monomer units split out from within the chain as n-mer containing A only is obtained by multiplying this probability by n/N, and summing over all possible values of i:

$$Y_n^{A} = [a/(1+a)]^n\alpha_A^2\ (1-\alpha_A)^{n-1}(n/N)\sum_{i=1}^{N-n-1} P_i^{A}$$

$$+[1/(1+b)]\ [a/(1+a)]^{n-1}\ \alpha_A\alpha_B(1-\alpha_A)^{n-1}(n/N)\sum_{i=1}^{N-n-1} P_i^{B}$$

Now, $(1/N)\sum_{i=1}^{N} P_i^{A}=F^{A}$ and $(1/N)\sum_{i=1}^{N} P_i^{B}=F^{B}$, so that when N tends to infinity,

$$Y_n^{A} = nF^{A}[a/(1+a)]^n\alpha_A^2(1-\alpha_A)^{n-1}+nF^{B}[1/(1+b)][a/(1+a)]^{n-1}\alpha_A\alpha_B(1-\alpha_A)^{n-1}.$$

But since[13] $F^{B}/(1+b)=F^{A}/(1+a)$ when the chains are infinitely long, this simplifies to:

$$Y_n^{A} = nF^{A}(1-\alpha_A)^{n-1}[a/(1+a)]^{n-1}[a\alpha_A^2/(1+a)+\alpha_A\alpha_B/(1+a)] \qquad (6)$$

Exchange of A for B and of a for b in all the symbols gives the corresponding equation for Y_n^{B}.

Proceeding to n-mers containing both monomers, it is evident that there are 2^n-2 different possible mixed sequences, and the yield of any particular one is readily arrived at by the same kind of reasoning as that just used. That of the dimer AB, for example, is given by

$$Y_2^{AB} = 2F^{A}[1/(1+a)](1-\alpha_A)\alpha_B[a\alpha_A/(1+a)+\alpha_B/(1+a)] \qquad (7)$$

while the corresponding equation for BA is obtained by exchanging the symbols as before.

In arriving at a general formula for the combined yield of all n-mers of mixed composition, it is convenient first to divide the expressions up into $(n-1)$ groups, according to the number (x) of units of B that they contain, and then to divide these groups into four subgroups, according to the identities of the first and last units in the sequence. Thus, the yield (Y_n^{x}) of all n-mers containing x units of B (and hence $n-x$ units of A) is written:

$$(Y_n^{x}) = (Y_n^{x})_{A\cdot A}+(Y_n^{x})_{A\cdot B}+(Y_n^{x})_{B\cdot B}+(Y_n^{x})_{B\cdot A} \qquad (8)$$

where the subscript A·B, for example, refers to all sequences beginning with A and ending with B. The separate yields of the four subgroups then follow

as a simple extension of the formulae derived earlier [14] for composition-distribution in randomly-degraded chains:

$$(Y_n^x)_{A\cdot A} = nF^A\left[\frac{a\alpha_A}{1+a}+\frac{\alpha_B}{1+a}\right]\left(\frac{a}{1+a}\right)^{n-x-1}\left(\frac{b}{1+b}\right)^{x-1}\left(\frac{1}{a}\right)\left(\frac{1}{1+b}\right)$$
$$(1-\alpha_A)^{n-x-1}(1-\alpha_B)^x\alpha_A\sum_{c=1}^{x}\left(\frac{1}{ab}\right)^{c-1}\binom{x-1}{c-1}\binom{n-x-1}{c} \quad (9)$$

$$(Y_n^x)_{A\cdot B} = nF^A\left[\frac{a\alpha_A}{1+a}+\frac{\alpha_B}{1+a}\right]\left(\frac{a}{1+a}\right)^{n-x-1}\left(\frac{b}{1+b}\right)^{x-1}\left(\frac{1}{1+a}\right)$$
$$(1-\alpha_A)^{n-x}(1-\alpha_B)^{x-1}\alpha_B\sum_{c=1}^{x}\left(\frac{1}{ab}\right)^{c-1}\binom{x-1}{c-1}\binom{n-x-1}{c-1} \quad (10)$$

