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Review

Polysialic acids: potential in improving the stability and pharmacokinetics of proteins and other therapeutics

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Abstract. Naturally occurring polymers of *N*-acetylneuraminic acid (polysialic acids) are biodegradable, highly hydrophilic and have no known receptors in the body. Following intravenous injection, polysialic acids exhibit long half-lives in the blood circulation and have therefore been proposed as carriers of short-lived drugs and small peptides. In addition, shorter-chain polysialic acids can be used as a means to increase the circulatory half-life of proteins and thus serve as an alternative to the nonbiodegradable monomethoxypoly(ethylene glycol). Recent work has shown that covalent coupling of a low molecular weight polysialic acid (colominic acid) to catalase and asparaginase leads to a considerable increase of enzyme stability in the presence of proteolytic enzymes or blood plasma. Comparative studies in vivo with polysialylated and intact asparaginase revealed that polysialylation significantly increases the half-life of the enzyme. The highly hydrophilic and innocuous nature of polysialic acids renders them suitable as a means to prolong the circulation of peptides and proteins.

Key words. Polysialic acids; colominic acid; catalase; asparaginase.

Introduction

The extensive presence of enzymes and other drugs within the vascular system or the extravascular space is often a prerequisite for their optimal use [1]. Many therapeutic peptides, enzymes, other proteins as well as small conventional drugs, for instance, are removed from the circulation rapidly and before effective concentrations in the blood or target tissues can be attained. It is generally accepted that such agents would be more effective, less toxic and also used in smaller quantities if their chances of interaction with corresponding receptors or substrates could be augmented. Progress to that end has recently been made by the conjugation of a number of short-lived proteins to low molecular weight (750–5000) monomethoxypoly(ethylene glycol) (mPEG) [2]. It appears the shell formed by mPEG around the surface of proteins sterically hinders their interaction with blood components that are thought to be responsible for their clearance [3]. Unfortunately, the low molecular weight of mPEG polymers used enables these to be excreted rapidly into the urine and thus renders them unsuitable for prolonging the half-life of small peptides (and conventional drugs). Moreover, mPEG is nonbiodegradable, and its chronic accumulation into tissues (where the pegylated proteins are expected to end up) may be toxic.

Polysialic acids

Recently, naturally occurring polymers of *N*-acetylneuraminic acid (NeuNAc) (polysialic acids) were shown to increase the half-life not only of proteins ([4]; M. Mital

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and G. Gregoriadis, unpublished observations) and microparticles such as liposomes [X. Zhang and G. Gregoriadis, unpublished observations] but also of small molecules [5] such as conventional drugs and peptides. Polysialic acids (fig. 1) include the serogroup B capsular polysaccharide from *Neisseria meningitidis* B and *Escherichia coli* KI, the serogroup C capsular polysaccharide C from *N. meningitidis* C and the polysaccharide K92 from *E. coli* K92, as well as shorter-chain derivatives. Because of the highly hydrophilic nature of polysialic acids and the absence of known receptors in the body for polysialic acids with α2,8 and α2,9-linked NeuNac, it was thought [5] that these biopolymers may exhibit long circulation times after intravenous injection and could, therefore, potentially serve as carriers of short-lived drugs or peptides. Results from experiments in mice injected with a variety of polysialic acids indicated that the clearance pattern of polysaccharide B (PSB) (see fig. 1) from the blood circulation was biphasic, with 50% of the dose removed soon after injection (fig. 2). The remainder exhibited a linear rate of clearance with a half-life of 20 h (fig. 2). However, the fully deacylated PSB exhibited a much reduced initial clearance that was followed by a linear rate of clearance with a half-life of 30 h. On the other hand, there was no apparent difference in the clearance patterns of PSK92 before and after deacylation. Following a relatively slow clearance during the first 6 h, clearance patterns became linear, with half-lives of 40 h [5]. It appears that removal of the acyl groups prevents PSB from forming micellar aggregates [6], which probably accounts for the initial rapid loss of the injected dose. The rate of clearance of polysialic acids from the blood after intravenous injection is thus likely to be dependent on the presence or absence in their structure of phospholipid acyl groups. However, as the α-(2-8)-linked PSB is cleared more rapidly than the α-(2-8)-α-(2-9)-linked PSK92 (see above), the rate of clearance also appears to depend on the structure of polysialic acids. Moreover, as polysialic acids are polydisperse, entities of low molecular weight are expected to contribute to the early rapid removal of some of the injected PSB and, to a

