

## Stabilization of Human $\beta$ -D-N-Acetylhexosaminidase A towards Proteolytic Inactivation by Coupling It to Poly(N-vinylpyrrolidone)

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Human hexosaminidase A was covalently bound to soluble poly(N-vinylpyrrolidone), and the effect of this binding on the enzyme inactivation by various procedures was investigated. Whereas the polymer-bound hexosaminidase underwent inactivation to the same extent as the free enzyme, when exposed to heat or acidic pH, the conjugation to polymer appeared to protect the enzyme towards proteolysis. Thus, the polymer-bound enzyme exhibited considerably higher resistance to treatment of both pronase and macrophage cathepsins. The clearance rate from rabbit blood, of the polymer-bound enzyme (expressed as enzyme activity), was shown to be significantly slower than that of the free enzyme.

The use of exogenous enzymes for the treatment of various enzyme deficiencies in humans has raised much interest in recent years. Special interest was devoted to enzyme replacement therapy in several inherited storage diseases; these disorders are characterized by the massive accumulation of a catabolite in the body, mainly in the lysosomes of the affected organs, which stems from the absence of a catabolic enzyme. Promising results were published by R. O. Brady and his group, who injected purified glucocerebrosidase into patients with Gaucher's disease. They showed that even as little as one or two injections of enzyme caused a remarkable decrease in the level of glucocerebroside in the liver and in the erythrocytes of the patients [1,2]. Encouraging results were also obtained by the same group in the use of ceramide trihexosidase for the treatment of Fabry's disease [3]. Enzyme replacement therapy was applied also in Tay-Sachs disease; this disease is characterized by the absence of the A isozyme of  $\beta$ -N-acetylhexosaminidase, which results in a massive accumulation of ganglioside GM<sub>2</sub> in the central nervous system.

*Abbreviations.* MeUmbGlcNAc, 4-methylumbelliferyl N-acetyl- $\beta$ -D-glucosaminide; MeUmbGalNAc, 4-methylumbelliferyl N-acetyl- $\beta$ -D-galactosaminide.

*Enzyme.* N-Acetylhexosaminidase (hexosaminidase), 2-acetamido-2-deoxy- $\beta$ -D-glucoside acetamidodeoxyglucohydrolase (EC 3.2.1.30).

*Definition.* 1 unit (U) hexosaminidase activity is the amount of enzyme which hydrolyses 1  $\mu$ mol substrate/min under the conditions of the assay system tested.

Attempts to infuse normal plasma into children suffering from Tay-Sachs disease have been made, but did not seem beneficial [4]. Tissue-culture experiments, in which Tay-Sachs fibroblasts were incubated with partially purified hexosaminidase A, failed to show incorporation of the exogenous enzyme into the cells [5]. Recent experiments in which purified human enzyme was introduced intravenously into Tay-Sachs patients (variant O) did not show any therapeutic effect by the introduced enzyme [6,7], probably due to its incapacity to cross the blood-brain barrier, on the one hand, and to its fast clearance from the blood and inactivation *in vivo*, on the other.

The latter point, namely the possible degradation of the enzyme in the body following its injection, is especially relevant for lysosomal enzymes (like hexosaminidase), which are exposed to the proteolytic action of lysosomal proteases in their expected site of action. It is envisaged that this problem might be overcome if means were found to protect the enzyme against proteolysis.

Attempts in this direction were made in previous studies in which various proteins, including trypsin, chymotrypsin, kallikrein and insulin, were covalently coupled to the soluble carrier poly(N-vinylpyrrolidone). It was observed that the coupling of the proteolytic enzymes to poly(N-vinylpyrrolidone) considerably protected them against autolysis and heat inactivation [8,9]. In the case of the insulin a higher resistance towards inactivation *in vivo* and *in vitro* was found (unpublished data).

In the present paper we wish to report on the successful stabilization of human hexosaminidase A towards proteolytic degradation by means of coupling it to poly(*N*-vinylpyrrolidone).

## MATERIALS AND METHODS

Hexosaminidase A was purified from human placenta as described previously [10,11]. The purification steps included chromatography on Sepharose-bound concanavalin A, followed by affinity chromatography on a ligand column (Sepharose-bound 2-acetamido-*N*-(6-aminohexanoyl)-2-deoxy- $\beta$ -D-glucopyranosylamine. Separation of isozymes A and B of hexosaminidase was obtained by chromatography on DEAE-cellulose. The pure enzyme migrated as a single band in polyacrylamide gel electrophoresis and as a single peak in the analytical ultracentrifuge.