$$(Y_n^x)_{B\cdot B} = nF_B\left[\frac{b\alpha_B}{1+b}+\frac{\alpha_A}{1+b}\right]\left(\frac{a}{1+a}\right)^{n-x-1}\left(\frac{b}{1+b}\right)^{x-1}\left(\frac{1}{b}\right)\left(\frac{1}{1+a}\right)$$
$$(1-\alpha_A)^{n-x}(1-\alpha_B)^{x-1}\alpha_B\sum_{c=1}^{x}\left(\frac{1}{ab}\right)^{c-1}\binom{x-1}{c}\binom{n-x-1}{c-1} \quad (11)$$

$$(Y_n^x)_{B\cdot A} = nF^B\left[\frac{b\alpha_B}{1+b}+\frac{\alpha_A}{1+b}\right]\left(\frac{a}{1+a}\right)^{n-x-1}\left(\frac{b}{1+b}\right)^{x-1}\left(\frac{1}{1+b}\right)$$
$$(1-\alpha_A)^{n-x-1}(1-\alpha_B)^x\alpha_A\sum_{c=1}^{x}\left(\frac{1}{ab}\right)^{c-1}\binom{x-1}{c-1}\binom{n-x-1}{c-1} \quad (12)$$

The combined yield (Y_n) of n-mers of every possible composition is therefore given by:

$$Y_n = Y_n^A + Y_n^B + \sum_{x=1}^{n-1}(Y_n^x) \quad (13)$$

Since, in the present model, $F^A=(1+a)/(2+a+b)$, and $F^B+F^A=1$ (Refs. 13, 14), eqn. (6) and eqns. (8)—(13) permit calculation of Y_n in terms of a, b, α_A, and α_B only. These equations, together with eqns. (4) and (5), were used to prepare a computer program to give a print-out of Y_n for given values of n, a, b, the ratio of the rate-constants k_A/k_B, and the overall degree of scission, α.

It must be noted that none of the foregoing equations takes account of the addition of the elements of water which occurs during the hydrolysis of a polysaccharide. The experimental yields were accordingly calculated on a basis of anhydrohexuronic acid throughout.

EXPERIMENTAL

Materials and analytical methods. The relative proportions of mannuronic and guluronic acid residues (M/G ratio) in all samples were determined as described by Haug and Larsen.[16] Total carbohydrate was measured by the phenol [17] and orcinol [18] methods, and calculated as anhydrohexuronic acid. The degrees of polymerisation (P_n) of oligosaccharides, and the degrees of scission (α) of partial hydrolysates were determined by measurement of total carbohydrate before and after reduction with sodium borohydride.[19] The heteropolymeric fragment (A), and the largely homopolymeric fragments (B and C)

which were used as model substances, were all prepared from *Laminaria digitata* alginate as previously described;[9] they all had P_n, determined by measurement of reducing power,[20] of 20 ± 1, and were isolated as their sodium salts.

Partial hydrolysis of fragment (*A*). A solution (1 % w/v) of *A* (85 ml) was acidified with sulphuric acid (1 N; 5.3 ml) and dialysed twice at 20° against water (600 ml) to remove sodium ions. A portion containing 480 mg of anhydrohexuronic acid (M/G = 1.2; $P_n = 20$) was brought to pH 2.8 with N sulphuric acid, and heated 8 h at 100° under nitrogen. The resultant solution, which contained 450 mg anhydrohexuronic acid ($\alpha = 0.474$) was brought to pH 7 with sodium hydroxide. A portion containing 300 mg was evaporated under diminished pressure to a volume of 3 ml, and the concentration of sodium sulphate was adjusted to 0.1 M by addition of the anhydrous salt (42 mg).

Partial hydrolysis of fragment (*B*). A solution of *B* (850 mg; M/G = 13.3; $P_n = 21$) in water (65 ml) was brought to pH 2.8 with N sulphuric acid, and dialysed twice against distilled water (600 ml). The pH was then again adjusted to 2.8, and the solution was heated 5 h at 100° under nitrogen. A portion of the solution, containing 300 mg of anhydrohexuronic acid ($\alpha = 0.324$) was neutralised with sodium hydroxide, concentrated to 3 ml, and made 0.1 M with respect to sodium sulphate.

Partial hydrolysis of fragment (*C*). A solution (1 % w/v; 60 ml) of *C* (M/G = 0.067; $P_n = 20$) was adjusted to pH 3.6 with N sulphuric acid, and heated 24 h at 100° under nitrogen. The pH was then adjusted to 3.2, and hydrolysis continued for 15 h. A sample (300 mg) of the product ($\alpha = 0.521$) was prepared as a solution in 0.1 M sodium sulphate (3 ml).

