Thus, if an acyl-thiol were absorbed onto a protein so that the steric relationship of the ester-bond to an imidazolyl group of histidine (or other appropriate nucleophile) was analogous to that in IV then the protein *would* undergo acylation at an "enzymic" rate. The value of V_m for the hydrolytic reaction and its pH dependence would depend on the rate of the intercomplex nucleophilic displacement, the rates of acid and base catalysis of acyl-protein hydrolysis and the pK_{app} of the nucleophile involved. If the process were no more efficient than the model studied here the rate of the acylation step would still be 10^6 greater than for the hydrolysis of a thiol-ester in water at neutrality. Furthermore, if an imidazolyl group were the nucleophile then the protein involved would be a specific acyl-thiol hydrolase because neither the methyl ester nor the amide of III undergo hydrolysis at room temperatures.[1]

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[CONTRIBUTION FROM THE LABORATORY OF CHEMICAL PHARMACOLOGY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Synthetic Polysaccharides. IV. Preparation of Carboxyl Derivatives of Polyglucose

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Synthetic polyglucose has been oxidized with sodium periodate to the dialdehyde derivative of polyglucose which, by further oxidation with different amounts of chlorous acid, has been converted to a series of derivatives containing different amounts of carboxyl groups.

The preparation of polyglucose sulfates was reported in the third paper of this series.[1] These highly branched polyanions were useful models to study macromolecular interactions with basic or amphoteric proteins.[2] For example, polyglucose sulfate preparations of different molecular weight and different degree of substitution had different enzyme inhibitory potency.[3]

Derivatives having known amounts of weaker dissociating anionic groups, such as carboxyl groups, but retaining the high molecular weight and the unique highly-branched structure of the polyglucose[4] have been sought for further studies. These carboxyl derivatives have the advantage in certain biological applications of being less toxic than the sulfates, which are strong anticoagulants.[1,5]

This paper reports the preparation of a series of non-dialyzable polyglucose carboxyl derivatives which have different carboxyl content. The derivatives were prepared by oxidation of polyglucose first with periodate ion to an aldehyde derivative, and then with different amounts of chlorous acid further converting a percentage of the aldehyde groups to carboxyl groups.[6]

The polyglucose was prepared by one of the previously reported polycondensation methods.[7] It was not fractionated, it had a highly branched structure[4,8] and an intrinsic viscosity of 0.06; a number average molecular weight of 13,000 was indicated by the reducing end group method.[7] The polyglucose, however, contained only 15% of a lower molecular weight fraction which dialyzed through cellophane in forty hours. This latter experiment showed that about 85% of the polymer had substantially higher molecular weight than 13,000. In fact, indication from previous measurements[4] was that the majority of such a polymer had a higher molecular weight than 30,000. The preparation was therefore highly polydisperse.

To choose the length of oxidation a preliminary periodate oxidation was carried out under previously employed experimental conditions[8] (0.03 M periodate, 2°, in the dark). The amounts of periodate consumed and of formic acid liberated were measured at intervals. These values were generally similar to those obtained from previous determinations on similar polyglucoses.[8] Most of the oxidation took place rapidly within the first 24 hours, and although after this time the amounts of periodate consumed and formic acid released still increased, the rate of increase was much lower and approximately linear. An oxidation period of 48 hours was selected for the preparative work, and the periodate concentration was increased to 0.188 M in order to reduce the volume.

Again the oxidation was followed by measuring the periodate consumed and the formic acid released (Fig. 1). After 48 hours of oxidation, 30% non-dialyzable product was obtained. It contained 63% dialdehyde and 24% unoxidized glucose. Apparently over-oxidation at a few sites of the polymer chain caused random scission which increased considerably the amount of dialyzable product.