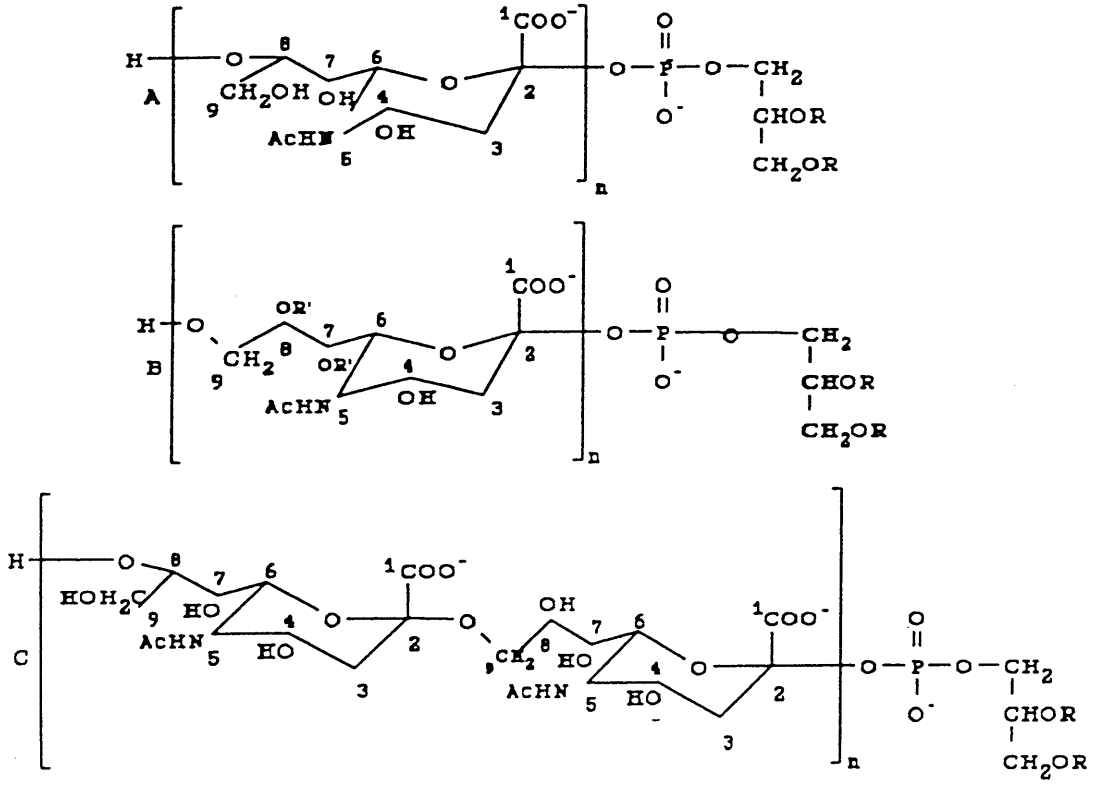

Figure 1. Structures of polysialic acids. (*A*) Serogroup B capsular polysialic acid B (PSB) from *N. meningitidis* or *E. coli* K1 is a homopolymer (average $n = 199$) of α-(2-8)-linked *N*-acetylneuraminic acid. (*B*) Serogroup C capsular polysialic acid (PSC) from *N. meningitidis* C is a homopolymer (average $n = 74$) of α-(2-9)-linked *N*-acetylneuraminic acid; R^1 is H or $OCOCH_3$. (*C*) Polysialic acid (PSK92) from *E. coli* K92 is a heteropolymer (average $n = 78$) of alternate units of α-(2-8)-α-(2-9)-linked *N*-acetylneuraminic acid. All three polysialic acids contain a phospholipid molecule covalently linked to the reducing end of the polymers. (From [5], with permission.)

