772. *An Attempt to Devise an Artificial Endo-polysaccharase System.*

By T. J. Painter.

When three different specimens (A, B, and C) of diethylaminoethyl ethers of starch, having degrees of substitution of 0·24, 0·14, and 0·074, respectively, were heated separately in water with a water-soluble polystyrenesulphonic acid, the rates of hydrolysis were initially much greater than in aqueous hydrochloric acid under the same conditions, but became very small when the reducing power of the reaction mixtures was still only 50—70% of that corresponding to complete hydrolysis. This "final" reducing power was highest for A, and lowest for C.

Large amounts of unsubstituted oligosaccharides were still present in the reaction mixtures at this stage, and their average molecular weight was highest for C, and lowest for A. The yields of these oligosaccharides were much higher than the maximum yields obtainable by mineral-acid hydrolysis of the same materials.

The selectivity of polystyrenesulphonic acid for the hydrolysis of the substituted starch could be increased by diluting the reaction mixture.

The results are explained in terms of the counterion-binding properties of polyelectrolytes. The behaviour of this system resembles that of certain natural enzyme–substrate mixtures.

Certain endo-enzymes convert polysaccharides into oligosaccharides in very high yields; for example, human salivary α-amylase can convert amylose into maltose and maltotriose with a yield exceeding 90%.[1] It appears that these enzymes hydrolyse larger fragments much faster than smaller ones, with the result that the lower oligosaccharides accumulate in the reaction mixture.[2]

Acidic catalysts, on the other hand, show no selectivity for large fragments, and indeed they often hydrolyse the smaller oligosaccharides faster than the larger ones. For example, cellobiose is hydrolysed about three times as fast, cellotriose about twice as fast, and cellotetraose about 1·5 times as fast as cellulose itself.[3] This clearly disfavours the accumulation of oligosaccharides in the reaction mixture, and it accounts for the low yields of oligosaccharides often obtained in the partial acid-hydrolysis of polysaccharides.*

The need for obtaining higher and more representative yields of oligosaccharides in the study of polysaccharide structure has aroused interest in enzymes having the properties noted above, but unfortunately few of them are known, and they attack only a limited number of polysaccharides. We have therefore attempted to devise an artificial enzyme system, which would simulate certain natural endo-polysaccharases in their ability to hydrolyse large fragments very much faster than small ones.

It is now well established [5–7] that, in aqueous solutions of polyelectrolytes, a large proportion of the counterions is closely bound to the surfaces of the polymeric ions. With a polymeric acid such as water-soluble polystyrenesulphonic acid [8] this phenomenon gives rise to a very high localised hydrogen-ion concentration in the vicinity of the polyanions, although

* Some polysaccharides are exceptional in having particularly acid-labile linkages at regular intervals along the chains. Agar is an example in which every second glycosidic linkage is very labile; partial methanolysis of agar affords agarobiose dimethyl acetal in 76% yield.[4]

[1] Whelan and Roberts, *J.*, 1953, 1298.
[2] Meyer and Gonon, *Helv. Chim. Acta*, 1951, **34**, 294.
[3] Freudenberg and Blomqvist, *Ber.*, 1935, **68**, 2070.
[4] Clingman, Nunn, and Stephen, *J.*, 1957, 197.
[5] Mock and Marshall, *J. Polymer. Sci.*, 1955, **13**, 263.
[6] Lapanje and Rice, *J. Amer. Chem. Soc.*, 1961, **83**, 496.
[7] Kotin and Nagasawa, *J. Amer. Chem. Soc.*, 1961, **83**, 1026.
[8] Painter, *Chem. and Ind.*, 1960, 1214.

the acidity of the solution as a whole may be quite low.[9] On potentiometric titration, polystyrenesulphonic acid behaves as a strong acid [10] and, as studies with Raman spectroscopy [6] and proton magnetic resonance [7] show, it is completely ionised in aqueous solution. Moreover measurements [5,6] of the apparent " dissociation constant " at different concentrations indicate that the degree of counterion binding is independent of concentration; hence at high dilutions, the difference between the average acidity of the solution and that in the neighbourhood of the polyanions may be very large.

Since it appears that the forces responsible for counterion fixation are purely electrostatic, it is reasonable to expect that any positively charged molecule would be attracted into the vicinity of the polyanions, where it would be exposed to the high localised acidity. Furthermore, if as a consequence of this, an electrically neutral fragment were split from the molecule by hydrolysis, it would be free to diffuse uniformly throughout the solution and would then be subject only to the average acidity of the system.

