379. *Partial Acid-hydrolysis of Glycoproteins in which the Oligosaccharides, once Liberated, are Resistant to Further Cleavage.*

By T. J. Painter.

A simple theoretical model is considered for partial acid-hydrolysis of a glycoprotein. On the assumption of random cleavage of the glycosidic linkages, the final yield of oligosaccharides is calculated for hydrolysis in which the oligosaccharides, once liberated, are resistant to further cleavage, and is shown to be much greater than the maximum possible yield when the oligosaccharides are susceptible to further cleavage.

A comparison is drawn between the theoretical model and the expected behaviour of a glycoprotein when it is hydrolysed by a strong polymeric acid, and by an ordinary mineral acid, and it is inferred that a higher yield of oligosaccharides should be obtained when the polymeric acid is used for hydrolysis.

Many naturally occurring glycoproteins appear to consist of a polypeptide moiety of high molecular weight, to which relatively short oligosaccharide side-chains are attached.[1,2] Several investigators have noted[2,3] that, when such materials are subjected to partial acid-hydrolysis, the yield of oligosaccharides is very low compared with that of monosaccharides; this constitutes a serious obstacle to structural analysis. A possible explanation is that the oligosaccharides, once liberated, are hydrolysed more rapidly than those still attached to peptide material.

It is suggested elsewhere[3,4] that, since all peptide-containing fragments in the reaction mixture would normally carry a positive charge, their rate of hydrolysis relative to that of neutral oligosaccharides could be increased by use of a strong polymeric acid, instead of a mineral acid, as a catalyst for hydrolysis. The influence of polyelectrolytes upon the rate of acid- and base-catalysed solvolysis of charged substrates is discussed by Morawetz and his co-workers.[5] Kinetic studies[3,4] of the hydrolysis of simple model substances by polystyrenesulphonic acid corroborate this idea, and show that the selectivity of this acid for the hydrolysis of positively charged fragments can be increased by dilution of the reaction mixture, until, at high dilutions, the neutral oligosaccharides, once liberated, are virtually resistant to further cleavage.[4]

A simple theoretical model for the partial acid-hydrolysis of a glycoprotein is now considered, in an attempt to determine the extent to which the use of a strong polymeric acid could improve the yield of oligosaccharides. In this model, it is assumed that (*a*) the oligosaccharide side-chains are unbranched, all of the same length, and joined glycosidically to the peptide moiety; (*b*) in polymeric-acid-catalysed hydrolysis, all glycosidic linkages in fragments containing peptide material are hydrolysed at the same rate, whereas fragments containing no amino-acid residues, once liberated, are completely resistant to further cleavage; (*c*) in mineral-acid-catalysed hydrolysis, all glycosidic linkages in all fragments are hydrolysed at the same rate; and (*d*) all monosaccharide residues have the some molecular weight.

[1] Neuberger, *Biochem. J.*, 1938, **32**, 1435; Jevons, *Nature*, 1958, **181**, 1346; Gottschalk, Murphy, and Graham, *ibid.*, 1962, **194**, 1051, and references cited therein; Aspinall, *Ann. Rev. Biochem.*, 1962, **31**, 96; Lee and Montgomery, *Arch. Biochem. Biophys.*, 1962, **97**, 9; Eylar and Jeanloz, *J. Biol. Chem.*, 1962, **237**, 622; Yamashina and Makino, *J. Biochem.* (*Japan*), 1962, **51**, 359; Izumi, Makino, and Yamashina, *ibid.*, p. 365; Clamp and Hough, *Chem. and Ind.*, 1963, 82.

[2] Bragg and Hough, *Biochem. J.*, 1961, **78**, 11; Cheese, Ph.D. Thesis, London, 1961.

[3] Painter and Morgan, *Chem. and Ind.*, 1961, 437.

[4] Painter, *J.*, 1962, 3932.

[5] Morawetz and Westhead, *J. Polymer Sci.*, 1955, **16**, 273; Morawetz, *ibid.*, 1960, **42**, 125; Morawetz and Shafer, *J. Phys. Chem.*, 1963, **67**, 1293; *idem*, *Biopolymers*, 1963, **1**, 71.

The mathematical principles are similar to those applied to the hydrolysis of polysaccharides by Kuhn,[6] and later extended by the present author.[7] The calculated yields of oligosaccharides are not corrected for the addition of the elements of water that occurs during hydrolysis.

