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Polypeptide-Polysaccharide Conjugates Produced by Spontaneous Non-Enzymatic Glycation[1]

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A fundamental dogma has developed over the past 20 years that non-enzymatic glycation involving saccharide chains of greater than 3 to 4 residues is an extremely unlikely reaction. Our investigations using glycosaminoglycans have shown that, given sufficient time, polypeptide-polysaccharide conjugates form *via* the Schiff base-Amadori rearrangement mechanism. Further, even though these straight chain polysaccharides are relatively charged and sterically hindered, spontaneous glycation can also occur *in vivo*. A complete reinvestigation of all aldose terminating polysaccharides is required to elucidate this new class of macromolecules, which is likely to contain unusual polypeptide-polysaccharide combinations and functions.

Key words: antithrombin, Amadori, glycation, glycosaminoglycan, heparin.

Spontaneous glycation of polypeptides by aldoses occurs by Schiff base formation between a protein amino group and the aldehyde from a sugar residue, followed by tautomeric Amadori rearrangement with the vicinal hydroxyl to produce a stable keto-amine (*1*). This reaction occurs *in vivo* wherever proteins encounter significant concentrations of free monosaccharides (*2*); such as in the blood of diabetics (*3*). *In vitro* experiments have shown an apparent decrease in yield of conjugate for several disaccharides, compared to monosaccharides, which led to the conclusion that coupling between proteins and oligo or polysaccharides was extremely unlikely, if not impossible (*4*). The presumption made was that since aldose aldehyde groups are already masked as cyclic hemiacetals, further restraints found in long carbohydrate chains (such as steric hindrance and lack of freedom of rotation of residues) would make product formation highly unfavourable. Another, common supposition was that since the aldose terminus exists in such a small proportion of the polysaccharide chain, its reactive functions would be relatively undetectable on a mass basis (*5*).

This report challenges and refutes these hypotheses. It was postulated that Schiff base-Amadori rearrangement between polymers can occur since there is no evidence that structural elements relating to chain length inhibit imine formation and the termini of polysaccharides may even be preferred sterically for approach by macromolecules. More importantly, if spontaneous glycation of polypeptides by polysaccharides is possible, a broad range of compounds exists, which is easily determinable by examination of reactions occurring with any aldose terminating polysaccharide. The possibility of product formation was studied *in vitro* using glycosaminoglycans (GAGs) and *in vivo* using the clinical anticoagulant heparin (H); which contains a subpopulation of aldose containing molecules (*6*).

MATERIALS AND METHODS

Complex Formation—Antithrombin (AT) and heparin cofactor II (HCII) (Bayer, Etobicoke, ON, Canada and Affinity Biologicals, Hamilton, ON, Canada) were from human plasma and of high purity. H (Leo, Ajax, ON, Canada; Organon, Toronto, ON, Canada; Sigma, grade 1A, St Louis MO, USA) and dermatan sulfate (DS; Mediolannum, Italy) were highly purified materials from porcine intestinal mucosa. A portion of the rabbit plasma albumin used (a kind gift from Mark Hatton) was iodinated by iodogen (*7*) before use. Covalent polypeptide-polysaccharide complexes were produced by incubation of protein and carbohydrate mixtures in 0.02-0.3 M phosphate buffered (pH 7-8) aqueous solution containing NaCl (0.15-1 M) at either 37 or 40°C for 2 days to 14 days. In some cases, a further incubation with added $NaBH_3CN$ (final concentration 0.05 M) in buffer was carried out for 5 h after the initial reaction. Polypeptide and polysaccharide concentrations, in the reaction mixtures, ranged from 0.5 to 3 mg/ml and 10 to 70 mg/ml respectively. Purification of the complexes formed was either by gel filtration or, in the case of antithrombin-heparin covalent complex (ATH), by the following two step procedure. Reaction mixtures were incubated with butyl agarose beads (Sigma) in 2.5 M $(NH_4)_2SO_4$ in 0.02 M phosphate pH 7.0 buffer. After

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Abbreviations: AT, antithrombin; ATH, antithrombin-heparin; DS, dermatan sulfate; GAG, glycosaminoglycan; H, heparin; HCII, heparin cofactor II; HCD, heparin cofactor II-dermatan sulfate; HCH, heparin cofactor II-heparin.

washing the beads to remove free GAG, bound AT and ATH were eluted with buffer, dialysed *vs.* 0.01 M Tris·HCl pH 8.0 buffer and incubated with pre-equilibrated DEAE Sepharose beads (Pharmacia, Uppsala, Sweden). After washing with 0.2 M NaCl in pH 8.0 buffer to remove bound AT, ATH was eluted with 2 M NaCl in pH 8.0 buffer. Purified conjugates were concentrated by pressure-dialysis.