The enzymic activity was determined with the fluorogenic substrate 4-methylumbelliferyl-*N*-acetyl- $\beta$ -D-glucosaminide (Pierce, U.S.A.) [12]. The  $K_m$  values of the free and of the poly(*N*-vinylpyrrolidone)-bound hexosaminidase A were determined with the two fluorogenic substrates: 4-methylumbelliferyl *N*-acetyl- $\beta$ -D-glucosaminide (MeUmbGlcNAc) and 4-methylumbelliferyl *N*-acetyl- $\beta$ -D-galactosaminide (MeUmbGalNAc).

Constant amounts of enzymes (in 0.04 M citrate buffer, pH 4.4) were added to various concentrations of substrates, ranging from 0.2 mM to 2 mM for MeUmbGlcNAc, and from 0.05 mM to 0.5 mM for MeUmbGalNAc. Calculations of  $K_m$  values were done according to a Lineweaver and Burk plot [13]. The pH optimum for the reaction was measured in phosphate-citrate buffer in the pH range of 3–8. The specific activity of the pure hexosaminidase A was 23.3 units/mg protein (5800-fold purification as compared to the crude homogenate).

### *Activation of Poly(N-vinylpyrrolidone) and Coupling to Hexosaminidase*

Poly(*N*-vinylpyrrolidone), K-15 (Roth, W. Germany), with an average molecular weight of 10000 was activated as described previously [9]. The activation included partial alkaline hydrolysis of the  $\beta$ -lactam rings, blocking of the resultant by formaldehyde and formation of the *N*-hydroxysuccinimide ester. About 5% of the lactam rings were found to be converted to the *N*-hydroxysuccinimide ester.

Coupling of the activated poly(*N*-vinylpyrrolidone) to hexosaminidase A was performed in 0.1 M sodium borate buffer, pH 8.4; activated dry polymer was added to the enzyme solution in 10-fold excess (on weight basis) and the pH of the reaction mixture was maintained constant by addition of sodium tetra-

borate. The solution was stirred at 4 °C for 16 h, then centrifuged to remove insoluble material and passed through a Sephadex G-200 (Pharmacia) column (2.6 × 80 cm) at a rate of 15 ml/h. The elution buffer was phosphate-buffered saline. Protein was determined according to Lowry *et al.* [14] using bovine serum albumin as a standard. The quantitation of the content of poly(*N*-vinylpyrrolidone) in the conjugate was performed using iodine reagent [15].

### *Immunochemical Methods*

Two antibody preparations were used in this study, those common to both hexosaminidases A and B, and those specific for hexosaminidase A exclusively. They were evoked in goats and prepared as described in detail elsewhere [12]. Radial immunodiffusion assays with both of these antisera were carried out in 1.5% agarose gels [11] containing the cross-reactive antiserum diluted 1:500 or specific anti-hexosaminidase-A diluted 1:100. The precipitin rings were visualized by reacting with the chromogenic substrate naphthol AS-BI-*N*-acetyl- $\beta$ -D-glucosaminide (Sigma, U.S.A.) in the presence of fast garnet GBC (Sigma, U.S.A.).

Iodination of both free hexosaminidase A or of the polymer-bound enzyme was carried out with lactoperoxidase according to Marchalonis [16]. The specific labeling of the free enzyme was 3 times higher than that of the polymer-bound. Antigen-binding experiments of the radioactive enzyme preparations with the cross-reactive or with the anti-A specific antisera were performed by the addition of various concentrations of antibody preparations to a constant amount of the labeled enzyme and subsequent precipitation of the complex with rabbit antiserum against goat immunoglobulin. The extent of enzyme binding was determined from the radioactivity in the precipitate.

### *Inactivation of Hexosaminidase A*

*a) Heat Inactivation.* Solutions exhibiting equal activities of either free or polymer-bound hexosaminidase A were diluted in 0.04 M sodium citrate buffer, pH 4.4, containing 1 mg/ml bovine serum albumin (grade A, Calbiochem, U.S.A.). Aliquots (100  $\mu$ l) of the enzyme solutions were incubated at various temperatures or at 50 °C for various periods. Thereafter, 200  $\mu$ l of substrate was added and the residual activity monitored as described above.

*b) Acid Inactivation.* This was performed by maintaining the enzymes in 0.05 M phosphate-citrate buffers in the pH range of 2.0–8.0. (The pH remained constant during the inactivation procedure.) The solutions were incubated at these pH values for 15 min at 37 °C, then diluted with the citrate buffer to pH 4.4 and the residual activity determined.