Earlier experiments [11] had already shown that basic carbohydrates were hydrolysed much faster by polystyrenesulphonic acid than by an equivalent concentration of mineral acid, and it seemed clear that, if it were possible to substitute a polysaccharide uniformly and partially with basic groups, its behaviour on hydrolysis by this acid would resemble that of starch on hydrolysis by α-amylase. Thus, if the degree of substitution were, for example, 0·1, any fragment containing ten or more monosaccharide residues would be rapidly hydrolysed, whereas neutral fragments split from the intermediate groups of contiguous unsubstituted sugar residues would subsequently undergo relatively slow hydrolysis.

TABLE 1.

Reduction (%).

Time (hr.)	A		B		C		Starch	
	PSS *	HCl	PSS *	HCl	PSS *	HCl	PSS *	HCl
½	7·3	0·26	5·8	0·34	4·9	0·41	0·37	0·45
1	12·4	0·38	11·3	0·45	9·4	0·62	0·54	0·66
2	22·1	0·85	20·2	0·80	15·0	1·09	0·88	1·22
3	32·4	1·11	26·9	1·21	19·8	1·51	1·32	1·81
4	40·2	1·30	34·3	1·59	25·0	2·18	1·75	2·47
5	47·0	1·75	39·0	1·93	29·6	2·75	2·23	3·22
7	56·3	2·20	45·8	2·99	37·7	4·23	3·30	4·90
10	65·4	3·50	52·2	5·04	44·4	7·75	6·10	8·70
13	69·6		56·8		48·5			
16	72·6		59·3		51·5			
19	74·7		61·4		52·8			
22	76·0		62·5		54·8			

* Polystyrenesulphonic acid.

Unfortunately, we could not devise a means of substituting a polysaccharide uniformly with basic groups, so we investigated the behaviour of randomly substituted polysaccharides. With these materials, there would be no maximum possible size for a neutral fragment, and the yield of the smaller oligosaccharides would be lower in consequence. However, the yield of oligosaccharides should be higher than that obtainable by mineral-acid hydrolysis of the same material, and it should be possible to control the average molecular weight of the hydrolysate by varying the degree of substitution. Starch was chosen as the polysaccharide, and diethylaminoethyl ether groups as the substituents.

Three different specimens (A, B, and C) of diethylaminoethyl ethers of starch, having degrees of substitution of 0·24, 0·14, and 0·074, respectively, were prepared by heating solutions of starch in aqueous sodium hydroxide with 2-chloroethyldiethylamine.[12] The products were isolated as their hydrochloride salts. Solutions (0·3% w/v) of these

[9] Kern, Herold, and Scherhag, *Makromol. Chem.*, 1955, **17**, 231.
[10] Signer, Demagistri, and Müller, *Makromol. Chem.*, 1956, **18—19**, 139.
[11] Painter and Morgan, *Chem. and Ind.*, 1961, 437.
[12] McKernan and Ricketts, *Chem. and Ind.*, 1959, 1490.

materials, and also of an unsubstituted starch for comparison, in (*a*) 0·02N-polystyrenesulphonic acid, or in (*b*) 0·02N-hydrochloric acid, were heated at 86·5°. In each instance, samples were withdrawn at intervals and neutralised. After appropriate dilution, the reducing power of each sample was measured with Nelson's reagents;[13] it is expressed in Table 1 as a percentage of that corresponding to complete hydrolysis.

For all three preparations of substituted starch, the initial rate of increase in reducing power, determined graphically, was about 40—50 times greater in the polystyrenesulphonic acid than in the hydrochloric acid. However, in the polymeric acid-catalysed hydrolyses, the rate decreased markedly as hydrolysis progressed, until after about 18 hours, it was very small indeed, in spite of the fact that the reducing power at this stage was still only 50—70% of that corresponding to total hydrolysis. In conformity with the theoretical principles considered above, this " final " reducing power was highest for A, lower for B, and lowest for C.

Chromatography of the samples showed that glucose and oligosaccharides were liberated much more rapidly in the polystyrenesulphonic acid-catalysed hydrolyses than in those catalysed by hydrochloric acid, thus qualitatively confirming the results of the Nelson estimations. (Control experiments had already shown that polystyrenesulphonic acid gives rise to no increase in reducing power when heated alone, and then tested under these conditions.) Furthermore, in the polymeric acid-catalysed hydrolyses, large amounts of oligosaccharides were still present after 22 hours, and they were also present even after the hydrolysis had been continued for a total of 36 hours. Control experiments indicated that this automatic decline in reaction rate was not due to any decrease in the acidity of the system, such as might arise from thermal decomposition of the polymeric acid or the absorption of basic substances from the laboratory atmosphere.

