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Purification and Properties of the Hexosaminidase A-Activating Protein from Human Liver

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Human liver extracts contain an activating protein which is required for hexosaminidase A-catalysed hydrolysis of the *N*-acetylgalactosaminyl linkage of G_{M2} ganglioside [*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)galactosylglucosylceramide]. A partially purified preparation of human liver hexosaminidase A that is substantially free of G_{M2} ganglioside hydrolase activity is used to assay the activating protein. The procedures of heat and alcohol denaturation, ion-exchange chromatography and gel filtration were used to purify the activating protein over 100-fold from crude human liver extracts. When the purified activating protein is analysed by polyacrylamide-gel disc electrophoresis, two closely migrating protein bands are seen. When purified activating protein is used to reconstitute the G_{M2} ganglioside hydrolase activity, the rate of reaction is proportional to the amount of hexosaminidase A used. The activation is specific for G_{M2} ganglioside and and hexosaminidase A. The activating protein did not stimulate hydrolysis of asialo-G_{M2} ganglioside by either hexosaminidase A or B. Hexosaminidase B did not catalyse hydrolysis of G_{M2} ganglioside with or without the activator. Kinetic experiments suggest the presence of an enzyme–activator complex. The dissociation constant of this complex is decreased when higher concentrations of substrate are used, suggesting the formation of a ternary complex between enzyme, activator and substrate. Determination of the molecular weight of the activating protein by gel-filtration and sedimentation-velocity methods gave values of 36000 and 39000 respectively.

An activating protein that stimulates the hydrolysis of G_{M2} ganglioside‡ catalysed by the human liver hexosaminidase A (*β*-*N*-acetylglucosaminidase, EC 3.2.1.30) has been identified in extracts of human liver (Hechtman, 1977). Li & Li (1976) have purified an activating protein from human liver that enhances the activity of a number of glycosphingolipid hydrolases. The requirement for specific proteins that activate the hydrolysis of ceramide-containing substrates *in vitro* has been reported by Ho & O'Brien, (1971) and Fischer & Jatzkewitz (1975).

Activating proteins may be important in the catabolism of gangliosides and glycolipids *in vivo*. The replacement of activating proteins in the glycolipid hydrolase incubation mixtures by bile salts (Wenger *et al.*, 1975) suggests that the protein activators may function as natural detergents.

In the present paper we report the purification of the hexosaminidase A-activating protein from human liver. In purest preparations two protein-staining bands migrate close together when subjected to polyacrylamide-gel electrophoresis. The molecular weight and some properties of the activating protein were determined.

‡ Abbreviations: G_{M2} ganglioside, *N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)galactosylglucosylceramide; asialo-G_{M2} ganglioside, *N*-acetylgalactosaminylgalactosylglucosylceramide [after nomenclature of Svennerholm (1963)]; 4MU-*N*-acetylglucosaminide, 4-methylumbelliferyl 2-deoxy-2-acetamido-*β*-D-glucopyranoside; SDS, sodium dodecyl sulphate.

Experimental

Materials

Adult human liver, obtained within 4h of death, was processed immediately. G_{M2} ganglioside, labelled with ^{3}H in the *N*-acetylgalactosamine moiety, was a gift from Dr. L. S. Wolfe of the Montreal Neurological Institute. The details of the tritiation procedure, proof of radiochemical purity, and confirmation of the location of the ^{3}H label have been previously described (Hechtman, 1977). The substrate asialo-G_{M2} ganglioside was prepared by mild acid hydrolysis of ^{3}H-labelled G_{M2} ganglioside followed by partition of the hydrolysate by the method of Folch *et al.* (1957).

The synthetic hexosaminidase substrate 4-methylumbelliferyl 2-deoxy-2-acetamido-*β*-D-glucopyranoside was purchased from Koch–Light (Edmonton, Alb., Canada). Gel-filtration and ion-exchange

materials were products of Pharmacia (Uppsala, Sweden).

Methods

Hexosaminidase was assayed by the method of Leaback & Walker (1961). Hydrolysis of the terminal *N*-acetylgalactosaminyl residue of G_{M2} ganglioside was measured by the method of Tallman & Brady (1972). One unit of activating protein activity is defined as the amount that increases the hexosaminidase A-catalysed hydrolysis of G_{M2} ganglioside by 1pmol/16h at 37°C. The standard incubation mixture for the assay of activating protein contained: 50mM-sodium citrate, pH4.0, 10mM-NaN_3, 10μM-^{3}H-labelled G_{M2} ganglioside (specific radioactivity 5.5×10^8 c.p.m./mmol), 600 units of partially purified human liver hexosaminidase A (a unit of hexosaminidase activity is that amount which catalyses the hydrolysis of 1mmol of 4MU-*N*-acetylglucosaminide/min at 37°C, pH4.4) and various amounts of activating protein in a volume of 200μl. All preparations of hexosaminidase A used in the assay of the activator were prepared by a three-step procedure described previously (Hechtman, 1977). The hexosaminidase A had an endogenous enzyme activity towards G_{M2} ganglioside of $<1.5\times10^{-7}$ mol/mol of 4MU-*N*-acetylglucosaminide. In each determination of activating protein activity several concentrations of activator were used to ensure that the velocity of substrate hydrolysis was proportional to activator concentration.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) as a standard. Protein in column eluates was monitored by measuring A_{280}.