Analyses—Aldose termini were measured by reaction with 3,4-dinitrobenzoic acid using D-xylose as a standard (*8*). Sulfate content was determined using $BaCl_2$ and Na rhodizonate (*9*). Chondroitinase ABC, used to analyze the albumin-dermatan sulfate complex, was from ICN (Costa Mesa, CA, USA). The linkage structure between H and AT in ATH was obtained by first incubating ATH (2.6 mg/ml in terms of AT) with protease P-5147 (Sigma; 0.2 mg added per ml of incubate every 24 h) in 0.5 M Tris·HCl pH 8.0 at 37°C for 98 h. After purification of the H containing species on DEAE Sepharose, as described above, the material, in 0.1 M NaCl, was reacted with heparinase (ICN, Costa Mesa, CA, USA; 0.4 mU per ml of reaction mixture) at 37°C for 24 h. The linkage product was isolated as the bound fractions from Dowex 50 (Baker, Phillipsburg, NJ, USA) cation exchange chromatography. IR spectroscopy of a KBr disk of the isolated linkage product was carried out on a Bio-Rad FTS-40 Fourier transform IR machine. ^{1}H-NMR spectra of the isolated linkage product were run in ^2H_2O on a 500 MHz machine (Bruker DRX-500). Insufficient fragment from the linkage region of AT and H in ATH were obtainable for 2 dimensional ^{1}H-NMR. Small amounts of linkage product were analyzed by standard 2,4-dinitrophenyl hydrazine and Tollen's tests (*10*). SDS PAGE and western immunoblotting of ATH products employed standard methods (*11*). Anti-factor Xa activities were determined using a Stachrom kit (Diagnostica Stago, France) on an ACL300. Human AT ELISA was done using a kit (Affinity Biologicals).

In Vivo Experiments—To study *in vivo* ATH generation, rabbits were injected with heparin (200 U/kg intravenously and 400 U/kg subcutaneously followed after 3 h by 100 U/kg intravenously and 400 U/kg subcutaneously) and then, at 5 h after initial injection, exsanguinated into Na citrate (0.38% final concentration). Similarly, 200 U heparin/kg were injected subcutaneously into a female human followed, after 5 h, by removal of 100 ml of blood into citrate. Saturated $(NH_4)_2SO_4$ was added to the resultant plasmas (1 μl/ml of plasma) to prevent any further Schiff base formation *ex vivo*. Covalent ATH generated *in vivo* was purified from plasma by initially collecting the supernatant after adding saturated $(NH_4)_2SO_4$ until 40% saturation was reached. After dialysis *vs.* 0.01 M Tris·HCl pH 8.0, chromatography was carried out on DEAE Sepharose as described above. This was followed by chromatography on butyl agarose using the same method as the one described earlier except that elution of bound ATH was with 1.2 M $(NH_4)_2SO_4$ in buffer. After concentration by pressure-dialysis, materials were analyzed by western immunoblotting using either an anti rabbit AT or anti human AT antibody raised in sheep (Affinity Biologicals). The half lives of ATH, AT, H, and AT+H were determined by I.V. injection of the materials into rabbits followed by AT ELISA and anti-factor Xa assays of the plasma from blood samples taken over time.

TABLE I. **GAG aldose and sulfate content.** GAG refers to glycosaminoglycan. Aldose termini are expressed as percent of GAG molecules containing reducing end groups with dermatan sulfate and heparin average molecular weights being taken as 36,300 and 15,000, respectively. Molecular weight estimates are according to the manufacturer and our own measurements (gel filtration).

