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Calculation of the Nearest-neighbour Frequencies in Fragments of Alginate from the Yields of Free Monomers after Partial Hydrolysis

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The chemical inhomogeneity of most heteropolysaccharides and glycoproteins implies that, in general, a quantitative description of the sequence of sugar residues and linkages in these materials can be given only in statistical terms.^{1,2} For linear heteropolysaccharides, it may be expressed as a series of conditional probabilities, or near-neighbour frequencies.^{2,3} A first step towards a total determination of sequence consists in measurement of the nearest-neighbour frequencies. Methods for doing this have so far entailed measurement of the total homopolymeric fraction,¹ and studies of composition-distribution³⁻⁵ and molecular-weight distribution,⁶ in partially degraded materials.

This communication describes a method that consists simply in the measurement of the individual yields of the free monomers after partial hydrolysis to a known degree of scission. Like the other methods, it requires that the kinetics of hydrolysis be understood, and the present application rests upon the finding⁷ that the acid-hydrolysis of soluble fragments of alginic

acid at 100° can be described, to a good approximation, by two first-order rate-constants, k_M and k_G . These represent the rates at which the two types of monomeric unit, D-mannuronic acid and L-guluronic acid, respectively, are liberated as non-reducing end-groups, and, at pH 2.8, the ratio (k_M/k_G) is 4.3 ± 0.3 (Ref. 7).

Theory. If a linear sequence of monosaccharide residues, A, B, C, D, etc. is depolymerised until fractions $\alpha_A, \alpha_B, \alpha_C, \alpha_D$, etc. of the respective monomers are exposed as a specified kind of end-group (i.e. either reducing or non-reducing), and if the rate of cleavage of any linkage is independent of the identity of the fragment in which it occurs, then the original sequence can be uniquely determined from the yields (Y_A, Y_B, Y_C, Y_D , etc.) of the free monosaccharides and the corresponding values of α , provided that the latter are all different. For example, if the values of α refer to reducing end-groups, and C is the reducing terminal unit, the tetrameric sequence B-A-D-C would give $Y_A = \alpha_A \alpha_B, Y_B = \alpha_B, Y_C = \alpha_D$ and $Y_D = \alpha_A \alpha_D$, where these yields are expressed as fractions of the amounts of A, B, C, and D, respectively, in the substrate.

When the sequence contains two or more linkages that are hydrolysed at identical rates, or the substrate contains a mixture of different sequences, the system becomes "degenerate", and a unique sequence cannot be determined in this way. Nearest-neighbour frequencies can still be calculated, however, and for relatively long chains, in which end-group effects can be neglected,⁶ the calculation is particularly simple.

For a binary linear copolymer, composed of monomeric units M and G, whose hydrolysis is described by two first-order rate-constants as found for the fragments of alginate,⁷ the following relationships hold good:^{1,4,6}

$$Y_M = F^M [p(MM)] \alpha_M^2 + (1 - F^M) [1 - p(GG)] \alpha_M \alpha_G \quad (1)$$

$$Y_G = F^M [1 - p(MM)] \alpha_M \alpha_G + (1 - F^M) [p(GG)] \alpha_G^2 \quad (2)$$

$$F^M = [1 - p(GG)] / [2 - p(GG) - p(MM)] \quad (3)$$

where Y_M and Y_G are the yields of monomeric M and G, respectively (calculated as anhydrosugar, and expressed as a fraction of the total weight of starting material); F^M is the mole fraction of M in the starting material; and $p(MM)$ and $p(GG)$ are nearest-neighbour frequencies, defined^{2,3} respectively as the fractions of

the total M or G units having a similar unit as a nearest neighbour in the starting-material.

Although these relationships are strictly valid only for infinitely long chains, they hold good with high accuracy for substrates having a number-average degree of polymerisation of about 20 (Ref. 6).

Experimental. The method was tested on two acid-soluble fragments (X and Y) of *Laminaria digitata* alginate, X being identical with the material described as "preparation A" in an earlier paper,⁸ while Y was obtained by further, mild acid-hydrolysis of X as described previously.³ Previous studies⁶ have indicated that these materials possess a block type of structure, in which virtually all the guluronic-acid residues are present in sequences of alternating guluronic- and mannuronic-acid residues, while the excess of mannuronic-acid residues (see F^M , Table 1) is present in homopolymeric sequences.

Partial hydrolysis, separation of the monomeric fraction by gel-permeation chromatography, correction of the total yield of monomers for losses occurring during hydrolysis, and measurement of the overall degree of scission (α) were carried out as described elsewhere.⁶ The ratio of the two hexuronic acids in the monomeric fraction was then determined by ion-exchange chromatography.⁹ It was unnecessary to correct this ratio because, under the conditions used for partial hydrolysis, the rates of decomposition of the two monomers, as determined from measurements of the changes in reducing power and absorbance at 260 m μ (Ref. 7), were closely similar.