Purification of activating protein

Dialysis. Liver samples (100g) were minced and homogenized in a Waring blender three times for 1 min with 200ml of 20mM-sodium phosphate buffer, pH5.5, containing 0.03% (v/v) Triton X-100. The homogenate was centrifuged at 13000*g* for 15min and the supernatant dialysed for 72h against 10vol. of 2.5mM-sodium citrate/phosphate, pH4.4, with three changes of buffer. Precipitated protein was removed by centrifugation at 16000*g* for 30min. All procedures were conducted at 4°C unless otherwise stated. The dialysed supernatant was stored at −20°C until further use.

Heat denaturation. Flasks containing 400ml of dialysed supernatant were placed in a boiling-water bath with rapid stirring until the temperature of the protein solution reached 57°C. The solution was then rapidly transferred to a 60°C bath and kept at this temperature for 30min. Heating was terminated by immersing the container in an ice–water bath. Denatured protein was removed by centrifugation at 16000*g* for 30min.

Ethanol precipitation. The supernatant solution from the previous step (376ml), chilled to 0°C, was rapidly added to 95% (v/v) ethanol at −15°C until the resulting mixture reached 40% ethanol concentration. The mixture was stirred at −15°C for 30min and then centrifuged at 16000*g* at −11°C for 30min. The supernatant was dialysed overnight at −10°C against 20mM-sodium phosphate, pH6.5, containing 20% (v/v) glycerol to prevent freezing. Glycerol was removed by two subsequent dialyses at 4°C in 20mM-sodium phosphate buffer, pH6.5. The dialysed ethanol supernatant was then concentrated to 17ml by ultrafiltration in an Amicon Diaflo pressure cell equipped with a PM-30 membrane filter (exclusion limit 30000mol.wt.).

Sephadex G-100 chromatography. Sephadex G-100 was swollen and equilibrated in 20mM-sodium phosphate, pH6.5, and packed into a column (53cm×2.5cm). Sucrose crystals (1g) were dissolved in the concentrated ethanol supernatant and the viscous sample was applied to the column at a flow rate of 21ml/h; 4ml fractions were collected. Fractions that contained the activating protein were pooled and used for further purification.

Ion-exchange chromatography. DEAE-Sephadex (A-50) was swollen and equilibrated in 20mM-sodium phosphate, pH7.5 and packed into a column (6.5cm×2.2cm). The activating-protein fraction obtained after Sephadex G-100 chromatography was adjusted to pH7.5 with NaOH and applied to the column at a flow rate of 8ml/h. Unadsorbed protein was removed by elution with the phosphate buffer and then a linear gradient was applied by the use of a mixing chamber containing 125ml of 20mM-sodium phosphate, pH7.5, and a reservoir containing 125ml of 20mM-sodium phosphate/0.3M-NaCl, pH7.5, and fractions (2.5ml) were collected. Fractions with activating-protein activity were pooled and concentrated against Aquacide II (Calbiochem, La Jolla, CA, U.S.A.) to a volume of 2.0ml.

Sephadex G-200 chromatography. Sephadex G-200 was swollen and equilibrated in 20mM-sodium phosphate, pH6.5, and packed into a column (57cm×1.5cm). Sucrose crystals (200mg) were dissolved in the concentrated activating-protein preparation obtained from the previous step and the sample was applied to the column at a flow rate of 6ml/h and fractions (1ml) were collected. The fractions with activating-protein activity were pooled and concentrated to 2.0ml against Aquacide II.

Analytical methods

Polyacrylamide-gel disc electrophoresis was carried out at pH8.3 by the method of Davis (1964) or at

pH 4.5 by the method of Reisfeld *et al.* (1962). The gels were stained with Amido Black and glycoproteins were stained by the periodate/Schiff reaction (Zacharius *et al.*, 1969). Hexosaminidase activity was detected by the staining procedure of Hayase *et al.* (1973). SDS/polyacrylamide-gel electrophoresis was by the method of Maizel (1969). Molecular-weight standards were ovalbumin and chymotrypsinogen (Pharmacia) and bovine serum albumin and cytochrome *c* (Sigma). Samples containing 100 μg of protein were boiled for 1 min in 2-mercaptoethanol (0.14 M) and SDS (1%). Gel electrophoresis was carried out at 15 mA/gel for 8 h at room temperature (21°C). Protein was stained with Amido Black.