Fractionation of partial hydrolysates. The sample (3 ml) was transferred to a column (2.6 × 170 cm) of Sephadex G-25 (fine), which was then eluted with 0.1 M sodium sulphate at a rate of 7 ml/h. Fractions (2 ml) were collected, and, after dilution with 0.1 M sodium sulphate when necessary, portions (0.2 ml) of them were taken for measurement of total carbohydrate. Portions were also withdrawn for monitoring the P_n of the eluted material, and at the end of the fractionation, aliquot parts of all the fractions were pooled for determination of the overall degree of scission (α).

RESULTS AND DISCUSSION

The equations given in the theoretical section resolve the determination of the nearest-neighbour frequencies in fragment *A* into a problem in the accurate measurement of the yields of mono- and oligo-saccharides. Despite the apparent complexity of the equations, it should be emphasised that their solution is a trifling matter in the hands of the digital computer, and constitutes no obstacle to the use of the method. On the other hand, attainment of the required accuracy in analysis of the partial hydrolysates met with a number of problems which merit discussion.

It is noteworthy that the success of the method did indeed rest solely upon the accurate analysis of mixtures of mono- and oligo-saccharides. This is because the yields of at least four different fragments were measured, namely, those of the mono- to tetra-saccharides inclusive. Thus, four equations were obtained in the four unknowns, a, b, α, and the k_A/k_B ratio. Strictly speaking, therefore, it was unnecessary to measure the M/G ratio of fragment *A* [which is simply $(1+a)/(1+b)$], or α, or the k_A/k_B ratio,* all these quantities being accessible from the yields of the four fragments. In practice, however, they were measured as carefully as possible, since this provided an independent check upon the accuracy of the measurement of the yields of oligosaccharides.

* A unit of A in the copolymer considered in the theoretical section is hereinafter regarded as a residue of mannuronic acid (M), and a unit of B as a residue of guluronic acid (G).

The measurement of the k_A/k_B (*i.e.* k_M/k_G) ratio has been described elsewhere,[11] and at pH 2.8 this was 4.3 ± 0.3.

Gel-filtration chromatography on Sephadex G-25 afforded the best means of fractionating the hydrolysates. Chromatography on charcoal, cellulose, or paper was unsatisfactory because the elution pattern on these materials is greatly complicated by fractionation on a basis of structure and composition as well as of molecular weight. Moreover, for reasons discussed below, the capacity of Sephadex to separate almost solely according to molecular weight largely overcame a major problem that besets all such work on polyuronides, namely, losses of uronic acid during acid hydrolysis, due to dehydration and decarboxylation.[11]

That these losses of uronic acid occur mainly as a result of the acid-catalysed modification of previously liberated monouronic acids is suggested by the recognition[21–23] that furfural, reductic acid, and 5-formyl-2-furoic acid are among the principal products, and the observation[11] that there is a very marked induction period in the formation of unsaturated material when polyuronides are heated in acid. Paper chromatography of the monomeric fraction from the Sephadex columns confirmed that the two monouronic acids were accompanied by substances of high chromatographic mobility, similar to those formed upon heating the pure monomers in acid under the same conditions. Similar examination of the oligomeric fractions indicated that a limited modification of reducing end-groups occurs to a significant extent even at the oligomeric level.

Since these modified monomers and oligomers migrated on Sephadex together with their respective parent fragments, and since it was expected that they were intermediates in the conversion of uronic acids into the chromogens that are formed under the acidic conditions of the phenol and orcinol methods, it was reasoned that, by following the elution with these analytical methods, the error would be largely eliminated. However, as the conditions of partial hydrolysis were very different from those employed in the phenol and orcinol methods of assay, complete elimination of error in this way could not be expected, and indeed it was found that, during partial hydrolysis, there was destruction of about 5—7 % of material capable of reacting with the phenol reagent. This loss of material was assumed to arise solely from reactions occurring at the monomeric level, and was accordingly added as a correction to the yield of monomers obtained from the Sephadex column.

In analysis by the phenol method, mannuronic acid gives a lower molar extinction than does guluronic acid, whereas the reverse is the case with the orcinol method.[16] However, as a result of the high proportion of alternating sequences in *A*, the M/G ratio of the material eluted from the column changed very little during fractionation. No appreciable error was therefore introduced in following the elution of total carbohydrate from the column by either method, and the phenol method was chosen for this because of its greater simplicity.