### Treatment of Hexosaminidase A with Proteolytic Enzymes

Preparations of polymer-bound and of free hexosaminidase A were incubated with pronase (B grade, Calbiochem, U.S.A.) in 0.1 M sodium borate HCl buffer, pH 7.5, containing  $\text{CaCl}_2$  (5 mM). To 1.9 ml of 0.5 mg/ml solution of polymer-bound or of free hexosaminidase A, 0.1 ml pronase solution (1 mg/ml) was added and incubated at 37 °C. At various intervals aliquots were withdrawn and the activity determined immediately.

Macrophage cathepsins were extracted from cultured murine macrophages. The cells were harvested from thioglycolate-stimulated DBA/1 mice. The culturing procedure and enzyme extraction were described previously [17]. The macrophage lysate was passed through the affinity column (used for the adsorption of hexosaminidase) prior to the experiment in order to remove inherently present hexosaminidase. The resultant cell extract was added to the free and to the polymer-bound enzyme and the hexosaminidase activity determined either directly or following 75 min of preincubation at 37 °C. The proteolytic capacity of the macrophage lysate was tested on bovine hemoglobin [18] (4% w/v in citrate buffer, pH 4.4) and was shown to release about 0.5  $A_{280}$  units/h to the trichloroacetic-acid-soluble fraction, under the reaction conditions used for degradation of the hexosaminidase.

### Clearance Experiments

Clearance of enzyme from the blood was tested on locally bred female rabbits, weighing approx. 2.5 kg. Solutions of free and of polymer-bound hexosaminidase A, containing 60  $\mu\text{g}$  and 130  $\mu\text{g}$  enzyme respectively, were injected (in 0.2 ml) into the marginal vein of the ear of the rabbits and blood samples were taken starting 2 min thereafter. The enzymic activity in the serum at the various intervals up to 48 h was measured. The discrimination between the injected human enzyme and the rabbit endogenous hexosaminidase was performed using rabbit antibodies to the human enzyme.

## RESULTS

### Binding of Poly(*N*-vinylpyrrolidone) to Hexosaminidase A

Pure hexosaminidase A was coupled to activated poly(*N*-vinylpyrrolidone) at pH 8.4. The reaction mixture was chromatographed on Sephadex G-200 to remove unbound polymer as well as other products of the coupling reaction. The fractions (3 ml) were tested for enzymic activity and monitored for absorbance at 280 nm. As seen in Fig. 1, the polymer-

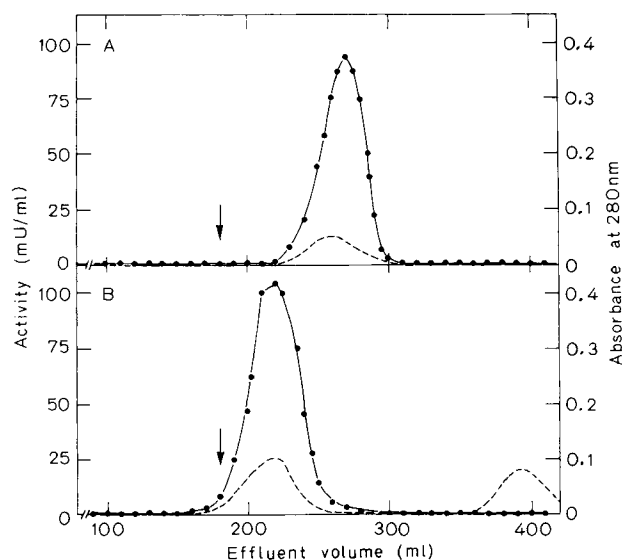


Fig. 1. Gel filtration of free (A) and polymer-bound (B) hexosaminidase A on Sephadex G-200 (2.6 × 88 cm). The arrow indicates the void volume as determined by the chromatography of dextran blue 2000. (●—●) Activity; (---) absorbance

bound enzyme was eluted from the column immediately following the void column, whereas free enzyme, when chromatographed separately on the same column (together with additional protein markers), was eluted by a larger volume as a sharp symmetrical peak. This indicated that all the enzyme in the reaction mixture was bound to the poly(*N*-vinylpyrrolidone).

Determination of enzymic activity before coupling and thereafter indicated that the binding to the polymer did not impair the enzymic activity towards the substrate and even caused some increase in activity (about 20%).