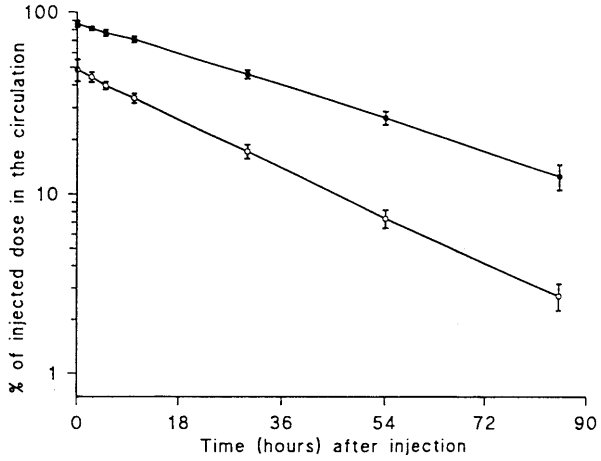

Figure 2. Clearance of PSB from the blood circulation. In six separate experiments, mice in groups of 3–4 animals were injected intravenously with 1.1–2.0 mg of intact (○) or deacylated (●) PSB and bled at time intervals. NeuNAc in the blood plasma samples was assayed as described [3] and expressed as % ± SD of the dose in total blood. (Values from all groups treated with intact and deacylated PSB respectively, were pooled.) Blood volume was estimated as 7% of the body weight. (From [5], with permission.)

lesser extent, PSK92 [5]. This is supported with data from experiments with a PSB of short chain length (15 NeuNAc units) where over 90% of the injected dose was removed from the circulation within 30 min [5].

The data of long half-lives in the circulating blood for the polysialic acids used here supported their use to extend the half-life of small drugs and small peptides. To that end, studies with a model drug (fluorescein) coupled to deacylated PSB of low molecular weight (82 NeuNAc units) indicated that whereas fluorescein as such was removed from the circulation very rapidly, removal of the polysialic acid-bound dye was slower, showing a half-life of 5 h [5]. Therefore, it is apparent that polysialic acids could potentially enable rapidly cleared drugs and small peptides to remain within the vascular and extravascular areas for extended periods of time. As polysialic acid clearance depends not only on the type used but also the molecular size of the polymer [5], it should be possible to tailor the rates of clearance of peptides or proteins optimally. Thus, polysialic acids of large molecular weight should be suitable for the delivery of one or more molecules (per molecule of polysialic acid) of low molecular weight drugs and peptides, whereas those of lower molecular weight could be used to coat large proteins as well as particulate drug delivery systems such as liposomes (fig. 3). Coupling of therapeutic entities to polysialic acids could be affected through the nonreducing end of the latter, which, on periodate oxidation, generates a reactive aldehyde, the

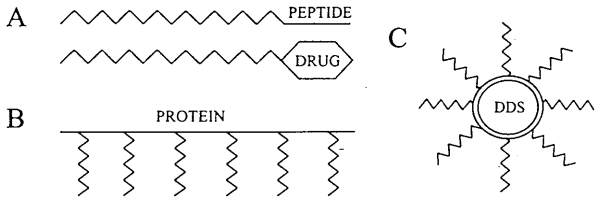

Figure 3. Schemes of polysialic acid use in drug delivery. Long polysialic acids can be used to prolong the circulation time of small drugs and peptides. Shorter polysialic acids bound to the surface of proteins or drug delivery systems (DDS) such as liposomes will render them more hydrophilic and extend their half-lives.

carboxyl and hydroxyl groups, as well as the amino groups obtained on deacetylation of the polymers [5].

Polysialylated enzymes

Protein use in therapy can be interfered with by proteolytic degradation and short half-lives in the circulation [2]. Furthermore, administration of large doses of proteins in order to maintain therapeutic efficacy can often lead both to toxicity and the promotion of adverse immune responses. As already discussed [2, 7], such problems can be circumvented by coupling proteins with hydrophilic macromolecules. These include dextrans and mPEG, which is by far the most successful and comprehensively studied.

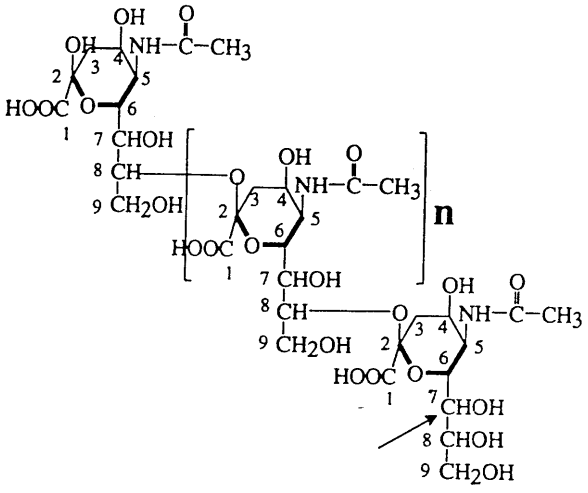

Figure 4. Structure of colominic acid. NeuNAC units are linked via α-(2→8) glycosidic linkages. Arrow indicates the carbon atom (C_7) at the nonreducing end of the sugar where periodate oxidation introduces an aldehyde group. (From [7], with permission.)