GAG (source, catalog #, lot #)	aldose termini (Mol %)	SO_4^{2-} (wt %)
Dermatan sulfate	95	16.6
Heparin (Leo, Z36A)	9	35.2
Heparin (Organon, T0080, 1VTC)	0	32.2
Heparin (Sigma, H-3393, 118F-0806)	6	42.7
Heparin (Sigma, H-3393, 124H0474)	18	38.1
Heparin (Sigma, H-5515, 53H09921)	14	36.1
Heparin (Sigma, H-9399, 24H0904)	9	40.1

RESULTS AND DISCUSSION

Amadori Rearrangement of Polypeptides and Polysaccharides In Vitro—DS GAG and rabbit albumin (containing a small amount of ^{125}I labeled albumin) at concentrations of 30 and 1 mg/ml, respectively, were incubated for up to 14 days. DS was used in these initial investigations because it has a high content of aldose termini (Table I) and circulates as a proteoglycan with anticoagulant activities in several physiological states (*12, 13*). Figure 1 shows the autoradiogram of a reduced SDS PAGE of the purified products. Labeled albumin in the product appeared as a polydisperse, high molecular weight band, characteristic of GAG containing proteoglycans. This material was associated with the DS as evidenced by the recovery of labeled albumin after chondroitinase ABC digestion of the GAG (Fig. 1, lane 2). Albumin and DS were likely together as a covalent complex since heating at 100°C in β-mercaptoethanol and SDS before electrophoresis did not cause dissociation.

Complexes were prepared between GAGs and either HCII and AT; serine protease inhibitors that are catalysed by noncovalent binding to GAGs *in vivo*. Conjugation of DS with HCII (HCD) was achieved, as well as linkage of H to HCII (HCH) and AT (ATH) as shown in Fig. 2. Covalent linkage of H to polypeptides provided stronger evidence that steric hinderance or charge interactions do not prevent formation of a stable product, since $-SO_3^-$ group substitu-

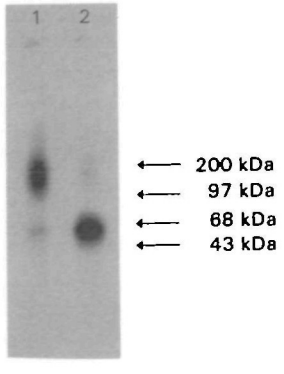

Fig. 1. **Autoradiogram of rabbit ^{125}I-albumin-dermatan sulfate covalent complex run on SDS PAGE.** Lane 1 shows the polydisperse high molecular weight complex while lane 2 shows the same material after pretreatment with chondroitinase ABC enzyme to degrade dermatan sulfate.

tion is increased over DS (Table I). Yield was not decreased by competition due to noncovalent binding to the protein moiety of other GAG molecules which lack reducing end groups. This was evident, as H and DS, both of which bind noncovalently to HCII at specific binding sites (*14*), gave similar yields of HCH and HCD respectively, even though H preparations have fewer aldose terminating molecules than DS (Table I). Conjugates were not dissociated by multiple freeze-thawing, lyophilization, high salt (3 M NaCl), treatment with detergents and heating at 100°C.

Chemical and spectral analyses showed that ATH contained a keto-amine formed by a lysine ε-amino and the open chain form of a sugar residue. A distinct structure, isolated by ion exchange chromatography of the product from exhaustive protease and heparinase digestion of ATH, was studied. Reaction with 2,4-dinitrophenyl hydrazine gave a positive result for the presence of a carbonyl, which was confirmed to be a ketone by a negative result with Tollen's test. Characteristic C=O and N-H stretches were observed on FT-IR scans (Fig. 3) which agreed with previous findings for Amadori conjugates of glucose and glycine (*15*). As found previously (*15*), it is likely that a significant proportion of the sugar-amino conjugate was in the hemi-ketal form, so that absorbance due to ketone C=O stretching was moderate. The ^{1}H-NMR spectrum was consistent with resonances for protons from ring or straight chain glucuronic acid and saturated alkyl chains such as lysine (Fig. 4). However, there where no anomeric proton signals (5-6 ppm) and a resonance at 3.1-3.2 ppm was present, compatible with protons on a carbon atom joined to an amino and an adjacent carbonyl group (Fig. 4D). These 2 characteristic features of the ^{1}H-NMR spectra of Amadori conjugates have been verified previously by other workers (*16*). The ^{1}H-NMR spectrum of the Amadori product formed by heating glucuronic acid and lysine (purified by gel filtration on Sephadex G-25 in H_2O) was comparable to that of the fragment isolated from ATH [single resonance at 3.2 ppm and no anomeric proton signals (Fig. 4C)]. Differences between the ^{1}H-NMR profiles of ATH fragment and purified glucuronic acid-lysine Amadori adduct are likely due to the fact that commercial heparin has other aldose termini [xylose, galactose, and glucosamine derivatives (*6*)] that could have linked with AT lysyl groups during ATH formation. These data verify that covalent bonding has occurred in ATH and an Amadori rearrangement is the likely mechanism.

