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Activator Proteins for Sulphatide Hydrolysis and G_{M1}-Ganglioside Hydrolysis. Probable Identity on the Basis of their Co-purification, Properties, Ligand Binding and Immunochemical Interactions

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The concurrent purification of the activator protein for sulphatide hydrolysis and for G_{M1}-ganglioside hydrolysis including chromatofocusing and hydrophobic chromatography stages is described. The purified preparation has a pI of 4.2 and the sub-unit M_r is 10 000. The stoichiometry of binding of sulphatide and ganglioside to the protein is very similar. Both activities are removed in similar proportions on binding to IgG purified from antisera raised against the activator protein. The probable identity of the activator protein for sulphatide hydrolysis with that for G_{M1}-ganglioside hydrolysis and a molecular explanation for this identity are discussed.

The sequential degradation of glycosphingolipids by specific lysosomal glycosidases and sulphatases is mediated by small non-enzymic proteins, termed activator proteins, whose function is to convert the lipid into a physical form suitable for enzymic hydrolysis (see [1]). The first such activator protein was shown in a study of the hydrolysis of cerebroside 3-sulphate by arylsulphatase A [2]. Subsequently the necessity for an activator protein in the degradation of gangliosides was demonstrated and a specific activator protein for G_{M1}-ganglioside and globotriaosylceramide hydrolysis was isolated [3].

These proteins are similar in their molecular weights, heat stability, isoelectric point and amino acid composition [4, 5]. On the basis of immunochemical studies it was postulated that the two proteins were identical [6] and a similar conclusion was reached from a study of the binding of activators to immunochemical affinity columns [7].

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The present study describes the concurrent purification and characterisation of the two proteins and shows that they are probably identical on biochemical criteria. A preliminary report of this work has already appeared [8].

Materials and Methods

Preparation of[^{3}H]-Cerebroside 3-Sulphate

Cerebroside 3-sulphate (sulphatide; Supelchem, Sawbridgeworth, U.K.) was tritiated using sodium [^{3}H]borohydride (Amersham International, Amersham, U.K.) with $PdCl_2$ as catalyst [9, 10]. The reaction mixture was purified using activated Florisil [11] (100-200 mesh, Sigma Chemical Co. Ltd., Poole, U.K.), the elution conditions being as previously described.

Preparation of [^{3}H]-G_{M1}-Ganglioside

An acetone powder of normal human brain obtained at autopsy was extracted with $CHCl_3/CH_3OH$ and partitioned as described by Suzuki [12]. The crude ganglioside fraction was treated with neuraminidase (*Clostridium perfringens*, Sigma) to convert some of the di- and tri-sialogangliosides to mono-sialogangliosides [13] and the reaction mixture purified by ion-exchange and gel permeation chromatography in organic solvents [14].

The purified G_{M1}-ganglioside was treated with galactose oxidase to convert the terminal galactose residue to an aldehyde, which was then reduced with sodium [^{3}H]borohydride [15] (Amersham). The labelled ganglioside was purified by chromatography on Sephadex LH-20 (Pharmacia, Milton Keynes, U.K.) using $CHCl_3/CH_3OH/H_2O$, 5/5/1 by vol, as eluting solvent. The final preparation had a specific activity of 20 mCi/mmol and TLC on silica gel plates with $CHCl_3/CH_3OH$/3.5 M NH_4OH, 60/40/9 by vol, gave a single band with only slight traces of contamination.

Partial Purification of β-Galactosidase

A partially purified preparation of β-galactosidase is required for use in the assay of G_{M1}-ganglioside activator. An extract of human liver was fractionated by batch ion-exchange chromatography on DEAE-cellulose followed by affinity chromatography on concanavalin A as previously reported [16]. The preparation had a specific activity of 250 units/mg protein when assayed with 1 mM methylumbelliferyl-β-D-galactoside, pH 4.3, as substrate. The unit of enzyme activity is defined as the amount of enzyme that catalyses the production of 1 nmol of methylumbelliferone per min under these conditions. Preparations of this purity were shown to be adequate for use in the activator assay.