Sucrose-density-gradient centrifugation was carried out at 49000 *g* for 17 h in a Beckman L-2 refrigerated ultracentrifuge with a SW 50L rotor. Molecular-weight determinations were made from sedimentation-velocity values with reference to molecular-weight standards by using the empirical formula of Martin & Ames (1961).

A molecular-weight determination was performed by using Sephadex G-200 by the method of Andrews (1964). Molecular-weight standards were ovalbumin, chymotrypsinogen, aldolase and ribonuclease (Pharmacia). Void volume and total column volume were determined from elution volumes of Blue Dextran and adenosine respectively.

Results

Purification of activating protein

The purification of the activating protein is summarized in Table 1. No activity was detectable in either the liver homogenate or in the supernatant. Activity was detectable only after the liver supernatant was dialysed at acidic pH and low ionic strength, which precipitated one-half to two-thirds of the supernatant protein. The final purification ratio may thus be underestimated by a factor of 2–3.

The activating protein was stable in the heat and ethanol steps of the purification. However, heating the activating-protein preparations for 5 min at 100°C destroys 80–90% of the activity. Considerable losses of activity occur also when ethanol fractionation is attempted at temperatures higher than 0°C.

The elution profile of activating protein from Sephadex G-100 is shown in Fig. 1. DEAE-Sephadex chromatography (Fig. 2) consistently resulted in activity losses of 60–80%. We did not investigate whether some of the activity was retained on the column at the end of the gradient elution or whether the column resolved the activity into two or more distinct fractions, all of which were necessary for complete recovery of activity. No activity was eluted with the non-adherent fraction that contains 85% of the applied protein.

The elution profile of activating protein from Sephadex G-200 is shown in Fig. 3. The peaks of activity and protein did not coincide exactly. However, gel electrophoresis of an equal amount of activating protein obtained from either side of the activity peak shown in Fig. 3 did not reveal contaminant protein in the activating-protein samples obtained from column fractions with lower specific activity.

Table 1. *Purification of activating protein*

A unit of activity is defined as that amount of activating protein that increases the rate of *N*-acetylgalactosamine release from G_{M2} ganglioside by 1 pmol/16 h in a reaction mixture containing 600 units of hexosaminidase A from which activating protein has been removed by Sephadex G-100 chromatography.

Procedure	Protein (mg)	Total activity (units)	Specific activity (units/mg)
Liver supernatant	13400	—	—
Dialysis	4990	1020000	204
Heat denaturation	1960	829000	423
Ethanol precipitation	1120	640000	572
Sephadex G-100 chromatography	271	502000	1850
DEAE-Sephadex chromatography	13.2	158000	12000
Sephadex G-200 chromatography	5.1	123000	24100

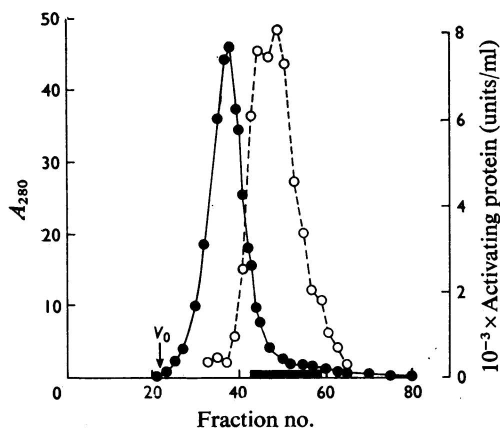

Fig. 1. *Sephadex G-100 chromatography of activating protein*

Activating protein (640000 units, 1120 mg in 17 ml) was applied to a Sephadex G-100 column (bed volume 260 ml) prepared as described in the Experimental section; fractions (4 ml) were collected by using 20 mM-sodium phosphate, pH 6.5, as eluent, and assayed for protein (A_{280}; ●) and activating protein (○). Area shown in black corresponds to fractions that were pooled for further purification. V_0, void volume.