TABLE 2.

		Saccharides obtained (%)							
Sample	Acid	Mono-	Di-	Tri-	Tetra-	Penta-	Hexa-	Hepta-	Higher
A	PSS *	24·2	15·6	11·8	8·0	5·5	4·8	4·5	18·6
,,	HCl	28·0	12·4	6·8	3·3	1·0	0·8	0·4	1·7
B	PSS *	21·5	13·2	10·0	8·5	6·9	5·6	5·0	25·2
,,	HCl	17·4	11·5	7·7	5·2	3·4	1·8	1·1	5·5
C	PSS *	17·9	9·3	7·8	7·1	7·0	6·1	5·3	33·6
,,	HCl	16·9	8·5	6·7	6·0	3·8	3·3	1·8	12·6

* Polystyrenesulphonic acid.

To investigate the yields of oligosaccharides in the hydrolyses catalysed by the polymeric acid, samples of A, B, and C were hydrolysed under the same conditions for 18 hours, that is, until the initial rapid phase of the degradation was judged complete. The acid was then removed by precipitation with cetyltrimethylammonium bromide, and diethylaminoethyl ethers of glucose and halide ions with a mixed-bed ion-exchange resin. The solutions were concentrated to yield syrupy mixtures of unsubstituted glucose and glucose-containing oligosaccharides. The yields of the syrups corresponded to approximately 95% of the total unsubstituted glucose residues in the starting materials.

The number-average degree of polymerisation of each hydrolysate, determined iodometrically,[14] was 2·37 for A, 2·57 for B, and 3·05 for C. The components of each hydrolysate were then resolved on paper chromatograms; each chromatographically distinct component was eluted separately, and was estimated with phenol–sulphuric acid.[15] The yields (Table 2) are expressed as a percentage of the total original unsubstituted glucose residues.

[13] Nelson, *J. Biol. Chem.*, 1944, **153**, 375.
[14] Ingles and Israel, *J.*, 1948, 810.
[15] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.

To obtain an indication of the yields of oligosaccharides during the mineral-acid hydrolysis of the substituted starches, solutions (0·6% w/v) of A, B, or C in 0·5N-hydrochloric acid were heated separately at 86·5°. Samples of each were withdrawn at intervals, treated with mixed-bed ion-exchange resin to remove hydrochloric acid and all basic carbohydrate material, and concentrated to yield syrupy mixtures of unsubstituted mono- and oligo-saccharides. On chromatography of the samples, the combined yield of di- to hepta-saccharides inclusive appeared to pass through a maximum after 95 minutes for A, 75 minutes for B, and 61 minutes for C. The neutral sugars in the reaction mixtures after these periods were determined by chromatographic separation, followed by estimation with phenol–sulphuric acid as before. The results, expressed as a percentage of total unsubstituted glucose residues in the starting materials, are in Table 2. Since the times corresponding to a maximum combined yield of di- to hepta-saccharides were determined merely by visual examination of the treated chromatograms, the validity of these estimates was checked by determining the components of samples taken before and after the stated times.

Table 2 shows that the combined yields of di- to hepta-saccharides are 50—100% higher in the polystyrenesulphonic acid-catalysed hydrolyses than in those catalysed by hydrochloric acid. The large yields of the higher saccharides in the polymeric acid-catalysed hydrolyses are also noteworthy in indicating that the distribution of diethylaminoethyl ether groups along the polysaccharide chains must have been highly random; if the molecules had been uniformly substituted, it would have been impossible to isolate any fragment larger than a trisaccharide from A, a hexasaccharide from B, or a dodecasaccharide from C.

It is interesting to consider the effect that uniform substitution should have upon the yields of the lower oligosaccharides in the polymeric acid–substituted starch system. For completely random substitution, the distribution of the various blocks of contiguous unsubstituted sugar residues can be calculated statistically (cf. Kuhn [16]). If the polysaccharide is unbranched, and the degree of substitution is s, the probability that a particular monomer unit is substituted is s, and the probability that it is not substituted is $(1 - s)$. It follows that the probability of a particular group of n consecutive unsubstituted monomer units existing between two substituted units is $s^2(1 - s)^n$, and if the degree of polymerisation of the polysaccharide is N, the weight-fraction of this group is $ns^2(1 - s)^n/N$. Since there are $(N - n - 1)$ different ways of selecting such a group from a chain N units long, the total weight-fraction of all such groups will be $n(N - n - 1)s^2(1 - s)^n/N$, and, if N is very large, this expression simplifies to $ns^2(1 - s)^n$. The corresponding fraction, based upon the weight of unsubstituted monomer units, will then be $ns^2(1 - s)^{n-1}$. In Table 3, the last expression has been used to calculate the percentages of the total unsubstituted carbohydrate in A, B, and C existing as discrete groups of 1—7 anhydroglucose units. (Since the amylopectin component of starch is branched, and the above reasoning assumes that a monomer unit may not carry

TABLE 3.