Our figure of 63% for the dialdehyde content of the non-dialyzable fraction of periodate oxidized polyglucose has to be taken with some reservation. In the determination we heated a sample of the aldehyde with p-nitrophenylhydrazine and measured the intensity of the color of the insoluble p-

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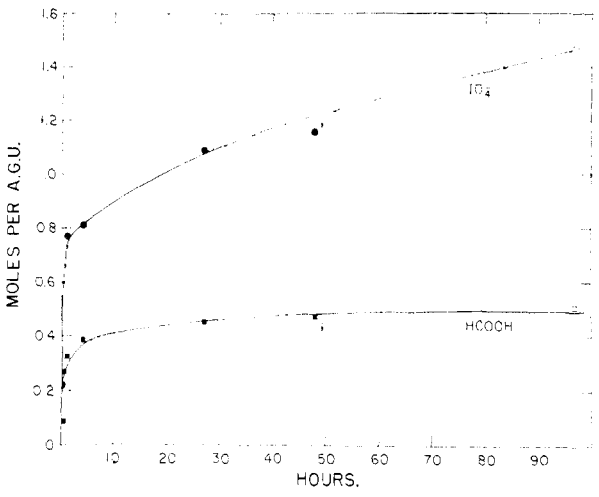

Fig. 1.—Periodate oxidation of polyglucose: moles of periodate per anhydro glucose unit (A.G.U.), preliminary determination, O; preparative run, ●; moles of formic acid per A.G.U., preliminary determination, □, preparative run, ■. The arrow shows the time at which the preparative run was stopped.

nitro-osazone. This method had been used to determine the dialdehyde content of periodate-oxidized starches.[9] The alkaline degradation of periodate oxidized polysaccharides favors, however, the production of glyoxal as an intermediary product,[10] which with the *p*-nitrophenylhydrazine forms an insoluble osazone. In our experiment on polyglucose aldehyde we indeed separated the *p*-nitro-osazone of glyoxal. Only sugars having an unsubstituted hydroxyl on the second carbon atom produce glyoxal when degraded by alkali.[10] Branches through the second carbon atom are not present in starch, but occur in polyglucose.[11] Since we compared the color produced by the polyglucose aldehyde with that produced by a sample of starch aldehyde with known aldehyde content,[12] the actual amount of oxidation in the non-dialyzable polyglucose fraction might be higher than 63%.

To convert different amounts of aldehyde residues to carboxyl, six equal samples of the non-dialyzable dialdehyde derivative were oxidized with different amounts of sodium chlorite. From similar work on starches[6] 20 to 100% conversion of the aldehyde to carboxyl was expected. After oxidation the samples were dialyzed and freeze-dried; losses upon dialysis ranged from about 20 to 50%. Since the solutions were not neutralized before freeze-drying and some of the carboxyl groups were present in the free acid form, the samples were redissolved in water, and after adjustment to *p*H 8.4 were dialyzed again for 24 hours. The losses which now occurred were high (up to 80%), undoubtedly due to alkaline degradation to which aldehyde derivatives of polysaccharides are known to be very susceptible.[10] The alkaline scission appears to be a rapid process, which reaches completion almost immediately, since a third dialysis of a sample from a solution of *p*H 8.4 resulted only in 13% additional loss.

Physical constants of the twice-dialyzed sodium carboxyl derivatives are given in Table I.

TABLE I

SODIUM CARBOXYL DERIVATIVES OF POLYGLUCOSE

Prepn.	Recovery (after two dialyses), %	$[\alpha]^{25}D \pm 1$ in H_2O	Carboxyl content,[a] %	Oxidation,[b] %	$[\eta]$ in 2 *M* NaCl, dl./g.
a	17	+37.9°	2.9	8.4	0.05
b	40	+41.7	5.4	15.6	.04
c	31	+35.0	10.5	30.4	.04
d	18	+36.1	16.9	48.9	.06
e	15	+33.6	19.0	54.9	.06
f	26	+27.6	28.0	80.9	.07

[a] Percentage of fragments having mol. wt. 45 (COOH) in 100 g. of material. The calculation is based on the percentage of sodium found by ashing. [b] The calculation was based on the assumption that the molecular weight of each carboxyl unit is 130; this figure was arrived at by taking into account the loss of molecular fragments measured as formic acid and formaldehyde, and a dialdehyde content of 63%. In this 130 molecular weight unit, 45 represents one carboxyl group and complete conversion would lead to 34.6% carboxyl. The actual carboxyl contents were divided by 34.6 to give the percentage oxidation.