Purification of Arylsulphatase A

Relatively pure arylsulphatase A is necessary for the accurate assay of the sulphatide activator protein since the assay procedure is subject to interference by large amounts of inert protein. This enzyme was purified from human liver by a method adapted from

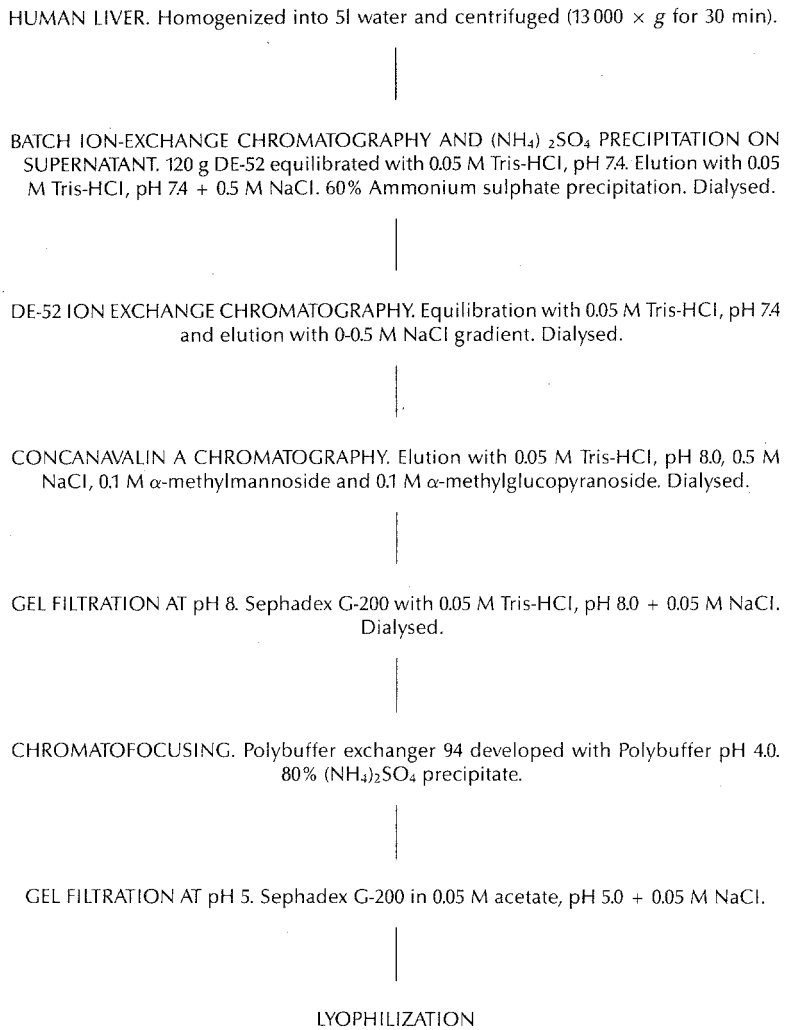

Figure 1. Scheme for the partial purification of arylsulphatase A from human liver. For 1.2 kg normal human liver, all stages being carried out at 4°C.

that of Fluharty and Edmond [17] and using a concanavalin A affinity chromatography stage based on that of Bishayee and Bachhawat [18] together with a chromatofocusing procedure. The purification scheme is summarised in Fig. 1 and the final preparation had a specific activity of 19.5 units/mg protein which represented a purification factor of 5 800 over the initial aqueous extract. The enzyme was assayed using 10 mM nitrocatechol sulphate, pH 5.0, as substrate [19], the unit of enzyme activity being defined as that amount of enzyme that catalyses the production of 1 μmol nitrocatechol per min under these conditions.

Assay of Sulphatide Activator Protein

The assay procedure used was adapted from that of Poulos and Beckman [20]. Prior to assay, all activator preparations were dialysed against the reaction buffer, 0.2 M sodium acetate buffer, pH 4.5. In a typical assay 45 μl of [^{3}H]-cerebroside 3-sulphate (10 nmol containing 200 000 d.p.m. dispersed in reaction buffer by solvent dilution [10], 25 μl of arylsulphatase A (0.1 units dissolved in reaction buffer) and varying amounts of activator preparation were diluted to 400 μl with reaction buffer. After incubation at 38°C for 1 h, the reaction was terminated by the addition of 800 μl of $CHCl_3/CH_3OH$, 1/1 by vol, mixed thoroughly with a vortex mixer and the phases separated by centrifugation at 12 000 $\times$ g for 2 min. After removal of the upper layer, the lower organic layer was dried under nitrogen, the residue redissolved in 0.5 ml toluene/ethanol, 2/3 by vol, and applied to a minicolumn (0.6 $\times$ 3 cm) of DE-52 (DEAE-cellulose pretreated with glacial acetic acid, washed with methanol and air dried prior to suspension in toluene/ethanol). The reaction vessel and the column were washed with 1.5 ml toluene/ethanol, the total eluate collected in a scintillation vial and the radioactivity determined, correcting for quenching. Controls where the incubation mixture was stopped immediately after the addition of enzyme were performed and the values deducted. Preliminary experiments showed that less than 2% of the total radioactivity separated into the aqueous layer using this technique and that the separation of cerebroside and cerebroside sulphate on the DE-52 was better than 96%.

