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Specific binding of ^{125}I-labeled β-hexosaminidase A to rat brain synaptosomes

(glycoproteins/receptor-mediated uptake/Tay-Sachs disease/β-*N*-acetylglucosaminidase)

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ABSTRACT **Purified human β-hexosaminidase A (β-*N*-acetylglucosaminidase; 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) has been labeled with ^{125}I to high specific activity with the retention of 80% of its enzyme activity. The binding of this enzyme to sonicated synaptosomes from rat brain was shown to be a saturable and specific process. Glycoproteins containing a sialic acid-terminal oligosaccharide or a galactose-terminal oligosaccharide (i.e., α_1-acid glycoprotein and fetuin and their asialo derivatives) were strong inhibitors of the binding. In contrast, ovalbumin, which contains a mannose-rich oligosaccharide, and mannans were poor inhibitors of the binding. Of the monosaccharides tested, sialic acid, galactosamine, mannose, galactose, and lactose were inhibitory in decreasing potency of inhibition. Optimal binding occurred at pH 7.0 in the presence of 3 mM calcium ions. The binding was a linear function of synaptosomal protein concentration between 25 and 200 μg of protein per assay and was directly proportional to time up to 3 hr, beyond which there was no further increase in specific binding. The data suggest a unique but complex mode of interaction of glycoproteins with receptors on synaptic membranes.**

The metabolism of glycoproteins has gained interest with the discovery of numerous systems involved in their uptake and sequestration in different tissues. The discovery, purification, and subcellular localization of a mammalian hepatic receptor capable of binding galactose-terminal glycoproteins by Ashwell and coworkers (1–3) has led to the elucidation of distinct receptor systems in avian and mammalian liver specific for *N*-acetylglucosaminyl-terminal glycoproteins (4–6), in mammalian liver for mannosyl- (7) and fucosyl- (8) terminal glycoproteins, in alveolar macrophages for mannose and *N*-acetylglucosaminyl-terminal glycoproteins (9), and in fibroblasts for mannose 6-phosphate-containing glycoproteins (10). These animal lectins may play a significant role in the removal of senescent erythrocytes, the turnover of cellular glycoproteins, and the clearance of exogenous glycoproteins from the circulation. Because a number of, if not all, lysosomal glycosidases are glycoproteins, their plasma levels and subcellular localization may depend upon their carbohydrate composition and ultimately upon their interaction with these various receptors. Specifically, it has recently been shown that native human placental glucocerebrosidase infused into rats is cleared by the liver with a half-life of 21 min and is distributed between hepatocytes and Kupffer cells (11). In contrast, the asialo derivative of glucocerebrosidase is cleared rapidly and is sequestered predominantly in hepatocytes. The agalactoglucocerebrosidase is also cleared rapidly, and it is found in increased association with Kupffer cells (12).

The following studies were undertaken to determine whether analogous receptor systems are present in brain tissue and to characterize the oligosaccharide recognition markers. The goal of these studies was to establish a theoretical basis for the infusion of purified enzymes into patients suffering from inherited lipid storage disorders affecting the central nervous system. Effective therapy for these individuals may require not only alteration of the blood–brain barrier so that the requisite enzyme may gain access to the brain (13) but also the presence of high-affinity receptor-mediated uptake systems for entry of the infused enzyme into the cells.

MATERIALS AND METHODS

Iodination of β-Hexosaminidase A. Human placental β-hexosaminidase A (β-*N*-acetylglucosaminidase; 2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) was purified and assayed according to Tallman *et al.* (14). Iodination of the purified enzyme was carried out by using Enzymobeads from Bio-Rad Laboratories. The reaction mixture contained, in a 1-ml plastic capped tube, 50 μg of β-hexosaminidase A, 25 μl of 0.5 M sodium phosphate buffer at pH 7.2, 25 μl of Enzymobeads prepared according to the manufacturer's instructions, 1.5 mCi of Na^{125}I (Amersham) (1 Ci = 3.7×10^{10} becquerels), and water in a volume of 175 μl. Iodination was initiated by the addition of 25 μl of 1% equilibrium D-glucose (64% β, 36% α) and the reaction was allowed to proceed at room temperature for 20 min. At the conclusion of the iodination, 5-μl aliquots were removed for determination of enzyme activity and incorporation of ^{125}I into protein. Free iodine was separated from iodinated protein by passing the entire reaction mixture over a 0.5-ml column of concanavalin A-Sepharose previously equilibrated with 10 mM phosphate buffer at pH 6.5 containing 1% human serum albumin. After the reaction vessel had been rinsed with buffer, the gel was washed with 15 ml of buffer to remove the free iodine. The enzyme was eluted with buffer containing 1 M NaCl, 0.5 M α-methyl D-mannoside and 1% human serum albumin and dialyzed against six changes, 500 ml each, of phosphate-buffered saline at pH 7.4.