		Weight-fraction (%)							
Sample	s	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n > 7$
A	0·24	5·76	8·76	9·98	10·11	9·61	8·76	7·77	39·25
B	0·14	1·96	3·37	4·35	4·99	5·36	5·53	5·55	68·89
C	0·074	0·55	1·01	1·41	1·74	2·01	2·24	2·42	88·62

more than one substituent, the results are not strictly valid. However, the effect of these deviations is unlikely to be large, and the conclusions drawn should be qualitatively correct.)

Table 3 shows that a very large part of the unsubstituted carbohydrate in A, B, and C

[16] Kuhn, *Ber.*, 1930, **63**, 1503.

is capable of yielding oligosaccharides containing more than seven glucose residues, which accounts satisfactorily for the high yields of such fragments obtained when A, B, and C were hydrolysed by polystyrenesulphonic acid. It is also noteworthy that a small part could not yield any fragment other than glucose. It therefore seems reasonable to expect that the effect of uniform substitution would be to increase very substantially the yields of the lower oligosaccharides.

The factors affecting the selectivity of polystyrenesulphonic acid for the hydrolysis of basic fragments were next considered, because, if the selectivity could be increased, so also would the yields of neutral oligosaccharides. It was evident that the degree of counterion binding would be important in determining selectivity, and temperature and the concentrations of the reactants were also likely to affect it. In this paper, we consider only the effects of varying the concentrations of the polymeric acid and substituted starch.

The degree of counterion binding in our preparation of polystyrenesulphonic acid [8] remained constant at about 60%, irrespective of the concentration.[17] At the high dilutions used in these experiments, intermolecular electrostatic repulsion should be slight, so that the molecular volume of the polyanions should not change appreciably with concentration. Thus, the hydrogen-ion concentration in the vicinity of the polyanions should be independent of the concentration of polystyrenesulphonic acid, while that in the bulk of the solution should be directly proportional to it. Therefore it should be possible to increase the selectivity by decreasing the concentration of polymeric acid.

Solutions (0·3% w/v) of substituted starch (preparation B) in polystyrenesulphonic acid at concentrations of 0·05N, 0·02N, or 0·01N, and also in hydrochloric acid at the same concentrations, were heated separately at 86·5°. The increase in reducing power of each solution was followed as before, and expressed as a percentage of the reducing power corresponding to complete hydrolysis (Table 4).

TABLE 4.

Normality of acid	Acid	Reduction (% of total) after times: ½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	7 hr.	10 hr.	Initial rate ($hr.^{-1}$)	Ratio of initial rates, PSS/HCl
0·05	PSS	6·8	11·8	18·4	25·6	30·3	36·4	43·9	48·2	11·5	10
,,	HCl	0·50	1·09	2·31	3·71	5·36	6·93	9·66	16·1	1·11	
0·02	PSS	5·8	11·3	20·2	26·9	34·3	39·0	45·8	52·2	12·4	40
,,	HCl	0·34	0·45	0·80	1·21	1·59	1·93	2·99	5·04	0·30	
0·01	PSS	5·8	10·4	17·6	23·8	30·3	33·5	39·8	45·8	11·2	140
,,	HCl	0·10	0·17	0·31	0·47	0·62	0·75	1·17	2·30	0·08	

In all the foregoing experiments, a large excess of acid was used. The equivalent weights of A, B, and C were 816, 1286, and 2323, respectively, from which is follows that, in the experiments summarised by Table 1, the molar excess of acid was about 5-fold for A, 9-fold for B, and 15-fold for C. It is to be expected that, as the substrate concentration is increased, increasing amounts of the hydrogen ions associated with the polyanions will be displaced, thereby diminishing the acidity in the neighbourhood of the polyanions. The acidity in the bulk of the solution will be correspondingly increased, with a resulting decline in selectivity. However, the results of experiments designed to confirm this were inconclusive; with low concentrations of substrate, significant differences in selectivity could not be detected, whereas with high concentrations of substituted starch, the solutions were too viscous for accurate measurement of volumes. It is hoped to investigate this problem by using a simpler substrate, such as a diethylaminoethyl ether of methyl α-D-glucopyranoside.