Assay of G_{M1}-Ganglioside Activator

Prior to assay, all activator preparations were dialysed against the reaction buffer, 0.2 M sodium citrate buffer, pH 4.0. In a typical assay 25 μl of [^{3}H]-G_{M1}-ganglioside (0.2 nmol containing 10 000 d.p.m. dispersed by sonication), 10 μl of β-galactosidase (0.3 units dissolved in the reaction buffer) and varying amounts of activator preparation were diluted to 100 μl with reaction buffer. After incubation for 1 h at 37°C, the assay was stopped by the addition of 0.9 ml ice-cold 1 mM galactose and the samples stored on ice. The assay mixture was applied to a mini-column (0.6 $\times$ 3 cm) of DE-52 (DEAE-cellulose in water pre-quilibrated with 1 ml of 1 mM galactose) and the [^{3}H]-galactose liberated in the reaction washed through the column into a scintillation vial with two 0.5 ml aliquots of unlabelled galactose. The radioactivity of the eluate was determined by standard methods and corrected for quenching. Controls where the incubation mixture was stopped immediately after the addition of enzyme were performed and the values deducted.

Units of Activator

In each case the unit of activator is defined as that amount of activator that will lead to the production of 1 nmol of product in 1 h in the presence of 1 unit of enzyme.

Protein Determination

Protein was determined by a dye-binding assay method [21] using bovine serum albumin as standard.

In view of the heat stability of this protein all operations were carried out at room temperature unless otherwise stated.

(1) Aqueous Extraction. After removal of the vascular structures and the surrounding connective tissue, 500 g of normal human liver were homogenised into 1 l distilled water in a Waring blender for 1 min at maximum speed. The cellular debris was removed by centrifugation (11 000 × *g* for 30 min) and resuspended by homogenisation in 500 ml distilled water. After centrifugation, the supernatants were combined.

(2) Heat Treatment. The method of Fischer and Jatzkewitz [22] was used. The extracts were heated in 200 ml aliquots to 80°C, held at this temperature for 30 min, allowed to cool to room temperature and centrifuged at 11 000 × *g* for 30 min, discarding the precipitate.

(3) Ammonium Sulphate Precipitation. The supernatant was brought to 70% saturation with $(NH_4)_2SO_4$ (calculated amount of solid at 4°C) and allowed to stand at 4°C for 18 h before centrifugation at 13 000 × *g* for 30 min. The precipitate was suspended in 250 ml distilled water, dialysed against running tap water until free of detectable sulphate and finally dialysed against 50 mM sodium acetate buffer, pH 6.0. Any precipitate forming during dialysis was removed by centrifugation at 30 000 × *g* for 30 min.

(4) DE-52 Ion Exchange Chromatography. The clarified dialysate was applied to a column (4.5 × 24 cm) of DE-52 (DEAE-cellulose, pretreated, fines removed and equilibrated with 50 mM sodium acetate buffer, pH 6.0) and unbound material was washed from the column with the same buffer. Adsorbed protein was eluted with 50 mM sodium acetate buffer, pH 6.0 containing 0.2 M NaCl, monitoring the effluent at 280 nm and collecting 10 ml fractions. Protein peaks were pooled, dialysed against distilled water and assayed for activator. These chromatographic conditions are preferable to elution at pH 7.0 as previously described [4], in that better separation of the activator protein from inert protein is achieved.

(5) Batch CM-52 Ion Exchange Chromatography. The activator preparation was dialysed against 50 mM sodium acetate buffer, pH 5.1 and 50 g CM-52 (CM-cellulose pre-equilibrated with this buffer) was added. After stirring for 1 h, the activator, which was not bound, was removed by filtration through a sintered glass funnel, the cellulose was washed with a further 100 ml of buffer and the filtrates combined. This method was found to be preferable to that of Li and Li [4] who bound the activator to CM-52 at pH 4.0 and eluted at pH 4.4 in the presence of salt. The yield of the batch method (see Table 1) was higher than that previously reported as were the purification achieved and the facility of the method.