Preparation of Synaptosomes. Rat brain synaptosomes were prepared by a modification of the method of Whittaker *et al.* (15) as modified by Colburn *et al.* (16). The synaptosomal fraction was aspirated from the centrifuge tube, dispersed with 0.32 M sucrose, and pelleted at $48,000 \times g$ for 20 min. The synaptosomes were taken up in a small volume of 0.32 M sucrose and 0.1-ml aliquots were individually stored at −20°C until use. Purity of the preparations was ascertained by enzymatic assay of the fractions using 5′-nucleotidase, ATPase, and β-hexosaminidase.

Binding Assay. The assay procedure was similar to that of Stahl *et al.* (9). Reactions were carried out in 12 × 75 mm plastic

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Abbreviations: Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

tubes. The standard assay buffer contained 20% fetal calf serum, 10 mM Hepes, 10 mM 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid (Bes), 15 mM 1,4-piperazinediethanesulfonic acid (Pipes), 3 mM $CaCl_2$ in Dulbecco's modified Eagle's medium at pH 7.0. Each reaction tube contained 9 ng of labeled protein (approximately 0.3–1.0 × 10^6 dpm), 100 μg of synaptosomal protein, and buffer in a final volume of 150 μl. Reaction blanks contained no synaptosomes and consistently gave backgrounds of less than 0.5% of the total radioactivity in the reaction mixture. When appropriate, various amounts of inhibitors were added in buffer to the reactions. The reaction was initiated by the addition of sonicated synaptosomes, and the mixtures were incubated for 3 hr with shaking at 37°C. The reaction was stopped by transferring the tubes to an ice bath for 2 min and then quickly transferring the contents of each tube to a 4.8 × 19.9 mm cellulose proprionate tube (Beckman). The reaction tubes were washed with 25 μl of buffer which was added to the reaction mixtures. The suspension was centrifuged for 30 sec at 100,000 × *g* in a Beckman Airfuge. The supernatant was aspirated and the pellets were washed once with 175 μl of buffer and recentrifuged. After aspiration, the bottoms of the tubes containing the pellets were cut off, placed in scintillation vials, shaken with 10 ml of Bio-Solv HP, and assayed for radioactivity in a Searle Mark III liquid scintillation system. Counting efficiency for ^{125}I was 41%. Duplicate samples were prepared for each reaction and each experiment was done in triplicate. Specific binding represents the difference between the total amount of label bound in the absence and presence of an excess of asialofetuin.

RESULTS

Labeling of β-Hexosaminidase A. The labeling of β-hexosaminidase A with ^{125}I by use of the Enzymobeads gave greater than 85% incorporation of iodine, with the retention of enzymatic activity and presumably conformational integrity. In two preparations of labeled enzyme, greater than 80% of the enzymatic activity remained in each case. Furthermore, the labeled enzyme was cleared from the circulation of the rat with a half-time indistinguishable from that of the native enzyme, and an excess of native enzyme was able to block the clearance of the labeled enzyme (unpublished observations).

Optimization of Binding. Optimum binding occurred using Dulbecco's modified essential medium supplemented with 20% fetal calf serum and buffered with Hepes, Bes, and Pipes. Other buffers such as 35 mM Tris or 25 mM Hepes, each supplemented with 20% fetal calf serum, were not as effective as Dulbecco's medium in causing a high degree of specific binding. Binding was maximal after incubation with shaking for 3 hr at 37°C (Fig. 1). Further incubation decreased the binding slightly. The addition of Ca^{2+} to the incubation buffer at a concentration of 3 mM enhanced binding about 1.5-fold over unsupplemented buffer. However, the presence of Ca^{2+} in the synaptosome preparation cannot be ruled out. Fig. 2 indicates that the pH optimum for binding is 7.0. The binding was linear over a range of 25 to 200 μg of synaptosomal protein. The amount of specific binding decreased slightly above 200 μg of protein per assay.