Other variables previously considered in studies of the glycation reaction with monosaccharides were examined. As expected, Schiff base formation occurred in the pH range of 7-8, which agreed with the data for monosaccharides (*2*). Ionic strength varying from 0.15 M NaCl in 0.01 M phosphate to 1 M NaCl in 0.3 M phosphate had little effect on product generation, which was consistent with the lack of binding competition between macromolecules. Reaction temperature was critical. Up to 37°C, maximal product yields of 10 to 15% of protein glycated were obtained, regardless of the incubation time or GAG concentration. At 40°C, yields of 50% were consistently observed. The temperature effect may be due to a reaction step energy barrier where steric hinderance of some group(s) requires more thermal motion to achieve the correct orientation of protein and GAG. In all conditions studied, significant glycation of proteins by GAG polysaccharide occurred, which refutes previous assumptions for macromolecular aldoses.

Amadori Rearrangement In Vivo—The *in vitro* results provided the rationale for investigating the possibility that non-enzymatic glycation of proteins by macromolecules may occur spontaneously *in vivo*. Glucation of a variety of plasma proteins in animals has been studied extensively (*17*). Albumin-glucose in diabetic rats and humans can reach levels in plasma of 6-10% of total protein (*18, 19*). Rate of formation of these adducts is dependent on a variety of factors including the concentration of D-glucose in the circulation. Since GAGs, as well as other polysaccharides, have a short half life intravenously, we were intrigued to determine if glycation of plasma proteins could occur before disappearance of the aldose from the vascular system. Therefore, H was injected into rabbits (simultaneous intravenous and subcutaneous administration) and

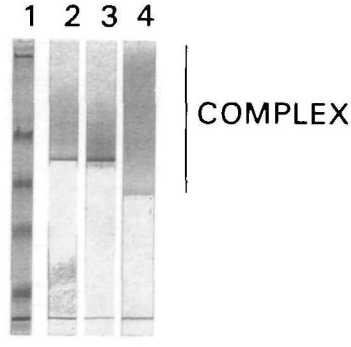

Fig. 2. **SDS PAGE of protein-glycosaminoglycan conjugates.** Each complex was electrophoresed on a 7.5% polyacrylamide gel and stained with alcian blue (for glycosaminoglycan) and Coomassie Blue (for protein). Molecular weight standards in lane 1 are from top to bottom: 250, 98, 64, 50, and 36 kDa. Preparations of heparin cofactor II-dermatan sulfate, heparin cofactor II-heparin, and antithrombin-heparin (see text for details) are shown in lanes 2, 3, and 4, respectively.

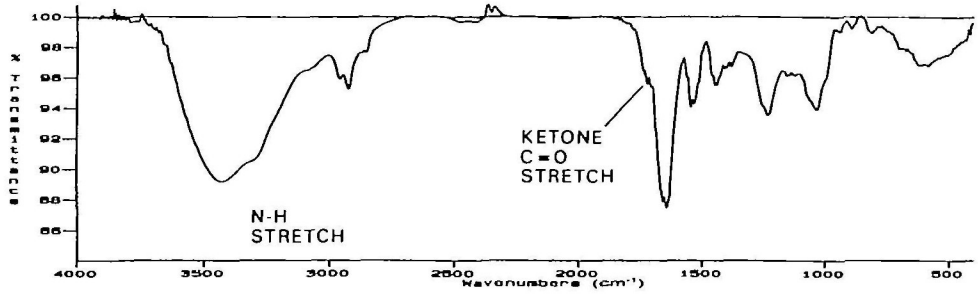

Fig. 3. **IR spectrum of the poly peptide-glycosaminoglycan linkage group isolated from antithrombin-heparin treated with protease and heparinase.** The linkage group was analyzed as a KBr disk.