The  $K_m$  values of the polymer-bound hexosaminidase A for both fluorogenic substrates were found to be practically identical to the values obtained for the unmodified enzyme. Thus, the  $K_m$  values of the free and of the polymer-bound enzyme towards MeUmbGlcNAc were  $5.18 \times 10^{-4}$  M and  $4.93 \times 10^{-4}$  M respectively. The  $K_m$  values of the free and bound hexosaminidases towards MeUmbGalNAc, were  $1.12 \times 10^{-4}$  M and  $1.29 \times 10^{-4}$  M respectively. The ratio between the activities towards the two substrates was not affected by the binding of poly(*N*-vinylpyrrolidone).

Quantitative determination of poly(*N*-vinylpyrrolidone) and protein in the peak of the bound enzyme eluted from the Sephadex G-200 column showed that the ratio of poly(*N*-vinylpyrrolidone) to protein in the conjugate was on the average 9.2:1 on a molar basis. Thus the molecular weight of the conjugated enzyme is on the average 200000. This value was verified by analytical gel filtrations on a Biogel A 1.5 column.

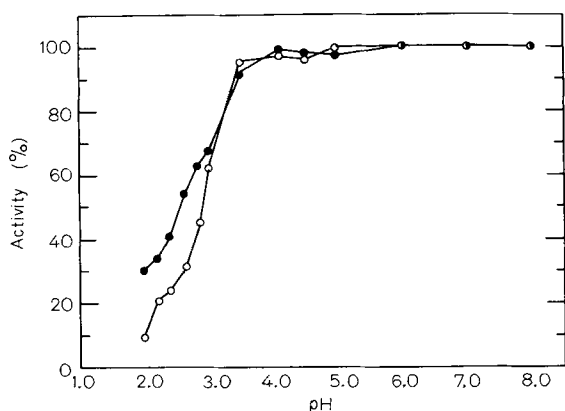


Fig. 2. pH inactivation curve of free (O) and bound (●) hexosaminidase A. The percentage of residual activity was calculated from the activity of untreated controls

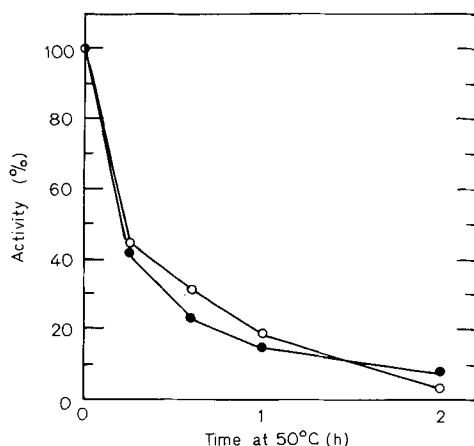


Fig. 3. Heat inactivation of free (O) and polymer-bound (●) hexosaminidase A. The enzyme solutions at pH 4.4 were heated for up to 2 h at 50 °C, then cooled and the activity determined. The percentage of residual activity was calculated from the activity of identical enzyme samples that were kept at 0 °C during the experiment

#### The Effect of Binding to Poly(*N*-vinylpyrrolidone) on Denaturation by Heat and pH

Hexosaminidase A was shown to be sensitive to both heat and acid [19,20]. The effect of polymer binding on this sensitivity was estimated as follows. The effect of short incubations at different pH values on the enzymic activity of both free and polymer-bound hexosaminidase A is shown in Fig. 2. The free enzyme underwent strong inactivation below pH 3.5 and reached a minimum value of about 8% of residual activity at pH 2.0. The polymer-bound enzyme showed a similar inactivation curve; it seemed to be slightly more resistant to acid treatment, but the difference between the free and the bound enzyme did not seem to be significant.

Heat inactivation was also unaffected by conjugation to poly(*N*-vinylpyrrolidone). When the two enzyme preparations were heated at pH 4.4 for

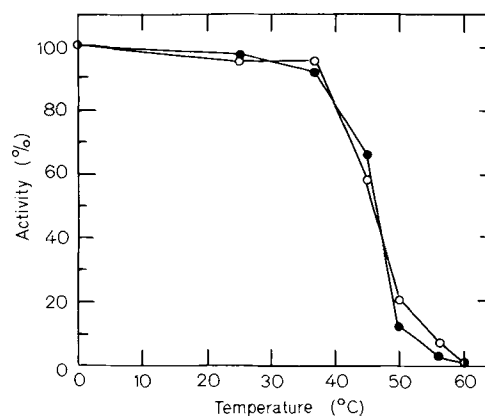


Fig. 4. Heat inactivation of free (O) and polymer-bound (●) hexosaminidase A. The enzyme samples were heated for 1 h at the various temperatures and the percentage of residual activity calculated as described in Fig. 3

periods up to 2 h at 50 °C, identical values were observed for the free enzyme as for the polymer-bound (Fig. 3). Similarly, heating of both enzymes at pH 4.4 for 1 h at various temperatures, up to 60 °C, also gave an identical pattern for the two preparations (Fig. 4), indicating that the binding of poly(*N*-vinylpyrrolidone) to the enzyme molecule did not alter its sensitivity to this physical denaturation.