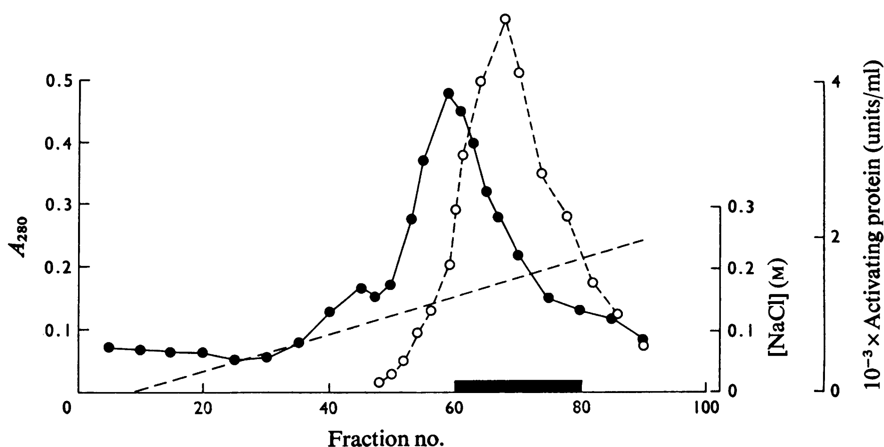

Fig. 2. *DEAE-Sephadex chromatography of activating protein*

The sample applied to the column contained 502000 units of activating protein and 271 mg of protein prepared as described in the legend to Fig. 1. The bed volume of the column was 25 ml, prepared as described in the Experimental section. After application of the sample, 230 mg of unadsorbed protein was removed by elution with 0.02 M-sodium phosphate, pH 7.5. A linear gradient (250 ml of 20 mM-sodium phosphate, pH 7.5, containing 0–0.3 M-NaCl) was then applied and fractions (2.5 ml) were collected. Fractions were assayed for protein (A_{280}; ●) and activating protein (○). Broken line represents NaCl concentration of eluate. Black bar indicates fractions that were pooled for further purification.

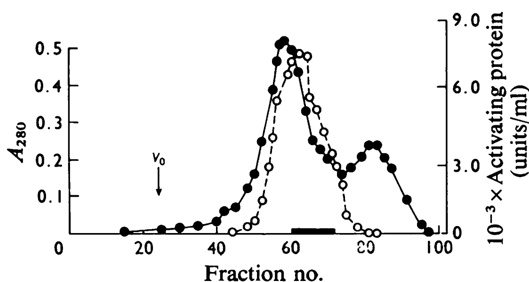

Fig. 3. *Sephadex G-200 chromatography of activating protein*

The sample applied contained 158000 units of activating protein and 12.4 mg of protein prepared as described in the legend to Fig. 2 in 2.0 ml of 10% sucrose. A Sephadex G-200 column (bed volume 100 ml) was prepared as described in the Experimental section. Fractions (1.3 ml) were collected and assayed for protein (A_{280}; ●) and for activating protein (○). Black bar indicates fractions that were pooled.

Polyacrylamide-gel-electrophoretic analysis of purified activating protein

The most purified activating protein preparation was subjected to polyacrylamide-gel electrophoresis (Fig. 4). Two bands migrating close together were seen at alkaline pH. Acidic electrophoresis conditions gave a single diffuse band. Staining of gels with the periodate/Schiff stain revealed no carbohydrate.

To find out whether one or both protein bands contained the activating-protein activity, attempts were made to elute activity from unstained gels. These attempts were unsuccessful. Water, buffers at various pH values, concentrated salt solutions, 20% (v/v) ethanol and neutral detergents were all unsuccessful in recovering activity or protein from the gels. These eluting solutions were used on whole gels, thin slices and gels ground in a Teflon–glass homogenizer.

In one experiment, gels that had been subjected to electrophoresis without sample were added to solutions containing the activating protein. After overnight incubation neither protein nor activity were detectable in solution. Control experiments ruled out both inactivation during overnight incubation and elution of a hexosaminidase A inhibitor from the gel, thus indicating a high affinity of the activating protein for polyacrylamide.

Since elution of activating protein from electrophoretic gels proved impossible, confirmation of the identity of the purified activating protein was performed by using an indirect method of analysis. Fig. 5 shows a comparison of the protein-staining bands of electrophoretic gels of the same number of units of activating protein but using preparations differing in specific activity. The protein bands present in the purest preparation stain with equal