Table 4 shows that, under the conditions used, the rate of hydrolysis of material B by polystyrenesulphonic acid is of zero-order with respect to acid, whereas with hydrochloric

[17] Painter, unpublished work.

acid as the catalyst the rate is very sensitive to changes in acid concentration.* Therefore, by working with an excess of polymeric acid at high dilutions, extremely high selectivities are theoretically obtainable. For example, when a solution (0·02% w/v) of material B in 0·002N-polystyrenesulphonic acid was heated at 95°, rapid hydrolysis occurred although the pH of the reaction mixture was about 3·5; with hydrochloric acid under the same conditions, the increase in reducing power could scarcely be detected, and it was estimated that the ratio of the initial rates must have been at least 500 : 1, and possibly as high a 1000 : 1.

Owing to the use of randomly substituted substrates, the polymeric acid–substituted starch system described here does not simulate enzymes such as α-amylase in their ability to hydrolyse large fragments faster than small ones. Nevertheless, the work has implications of immediate practical importance.

It has been shown that it is possible to set up a simple, self-controlling system for the partial hydrolysis of a polysaccharide, which will give a relatively high yield of oligosaccharides of a size suitable for structural study. If the hydrolysis is carried out in a sufficiently dilute solution, the reaction will, for practical purposes, stop automatically at a point which is precisely pre-determined by the degree of substitution of the substrate, yielding a hydrolysate whose average molecular weight is also pre-determined in the same way. The overall yield of oligosaccharides in a given range of molecular weights will not necessarily be higher than that obtainable by standard methods for partial hydrolysis, since the yield will depend upon the way in which the diethylaminoethyl ether groups distribute themselves in the molecule, and this in turn will depend upon the structure of the polysaccharide. However, since the kinetics of the degradation are different from those which apply in hydrolysis by mineral acid, the relative yields of the different possible oligosaccharides should also be very different. Moreover, since every neutral fragment is protected almost instantaneously and completely from further degradation from the moment it is liberated, acid-labile fragments liberated in the early stages of hydrolysis, which would have only a transient existence in a medium of uniformly distributed acidity, would be preserved and could perhaps be recovered intact at the end of the reaction. Since very dilute solutions of extremely low average acidity are used, it could also be expected that the formation of artifacts by the acid-reversion of neutral sugars would be greatly diminished.

The system therefore constitutes a useful alternative method for the partial hydrolysis of polysaccharides, which, used in conjunction with conventional methods, should, it is hoped, provide additional information in many instances.

The work also has interesting implications in the field of mucopolysaccharide chemistry, with which we are particularly concerned.[18,19] When a polysaccharide containing residues of *N*-acetylhexosamine (or hexosamine *N*-sulphate) is subjected to partial acid-hydrolysis, scission of the glycosidic linkages is accompanied by *N*-deacetylation (or *N*-desulphation) of the hexosamine residues.[20] This results in the appearance of basic amino-groups in the molecules, probably in a random manner. Also of course, if the material is a glycopeptide, basic groups may pre-exist in the peptide moiety, or may appear by the scission of peptide or amide linkages. It has already been shown [11] that methyl 2-amino-2-deoxy-β-D-glucopyranoside is hydrolysed much more rapidly by polystyrenesulphonic

* It is not possible to determine the order of this reaction from the figures in Table 4, because the Nelson reagents do not react stoicheiometrically with all reducing sugars. The molar extinction is generally lower for large oligosaccharides than for smaller ones, with the result that differences in rate of hydrolysis are exaggerated. For the same reason, the figures in Tables 1 and 4 do not represent percentages of glycosidic linkages cleaved; they are merely a convenient arbitrary measure of the extent of hydrolysis.

[18] Morgan, *Proc. Roy. Soc.*, 1960, *B*, **151**, 308; Côté and Morgan, *Nature*, 1956, **178**, 1171.
[19] Painter and Morgan, *Nature*, 1961, **191**, 39.
[20] Moggridge and Neuberger, *J.*, 1938, 745; Foster, Horton, and Stacey, *J.*, 1957, 81; Johansen, Marshall, and Neuberger, *Biochem. J.*, 1960, **77**, 239.