(6) Hydrophobic Interaction Chromatography. Preliminary experiments revealed that, although activator protein bound to both Phenyl-Sepharose and Octyl-Sepharose, elution from the former matrix required the use of extreme conditions such as chaotropic agents whereas elution from the latter could be effected with octyl glucose. The activator preparation was dialysed against distilled water and centrifuged to clarify before application to a column (1 × 25 cm) of Octyl-Sepharose CL-4B (Pharmacia) which had been pre-equilibrated with water. After thorough washing with water and a 1% (w/v) solution of octyl sulphate (Sigma) to remove unbound material, the activator was eluted

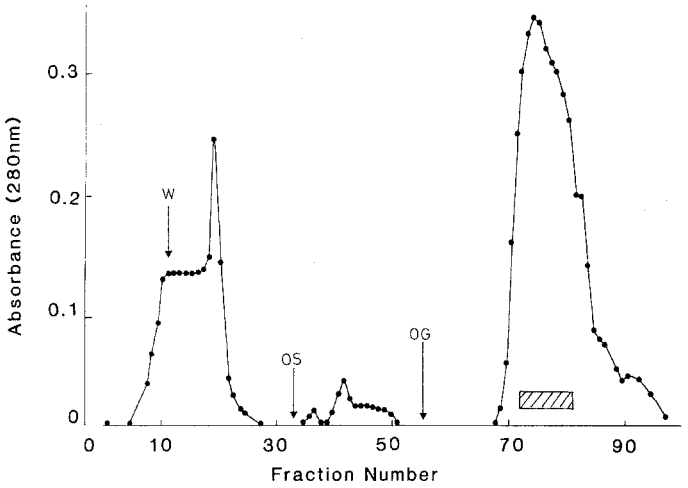

Figure 2. Elution profile of partially purified activator protein on Octyl Sepharose.
The column (1 × 25 cm) of Octyl-Sepharose CL-4B was equilibrated with water and the protein concentration of the eluate monitored by absorbance at 280 nm (●). Eluting solvents: W, water; OS, octyl sulphate; OG, octyl glucose. The flow rate was 20 ml/h and the fraction volume was 2.5 ml. The cross-hatched area represents the elution position of the ganglioside activator protein.

with 100 ml of 1% (w/v) octyl glucose (Sigma). The elution profile is shown in Fig. 2. Only the peak that eluted with octyl glucose contained activator.

(7) Chromatofocusing. The Polybuffer exchanger (PBE 94; Pharmacia), was poured into a column (1 × 25 cm) and equilibrated with 500 ml of 25 mM piperazine-HCl buffer, pH 5.5, at a flow rate of 100 ml/h. After dialysis against this buffer, the activator preparation was applied and washed into the column with 3 ml of buffer. The column was developed with 150 ml of Polybuffer (Polybuffer 7-4, Pharmacia; 37.5 ml of concentrated solution adjusted to pH 4.0 with 0.5 M HCl and diluted to 150 ml) at a flow rate of 50 ml/h, monitoring the pH of the effluent continously. When the pH dropped to 3.8, any residual protein was washed from the column with 1 M NaCl. The elution profile is shown in Fig. 3. Protein peaks were pooled and dialysed against distilled water to remove salts. Dialysis does not remove Polybuffer but furtunately this buffer does not interfere in the assay of activator.

(8) Gel Filtration. After lyophilisation, the activator preparation was dissolved in 2 ml of 50 mM sodium acetate buffer, pH 5.0, containing 50 mM NaCl and applied to a column (2.2 × 56 cm) of Sephadex G-100 (Pharmacia; pre-equilibrated with this buffer and calibrated with Dextran Blue, bovine serum albumin, soya bean trypsin inhibitor, α-lactalbumin and potassium chromate) continuing the elution in the same buffer. The final preparation was dialysed against distilled water, prior to lyophilisation, and was stored in a desiccator at room temperature.

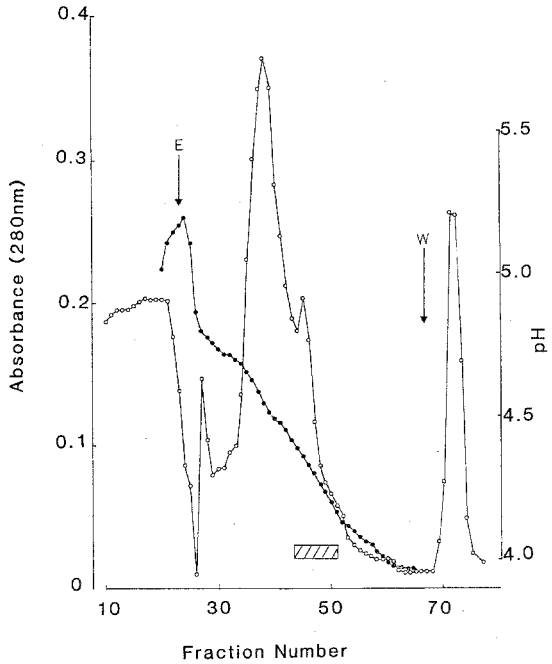

Figure 3. Partial purification of activator protein by chromatofocusing.
A column (1 × 25 cm) of PBE 94 was equilibrated with 25 mM piperazine-HCl buffer, pH 5.5 and developed with Polybuffer 7-4, pH 4.0, (E) and 1 M NaCl (W). The flow rate was 50 ml/h and the fraction volume was 3.5 ml. The cross-hatched area represents the elution position of the ganglioside and sulphatide activator protein. (○) = absorbance at 280 nm. (●) = pH.