#### Immunochemical Analysis of Polymer-Bound Hexosaminidase A

Two immunochemical methods were used for testing the overall changes in antigenic specificity conferred on the enzyme by coupling to poly(*N*-vinylpyrrolidone). The first technique was radial immunodiffusion in agarose gels containing either anti-hexosaminidase-B, which cross-reacts strongly with hexosaminidase A, or specific anti-hexosaminidase-A, an antiserum which reacts with antigenic determinants which are specific to hexosaminidase A and are not present on hexosaminidase B. As seen in Fig. 5, the polymer-bound enzyme retains its antigenic activity and can still react with the cross-reactive anti-hexosaminidase-B. On the other hand, the reaction with specific anti-hexosaminidase-A following coupling to poly(*N*-vinylpyrrolidone) was drastically impaired and the precipitin rings which formed can hardly be visualized.

It is worth mentioning that the precipitin rings formed between the anti-hexosaminidase-B and the polymer-bound enzyme appear more diffuse when compared to the rings of free hexosaminidase A and B, suggesting a less uniform reaction between the bound enzyme and the antibodies. This may indicate heterogeneity in the polymer-bound enzyme.

Similar results were obtained using antigen-binding experiments with <sup>125</sup>I-labelled free and bound enzymes. As depicted in Fig. 6, maximal binding of

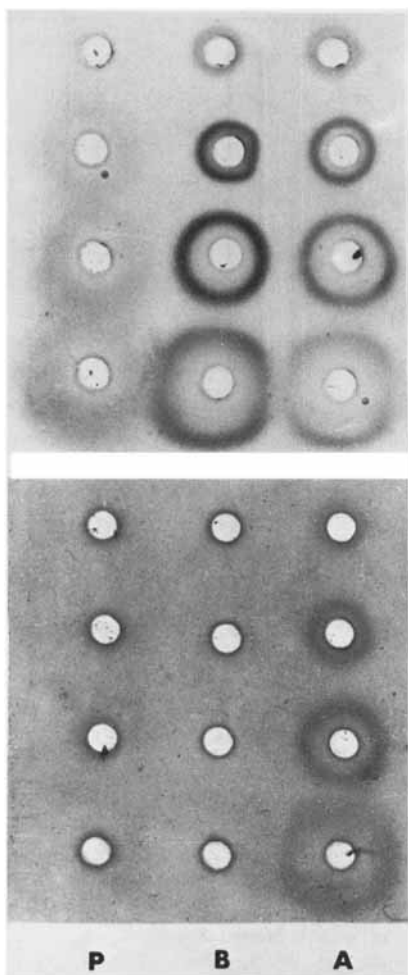


Fig. 5. Radial immunodiffusion of free and polymer-bound hexosaminidase with anti-hexosaminidase-B (top) or with specific anti-hexosaminidase-A (bottom). (A) Pure hexosaminidase A; (B) pure hexosaminidase B; (P) poly(*N*-vinylpyrrolidone)-bound hexosaminidase A. Four concentrations of each enzyme sample (1:3 dilutions) were applied to the gel

70–75% for either the poly(*N*-vinylpyrrolidone)-conjugated or the free enzyme were obtained with the anti-hexosaminidase-B antiserum, whereas specific anti-hexosaminidase-A bound 65% of the free enzyme as compared to 20–25% of the polymer-bound.

#### Stabilization of Hexosaminidase A towards Proteolytic Inactivation by the Coupling to Poly(*N*-vinylpyrrolidone)

The effect of coupling to poly(*N*-vinylpyrrolidone) on the susceptibility of hexosaminidase A to enzymic degradation was studied by comparing the inactivation of bound and free enzyme preparations following their subjection to pronase on the one hand, or to macrophage enzymes on the other. The results obtained with pronase are presented in Fig. 7.