acid than by hydrochloric acid under the same conditions, whereas the *N*-acetyl derivative of this compound is hydrolysed at the same rate in both acids. Consequently, our preparations A, B, and C of substituted starch can be considered as representing a mucopolysaccharide at various stages of its hydrolysis, that is, in various states of *N*-deacetylation (or *N*-desulphation). The greater yields of neutral oligosaccharides when A, B, or C was hydrolysed by polystyrenesulphonic acid imply that, if a mucopolysaccharide is hydrolysed by this acid, the maximum yield of oligosaccharides containing neutral sugar residues and/or intact *N*-substituted hexosamine residues will be greater than that obtainable with mineral acid as catalyst. This prediction has been found correct in our studies of the blood-group specific mucopolysaccharides.[11,19]

The possibility of devising artificial enzyme systems for the selective degradation of polysaccharides is attractive, and the simple system described here could be improved in many ways. From the point of view of obtaining higher yields of oligosaccharides, the expedient of first introducing basic groups into the substrate is not desirable, since not only does it decrease the total amount of carbohydrate recoverable as neutral oligosaccharides, but also it diminishes the number of ways in which a particular fragment may be split from a long chain, resulting in a lower overall yield of the fragment.[16,17] To overcome this drawback, the synthetic catalyst should contain two types of functional group, namely, one to provide the high localised acidity, and a different type to " bind " the substrate molecule to the " enzyme." If prior introduction of substituents into the substrate is to be avoided, the binding mechanism would have to utilise the hydroxyl groups of the polysaccharide. This might be accomplished by hydrogen bonding,[21] by chelation through a metallic ion, or by ester formation.

Previous examples of the use of acidic colloids or resins as selective catalysts in organic chemistry include the hydrolysis of proteins by anionic detergents [22] or by polyethylenesulphonic acid,[9] the use of Dowex-50 cation-exchange resin as an aminopeptidase,[23] the stepwise degradation of oligosaccharide phenylosazones by Amberlite IR-120 resin,[24] and the selective hydrolysis of an unsubstituted amide group by Amberlite IR-120 resin.[25]

Experimental

General Methods.—Paper chromatography was carried out by the descending method, on Whatman No. 1 filter paper. The solvent was ethyl acetate–pyridine–water (10 : 4 : 3 v/v). Sugars were located on the chromatograms by spraying with silver nitrate in acetone, followed by ethanolic sodium hydroxide.[26] For the quantitative estimation of mixtures of mono- and oligo-saccharides, a solution of the mixture (20 mg.) in water (1 ml.) was streaked across the top of a sheet (46 × 57 cm.) of filter paper and allowed to dry. The chromatogram was irrigated for 48 hr. and then dried. The components were located by spraying guide-strips cut from each side, and from the centre of the chromatogram. The bands of the individual components were excised, cut into small pieces, and shaken for 1 hr. with water (10 ml.). The mixtures were filtered, and the filtrates analysed by the phenol–sulphuric acid method.[15] To correct for carbohydrate material extracted from the paper, a " blank " chromatogram was irrigated under identical conditions; the guide-strips from the " test " chromatogram were used to mark the positions of the original bands, and the " blank " bands were excised, extracted, and analysed as before. A calibration curve was used to convert the corrected results into concentrations of anhydroglucose ($C_6H_{10}O_5$) units, from which the percentage composition of the mixture was calculated.

Colorimetric analysis was carried out with an EEL single-cell colorimeter and Ilford filters. The probable error of the results in Tables 1 and 4 is $\pm 2\%$, and that of the figures in Table 2 is $\pm 5\%$.

Unless otherwise stated, solvents were evaporated at 30° under diminished pressure in a

[21] Foster, *Ann. Rev. Biochem.*, 1961, **30**, 57.
[22] Steinhardt and Fugitt, *J. Res. Nat. Bur. Stand.*, 1942, **29**, 315.
[23] Whitaker and Deatherage, *J. Amer. Chem. Soc.*, 1955, **77**, 5298.
[24] Finan and O'Colla, *Chem. and Ind.*, 1955, 1387.
[25] Collins, *Chem. and Ind.*, 1957, 736.
[26] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.

rotary evaporator. The water-soluble polystyrenesulphonic acid was prepared as described previously,[8] from polystyrene having a number-average molecular weight (determined by osmometry) of 50,000, and a weight-average molecular weight (determined by light-scattering) of 300,000.

The degree of substitution in preparations of diethylaminoethyl ethers of starch was determined by the volumetric estimation of chloride ions, according to the following modification of Volhard's method.[27] A brief pre-hydrolysis of the material with nitric acid was necessary, as the substituted starch inhibited coagulation of silver chloride. A solution of the sample (5 ml.; *ca.* 12 mg./ml.) was heated with 3N-nitric acid (5 ml.) at 100° for 30 min., and then cooled. 0·01M-Silver nitrate (10 ml.) and then nitrobenzene (1 ml.) were added, and the mixture was set aside with periodic shaking for 10 min., then titrated with 0·01M-potassium thiocyanate to a faint pink end-point (ferric nitrate as an indicator).