Lipid-Activator Binding Studies

Both cerebroside 3-sulphate and G_{M1}-ganglioside form relatively large vesicles on aqueous dispersion and these can be separated from activator-lipid complexes by gel filtration. Since the molecular weight of activator is approximately 20 000, the gel filtration medium of choice would be Sephadex G-100. This medium is satisfactory for G_{M1}-ganglioside, but unfortunately sulphatide vesicles tend to block columns of this material leading to very high back pressures and leakage. For this reason the experiments with sulphatide were carried out using Sepharose 4B.

Columns (1 × 30 cm) of either Sephadex G-100 or Sepharose 4B (Pharmacia) were equilibrated with 200 mM sodium acetate buffer, pH 4.5 and at least 5 mg of lipid in aqueous dispersion were passed through the column to coat the column and avoid any non-specific adsorption during the course of the experiments. Trial experiments were then conducted to check that quantitative recovery of [^{3}H]-sulphatide or G_{M1}-ganglioside could be achieved. Varying proportions of activator and tritiated lipid, as specified in the legends to Figs. 4 and 5, were incubated for periods ranging from 0-24 h and an aliquot (0.1 ml) of the incubation mixture applied to the column and eluted at a flow rate of 12 ml/h. The radioactivity of each fraction (250 μl) was determined by liquid scintillation counting.

Antibody Production

Antibodies to the activator were raised in a New Zealand White rabbit. Lyophilised activator was suspended in sterile isotonic saline and sterilised by filtration through a 0.2 μm filter to give a final concentration of 3 mg/ml. An aliquot (150 μl) was mixed thoroughly by vortexing with an equal volume of Freund's complete adjuvant and injected intradermally into twenty sites along the rabbit's back. Two booster intramuscular injections were given at two week intervals and contained Freund's incomplete adjuvant.

Blood was collected prior to the initial injection and subsequently at weekly intervals by catheterisation of the central ear artery. The blood was allowed to clot at 37°C for 1 h and, after easing the clot from the sides of the tube with a glass rod, the clot was allowed to contract at 4°C for 18 h. The serum was collected after centrifugation (12 000 × g for 10 min) and stored at -20°C in 0.25 ml aliquots.

Partial Purification of Immunoglobulins from Antisera

Two volumes of 60 mM sodium acetate buffer, pH 4.0, were added to the antiserum at room temperature and the pH adjusted to 4.8 with 60 mM sodium acetate. Octanoic acid (74 μl/ml of serum) was added dropwise with continuous stirring and the precipitate allowed to develop, without stirring, for 15 min. After centrifugation at 12 000 × g for 10 min, the supernatant was dialysed against 20 mM Tris-HCl buffer, pH 8.0 containing 28 mM NaCl and 0.02% (w/v) sodium azide. The dialysed crude IgG preparation was loaded on a column (3.8 × 9 cm) of DEAE Affi-Gel Blue (Bio-Rad Labs., Watford, U.K.; pre-washed with 20 mM Tris-HCl buffer containing 28 mM NaCl and 0.02% (w/v) sodium azide) and the IgG, which does not bind, washed through the column with the same Tris buffer. The IgG was stored in a lyophilised state at -20°C.

Depletion of Activator by Antibody Binding

The wells of a microtitre plate (Immulon 1; Dynatech Labs. Ltd., Billinghurst, U.K.) were coated with 200 μl of purified IgG (30 μg/ml) in 0.1 M sodium carbonate buffer, pH 9.6 for 18 h at room temperature. After washing thoroughly with 10 mM sodium phosphate buffer, pH 7.2, containing 0.145 M NaCl and 0.01% (v/v) Tween 20, any remaining binding sites were blocked by incubation with 400 μl of bovine serum albumin (1 mg/ml) dissolved in the washing buffer. Aliquots of a purified activator preparation (200 μl) were added to each well and, after standing for 1 h at room temperature, any unbound activator was removed from the well and assayed for activity in both the sulphatase and β-galactosidase systems. Control experiments, in which IgG was omitted, were also performed.