The carboxyl content varied from about 3 to 28% in the six preparations, representing from 8 to 80% conversion of the aldehyde to carboxyl groups. Since the carboxyl groups were introduced in the polymer at random these polyglucose carboxyl preparations can be taken as a homologous series with difference in charge density. The intrinsic viscosities of these non-dialyzable carboxyl derivatives, measured in 2 *M* NaCl, were similar to those of the starting polyglucose, indicating that the molecular weights are approximately the same as those of the starting material and that therefore no extensive breakdown occurred in the non-dialyzable portion of the end-products, retaining the highly branched structure.

We cannot speculate on the mechanism of oxidation of a polysaccharide with such a complex structure as polyglucose.[8] It is interesting to note that about 10% of the glucose units in the polyglucose gave rise to formaldehyde during periodate oxidation. Formaldehyde can be produced only from hexose in the furanose form, in which neither the 5th nor the 6th hydroxyl participate in linkage. No naturally occurring polysaccharide is known to exist containing glucose residues in the furanose form.

These carboxyl derivatives of polyglucose were used to obtain various biological effects. For example, low concentrations of the polyglucose carboxyl derivatives inhibited lysozyme; this inhibition was reversed with protamine or with salts.[3] Similarly, they inhibited the depolymerizing activity of ribonuclease but did not affect the activity of the enzyme on uridine-2′:3′-(cyclic) phosphate.[3] Preliminary experiments[13] indicated

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that the polyglucose derivatives block the attachment of the bacteriophage to T-2 *E. coli*. The derivatives have also been used for *in vivo* detoxification of basic macromolecules, for example, polymyxin-B, and it was shown that the effectivity in detoxification increased with the charge density of the polyanionic derivatives.[14] The activity of ACTH *in vivo* was increased by carboxylpolyglucose.[15] Details of the biological experiments, and of the effect of differences in charge density upon them, will be reported separately.

Experimental

The polyglucose was prepared by the "two-stage powder" polycondensation method; the first stage of the preparation was reported in expt. 8, Table I, in ref. 7; the conditions of the second stage were similar to those of expt. 6 and 7, with the exception that the average temperature was 160° and the time 23 hours. The polymer was precipitated[8] by adding to an aqueous solution (16.5% vol./wt.) absolute ethanol to an alcohol concentration of 90%.[7] The yield of polyglucose was 96.4%. Number average molecular weight by the reducing end-group method[7] was $\bar{M}_n \approx 13{,}000$, $[\eta] \approx 0.06$.

A preliminary oxidation of the polyglucose with 0.030 *M* periodate in the dark at 2° (method presented in ref. 8) gave the following results: moles of IO_4^- consumed per $C_6H_{10}O_5$ anhydro glucose unit (A.G.U.): 28 hours, 1.10; 52 hours, 1.23; 96 hours, 1.47. Mole of HCO_2H released per $C_6H_{10}O_5$ unit: 25 hours, 0.455; 49 hours, 0.478; 96 hours, 0.506. Mole of HCHO released per $C_6H_{10}O_5$ unit[16]: 25 hours, 0.058; 72 hours, 0.096; 96 hours, 0.100.