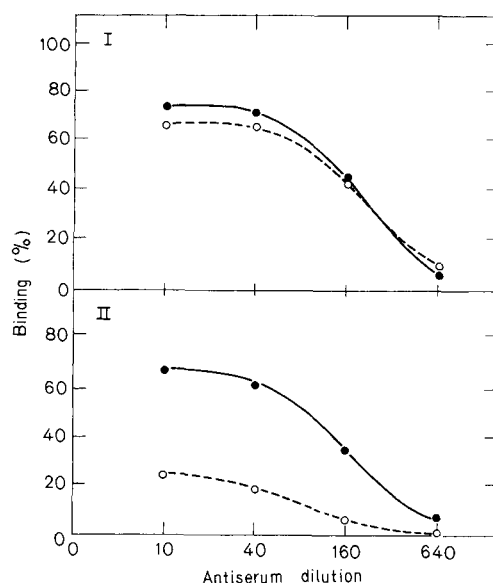


Fig. 6. Antigen-binding experiment of free (●) or polymer-bound (○) hexosaminidase A, tested with anti-hexosaminidase-B (I), or with specific anti-hexosaminidase-A (II). The percentage of bound radioactive enzyme found in the immune precipitate was calculated from the total radioactivity introduced after subtraction of non-specific binding (as tested with non-relevant goat antiserum)

The free enzyme loses its activity very fast (actually most of the inactivation is achieved even without preincubation of the enzyme with pronase prior to the hexosaminidase assay, but merely during the co-incubation with the hexosaminidase substrate). The polymer-bound enzyme, on the other hand, showed a marked stability, maintaining about 90% of the original activity after 30 min of preincubation. Addition of free poly(*N*-vinylpyrrolidone) to the incubation mixture of pronase and unbound hexosaminidase A had no protective effect. In this experiment the proper control, namely the level of hexosaminidase activity present in the pronase preparation (which was very low), was determined and subtracted from the experimental values.

Similar stabilization by polymer binding to hexosaminidase A was observed towards degradation by macrophage enzymes. The enzyme solutions (containing mouse lysosomal cathepsins) were tested for proteolytic activity on dialyzed bovine hemoglobin [17] and were shown to degrade it efficiently. The mixing of free hexosaminidase with the mouse cathepsins, even for the period of the hexosaminidase assay only (10 min), resulted in a strong inactivation of about 50% (Fig. 8). Longer preincubation periods resulted in slightly higher inactivation values. In contrast, polymer-bound enzyme was stable towards this treatment maintaining most of its activity, even following 75 min of preincubation with the cathepsin mixture.

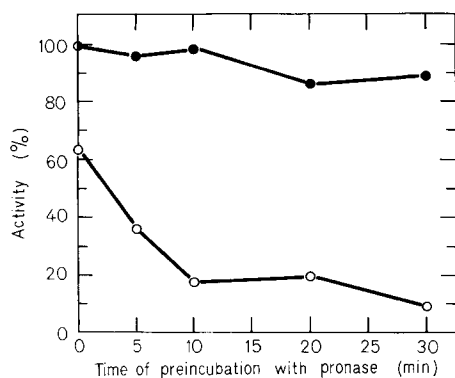


Fig. 7. Proteolytic inactivation of free (○) and polymer-bound (●) hexosaminidase A by pronase. The duration of the preincubation of the hexosaminidase preparation with the pronase, prior to the addition of the hexosaminidase substrate, is given on the abscissa. The percentage of residual activity was calculated from the activity of untreated hexosaminidase samples

#### Clearance of Polymer-Bound Hexosaminidase from Rabbit Blood

Solution of free hexosaminidase A and of poly(*N*-vinylpyrrolidone)-bound enzyme (60  $\mu$ g and 130  $\mu$ g respectively) were injected intravenously into rabbits and the clearance rate from the blood was followed for 48 h after the injection. The total hexosaminidase activity, which was found in the sera of the injected rabbits 2 min following the injection, was elevated approximately 40-fold as compared to the level of the endogenous rabbit enzyme, and this value of enzymic activity corresponding to the exogenous human enzyme which was present in the serum 2 min after the injection was taken as the 100% activity in the calculations of the clearance rate. Immunochemical analysis, in which rabbit antibodies to human hexosaminidase A were used for the selective determination of human *versus* rabbit enzyme, proved that the level of the remainder rabbit enzyme in the serum was constant throughout the experiment.

As seen in Fig. 9 the free human enzyme was cleared rapidly from the rabbit blood within 20–60 min after injection. In contrast, the polymer-bound hexosaminidase A remained in the blood for long periods with significantly slower clearance rate. This is manifested both in the slope of the initial clearance, and in the levels of remaining enzyme (approximately 20%) as long as 48 h following the injection.