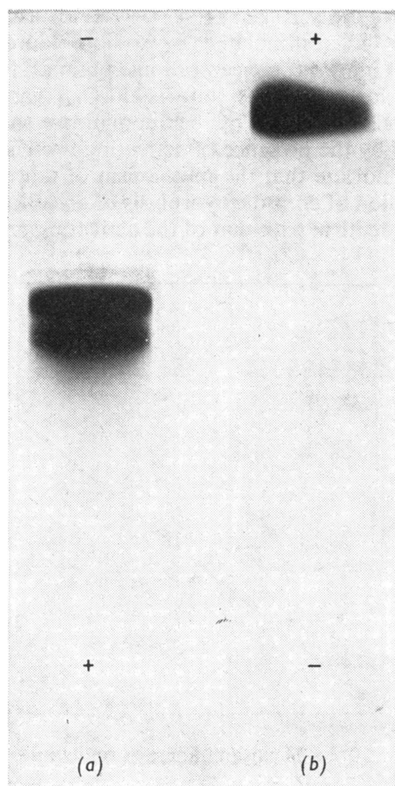

Fig. 4. *Polyacrylamide-gel electrophoresis of activating protein*

The sample applied to each gel contained 50 μg of protein and 1200 units of activating protein. Gel (*a*) was prepared and run at pH 8.3 under the conditions described by Davis (1964). Gel (*b*) was run under the conditions of Reisfeld *et al.* (1962). Both gels were electrophoresed at 4 mA/gel until the tracking dyes (Bromophenol Blue and Methyl Green respectively) migrated to the end of the gel. Gels were removed and fixed in 12% (w/v) trichloroacetic acid for 45 min and stained for 20 min in 0.1% Amido Black in 7% (v/v) acetic acid. Destaining was performed by successive rinsing with 7% acetic acid.

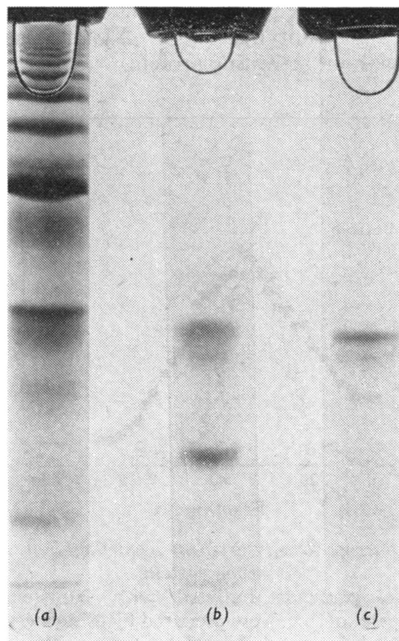

Fig. 5. *Electrophoresis of crude and purified activating-protein preparations*

Samples all contained 250 units of activating protein in a volume of 50 μl. The sample applied to gel (*a*) contained 1.2 mg of dialysed supernatant protein. The sample applied to gel (*b*) contained 20 μg of the DEAE-Sephadex fraction prepared as described in Fig. 2 and gel (*c*) contained 10 μg of the Sephadex G-200 preparation described in Fig. 3. Electrophoresis and staining were performed by the Davis (1964) procedure and as indicated in the legend to Fig. 4.

intensity in all preparations when the samples contain equivalent amounts of activity units.

Molecular weight of activating protein

The molecular weight of the activating protein was determined by three different methods.

Molecular-weight determination on Sephadex G-200 calibrated with standards indicated elution of activating protein at a $K_{av.}$ corresponding to mol.wt. 36000. After denaturation of the protein in SDS and 2-mercaptoethanol, SDS/polyacrylamide-gel electrophoresis indicated a single protein band of migration rate corresponding to mol.wt. 15000.

The sedimentation-velocity profile (Fig. 6) indicated a mol. wt. of approx. 39000 for the native protein.

Interaction of activating protein with substrate and hexosaminidase

Table 2 shows the effect of activating protein on hydrolysis of G_{M2} ganglioside in the presence of hexosaminidase A and hexosaminidase B. The stimulation of G_{M2} ganglioside hydrolysis by activating protein is specific for the hexosaminidase A component. When hexosaminidase A is incubated with merthiolate [ethyl-(2-mercaptobenzoato-*S*)-mercury sodium salt] under conditions developed by Carmody & Rattazzi (1974), the enzyme activity acquired the heat stability and electrophoretic-mobility characteristic of hexosaminidase B. This

merthiolate-induced hexosaminidase B did not catalyse significant hydrolysis of G_{M2} ganglioside with or without activating protein.