Results

Purification of Activator Protein from Human Liver

The purification of the activator protein is summarized in Table 1. The overall recovery, assayed for G_{M1}-ganglioside activator was typically 30-35%, with a purification of approximately 2500-fold. The apparently anomalous yields in the early stages were due to

Table 1. Summary of the purification of G_{M1}-ganglioside activator.
For full experimental details see text. Purification stages: I. Heat treatment; II. Ammonium sulphate precipitation; III. DE-52 Ion exchange chromatography; IV. Batch CM-52 ion exchange chromatography; V. Octyl-Sepharose hydrophobic interaction chromatography; VI. Chromatofocusing; VII. Gel filtration chromatography.

			β-Galactosidase assay			Sulphatidase assay		
Stage	Volume (ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
I	210	61	0.0059	100	1	N.D.[a]	N.D.	N.D.
II	25.2	74.5	0.0757	188	13	N.D.	N.D.	N.D.
III	21.6	25	0.277	198	47	N.D.	N.D.	N.D.
IV	13.1	2.5	1.912	83	324	2.49	100	1
V	46	0.21	7.238	92	1226	10.57	125	4.2
VI	22	0.28	13.93	113	2360	20.32	153	8
VII	3.6	0.47	15.2	34	2576	26.42	55	10.6

[a]Not determined because of interference by foreign protein in the sulphatidase assay.

the removal of contaminating protein which interferes to some extent with the assay. The purified protein gave a single major band on sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis with a minor contaminant of very similar molecular weight. Calibration of the gels with a series of molecular weight markers showed that the mobility of the major component was greater than α-lactalbumin (M_r = 14 200) and the M_r was determined as 10 000 by extrapolation.

Unfortunately, the interference of inert protein in the sulphatide assay was too great to allow any quantitative comparisons to be made in the early stages, but qualitatively, no separation of the two activities was observed. Table 1 shows that in the later stages of the purification there was no separation of the activator activities towards G_{M1}-ganglioside and sulphatide. The yields and purification in each of the stages following batch ion-exchange chromatography were within experimental error for each substrate.

Molecular Properties of the Activator Protein

The M_r of the activator protein as determined under native conditions by gel filtration was 16 000 ± 750 using bovine serum albumin, soya bean trypsin inhibitor and α-lactalbumin as markers.

The pI of the activator protein as measured by chromatofocusing was 4.2 (Fig. 3). A small amount of activator was not eluted during the pH gradient and needed high ionic strength for elution, suggesting that some non-specific interaction with the Polybuffer exchanger was taking place. This non-specific interaction depended on the state of purification of the protein and varied with liver sample.

There is a strong hydrophobic domain in the protein as shown by its interaction with Octyl-Sepharose (Fig. 2) and the need to use chaotropic agents to elute from Phenyl-Sepharose. Such behaviour would be predicted from the physiological function of the protein in binding glycolipids.

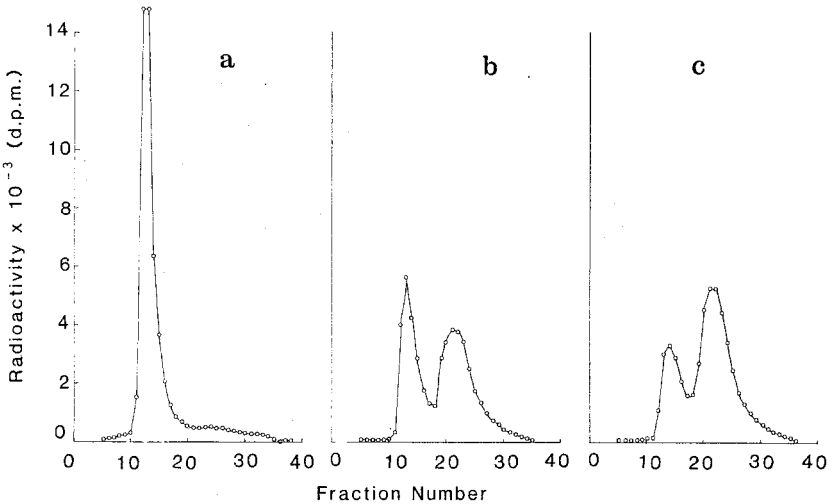

Figure 4. Time dependence of the binding of G_{M1}-ganglioside to activator protein.
Activator protein (3.5 nmol) and G_{M1}-ganglioside (5 nmol) incubated at room temperature for (a) 0 h, (b) 1 h and (c) 5.5 h, prior to separation on Sephadex G-100. For full experimental details see the text.