The different molar extinctions given by the two monomers did, however, cause some difficulty in monitoring the P_n of the fractions. The method used was that entailing measurement of the total carbohydrate before and after reduction with borohydride,[19] and, since the guluronic-acid residues were

the more rapidly exposed as reducing end-groups,[11] the M/G ratio was not the same after reduction as it was before. However, by using both the phenol and the orcinol methods together, a clear identification of the four major peaks was achieved. Table 2 compares the results obtained by the phenol

Table 2. Degrees of polymerisation of dimer fractions from fragments *A*, *B*, and *C*, determined by analysis for total carbohydrate, before and after reduction with sodium borohydride.[19]

Analytical method	Dimer fraction from fragment		
	A	*B*	*C*
Phenol [17]	1.82[a]	2.32	2.11
Orcinol [18]	1.98[a]	2.12	1.86

[a] Calculated on the assumption that the dimer fraction consisted solely of M—G.

and orcinol methods, when applied to the disaccharide fractions from hydrolysates of fragments *A*, *B*, and *C*. Determination of the P_n of the fractions by the measurement of reducing power [20] was inaccurate because of the presence of the acid-modified uronic acids referred to above.

The yields of the individual fractions were determined by plotting the elution pattern on paper (Figs. 1, 2, and 3) and measuring the area under each peak by cutting it out and weighing it. Even with a column length of 1.7 m, there was some overlapping of the peaks. The peaks were therefore divided by lines drawn from the lowest points of the troughs perpendicularly to the baseline. This procedure, which is based upon the assumption that the peaks are approximately triangular, is known to give quite high accuracy in gas chromatography.[24,25]

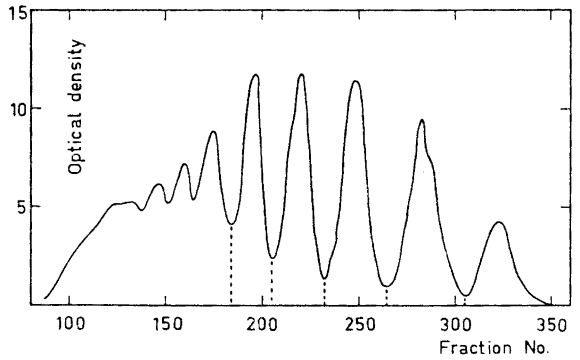

Fig. 1. Elution pattern of hydrolysed fragment *B* ($\alpha = 0.324$) from Sephadex G 25 irrigated with 0.1 M sodium sulphate. The ordinates represent the total optical density for the 2 ml fraction.

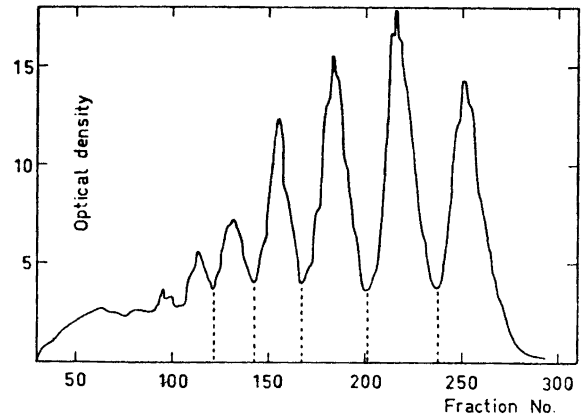

Fig. 2. Elution pattern of hydrolysed fragment *C* ($\alpha = 0.521$).

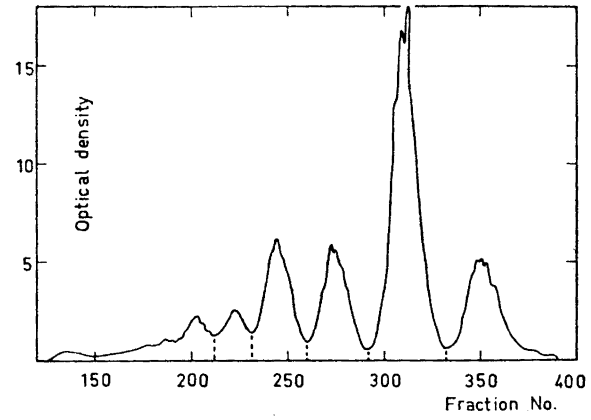

Fig. 3. Elution pattern of hydrolysed fragment *A* ($\alpha = 0.474$).