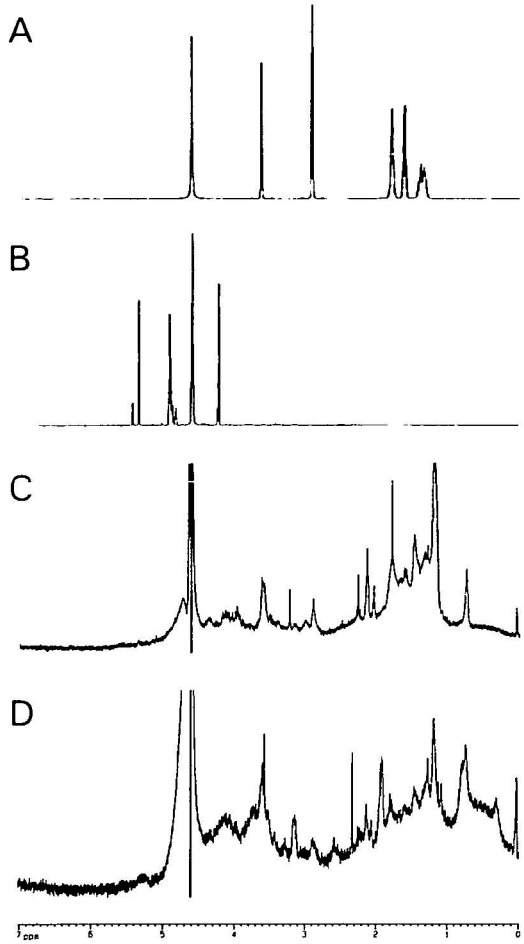

Fig. 4. **^{1}H-NMR spectrum in ^2H_2O of the polypeptide-glycosaminoglycan linkage group isolated from antithrombin-heparin treated with protease and heparinase.** The ^{1}H-NMR spectrum of the linkage region between antithrombin and heparin in covalent antithrombin-heparin complex is shown in scan D. ^{1}H-NMR spectra of lysine, D-glucuronic acid, and the Amadori adduct of lysine+glucuronic acid (scans A, B, and C, respectively) are shown for comparison. The resonance at 4.60 is from $^1HO^2H$.

blood samples collected several hours later into citrate and $(NH_4)_2SO_4$ (to prevent further Schiff base formation *in vitro*).

In plasma, H acts as an anticoagulant by selectively interacting non covalently with AT *via* a pentasaccharide sequence and catalyzing AT's inhibition of generated thrombin (*20*). Thus, since H polysaccharide aldoses do not randomly associate with plasma polypeptides, it was concluded that initial discriminatory binding (by ionic, hydrogen bonding, or other interactions) could moderate the variety of protein conjugates that may form with a polysaccharide aldose (H). Therefore, due to non covalent binding of H by AT, the effective local concentration of H around AT molecules would be markedly increased, leading to a significant increase in the probability of AT conjugation with H compared to linkage to other plasma proteins. Consequently, we attempted to purify AT, which was covalently bonded to H, from the plasma of the heparinized animals.

Figure 5A shows the results for a western blot, developed

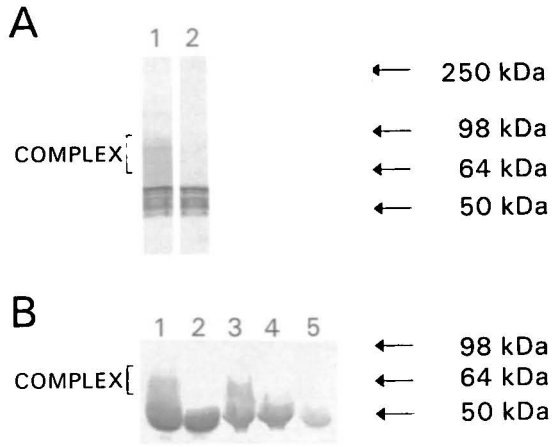

Fig. 5. **Western immunoblots of materials isolated from plasma of rabbits (A) and a human (B) injected with heparin.** Western blots were probed with either anti-rabbit antithrombin (A) or anti-human antithrombin (B) antibodies. Antithrombin containing, polydisperse, high molecular weight complexes were isolated from either rabbits (A, lane 1) or a human (B, lane 3) injected with heparin, which diminished on treatment with heparinase (A, lane 2 and B, lane 4, respectively). A similar polydisperse complex could be isolated from human plasma incubated with heparin *in vitro* (B, lane 1) which was sensitive to heparinase (B, lane 2). Complex was found to be absent in normal human plasma pool (B, lane 5).

using anti-rabbit AT antibody, of material recovered from the plasma of heparinized rabbits using a method adapted from the procedure for purification of covalent ATH produced *in vitro*. Polydisperse material, higher in molecular weight than rabbit AT, was recognized by the antibody (Fig. 5A, lane 1), which disappeared on treatment with heparinase (Fig. 5A, lane 2). These data confirmed the presence of a species of ATH, produced *in vivo*, with the characteristics of a covalent complex. Western blots of AT, H, and non covalent mixtures of AT+H showed no high molecular weight band, whereas an identical band compared to the *in vivo* material was observed with covalent ATH produced *in vitro* (data not shown).