For kinetic experiments (Tables 1 and 4), a well-insulated water-bath fitted with a "Techne" thermostatic heating unit was used. The surface of the water was covered with oil to minimise evaporation and heat losses. A temperature of 86·5° ± 0·2° was used throughout. The hydrolyses were carried out in "Quickfit" micro-apparatus, consisting of a 25-ml. 3-necked flask, fitted with an air-turbine stirrer, reflux condenser, and thermometer.

Solutions were de-ionised with "Biodeminrolit," a mixed-bed resin consisting of strongly acidic and basic components. Before use, the basic component was converted into the carbonate form by stirring the resin in water saturated with carbon dioxide. This prevented any significant absorption of neutral sugars.[28]

Preparation of Substituted Starches.—Sample A. A solution of starch (12 g.) in aqueous 10% w/v sodium hydroxide (80 ml.) was stirred at 85° with 2-chloroethyldiethylamine hydrochloride (7·0 g.) for 30 min. The mixture was then cooled rapidly, neutralised with N-hydrochloric acid (*ca.* 90 ml.), and diluted to 300 ml. with water. This solution was dialysed three times against distilled water (5 l.) for 24 hr., and then made up to 1 l. Evaporation of a measured sample, followed by weighing of the residue after drying over phosphorus pentoxide, indicated that the solution contained 11·5 mg./ml. of product.

Sample B. Starch (12 g.) in aqueous 5% w/v sodium hydroxide (80 ml.) was heated at 85° with 2-chloroethyldiethylamine hydrochloride (3·5 g.) for 30 min. The product was isolated in the same way as sample A, and the final solution contained 11·6 mg./ml. of material.

Sample C. To a solution of starch (12 g.) in N-sodium hydroxide (100 ml.), 2-chloroethyldiethylamine hydrochloride (2 g.) in water (10 ml.) was added, and the mixture was stirred at 85° for 20 min. The final solution of product, prepared as for sample A, contained 11·3 mg./ml. of substituted starch.

To Follow the Hydrolysis of the Substituted Starches by the Measurement of Reducing Power.—The stock solutions of A, B, or C, prepared as described above, were diluted with an equal volume of water and heated at 86·5° ± 0·2°. Portions (10 ml.) of these solutions, or of starch (0·6% w/v), were added with vigorous stirring to 0·04N-polystyrenesulphonic acid (10 ml.) or to 0·04N-hydrochloric acid (10 ml.), which was contained in the reaction vessel and had previously been heated to the same temperature. At intervals, samples (2 ml.) were withdrawn, cooled, and neutralised with aqueous pyridine (16·2 ml./l.; 0·3 ml.). Portions (1 ml.) of the neutralised samples were withdrawn and diluted with water (2 ml. when hydrochloric acid was the catalyst; 8 ml. when polystyrenesulphonic acid was used). The diluted samples were then used for the measurement of reducing power by Nelson's method.[13] The optical density was read at 520 mμ (Ilford filter No. 624), against controls prepared from neutralised acid and unhydrolysed substrate at the appropriate concentrations. In the control samples, and also in those from the early stages of the hydrochloric acid-catalysed hydrolyses, unhydrolysed substrate formed a white precipitate on addition of the Nelson arsenomolybdate reagent; these precipitates were removed by centrifugation before the colour was measured. Control experiments showed that no colour was lost in this way. Control experiments were also carried out to correct the results for any changes in the absorbance of the polystyrenesulphonic acid which occurred upon heating; such changes were, however, very slight under these conditions. (Very prolonged heating led to a decrease in the absorbance of the acid at 520 mμ.)

The reducing powers corresponding to complete hydrolysis were determined as follows. Portions of the stock solutions of A, B, or C were heated with an equal volume of N-hydrochloric

[27] Vogel, "Quantitative Inorganic Analysis," Longmans, Green and Co., 2nd edn., 1951, p. 258.
[28] Roseman, Abeles, and Dorfman, *Arch. Biochem. Biophys.*, 1952, **36**, 232.

acid at 100° for 15 hr. Samples (2 ml.) were withdrawn, neutralised with N-sodium hydroxide (1 ml.), and diluted with water (25 ml.) before measurement of the reducing power with Nelson's reagents.