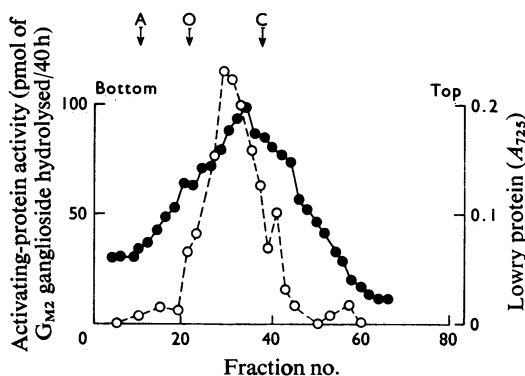

Fig. 6. *Sucrose-density-gradient centrifugation of activating protein*
Sucrose gradients buffered with 20mM-sodium phosphate, pH6.5, were prepared by using a gradient mixer with 2.4ml of 5% (w/v) sucrose in the mixing chamber and 2.4ml of 20% (w/v) sucrose in the reservoir. Nitrocellulose tubes containing gradients were kept at 4°C for 3h before application of sample. Samples were applied as 0.2ml of 2% sucrose solutions. The samples containing calibration standards had 2mg of human serum albumin (A), 4mg of ovalbumin (O) and 8mg of chymotrypsinogen (C). Tubes were centrifuged at 48000rev./min in an SW 50L Spinco rotor for 17h. The bottom of the tube was punctured and fractions (two drops) were collected. Fractions were analysed for Lowry protein (A_{725}; ●) and activating protein (○).

The hydrolysis of both G_{M2} and asialo-G_{M2} ganglioside is stimulated by sodium taurocholate (Koch–Light) at concentrations optimal for G_{M2} ganglioside hydrolysis, but asialo-G_{M2} ganglioside hydrolysis catalysed by hexosaminidase A is unaffected by the presence of activating protein. These results indicate that the mechanism of taurocholate stimulation of enzymic hydrolysis of glycolipids may not be identical with that of the activating protein.

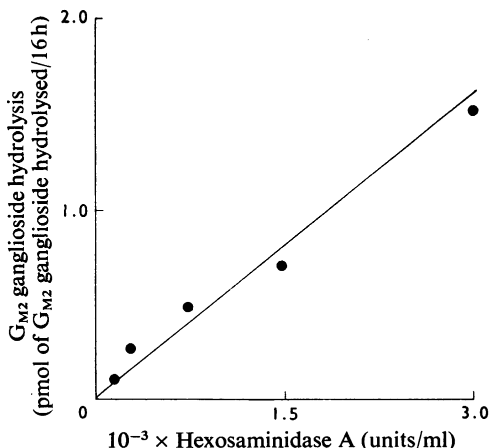

Fig. 7. *Effect of hexosaminidase A concentration on G_{M2} ganglioside hydrolysis*
Reaction mixtures were incubated as described for the determination of activating protein, except that 6000 units of activating protein were used in each incubation. Partially purified hexosaminidase A was used as the source of enzyme.

Table 2. *Interaction of activating protein with hexosaminidase isoenzymes and substrates*
Concentrations of substrates and activators were as follows: sodium taurocholate (5mg/ml), activating protein (0.5mg/ml, saturating concn.), G_{M2} ganglioside (10μM) and asialo-G_{M2} ganglioside (5μM). Partially purified hexosaminidases A and B were prepared as described previously (Hechtman, 1977).

Hexosaminidase	Substrate	Activator	10^7 × mol of substrate hydrolysed/mol of 4MU-*N*-acetylglucosaminide hydrolysed
A	G_{M2} ganglioside	—	1.1
A	G_{M2} ganglioside	Activating protein	26
A	G_{M2} ganglioside	Sodium taurocholate	11
B	G_{M2} ganglioside	—	1.2
B	G_{M2} ganglioside	Activating protein	1.2
B	G_{M2} ganglioside	Sodium taurocholate	0.9
B*	G_{M2} ganglioside	—	1.2
B*	G_{M2} ganglioside	Activating protein	1.1
B*	G_{M2} ganglioside	Sodium taurocholate	1.6
A	Asialo-G_{M2} ganglioside	—	1.8
A	Asialo-G_{M2} ganglioside	Activating protein	1.8
A	Asialo-G_{M2} ganglioside	Sodium taurocholate	3.5

* Hexosaminidase B in these experiments was prepared by overnight incubation of partially purified hexosaminidase A with merthiolate.

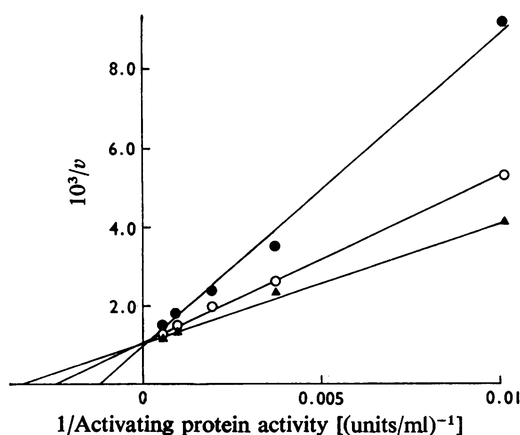

Fig. 8. *Effect of substrate concentration on activation of G_{M2} ganglioside hydrolysis*
Activating protein was incubated with hexosaminidase A (3000 units/ml) and G_{M2} ganglioside at concentrations of 10μM (●), 30μM (○) and 100μM (▲). Units of v are pmol of G_{M2} ganglioside hydrolysed/16h.