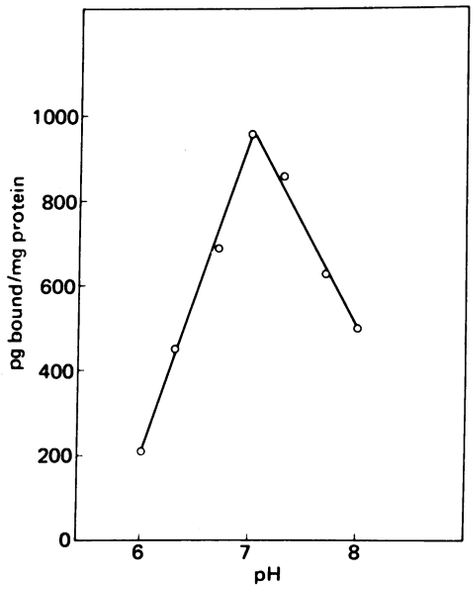

FIG. 2. Effect of pH on specific binding of hexosaminidase to rat brain synaptosomes. Reactions were carried out as described in *Materials and Methods* except that the pH of the buffer was varied between 6.0 and 8.0

Kinetics. The binding of β-hexosaminidase to rat brain synaptosomes was saturable as indicated in Fig. 3, and although the kinetics appear to be complex, the dissociation constant K_d was estimated to be approximately 1 nM. The binding was in-

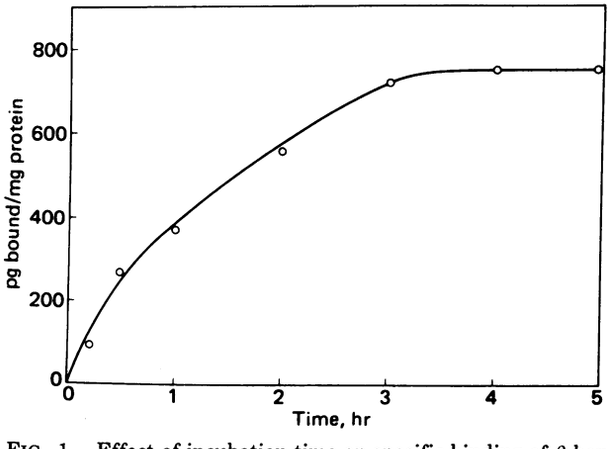

FIG. 1. Effect of incubation time on specific binding of β-hexosaminidase A to rat brain synaptosomes. Reactions were carried out as described in *Materials and Methods* except that the length of incubation was varied between 15 min and 5 hr. The points on the graph are the averages of three experiments.

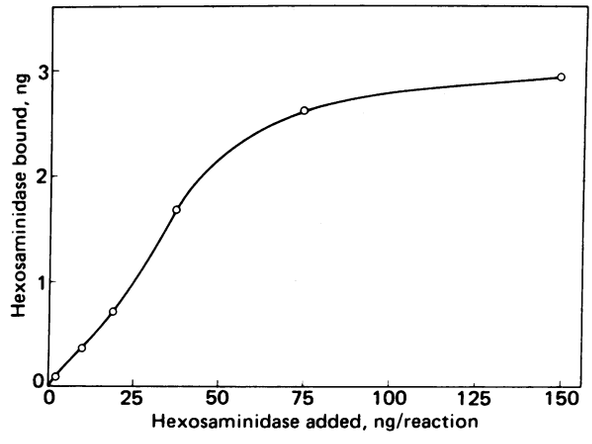

FIG. 3. Effect of substrate concentration on amount of specific binding. The procedure outlined in *Materials and Methods* was followed except that the substrate was varied between 2 and 150 ng per assay.