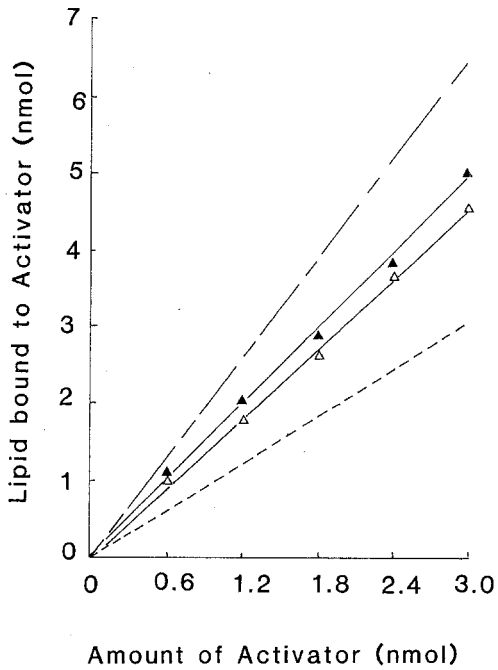

Figure 5. The stoichiometry of binding of sulphatide and G_{M1}-ganglioside to activator protein.
Varying amounts of activator protein were allowed to bind to 5 nmol of either sulphatide (▲) or G_{M1}-ganglioside (△) for 24 h at room temperature before gel filtration as described in the text. (----) Calculated for lipid to activator ratio of 1:1, (----) calculated for lipid to activator ratio of 2:1.

Table 2. Binding of activator protein to immobilized antisera.
Microtitre plate wells coated with purified IgG from control and experimental sera and blocked with bovine serum albumin. Purified activator was allowed to bind for 1 h and residual activator assayed. For full experimental details see text.

Serum	Residual activator (% of activator added ± S.D.)	
	Sulphatidase activity	β-Galactosidase activity
Experimental	19.1 ± 5.6 (4)	27.9 ± 3.6 (4)
Control	79.0 ± 7.0 (3)	90.3 ± 5.2 (3)

The protein is relatively unstable when stored at -70°C and as it binds strongly to ultrafiltration membranes such as PM 10 (Amicon Ltd., Stonehouse, U.K.), such techniques should be avoided during its preparation.

Formation of Lipid-Protein Complexes

The binding of G_{M1}-ganglioside to activator protein is time-dependent as shown in Fig. 4. There was a clear cut separation of vesicles of ganglioside from protein-bound lipid on Sephadex G-100 and the amount of lipid bound increased steadily over several hours and was only complete after 24 h (results not shown). Although the binding of sulphatide was somewhat more rapid, several hours were still necessary for the system to come to equilibrium.

In another series of experiments the binding time was increased to 24 h while allowing the ratio of protein to lipid to vary. The binding ratios for sulphatide and G_{M1}-ganglioside were very similar (Fig. 5) being calculated as 1.4 and 1.5 nmol lipid bound/nmol activator, respectively. It is difficult to calculate the amount of activator present with any accuracy. The present calculation was based on a M_r of 16 000 and protein estimated by dye-binding using bovine serum albumin as standard. Serum albumin is an atypical standard for this method (Bio-Rad Protein Assay Instruction Manual) having a colour yield of nearly twice that of the average of 23 other proteins. It is likely, therefore, that the amount of activator in these experiments has been underestimated and that the true ratio was nearer to 1 nmol lipid/nmol activator. However, the striking similarity in the binding of sulphatide and G_{M1}-ganglioside is not affected by this uncertainty in activator determination.

Depletion of Activator by Antibody Binding

While no antibody to activator protein was present in rabbit serum prior to inoculation, serum obtained one week after the second booster injection showed a strong precipitin line on immunodiffusion against the purified activator. When purified IgG was bound to the wells of a microtitre plate and allowed to react with purified activator, the activator bound specifically to the immunoglobulin (Table 2). There was a marked similarity between the reductions for sulphatide activation and G_{M1}-ganglioside activation.

Discussion

The evidence presented here gives further weight to the postulate that the activator proteins for sulphatide and for G_{M1}-ganglioside hydrolysis are identical. Throughout the extensive purification scheme, no evidence of the separation of the two activities was observed and the similarities in the yields and purification factors lead us to believe that they are the same protein. While the separate activities have been purified previously [2, 4] this is the first detailed study of the co-purification of the two activities.

The pI of the sulphatide activator has been determined by iso-electric focusing to be 4.3 [22] while that of the G_{M1}-ganglioside activator was 4.1 [4] with some evidence of material with a pI of 4.6 [23]. The present value of 4.2 obtained with chromatofocusing is in good agreement with these previous values and again emphasizes the similarities and probable identity.