A single, subcutaneous injection of H to a human gave results similar to rabbits. A polydisperse, high molecular weight ATH complex was obtained which was not present in plasma from untreated humans (Fig. 5B). ATH isolated from heparinized (10 μg/ml) plasma, heated at 37°C for 30 h *in vitro*, gave a similar pattern to that of material formed *in vivo* (Fig. 5B, lane 1). Laser densitometry of the blots was used to determine the amount of ATH generated (compared to a standard curve of ATH produced *in vitro*) from the H injected. In rabbits, 0.005% (by mass) of the peak level of H in plasma was recovered as ATH. Thus, injection of 7.5 mg of H subcutaneously in a rabbit yielded, after 4 h, 0.25 to 0.4 μg of ATH in terms of H. Comparable results were seen for ATH formed in the human subject. These findings are the first demonstration of spontaneous formation of covalent polypeptide-polysaccharide complexes in an organism.

Clinical Implications for Heparin Therapy—Formation of covalent ATH from intravenous heparin may explain, in part, some of the observed persistence of activity following cessation of H therapy (*21*). Anti-Factor Xa activity assays for H in humans given low molecular weight heparin

intravenously still show significant plasma activity 8 h after administration of the drug has been discontinued (*22*). Although H can be degraded in the liver to penta or hexasaccharides which are released to recirculate over a long period of time (*23*), H molecules of this size do not have anti-thrombin activity (*24*) and would not explain a prolonged increase in activated partial thromboplastin time values. It has been shown that H binds to plasma proteins other than AT (*25*) as well as endothelium (*26*). Slow displacement from these sites over time could give rise to a lingering activity but it is unclear what agents are present to supplant H. Conceivably, formation of ATH from injected H and endogenous AT may contribute to the prolonged anticoagulant effect. The disappearance of ATH, prepared *in vitro*, was compared to AT, H and non covalent mixtures of AT+H in rabbits. ATH was cleared from the circulation at a rate 7 to 8 times slower than H alone and 4 to 5 times faster than AT. Since AT's half-life is 66 h in humans (*27*), the theoretical half-life for ATH in humans may be 13 h to 17 h. These projected values agree with the clinical data for patients with repeated heparin administration.

Possible Spectrum for Occurrence of Conjugates—Potentially, any polysaccharide terminating in an aldose, with an unsubstituted hydroxyl at C_2, could undergo a spontaneous conjugation with accessible proteins. As initiation of synthesis for many polysaccharides is still unknown, a large proportion may have reducing end groups. Further, *in vivo* catabolism can give rise to polysaccharide metabolites terminating in an aldose. Although GAGs in plasma are present mainly in their parent proteoglycan form, a portion of the species exist as free GAG chains (*28*).

In vivo non-enzymatic glycation of proteins with polysaccharides most likely occurs where the two species are sequestered together, as the reaction is a time dependent and high local concentration requiring event. Since there is a wide variety of conjugates that could exist, it is difficult to assess what may be the most probable systems for the formation of significant compounds. We anticipate that, in most cases, absolute amounts of conjugate produced would be low. This conclusion is based on the fact that occurrence of large local concentrations of high molecular weight sugar polymers often is accompanied by a large hydration sphere which either prevents the approach of other macromolecules or ties them up by hydrogen bonding. Nevertheless, spontaneous modification of a polysaccharide, by covalent linkage to a polypeptide, would significantly change its properties. Even small amounts of these altered molecules may have important biological functions.

All animal experimentation conformed with the institutional guidelines and informed consent was obtained in the experiments involving the human subject. We are indebted to Mark Hatton, Department of Pathology, McMaster University for supplying ^{125}I labeled, nonglycated rabbit albumin and for giving constructive criticism of this manuscript. We also wish to thank Sue Smith for help with the animal experiments.

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