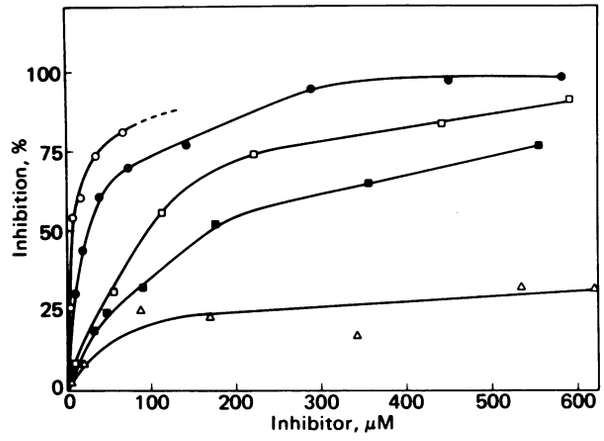

FIG. 4. Inhibition of binding by various glycoproteins and their derivatives. Increasing amounts of glycoproteins dissolved in buffer were added to the standard binding reaction and percent inhibition of binding was calculated as the ratio of label bound in the presence of inhibitor to the amount bound in the absence of inhibitor × 100. ○, Asialo-α_1-acid glycoprotein; ●, α_1-acid glycoprotein; □, asialofetuin; ■, fetuin; △, mannan.

hibited by a number of glycoproteins and their derivatives (Fig. 4). The asialo derivatives of orosomucoid and fetuin, both galactose-terminal glycoproteins, were more potent inhibitors of binding than the intact glycoproteins. Mannans and ovalbumin, which contains mannose-rich oligosaccharides, both exhibited the same poor inhibition of binding. The inhibition by various sugars was also examined (Fig. 5). Sialic acid was the best inhibitor, followed in order of decreasing potency by galactosamine, mannose, galactose, and lactose.

DISCUSSION

The results indicate that a high-affinity, saturable binding system that is capable of binding glycoproteins is present in rat brain synaptosomes. The binding appears to be complex in that a number of different oligosaccharides and monosaccharides will effectively block the binding of β-hexosaminidase A. The inhibition of binding by asialo derivatives of α_1-acid glycoprotein and fetuin and by galactose is reminiscent of the properties of the binding protein present on mammalian liver hepatocytes that binds galactose-terminal glycoproteins (1). It

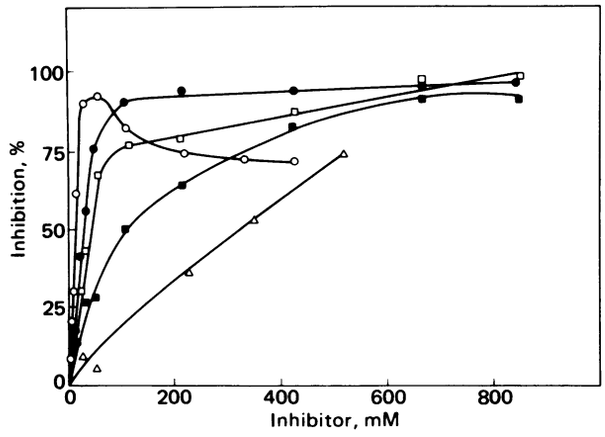

FIG. 5. Inhibition of binding by carbohydrates. Increasing amounts of carbohydrates were added to the standard binding reaction and the percent inhibition of binding was calculated as described in the legend to Fig. 4. ○, Sialic acid; ●, galactosamine; □, mannose; ■, galactose; △, lactose.

appears that the mannan-binding protein isolated from mammalian liver (7) is not functional in the synaptosomal system, because mannans and ovalbumin were only weak inhibitors of the binding.

It is difficult to reconcile the relative inhibitory capacity of the asialo derivatives and the intact glycoproteins with the monosaccharides sialic acid and galactose, because the asialoglycoproteins are more potent inhibitors than the respective sialic acid-terminal glycoproteins, whereas sialic acid is a more potent inhibitor than galactose. On the other hand, concentration of monosaccharides causing approximately 50% inhibition of binding is about 2500-fold higher than that of the inhibitory glycoproteins, and the monosaccharide inhibition patterns may not be representative of the physiologically important ligand–receptor interactions. Furthermore, the conformation of the individual monosaccharides in the oligosaccharide chain might be crucial for interaction with the receptor and it may differ from that exhibited by the free monosaccharides in solution. The possibility also exists that multiple sites on the receptor are involved in the binding of more than one monosaccharide unit in an oligosaccharide chain. This might explain why, although mannans and ovalbumin are weak inhibitors of binding, mannose alone is a rather potent inhibitor. Mannose may be interacting with one part of the receptor binding site, preventing ideal interaction of the intact oligosaccharide with the receptor.

The inhibition of binding by lactose and galactosamine was examined in view of the recent finding (17) that these sugars were capable of blocking the agglutination of erythrocytes by various proteins of brain, implying the presence of specific lectins on the surface of brain cells. Our results substantiate the presence of a high-affinity galactosamine-specific lectin molecule and perhaps a lower-affinity lactose-specific lectin. On the other hand, these results might reflect a relaxed specificity of a galactose receptor system, allowing both of these sugars to inhibit the binding.