A low molecular weight polysialic acid, namely colominic acid (fig. 4), has been employed in our laboratory as a means to render two enzymes, catalase and asparaginase, more hydrophilic [4, 7]. It was thought that by coating the proteins with colominic acid, their pharmacokinetics and stability would be improved and their immunogenicity reduced. Catalase was chosen because of its increasing use as an oxygen radical scavenger or in enzyme replacement therapy [8], and asparaginase because it is currently in clinical use for the treatment of acute lymphoblastic leukaemia [9]. Results with the polysialylated asparaginase are discussed below.

Polysialylated asparaginase

A variety of attempts have been made to increase the half-life of asparaginase in the blood circulation. They include entrapment into liposomes [10] or erythrocytes [11], and conjugation to mPEG [12]. In our recent work with polysialylation, activated colominic acid (see legend to fig. 4) was coupled covalently to asparaginase by reductive amination in the presence of $NaCNBH_3$ [4] as previously applied for catalase [7]. Results [4] from experiments using three different molar ratios of colominic acid:asparaginase (50:1, 100:1 and 250:1) in the coupling reaction revealed that the degree of polysialylation was directly dependent on the molar ratio of colominic acid and enzyme in the coupling reaction. For instance, the highest degree of polysialylation (8.1 $\pm$ 1.7 mol of colominic acid/mol asparaginase) was achieved when an excess (250-fold) of colominic acid was present in the reaction mixture, corresponding to an average of 11% of the available lysine ϵ-amino groups [4]. It should be noted however that as colominic acid is polydisperse, values of degree of polysialylation would only be average.

Asparaginase conjugates prepared by other methods are known to suffer substantial losses of enzyme activity, e.g. 70–90% in the case of pegylated asparaginase [12]. Polysialylation, on the other hand, led to only a modest loss (14–18%) of initial asparaginase activity [4]. This strongly suggests that the coupling procedure used here (possibly the presence of colominic acid in the reaction mixture) protects the enzyme from inactivation. Thus, as with catalase [7], only a minor fraction (17%) of asparaginase activity was retained by the enzyme when subjected to identical reaction conditions in the absence of colominic acid.

Enzyme kinetics

Enzyme kinetics studies with the native and polysialylated asparaginase suggested a modest but not significant increase in the K_m value of the polysialylated enzyme. Thus, K_m values for the polysialylated constructs made with colominic acid to enzyme ratios of 50:1, 100:1 and 250:1 were 1.90×10^{-5} M, 2.15×10^{-5} M, and 2.29×10^{-5} M, respectively [4]. These values were similar to those of native asparaginase (1.68×10^{-5} M) [4] and the K_m values reported [13] for the clinically useful asparaginases (e.g. 10^{-5} M). Corresponding V_{max} values for the polysialylated enzyme constructs were 0.901, 0.910 and 0.919 μmol min^{-1} U^{-1}, and 0.847 μmol min^{-1} U^{-1} for the native enzyme [4]. Results indicate that as with catalase [7], covalent coupling of colominic acid to asparaginase has no significant influence on enzyme activity despite the polysaccharide's very acidic nature. This could be attributed to the limited number of polysialic acid molecules bound per molecule of enzyme. Polysialylation of additional enzymes should reveal whether the acidity of bound polysialic acid can alter the activity of certain enzymes.

The effect of blood plasma on the stability of polysialylated asparaginase

Incubation of native and polysialylated asparaginase with mouse plasma at 37 °C revealed that polysialylation improves enzyme stability. Thus, whereas only about 13% of the activity of the native enzyme was retained (fig. 5) at 6 h, most (65–83%) of the activity of