#### DISCUSSION

In the present study it was demonstrated that hexosaminidase A may be conjugated to poly(*N*-vinylpyrrolidone) without any loss or change of its catalytic activity, at least towards the tested synthetic substrates. This finding enabled the investigation of

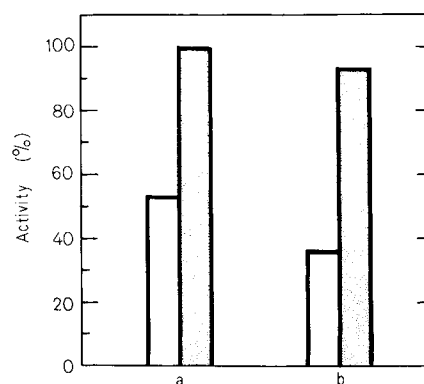


Fig. 8. Proteolytic inactivation of free (□) and polymer-bound (■) hexosaminidase A with macrophage enzymes. The residual activity was measured without preincubation (a) or after 75 min of preincubation (b). The calculation were done as described for Fig. 7

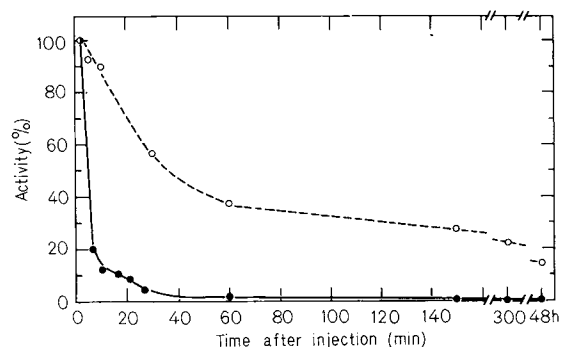


Fig. 9. Clearance of free hexosaminidase A (●) and of polymer-coupled hexosaminidase A (○) from rabbit serum. Each value represents the mean values obtained in groups of two animals. The value obtained in the first measurement following the injection (2 min) was taken as 100%

the effect of the conjugation on the behaviour of the enzyme towards external conditions.

An interesting observation is that the poly(*N*-vinylpyrrolidone) did not change the resistance of hexosaminidase towards physical denaturation by heat or acid. This is in contrast to the effect of antibody binding to the enzyme, as shown in a previous publication [12]. In that study we have shown that antibodies, and even their monovalent Fab fragment, protect hexosaminidase towards heat inactivation. This difference between antibody binding and poly(*N*-vinylpyrrolidone) conjugation is in accord with another finding reported here, namely that the attachment of polymer does not impair the antigenic activity. Nor does it enhance the immunogenicity; recent work has demonstrated that conjugation of this polymer to haptens decreases the immune response to the latter (Specht and Segal, unpublished data). This is an indication that the sites of binding of the antibodies on the one hand, and of the poly(*N*-vinylpyrrolidone) on the other, do not coincide or overlap.

Furthermore, this information may give us an insight into the mode of binding of antibodies and poly(*N*-vinylpyrrolidone) respectively, to hexosaminidase. This polymer is known to be conjugated *via* the amino groups of the protein [9]. On the other hand, antigenic determinants of globular proteins are mostly conformational [21,22] and may comprise regions which are distant in the sequence of the polypeptide, but happen to be in juxtaposition in the folded native protein. It is, therefore, logical to assume that antibodies exert their stabilization effect by locking the molecular conformation in an active state, whereas poly(*N*-vinylpyrrolidone) cannot provide such protection.

Conjugation to poly(*N*-vinylpyrrolidone) stabilized the hexosaminidase toward proteolysis. The proteolytic enzymes which were selected for this investigation are pronase and cathepsins; the former for its known capacity to hydrolyze, to a drastic extent, proteins even in their native state, and the latter for their relevance to potential degradation of hexosaminidase in the lysosomes. Both proteases were found to have a much lower effect on polymer-bound hexosaminidase than on the free enzyme. The criterion used for the proteolytic action was the loss of enzymic activity of hexosaminidase, namely the damage in region(s) which partake in the enzyme activity. It has been reported already that binding of enzymes to solid support increases their stability and resistance to proteolytic degradation (for review see [23]). The novelty in the present approach is that the conjugate with poly(*N*-vinylpyrrolidone) is a soluble product and is thus amenable for biological application.