Measurement of the overall degree of scission (α) by chemical means was subject to the same source of error as that encountered in determination of the P_n of the individual fractions. As an independent check, α was also calculated from the measured yields of mono- and oligo-saccharides. The relationship for this is clearly $\alpha = \sum_{n=1}^{\infty} Y_n/P_n$. However, as it was possible to measure accurately only the individual yields of the monomers to pentamers inclusive, a P_n of 8 was arbitrarily assigned to all fragments larger than pentamer, thus giving the following approximation:

$$\alpha = \tfrac{1}{8} \sum_{n=6}^{\infty} Y_n + \sum_{n=1}^{5} Y_n/P_n$$

Since the combined yield of oligomers larger than the pentamer is quite small (*ca.* 10 % when $\alpha=0.5$), and these fragments contribute very little to the overall degree of scission in comparison with the lower oligomers, the error in this approximation is very small (<2 %).

The complete procedure was first tested on the essentially homopolymeric fractions from degraded alginic acid, *B* and *C*. Kinetic studies had shown [11] that hydrolysis of these materials at pH 2.8 obeyed first-order kinetics, so that the degradation should be random, and the yields of the various fragments should agree with those predicted by the Kuhn equation. The elution patterns for partially hydrolysed fragments *B* and *C* are shown in Figs. 1 and 2, respectively.

Table 3. Experimental yields of mono- and oligo-mers from fragment *B* ($\alpha=0.324$) and fragment *C* ($\alpha=0.521$), compared with the theoretical yields, calculated with Kuhn equation (2).

	Yield (%) from Fragment B		Yield (%) from Fragment C	
	Found	Calc.	Found	Calc.
Monomer	8.9	10.8	28.5	27.1
Dimer	14.7	14.2	25.3	26.0
Trimer	14.0	14.4	18.1	18.7
Tetramer	13.6	13.1	11.2	11.9

Table 3 gives the results, together with the corresponding figures, calculated from the Kuhn equation. The accuracy of the method is seen to be satisfactory.

In applying the method to fragment *A*, the object is to determine *a* and *b* separately. For a given M/G ratio, there is an infinite number of different possible pairs of values of *a* and *b*. The physical significance of *a* and *b* is discussed in detail elsewhere,[13–15] but for present purposes, it is useful to recall simply that the average length of the groups of contiguous mannuronic acid units is $(1+a)$, and that that of the groups of contiguous guluronic acid units is $(1+b)$.

Table 4. Experimental yields (%) of mono- and oligo-mers from fragment *A* ($\alpha=0.474$), compared with theoretical yields, calculated for different pairs of values of *a* and *b*, all corresponding to an M/G ratio of 1.2. The k_M/k_G ratio is set at 4.3.

		Calc. for							
	Found	a = 0.200 b = 0.001	0.220 0.010	0.320 0.100	0.440 0.200	0.560 0.300	0.800 0.500	1.100 0.750	1.200 0.832
Monomer	18.5	18.4	18.5	19.2	19.9	20.5	21.4	22.3	22.5
Dimer	35.5	35.3	34.9	32.4	30.2	28.4	26.0	24.1	23.7
Trimer	13.9	14.3	14.7	16.7	18.1	18.8	19.1	18.8	18.7
Tetramer	14.4	15.6	15.4	14.0	13.4	13.1	13.1	13.1	13.1

Fig. 3 shows the elution pattern obtained in an experiment with fragment *A*, and the first column in Table 4 gives the results. The remaining columns in Table 4 were all computed from the experimentally determined values of the M/G ratio, the k_M/k_G ratio, and α, these being 1.2, 4.3, and 0.474, respectively. Each column was calculated for an arbitrary pair of values of a and b, chosen to give the correct M/G ratio of 1.2. The columns are arranged, from left to right, in the order of increasing values of a and b. The smallest possible pair of values is $a=0.200$ and $b=0.000$, and the second column, with $a=0.200$ and $b=0.001$, corresponds almost exactly to this. It is interesting to note that the ninth column corresponds to the situation in which the mannuronic- and guluronic-acid residues are randomly distributed along the chains.