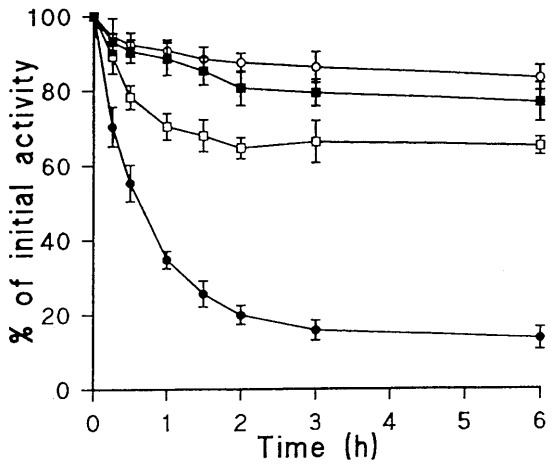

Figure 5. Retention of asparaginase activity in the presence of plasma. Native (●) and polysialylated asparaginase preparations 50:1 (□) (A), 100:1 (■) (B), 250:1(○) (C) molar ratios of CA:asparaginase were incubated in the presence of mouse plasma at 37 °C; values denote means $\pm$ SD (three different preparations). *Statistics:* Results obtained at 6 h were compared by ANOVA (analysis of variance) and p values corrected by the Bonferroni test. Native vs. A, B and C, $p < 0.001$; A vs. B, n.s.; B vs. C, n.s.; A vs. C, $p < 0.05$; n.s., nonsignificant. (From [4], with permission.)

polysialylated asparaginase was still present. Furthermore, retention of activity by the polysialylated asparaginase was significantly higher for the preparation obtained by the use of the greatest number of colominic acid molecules (250:1 molar ratio of CA:asparaginase; fig. 5). The effect of polysialylation on asparaginase stability (also observed previously for polysialylated catalase; [7]) could be tentatively attributed to changes in the microenvironment of the enzyme as a result of the presence of the highly hydrophilic, negatively charged colominic acid molecules. It is conceivable that this, in conjunction with a shielding effect of the colominic acid chains, interferes with the access of plasma proteases to their target sites on the enzyme molecule.

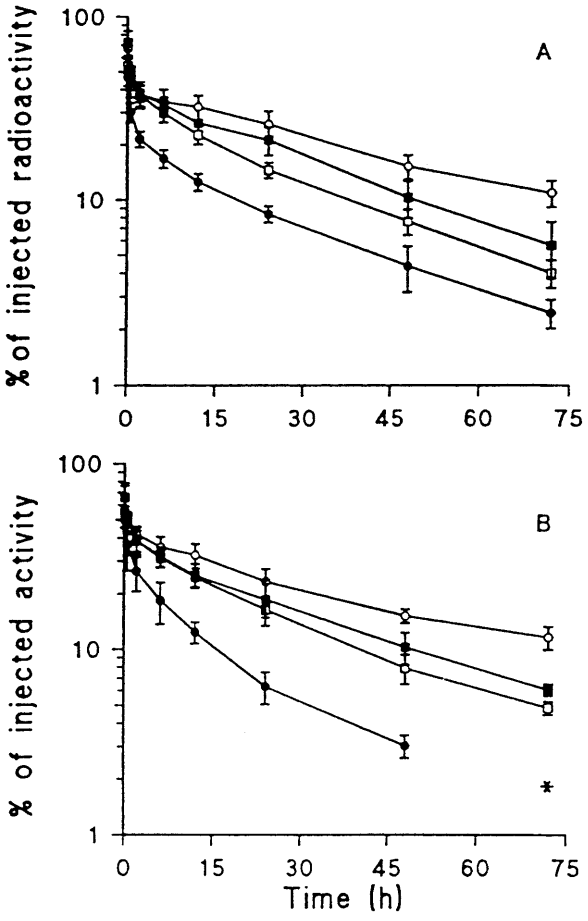

Figure 6. Clearance of asparaginase from the circulation. Mice were injected intravenously with 1 mg (550 U) tritiated native (●) and 1 mg (450–475 U) tritiated polysialylated asparaginase prepared by the use of 50:1 (□), 100:1 (■) and 250:1 (○) molar ratios of CA:asparaginase in the coupling reaction. Blood plasma obtained at time intervals was assayed for ^3H (*A*) and asparaginase activity (*B*). The pharmacokinetics profiles demonstrate biphasic patterns of clearance which are consistent with a two-compartment model. Values denote means ± SD; $n = 4$ animals. *Native asparaginase activity was not detectable at 72 h. (From [4], with permission.)

Effect of polysialylation on asparaginase removal from the circulating blood

The clearance of tritiated asparaginase after intravenous injection was compared with that of tritiated polysialylated constructs. Results indicated that the latter were removed from the circulation at slower rates than the native enzyme, in terms of both radioactivity (fig. 6A) and enzyme activity (fig. 6B). However, as with enzymes conjugated to other hydrophilic polymers (e.g. [2]), much of the administered dose (about 75% for the native enzyme and 60–65% for the three polysialylated constructs) was removed from the circulation within 2 h after injection, the remainder exhibiting slower, linear clearance rates (fig. 6A and B). Terminal half-lives ($t_{\frac{1}{2}\beta}$) estimated from the linear portions of asparaginase activity and ^3H radioactivity clearance patterns, were about 15 h for the native and about 38 h for the polysialylated asparaginase. Terminal half-life values were also independent of the dose injected (at least for the range of doses tested; fig. 6) and similar in terms of radioactivity or enzyme activity for each of the polysialylated constructs tested.