Preparative Periodate Oxidation of Polyglucose.—One hundred and sixty grams of polyglucose was oxidized with eight liters of 0.188 *M* $NaIO_4$ at 2° in the dark. The periodate consumed and the formic acid released were measured; and the results are reported in Fig. 1. Moles of IO_4^- consumed per $C_6H_{10}O_5$ unit: 0.25 hour, 0.21; 0.75 hour, 0.67; 1 hour, 0.77; 4 hours, 0.81; 27 hours, 1.09; 48 hours, 1.16. Mole of HCO_2H released per $C_6H_{10}O_5$ unit: 0.25 hour, 0.082; 0.75 hour, 0.261; 1 hour, 0.321; 4 hours, 0.383; 27 hours, 0.450; 48 hours, 0.478. The amount of formic acid liberated corresponded approximately to one mole per two glucose residues. The oxidation was stopped by addition to the reaction mixture of 100 ml. of ethylene glycol. The solution was dialyzed in a cellophane bag against running distilled water for 48 hours and "freeze-dried." Recovery of the periodate-oxidized polyglucose aldehyde was 53 g. or 30% (dry weight).

Loss on Dialysis.—Due to the large loss incurred on dialysis the following experiments were carried out to demonstrate that degradation during oxidation is the cause of this loss: the original unoxidized polyglucose lost 15% of its weight during 40-hr. dialysis when the initial *p*H of the solution was 4.8 and 16% when the initial *p*H was 8.6. Two aliquots of the dialyzed polyglucose aldehyde, one dissolved in distilled water (*p*H 5.8) and the other in dilute sodium hydroxide (*p*H 8), were dialyzed a second time in cellophane bags against running distilled water for 40 hours. The recovery of material in both experiments was 90%.

Properties of Polyglucose Aldehyde Derivative.—The polyglucose aldehyde was very sparingly soluble in cold water. Salt, urea or dimethylformamide did not increase the solubility in water. However, a 1% stable aqueous solution was obtained after a few minutes of heating at approximately 70°.

The dialdehyde content of the polyglucose aldehyde was 63%, determined by heating a sample with *p*-nitrophenylhydrazine and measuring the optical density of the *p*-nitroosazone derivative at 445 mμ.[9] The solution was evaporated to a small volume and the crystals filtered. The compound was recrystallized from ethanol and had m.p. 306–308° (uncor.). The *p*-nitro-osazone of glyoxal has m.p. 311° (cor.).[17]

The optical rotation of the polyglucose aldehyde $[\alpha]^{25}D$ was +36.3° (in H_2O). Hydrolysis of a sample with *N* sulfuric acid for 6 hours, and estimation of the glucose[18] indicated the presence of 24% glucose.

Sodium Chlorite Oxidation of Polyglucose Aldehyde.—To prepare derivatives with different amounts of carboxyl groups, 5.6-g. aliquots of polyglucose aldehyde were treated with: a, 0.01; b, 0.02; c, 0.04; d, 0.06; e, 0.08; and f, 0.33 mole of chlorous acid buffered at *p*H 3.3 with sodium acetate. The reaction mixture was left at room temperature for 24 hours.[6] The products were precipitated by the addition to the reaction mixture of six volumes of ethanol; the precipitates were dissolved in water and the solutions were dialyzed against running distilled water for 24 hours. Finally, the solutions were "freeze-dried." The recovery of material was: a, 25% (mechanical losses); b, 80%; c, 73%; d, 97%; e, 72%; and f, 49%.

These samples, when dissolved in water, gave solutions having *p*H values between 4.8 and 5.8. To prepare the sodium salts, each sample was brought to *p*H 8.4 by careful addition of dilute sodium hydroxide. Dialysis of the solutions was begun immediately in running distilled water and was continued for 24 hours, after which the contents of the cellophane bags were "freeze-dried." The recovery of the sodium salt of the polyglucose carboxylic acids was: a, 70%; b, 52%; c, 45%; d, 20%; e, 22%; f, 50%. Physical constants of these preparations are reported in Table I.

To check the contribution of alkaline degradation to the losses upon dialysis, two aliquots of sample b were redialyzed for 40 hours, one aliquot dissolved in distilled water and the other in a solution of *p*H 8.4 sodium hydroxide. In both cases 87% of the starting material was recovered.

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