Owing to the difficulty in controlling the pre-treatment and partial hydrolysis of *A* so precisely as to obtain always the same M/G ratio and α, it was not practical to attempt an exact duplication of the results in Table 4, in order to check their reproducibility. However, other experiments with partial hydrolysates differing in M/G ratio and α all pointed equally clearly to the conclusion that the smallest possible pair of values of a and b gave the closest agreement between theory and experiment. In the earlier experiments, it was invariably found that a value of a lower than the smallest permitted by the experimentally determined M/G ratio would have given a better fit. Thus, whereas the M/G ratio of fragment *A* as isolated from *Laminaria digitata* alginate, usually had a value near 1.6 (Ref. 9), the yields of mono- and oligosaccharides indicated that it should be nearer to 1.2. This discrepancy subsequently led to the discovery [15] that, during deionisation of *A* which was carried out prior to hydrolysis, by dialysis against cold, dilute acid as described in the experimental section, there had been a selective loss through the membrane of material rich in mannuronic acid. The material remaining inside the membrane did indeed have an M/G ratio very close to 1.2.

Table 5. Theoretical yields of mono- and oligo-mers for different M/G ratios, with b set at 0.001, k_M/k_G at 4.3 and α at 0.474.

M/G ratio	a	Yield (%) of			
		Monomer	Dimer	Trimer	Tetramer
1.1	0.100	17.9	37.0	13.1	16.4
1.2	0.200	18.4	35.3	14.3	15.6
1.3	0.300	18.8	33.9	15.2	15.1
Experimental yields		18.5	35.3	13.9	14.4

In this connection, it is of interest to examine the sensitivity of the method to small variations in the M/G and k_M/k_G ratios. For a given k_M/k_G ratio of 4.3, and $\alpha=0.474$, Table 5 shows the effect of variation in the M/G ratio, the smallest possible pair of values of a and b being chosen in each case. Similarly, Table 6 illustrates the effect of variation in the k_M/k_G ratio for a given M/G ratio of 1.2, again with the smallest possible values of a and b, and with α set at 0.474.

Table 6. Theoretical yields of mono- and oligo-mers for different k_M/k_G ratios, with a set at 0.200, b at 0.001 (M/G=1.2), and α at 0.474.

Ratio k_M/k_G	Yield (%) of Monomer	Dimer	Trimer	Tetramer
4.0	18.7	34.4	14.6	15.5
4.3	18.4	35.3	14.3	15.6
4.6	18.1	36.2	14.0	15.8

The physical meaning of the results obtained with fragment *A* may now be considered. With a value of *b* close to zero, it is clear that contiguous residues of guluronic acid are virtually absent in the material. Stated in statistical terms, the probability [p(BB)] that a residue of guluronic acid has another residue of guluronic acid as its nearest neighbour is zero, and the probability that it has a residue of mannuronic acid as a nearest neighbour is unity. With a value of *a* of 0.2, the average length of the groups of contiguous mannuronic-acid residues is 1.2 units, or, expressed statistically, the probability that a residue of mannuronic acid has a similar residue as a nearest neighbour [p(AA)] is 0.167.

These results create a picture of the structure of fragment *A* as an essentially alternating sequence of mannuronic and guluronic acid residues, in which one additional residue of mannuronic acid is inserted for every 10 residues in the sequence. Where exactly they are inserted relative to each other cannot be ascertained from the present data alone, since it is not known that the copolymer is fully defined by its nearest-neighbour frequencies alone. The required information is, in principle, readily accessible from the yields of the individual constituents of the various oligosaccharide fractions, but this will have to await the development of a sufficiently accurate analytical procedure.

For the present, however, there appears to be sufficient information from other sources to justify the speculation that the "additional" residues of mannuronic acid are all grouped together as small homopolymeric blocks, attached to the ends of the alternating sequences. It is established [8–10] that, in the original alginate, the sequences giving rise to fragment *A* upon hydrolysis are joined to long homopolymeric blocks of mannuronic acid residues, and also to similar blocks of guluronic acid residues. These homopolymeric blocks are responsible for the acid-insolubility of alginates, and precipitate out as insoluble material during acid hydrolysis, leaving the soluble fragment *A* in solution. It is to be expected that small segments of these homopolymeric blocks would be left attached to the ends of the alternating sequences in *A*. That these appear to be derived solely from the homopolymeric blocks that are composed of mannuronic acid only could be explained by the fact [10] that these blocks are much less insoluble in acid than those composed of guluronic acid only, and are therefore probably more accessible to hydrolysis.

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