The functional significance of the receptor system bears consideration. Because the levels of certain brain lectins seem to vary with age (17), it has been suggested that they play a role in developmental processes and may be responsible for cell–cell recognition and the formation of specific neurological pathways in the developing brain. Alternatively, the presence of these receptors on the membranes forming synaptic junctions between neuronal cells may mediate the reuptake of glycoproteins that are released into the synaptic cleft during nerve transmission (18). A third functional possibility for this system may be a role in scavenging of molecules that have escaped from dying brain cells or that have passed the blood–brain barrier in pathological conditions.

The results reported here provide a theoretical basis for therapeutic trials in individuals suffering from inherited storage disorders affecting the central nervous system such as Tay–Sachs disease. A number of investigators (13, 19) are examining the opening of the blood–brain barrier to permit entry of proteins into the substance of the brain. We have also found that the blood–brain barrier can be temporarily opened in rats, permitting entry of β-hexosaminidase A into the brain cells (unpublished observations). However, for effective reduction of pathological quantities of accumulating substances, a high-affinity receptor system would seem to be required to ensure the rapid uptake of a significant amount of enzyme within the cells and ultimately within the lysosomes. In this regard we have also found that rat brain lysosomes, free from membrane marker enzymes, contain binding activity with a specificity similar to that of the synaptosomes reported here (unpublished observations).

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1. Morell, A. C., Gregoriadis, G., Scheinberg, I. H., Hickman, J. & Ashwell, G. (1971) *J. Biol. Chem.* **246,** 1461–1467.
2. Hudgin, R. L., Pricer, W. E., Jr., Ashwell, G., Stockert, R. J. & Morell, A. C. (1974) *J. Biol. Chem.* **249,** 5536–5543.
3. Pricer, W. E., Jr. & Ashwell, G. (1976) *J. Biol. Chem.* **251,** 7539–7544.
4. Lunney, J. & Ashwell, G. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 341–343.
5. Kawasaki, T. & Ashwell, G. (1977) *J. Biol. Chem.* **252,** 6536–6543.
6. Stockert, R. J., Morell, A. G. & Scheinberg, I. H. (1976) *Biochem. Biophys. Res. Commun.* **68,** 988–993.
7. Kawasaki, T., Etoh, R. & Yamashina, I. (1978) *Biochem. Biophys. Res. Commun.* **81,** 1018–1024.
8. Prieels, J. P., Pizzo, S. V., Glasgow, L. R., Paulson, J. C. & Hill, R. L. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2215–2219.
9. Stahl, P. D., Rodman, J. S., Miller, M. J. & Schlesinger, P. H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1399–1403.
10. Kaplan, A., Achord, D. T. & Sly, W. S. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2026–2030.
11. Furbish, F. S., Steer, C. J., Barranger, J. A., Jones, E. A. & Brady, R. O. (1978) *Biochem. Biophys. Res. Commun.* **81,** 1047–1053.
12. Steer, C. J., Furbish, F. S., Barranger, J. A., Brady, R. O. & Jones, E. A. (1978) *FEBS Lett.* **91,** 202–205.
13. Barranger, J. A., Rapoport, S. I., Fredericks, W. R., Pentchev, P. G., MacDermot, K. D., Steusing, J. K. & Brady, R. O. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 481–485.
14. Tallman, J. F., Brady, R. O., Quirk, J. M., Villalba, M. & Gal, A. E. (1974) *J. Biol. Chem.* **249,** 3489–3499.
15. Whittaker, V. P., Michaelson, I. A. & Kirkland, R. J. A. (1964) *Biochem. J.* **90,** 293–303.
16. Colburn, R. W., Goodwin, F. K., Murphy, D. L., Bunney, W. E., Jr. & Davis, J. M. (1968) *Biochem. Pharmacol.* **17,** 957–964.
17. Simpson, D. L., Thorne, D. R. & Loh, H. H. (1977) *Nature (London)* **266,** 367–369.
18. Wallace, E. F. & Lovenberg, W. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 3217–3220.
19. Rapoport, S. I. (1976) *Blood-Brain Barrier in Physiology and Medicine* (Raven, New York).