Factors influencing the clearance of injected proteins include [14] non-specific uptake by the reticuloendothelial system, receptor-mediated endocytosis, protein molecular mass, shape and charge [15–17], which determine the extent of transcapillary passage or renal filtration [15], and protein degradation by plasma proteases. In the case of polysialylated asparaginase, for instance, greater resistence to plasma proteases (fig. 5) may have contributed to its increased half-life. In addition, it is conceivable that loss of some of the free-amino groups of asparaginase on polysialylation has rendered the modified protein intrinsically more negatively charged. This and a shielding effect of the colominic acid chains discussed above and elsewhere [4, 7] could interfere with the interaction of the enzyme with blood or tissue components that are responsible for its removal from the circulation.

The reduction in the clearance rates of the polysialylated asparaginase (fig. 6) is not as great as that claimed for the pegylated enzyme (e.g. [12]). However, the following should be taken into account: (i) the amounts of injected pegylated enzyme in the studies by Cao et al. [12] were too low (up to 40 U per animal) compared with those (450–550 U) used in the present work, and measurements of enzyme activity may not have been as accurate and may have led to overestimation of values; (ii) asparaginase conjugates with mPEG [12] are known to suffer quantitative loss of enzyme activity. Thus, polysialylation (which affects activity only modestly) [4, 7] may become a preferred alternative as it would limit wastage of proteins; (iii) there is no information regarding the fate and effect of the mPEG moiety of pegylated proteins subsequent to their uptake by tissues. As mPEG is nonbiodegradable, its intracellular accumulation, especially on chronic use, may prove undesirable.

Physiological properties of polysialic acids

Unlike other hydrophilic polymers (e.g. dextran, mPEG), polysialic acids are biodegradable, and their catabolic products (e.g. NeuNAc) are not known to be toxic. Moreover, polysialic acids are T-independent antigens and thus do not induce immunological memory. PSB, for instance, is nonimmunogenic [18], although other polysialic acids (e.g. PSC and PSK92) can be immunogenic in humans but only when their molecular weight is in excess of 50,000 Da (average chain length greater than 170 NeuNAc units). However, polysialic acids can become T-cell-dependent antigens (with induction of memory) when coupled to proteins, in which case there is no restriction on the size of the polymer. Nonetheless, polysialic acid-induced immune responses are difficult to achieve, especially for PSB. On the other hand, a more important consideration in selecting a polysialic acid for drug or enzyme delivery may be antigenicity (i.e. binding of the antigen to its antibodies). For instance, although antibodies against some of the polysialic acid structures do exist at low levels in the blood circulation, such antibodies, especially those against the α-(2-8)-linked structures [19] which are present on host cell surfaces, are generally of low affinity, thereby limiting any immunological response [20].

Two other aspects of polysialic acids that may have some relevance in their use in drug delivery are, on the one hand, the state in which oligomers of polysialic acid occur in solution, and on the other, the role that polysialic acids play in neural cell adhesion molecule (N-CAM) binding to proteoglycans. Thus, it has been observed [21] recently by atomic force microscopy that polysialic acid molecules with at least 12 sialyl residues tend to occur, at the concentration examined (1 pmol in 1 µl), as filament network bundles. However, it is not known at present whether the colominic acid used for polysialylation of proteins retains the filament bundle network state following periodate oxidation to form the aldehyde group at carbon 7, or whether if does retain that state, the ensuing Schiff base reaction with the ϵ-amino groups of the lysine residues occurs with single molecules of the sugar polymer or bundles of it.

Regarding the interaction of N-CAM polysialic acids with proteoglycans, it is known [22] that soluble polysialic acid fragments at appropriate concentrations inhibit such interaction in vitro. However, the biological significance of this interaction, potentially [21] occurring via the filament bundle networks, is unknown at present. Moreover, it is feasible that polysialic acid molecules on the surface of proteins are not in an appropriate configuration so as to interfere with physiological processes in vivo involving the heterophilic binding of N-CAM molecules to proteoglycans.

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