Previous results (Hechtman, 1977) showed that at constant enzyme and substrate concentrations the relationship between activating-protein concentration and the velocity of G_{M2} ganglioside hydrolysis was described by a Michaelis–Menten saturation curve. Fig. 7 shows the dependence of G_{M2} ganglioside hydrolysis on enzyme concentration in the presence of saturating concentrations of activating proteins. Increase in substrate concentration leads to a decrease in the dissociation constant between the activator and hexosaminidase A (Fig. 8). Lineweaver–Burk plots suggest that the binding of activating protein to the enzyme is dependent on the concentration of substrate. We attempted to demonstrate, by using gel-chromatography procedures, a physical association between activating protein and hexosaminidase A. The two activities are readily separable by gel filtration over a wide range of pH values when either partially purified or crude mixtures of hexosaminidase A and activating protein are used. The results presented in Fig. 8 suggest that a ternary complex of substrate, hexosaminidase A and activating protein might exist.

Mechanism of activation of hexosaminidase A

Gangliosides are known to form mixed micelles with certain detergents (Gammack, 1963). The mode of action of activating protein or bile-detergent stimulation of G_{M2} ganglioside hydrolysis may be due to physicochemical alteration of the substrate rather than to a direct effect on the enzyme. Since gangliosides exist in aqueous solution as aggregates, the action of activating protein or bile detergents might be to disperse these aggregates to smaller micelles. In an experiment, a 10μM solution of ^{3}H-labelled G_{M2} ganglioside was placed in each of three chambers of a Perspex equilibrium-dialysis block. Two chambers had amounts of activating protein and sodium taurocholate respectively that were optimal for stimulation of hexosaminidase A-catalysed hydrolysis of G_{M2} ganglioside. Opposite sides of the chamber were separated by cellulose dialysis membranes (exclusion limit mol.wt. <10000). After overnight shaking at 4°C, no radioactivity diffused across the membrane in either the control chamber or chambers containing taurocholate or activating protein. Although the experiment does not exclude the possibility of subtle changes in the size or conformation of the G_{M2} ganglioside micelle, the presence of activating protein or bile detergent does not cause dispersion of large ganglioside micelles into a true solution.

Activating-protein stimulation of G_{M2} ganglioside hydrolysis may be related to the *N*-acetylneuraminic acid group that is present in the ganglioside molecule. The enzymic hydrolysis of asialo-G_{M2} ganglioside is not stimulated by the activating protein.

Since the rate of hydrolysis of asialo-G_{M2} ganglioside is known to be greater than that of G_{M2} ganglioside (Frohwein & Gatt, 1967), it is possible that the preparations of activating protein may contain a neuraminidase that converts G_{M2} ganglioside into asialo-G_{M2} ganglioside and thus enhances the rate of hydrolysis of the *N*-acetylgalactosaminyl linkage.

This possibility was eliminated by incubation of ^{3}H-labelled G_{M2} ganglioside overnight with purified preparations of activating protein. After incubation, the reaction mixture was mixed with 5 vol. of chloroform/methanol (2:1, v/v) and centrifuged at 600*g* for 5min in the cold. Analysis of both phases showed that all the radioactivity was recovered in the aqueous phase when the incubation mixtures contained substrate alone or substrate and activating protein. In contrast, when solutions of substrate were hydrolysed for 2h at 80°C in 0.03M-HCl, 75% of the radioactivity was recovered in the lower phase.

The absence of stimulation of enzymic hydrolysis of asialo-G_{M2} ganglioside by the activating protein does not appear to be related to a specific binding site on the activator for *N*-acetylneuraminic acid. When *N*-acetylneuraminic acid was included in standard incubation mixtures for the hydrolysis of synthetic substrate or for the activating-protein-stimulated hydrolysis of G_{M2} ganglioside, no inhibition of hydrolysis occurred with *N*-acetylneuraminic acid concentrations up to 10mM. The inclusion of 10mM-*N*-acetylgalactosamine in these reaction mixtures caused 80–90% inhibition of the rate of hydrolysis of both substrates.

We conclude that, whereas a common site for the binding of the *N*-acetylgalactosamine residue of both

substrates exists on the enzyme, a specific site for the binding of the *N*-acetylneuraminyl residue of G_{M2} ganglioside is not demonstrable by competitive inhibition.