The finding that polymer conjugation (but not merely its presence in the reaction mixture) protects hexosaminidase A against proteolysis may imply that it either interferes with the accessibility of the relevant sites to the proteolytic enzymes by steric hindrance, or that it directly blocks lysine side-chains which may serve as potential cleavage points of the pronase or the cathepsins. The first of these two hypotheses seems more plausible, since cleavage by neither protease is specific for particular amino acid residue.

Another factor which may be highly relevant for potential enzyme replacement therapy besides the stability against proteolytic inactivation is the rate of clearance of injected enzyme from the blood stream. Results of intravenous injection of hexosaminidase A into Tay-Sachs patient, reported by Brady and his group [6,7] showed that the enzyme is taken up rapidly from the blood, probably by the liver, and is almost completely cleared within 20 min. Our findings in rabbits confirm these results and show that indeed the free injected enzyme is cleared from the blood

circulation rapidly. Of the polymer-bound hexosaminidase A, in contrast, a considerable portion (approx. 20%) is retained in the blood stream for longer periods, of hours and days, and thus may be available for uptake as an enzymically active moiety.

In view of the data reported in this presentation it seems plausible that by binding to the soluble polymer polyvinylpyrrolidone, two major difficulties in enzyme replacement therapy, which have been discussed in the Introduction, may be overcome, namely, the fast clearance of enzyme by the liver, and its degradations *in vivo*. This may promote in the future the replacement of missing and defective enzymes in some metabolic disorders.

## REFERENCES

1. Pentchev, P. G., Brady, R. O., Gal, A. E., Hibbert, S. R. & Dekaban, A. S. (1974) *Fed. Proc.* 33, 1300.
2. Brady, R. O., Pentchev, P. G., Gal, A. E., Hibbert, S. R. & Dekaban, A. S. (1974) *New Engl. J. Med.* 291, 989–93.
3. Brady, R. O., Tallman, J. F., Johnson, W. G., Gal, A. E., Leahy, W. R., Quirk, J. M. & Dekaban, A. S. (1973) *New Engl. J. Med.* 289, 9–14.
4. O'Brien, J. S. (1973) in *Lysosomes and Storage Diseases*, Hers & Van-Hoof, eds p. 342, Academic Press, N.Y.
5. Schneck, L., Amsterdam, D., Brooks, S. E., Rosenthal, A. L. & Volk, B. W. (1973) *Pediatrics*, 52, 221.
6. Johnson, W. G., Desnick, R. J., Long, D. M., Sharp, H. L., Krivit, W., Brady, B. & Brady, R. O. (1973) *Enzyme Therapy in Genetic Diseases*, vol. 9, pp. 120–124, Williams & Wilkins, Baltimore.
7. Brady, R. O., Pentchev, P. G. & Gal, A. E. (1975) *Fed. Proc.* 34, 1310–1315.
8. von Specht, B. U., Wahl, M., Kolb, H. J. & Brendel, W. (1975) *Arch. Int. Pharmacodyn. Ther.* 213, 242–256.
9. von Specht, B. U., Seinfeld, H. & Brendel, W. (1973) *Hoppe Seyler's Z. Physiol. Chem.* 354, 1659–1660.
10. Geiger, B., Ben Yoseph, Y. & Arnon, R. (1974) *FEBS Lett.* 45, 276–281.
11. Geiger, B., Navon, R., Ben Yoseph, Y. & Arnon, R. (1975) *Eur. J. Biochem.* 56, 311–318.
12. Ben Yoseph, Y., Geiger, B. & Arnon, R. (1975) *Immunochimistry*, 12, 221–226.
13. Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* 56, 658–666.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1971) *J. Biol. Chem.* 193, 265–275.
15. Poullain, P. & Pielte, M. (1948) *Bull. Soc. Chim. Biol.* 30, 496.
16. Marchalonis, J. J. (1969) *Biochem. J.* 113, 299–305.
17. Geiger, B. & Gallily, R. (1974) *Clin. Exp. Immunol.* 16, 643–655.
18. Barrett, A. J. (1967) *Biochem. J.* 104, 601–608.
19. Robinson, D. & Stirling, J. L. (1968) *Biochem. J.* 107, 321–327.
20. Saifer, A. & Rosenthal, A. (1973) *Clin. Chim. Acta*, 43, 417–421.
21. Sela, M., Schechter, B., Schechter, I. & Borek, F. (1967) *Cold Spring Harbor Symp. Quant. Biol.* 32, 537–545.
22. Crumpton, M. J. (1974) *The Antigens* (Sela, M., ed.) vol. 2, p. 1, Academic Press, N.Y.
23. Zabrosky, O. (1973) *Immobilized Enzymes*, CRC Press.