The Nelson reagents were calibrated against glucose in the presence of the neutralised acids at the appropriate concentrations. Beer's law was observed to be strictly obeyed, and the neutralised acids did not affect the amount of colour developed. It was therefore unnecessary to convert the results of the kinetic experiments into equivalent concentrations of glucose before calculating them as a percentage of the reducing power corresponding to complete hydrolysis.

The experiments in which the normalities of the acidic catalysts were varied were carried out in an identical manner, except that the amount of aqueous pyridine used for neutralisation was varied proportionately with the strength of acid used.

To Follow the Hydrolysis of the Substituted Starches Chromatographically.—Chromatograms were prepared from the samples remaining after the withdrawal of 1-ml. portions for the Nelson estimations. For hydrolyses catalysed by polystyrenesulphonic acid, the samples (1·3 ml.) were evaporated in a vacuum-desiccator over phosphorus pentoxide. The residues were redissolved in water (0·2 ml.), and 0·05-ml. portions of these solutions were spotted on the chromatograms. Since polystyrenesulphonic acid is insoluble in most organic solvents, it remained at the starting line of the chromatogram and did not interfere with the separation of the sugars.

For hydrolyses catalysed by hydrochloric acid, the samples (1·3 ml.) were shaken with " Biodeminrolit " resin (1 ml.), and filtered. The filtrates and washings were evaporated and spotted on chromatograms as described above.

Estimation of Yields of Oligosaccharides in Polystyrenesulphonic Acid-catalysed Hydrolyses.—Samples (2·5 ml.) of the stock solutions of A, B, or C were heated at 86·5° ± 0·2° with water (2·5 ml.) and 0·04N-polystyrenesulphonic acid (5 ml.) for 18 hr. The solutions were then cooled, neutralised with aqueous pyridine (16·2 ml./l.; 1·5 ml.), and concentrated to 5 ml. Aqueous 10% w/v cetyltrimethylammonium bromide (*ca.* 1 ml.) was then added dropwise, with shaking, to each concentrate, and the mixtures were centrifuged. The centrifugates were collected, and the residues were washed three times by decantation with water (5 ml.). Each centrifugate was combined with the appropriate washings and diluted to 50 ml. with water. The resultant solutions were shaken with chloroform (5 × 50 ml.) to remove the excess of cetyltrimethylammonium bromide, treated with " Biodeminrolit " resin (5 ml.), and filtered. The filtrates and washings were evaporated to dryness, and the residues were weighed after drying over phosphorus pentoxide. The yield of syrup from A was 17·0 mg., from B 21·4 mg., and from C 23·2 mg. These products were analysed as described above.

Estimation of Yields of Oligosaccharides in Hydrochloric Acid-catalysed Hydrolyses.—Portions (10 ml.) of the stock solutions of A, B, or C were heated at 86·5° ± 0·2° with N-hydrochloric acid (10 ml.). Samples (2 ml.) were withdrawn after 10, 20, 30, 45, 61, 75, 95, 125, and 180 min., respectively, and were run immediately into wet, ice-cold " Biodeminrolit " resin (17 ml.). The mixtures were filtered, and the filtrates and washings were evaporated to dryness. The residues were spotted on chromatograms, which were developed for 48 hr. Visual examination of the treated chromatograms suggested that the maximum combined yields of di- to hepta-saccharides inclusive occurred after times nearest to 95 min. for A, 75 min. for B, and 61 min. for C.

The hydrolyses were then repeated in the same way, except that only three 5-ml. samples were taken in each case, and neutralised with 42 ml. of resin. These samples were taken after 75, 95, and 125 min., respectively, for A; after 61, 75, and 95 min., respectively, for B; and after 45, 61, and 75 min., respectively, for C. All nine samples, upon evaporation, yielded residues representing only 40—60% of the total unsubstituted glucose residues in the starting materials. Control experiments showed that no appreciable absorption of neutral sugars by the resin occurred under these conditions. The components of each residue were estimated as described above. The results confirmed the finding that the maximum combined yields of neutral di- to hepta-saccharides occurred after times close to 95 min. for A, 75 min. for B, and 61 min. for C, and the figures from the appropriate samples are those in Table 2.

The author is indebted to Professor W. T. J. Morgan, F.R.S., and Dr. W. J. Whelan for their interest. Most of the analytical work was carried out by Mr. P. M. Gaylarde. The work was supported by a grant from the Medical Research Council.

THE LISTER INSTITUTE OF PREVENTIVE MEDICINE,
CHELSEA BRIDGE ROAD, LONDON, S.W.1.

[*Received, February 15th,* 1962.]