Discussion

Degradation of glycosphingolipids and mucopolysaccharides proceeds by a sequence of hydrolytic reactions catalysed by enzymes located in lysosomes. The specific catabolic role of many of the lysosomal hydrolases has been established by investigation of enzyme deficiencies in the tissues of individuals with hereditary diseases in which excessive amounts of glycolipid or mucopolysaccharide are stored or excreted. Comparison of the rates of enzymic hydrolysis of the synthetic chromogenic or fluorogenic substrates with hydrolysis of the natural sphingolipid substrates has led to the recognition that hydrolysis of the natural substrates requires the presence of cofactors. Thus the hydrolysis of lactosylceramide by human liver β-galactosidase requires sodium taurodeoxycholate, whereas hydrolysis of the same substrate by brain β-galactosidase requires sodium taurocholate (Wenger *et al.*, 1975). Naturally occurring activators have been isolated that stimulate respectively the hydrolysis of cerebroside sulphate by arylsulphatase A (Fischer & Jatzkewitz, 1975), glucocerebroside by β-glucosidase (Ho & O'Brien, 1971), G_{M2} ganglioside by hexosaminidase A (Li *et al.*, 1973) and ceramide α- and β-galactosides by their appropriate lysosomal hydrolases (Li *et al.*, 1974).

It is unclear whether a common activating protein is being isolated by different workers or whether each of the enzymes studied requires a specific effector. A comparison of some of the physical properties of the activating protein reported in the present paper with the properties of activators studied by other groups shows differences in molecular weight, thermolability and carbohydrate content (Li & Li, 1976; Fischer & Jatzkowitz, 1975; Ho *et al.*, 1973). On the other hand, Li *et al.* (1974) have reported that their hexosaminidase A-activating protein also stimulates hydrolysis of the substrates of lysosomal α- and β-galactosidases.

The mechanism of activation of lysosomal hydrolases by activating proteins is of considerable interest. On the basis of results in the present paper and those of Ho & Rigby (1975), it would be premature to draw conclusions on whether the activating proteins primarily effect changes in the properties of the glycolipid substrates or whether the activation of hydrolytic enzymes is related to alteration of the binding or catalytic properties of the enzymes.

The relationship between the requirement for activating proteins by lysosomal hydrolases *in vitro* and the metabolism of the glycolipid substrates *in vivo* is also unexplained.

The reported rates for hydrolysis of G_{M2} ganglioside differ widely. It is agreed, however, that the rate of hydrolysis of the asialo-G_{M2} ganglioside is many times greater than that of G_{M2} ganglioside (O'Brien *et al.*, 1977; Frohwein & Gatt, 1967; Bach & Suzuki, 1975) when either crude or purified hexosaminidases are present.

When relative rates of hydrolysis of hexosaminidase substrates, expressed as mol of G_{M2} ganglioside hydrolysed/mol of synthetic *N*-acetylglucosaminide substrate hydrolysed, are compared, published ratios vary from 10^{-6} (Bach & Suzuki, 1975; Sandhoff & Wassle, 1971) with purified enzymes to 1.6×10^{-3} (O'Brien *et al.*, 1977; Tallman & Brady, 1972) with freeze–thaw extracts of human fibroblasts and rat brain lysosomes respectively. The maximum ratio of activity of human liver hexosaminidase A obtained in our experiments is 2.5×10^{-6} mol of G_{M2} ganglioside hydrolysed/mol of synthetic glucosaminide hydrolysed. This ratio is a comparison of rates, measured under specific conditions, that monitors activity toward each substrate and does not represent a ratio of $V_{max.}$ for each substrate.

It is not yet clear whether the differences in G_{M2} ganglioside hydrolase activity of hexosaminidase A obtained from fibroblast extracts or rat brain lysosomes on the one hand or purified enzymes on the other can be attributed to a requirement for additional components present in the crude enzyme preparation, or if optimal G_{M2} ganglioside hydrolase activity depends on the conservation of the integrity of subcellular organelles during extraction of the tissue.

Among the G_{M2} gangliosidosis variants are conditions in which: (*a*) G_{M2} ganglioside hydrolysis is normal but activity toward fluorogenic substrates is absent or greatly diminished (Dreyfus *et al.*, 1975); (*b*) activity towards synthetic substrate is normal but G_{M2} ganglioside hydrolase activity is absent (AB variant) (Sandhoff, 1970); (*c*) activity towards both substrates is decreased, leading to later onset of neurological symptoms (the juvenile variant) (Okada *et al.*, 1970; Wood & MacDougall, 1976), as well as the classical forms involving absence of activities towards both substrates. It would be of interest to know whether the last two variant phenotypes could be related to either a deficiency of activating protein or an inability of hexosaminidase A to be stimulated by an activating protein.

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