Theory

Hydrolysis when the Oligosaccharides are Subject to Further Cleavage.—From an oligosaccharide side-chain, consisting of N contiguous, neutral monosaccharide residues attached glycosidically to the polypeptide, there are $(N - n)$ ways of choosing a neutral, n-membered oligosaccharide (*i.e.*, an n-mer) by the scission of two glycosidic linkages, and one way involving the scission of only one glycosidic linkage. If the side-chain is randomly depolymerised until a fraction s of the total glycosidic linkages is cleaved, the probability that a particular n-mer is split from within the chain is $s^2(1 - s)^{n-1}$, and the probability that one is split from the end of the chain is $s(1 - s)^{n-1}$. Therefore, the total probability of an n-mer's existing is $(N - n)s^2(1 - s)^{n-1} + s(1 - s)^{n-1}$, and the yield (Y_n) of n-mer, expressed as a fraction of the total sugar residues in the side-chain, is given by:

$$Y_n = (n/N)s(1 - s)^{n-1}[(N - n)s + 1]. \quad (1)$$

From the first derivative of equation (1),

$$dY_n/ds = (n/N)(1 - s)^{n-1}[1 + 2(N - n)s] - (n/N)(n - 1)s(1 - s)^{n-2}[1 + (N - n)s], \quad (2)$$

it follows that Y_n reaches a maximum when

$$(n^2 + n - Nn - N)s^2 + (2N - 3n)s + 1 = 0.$$

The maximum yield is then obtained by substitution for s in equation (1) (see Table 1).

Table 1.

Maximum yields of n-mer for different values of N and n; figures in parentheses are the optimum values of s.

n	$N = 2$	$N = 3$	$N = 4$	$N = 5$	$N = 6$	$N = 7$
2	0·250 (0·500)	0·257 (0·577)	0·264 (0·608)	0·270 (0·623)	0·274 (0·632)	0·277 (0·638)
3	—	0·148 (0·333)	0·151 (0·390)	0·156 (0·422)	0·160 (0·440)	0·163 (0·451)
4	—	—	0·106 (0·250)	0·107 (0·285)	0·110 (0·316)	0·113 (0·333)
5	—	—	—	0·082 (0·200)	0·083 (0·229)	0·085 (0·250)
6	—	—	—	—	0·067 (0·167)	0·068 (0·188)
7	—	—	—	—	—	0·057 (0·143)

Table 2.

Maximum possible yields of neutral oligosaccharides.

	$N = 2$	$N = 3$	$N = 4$	$N = 5$	$N = 6$	$N = 7$
Total	0·250	0·376	0·458	0·517	0·562	0·598
Dimer	0·250	0·245	0·235	0·226	0·213	0·204
Trimer	—	0·131	0·148	0·156	0·159	0·160
Tetramer	—	—	0·075	0·091	0·102	0·109
Pentamer	—	—	—	0·045	0·058	0·067
Hexamer	—	—	—	—	0·029	0·038
Heptamer	—	—	—	—	—	0·019
Optimum s	0·500	0·475	0·450	0·433	0·410	0·397

For several values of N, the maximum possible combined yield of all neutral oligosaccharides, from the dimer up to and including the N-mer, were calculated. The optimum

[6] Kuhn, *Ber.*, 1930, **63**, 1503.
[7] Painter, *J.*, 1963, 779.

values of s were computed graphically from equation (1). Table 2 gives the results, together with the composition of the reaction mixture in each instance.

Hydrolysis when the Oligosaccharides are Resistant to Further Cleavage.—The positive term in the right-hand side of equation (2) represents the increase in the amount of n-mer in the reaction mixture due to the break-down of all larger fragments containing n or more contiguous monosaccharide residues. Integration of this term, between the limits $s = 0$ and $s = 1$, gives a quantity,

$$(2N - n + 1)/N(n + 1), \qquad (3)$$

which represents [6,7] the fraction of the monosaccharide residues in the side-chain that exist at some stage as free n-mer during the total degradation of the side-chain to monosaccharides. However, when the n-mer, once liberated, is resistant to further cleavage, this quantity (3) represents the total yield of the resistant n-mer at the end of the reaction, provided that no larger oligomer is also resistant.[7]

Since all neutral fragments are resistant to further cleavage, the final yield of the largest possible neutral fragment, the N-mer, is $1/N$[*i.e.*, expression (3) when $n = N$].

The final yield of the $(N - 1)$-mer is $(N + 2)/N^2$[*i.e.*, expression (3) when $n = (N - 1)$], less the amount of $(N - 1)$-mer that would have been obtained by further cleavage of the N-mer, had the N-mer not been resistant. It is shown elsewhere [7] that, when a linear oligomer is randomly cleaved, the yield of a resistant n-membered fragment is $2/(n + 1)$, provided that no larger fragment is also resistant. In the present calculation, therefore, the amount by which the yield of the $(N - 1)$-mer is decreased owing to the resistance of the N-mer is obtained by multiplying the yield of N-mer by $2/(n + 1)$, where $n = (N - 1)$. The final yield of the $(N - 1)$-mer is therefore