Results and discussion. Hydrolysis of X and Y under the stated conditions for 25 and 8 h, respectively, afforded the data summarized in Table 1. Two different sets

of nearest-neighbour frequencies are shown in each case, the first being calculated from eqns. (1) and (3), and the second from eqns. (2) and (3).

It is clearly impossible to have a negative nearest-neighbour frequency, and for both X and Y, the value of p(GG) must be taken as zero within the limits of experimental error. The higher values of p(MM) found for X clearly reflect the larger excess of mannuronic-acid residues in this material.

The nearest-neighbour frequencies can be used^{1,3} to calculate the number-average sequence-lengths of the groups of contiguous M and G units in the chains, and, when the latter are small compared to the length of the chains, as in the present case, they are given to a good approximation by $1/[1-p(\text{MM})]$ and $1/[1-p(\text{GG})]$, respectively.

The results are thus seen to be in excellent agreement with the evidence obtained earlier, that these acid-soluble fragments of alginate contain a high proportion of alternating mannuronic- and guluronic-acid residues. In particular, the results for fragment Y agree well with the values of p(MM) and p(GG), 0.167 and zero respectively, obtained earlier, with essentially identical material, from the molecular-weight distribution of a partial hydrolysate.⁶

The present method thus provides a further example of how, once the kinetics of hydrolysis of a heteropolysaccharide are understood, valuable structural information can be obtained from measurements of the simplest kind. As pointed out previously,⁶ this information is of a different kind from that accessible through the qualitative identification of oligomeric fragments, because it provides a quantitative picture of the whole macromolecule.

Table 1.

Fragment	F^M	α	α_M^a	α_G^a	Y_M	Y_G	p(MM)		p(GG)	
X	0.620	0.776	0.942	0.510	0.392	0.184	0.373, ^b	0.377 ^c	-0.024, ^b	0.016 ^c
Y	0.555	0.520	0.728	0.261	0.137	0.084	0.164, ^b	0.216 ^c	-0.043, ^b	0.022 ^c

^a Calculated from α and the k_M/k_G ratio (4.30).

^b Calculated from eqns. (1) and (3).

^c Calculated from eqns. (2) and (3).

It must be recognised, however, that the kinetics of acid-hydrolysis of these fragments of alginate are probably exceptionally simple in requiring only two rate-constants for their description, a fact which may be connected with their intramolecular, autocatalytic mechanism of hydrolysis.⁷ In general, significant differences can be expected between the rates of hydrolysis of internal and terminal linkages in the chains,¹⁰ and in some cases the rate of hydrolysis of a given linkage may also depend upon the identities of both the units that it adjoins.

Work is in progress on an expanded theoretical treatment which takes account of such differences, and which should extend the possible range of materials to which the method can be applied.

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Isolation of ³²P-Labelled Phosphorylthreonine from Ehrlich Mouse-ascites Tumour Cells Suspended in an Isotonic Medium Containing ³²P-Labelled Adenosine Triphosphate

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It was reported in a previous paper¹ that intact Ehrlich tumour ascites cells are capable of forming extracellular ATP in an isotonic medium containing the necessary substrates and cofactors. This supports the view that the enzymes concerned are located at the surface of the cells. The newly formed ATP cannot penetrate into the interior of the cells.¹ During efforts to find out possible functions for the *de novo* formed ATP in enzyme reactions at the cell surface, [³²P]phosphorylserine was isolated from the tumour cells.² Maximum labelling of phosphorylserine took place after relatively short incubation times. It is well known that the serine residue of the active center of several enzymes can be phosphorylated.³ It has also been possible to isolate phosphorylserine from such enzymes.³

The present paper describes the isolation of phosphorylthreonine from Ehrlich tumour ascites cells incubated with [³²P]ATP under similar conditions. The details of the incubation procedure have been described previously.^{1,2} The acid hydrolysate of the cells was chromatographed on a Dowex 50 column.⁴ A labelled peak was observed in the expected position for phosphorylthreonine.^{5,6} This labelled fraction was further purified by a second chromatography on Dowex 50. Fig. 1 illustrates the parallelism between the radioactivity and the ninhydrin reaction from a typical experiment. Further identification was obtained by high voltage paper electrophoresis using 0.1 N pyridine-acetic acid buffer as solvent, pH 5.0. A radioautogram was made of the electropherogram. As is seen from Fig. 2 one single ninhydrin spot was found on the electropherogram which had the same location as the radioactive spot.