$$(N + 2)/N^2 - (2/N)(1/N) = 1/N.$$

The final yield of the $(N - 2)$-mer is obtained by putting $n = (N - 2)$ in expression (3), and subtracting that fraction of the combined yields of N-mer and $(N - 1)$-mer that would have given $(N - 2)$-mer if these oligomers had not been resistant to further cleavage. The latter quantity is obtained, as before,[7] by multiplying the combined yield of N-mer and $(N - 1)$-mer by $2/(n + 1)$, where $n = (N - 2)$. The final yield of the $(N - 2)$-mer is, therefore,

$$(N + 3)/N(N - 1) - [2/(N - 1)](2/N) = 1/N.$$

Further calculation confirms that the final yield of any neutral oligosaccharide from a side-chain N units in length is, simply, $1/N$. For several values of N, the final combined yield of all neutral oligosaccharides, from the dimer up to and including the N-mer, and the composition of the reaction product in each instance, were calculated (Table 3).

TABLE 3.

Final combined yields of neutral oligosaccharides, and composition of products.

	$N = 2$	$N = 3$	$N = 4$	$N = 5$	$N = 6$	$N = 7$
Total	0·500	0·667	0·750	0·800	0·833	0·857
Each oligomer	0·500	0·333	0·250	0·200	0·167	0·143

DISCUSSION

The Tables show that, in the theoretical model, the maximum combined yield of oligosaccharides is much the greater when the oligosaccharides are resistant to further cleavage, and that the greatest improvement in yield occurs for the largest resistant oligosaccharide. It is noteworthy that, as the lengths of the carbohydrate side-chains in the parent glycoprotein increase, the improvement in the yield of the lower oligosaccharides diminishes, so that, for structural work, hydrolysis in which all neutral fragments are resistant would

probably cease to be advantageous when the side-chains are more than about 15 sugar residues in length.

In practice, the behaviour of a glycoprotein during acid-hydrolysis is likely to differ from that of the theoretical model in that the depolymerisation would not be strictly random. In some cases, the improvement in yield obtainable by use of a strong polymeric acid as catalyst may be greater than that predicted by these calculations. For example, if, in hydrolysis by mineral acid, the neutral oligosaccharides were hydrolysed faster than those attached to peptide material, or, in peptide-containing fragments, glycosidic linkages close to positively charged groups were hydrolysed less rapidly than those more remote from them, the yields of neutral oligosaccharides would be lower than those shown in Tables 1 and 2. On the other hand, in the hydrolysis of diethylaminoethyl ethers of starch by polystyrenesulphonic acid,[4] the yields of the larger neutral oligosaccharides are higher, and those of the smaller ones lower, than the yields calculated [8] on the assumption of random cleavage. Therefore, in polymeric-acid-catalysed hydrolysis, glycosidic linkages near a positively charged group may be hydrolysed faster than those more distant from it, in which case the combined yield of neutral oligosaccharides would be higher than that shown in Table 3.

It is not always convenient in practice to carry out hydrolysis in solutions so dilute that the rate of hydrolysis of neutral fragments relative to that of charged ones is negligibly small, but, since the polymeric acid is non-dialysable, protection of the neutral oligosaccharides from further scission can be made complete by dialysing them continuously from the acidic environment as they are liberated.[9,10] By application of this technique to a glycoprotein isolated from hog gastric mucin, a three- to four-fold improvement in the yield of oligosaccharides was obtained.[10]

The foregoing calculations take no account of the fact that positive charges can arise in the carbohydrate moiety of the glycoprotein, for example, by the *N*-deacetylation of hexosamine residues. However, the rate of *N*-deacetylation of hexosamine residues by acid is small compared with the rate of scission of the glycosidic linkages,[11] and in our experience with several glycoproteins [10,12] about 90—95% of the total carbohydrate moiety can be recovered as mono- and oligo-saccharides in which the *N*-acetyl groups are still intact. The effect of this deviation from the conditions assumed in the theoretical model may not, therefore, be large.

I am grateful to Professor W. T. J. Morgan, F.R.S., for his interest. The arithmetic was done, with a calculating machine, by Miss Patricia Henson.

THE LISTER INSTITUTE OF PREVENTIVE MEDICINE,
CHELSEA BRIDGE ROAD, LONDON S.W.1. [*Received, October 10th*, 1963.]

[8] Painter, following paper, and unpublished results.
[9] Painter, *Chem. and Ind.*, 1960, 1214.
[10] Painter and Morgan, *Nature*, 1961, **191**, 39.
[11] Moggridge and Neuberger, *J.*, 1938, 745; Foster, Horton, and Stacey, *J.*, 1957, 81; Johansen, Marshall, and Neuberger, *Biochem. J.*, 1960, **77**, 239.
[12] Painter, Rege, and Morgan, *Nature*, 1963, **199**, 569.