The molecular weight is less clear. Previous reports have suggested a M_r of 21 500 for the sulphatide activator [22] and 22 000 for the G_{M1}-ganglioside activator [4]. The M_r as calculated from the amino acid sequence is 8 955 [24], which corresponds well with the M_r found on polyacrylamide gel electrophoresis under denaturing conditions in this report. It seems likely that the native molecule is a dimer with M_r in the range 18 000 to 22 000 depending on the amount of glycosylation. The reason for the anomalously low molecular weight on gel filtration on Sephadex G-100 is not clear.

The reversible formation of a 1:1 complex has been demonstrated by studying the behaviour of mixtures of sulphatide and its activator protein on electrophoresis in a discontinuous polyacrylamide gel [25]. Similar results were obtained with G_{M1}-ganglioside and its activator [26]. In the present study the similarity between the stoichiometry of the complex with sulphatide and ganglioside is again indicative of the identity of the activator proteins.

Further confirmation of this identity is provided by the finding that both the activity when sulphatide is the substrate and the activity with ganglioside as substrate are reduced when the activator preparation is bound to IgG purified from an antiserum raised against the preparation. The parallel reduction in these activities would appear to rule out the possibility that co-purification of two different proteins has occurred. Similar interaction between antibody and activator was observed in a study of the retention of activator activities by anti-G_{M1}-activator IgG coupled to Sepharose 4B [7].

The lysosomal storage disease, metachromatic leucodystrophy, is generally associated with a severe deficiency of arylsulphatase A but a case has been reported where the arylsulphatase A dysfunction was attributed to the deficiency of the sulphatide activator rather than the enzyme itself [27]. The accumulation of sulphatide by fibroblasts of this patient in a sulphatide loading test was reversed by the addition of sulphatide activator and also by the addition of G_{M1}-ganglioside activator [28]. The fibroblasts were lacking in cross-reactive material to antibody against G_{M1}-ganglioside activator. These results together with those reported here would seem to confirm the identity of these activator proteins, named SAP-1 by Inui and Wenger [6].

A molecular explanation of the specificities of activator proteins towards gangliosides has recently been put forward [29]. By comparing the minimum energy conformers of G_{M1}- and G_{M2}-ganglioside, obtained by computation, it has been possible to show that

the orientation of the charge and sites of hydrophilic interaction with respect to the lipid moiety are different in the two gangliosides [30, 31]. Such differences may well determine the interaction with their specific activator proteins and work at present in progress in this laboratory on the structure of the activator protein SAP-1 seeks to confirm the complementarity of its interaction with G_{M1}-ganglioside.

The finding that the formation of glycosphingolipid-activator complexes is time-dependent has implications in the assay of cerebroside sulphatase and G_{M1}-ganglioside β-galactosidase. In order for equilibrium to be established the activator and lipid should be incubated for at least 5 h, and preferably for 18 h, before addition of enzyme. Under these conditions the enzyme shows classical Michaelis-Menten kinetics for the activator-lipid concentration.

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References

1 Sandhoff K, Conzelmann E (1979) Trends Biochem Sci 4:231-33.
2 Mehl E, Jatzkewitz H (1964) Hoppe-Seylers Z Physiol Chem 339:260-76.
3 Li Y-T, Mazzotta MY, Wan C-C, Orth R, Li S-C (1973) J Biol Chem 248:7512-15.
4 Li S-C, Li Y-T (1976) J Biol Chem 251:1159-63.
5 Jatzkewitz H (1978) Adv Exp Med Biol 101:561-71.
6 Inui K, Wenger DA (1984) Arch Biochem Biophys 233:556-64.
7 Li S-C, Kihara H, Serizawa S, Li Y-T, Fluharty AL, Mayes JS, Shapiro LJ (1985) J Biol Chem 260:1867-71.
8 Banks MN, Gutteridge CL, Wynn CH (1985) Biochem Soc Trans 13:1150-51.
9 Schwarzmann G (1978) Biochim Biophys Acta 529:106-14.
10 Wynn CH, Banks MN, Grundy SM, Roy AB (1986) Glycoconjugate J 3:15-25.
11 Mehl E, Jatzkewitz H (1963) Naturwissenschaften 50:277.
12 Suzuki K (1965) J Neurochem 12:629-39.
13 Gatt S, Gazit B, Cestaro B, Barenholz Y (1980) Adv Exp Med Biol 125:137-46.
14 Byrne MC, Sbaschnig-Agler M, Aquino DA, Sclafani JR, Ledeen RW (1985) Anal Biochem 1480:163-73.
15 Radin NS (1972) Methods Enzymol 28:300-9.
16 Heyworth CM, Neumann EF, Wynn CH (1981) Biochem J 193:773-79.
17 Fluharty AL, Edmond J (1977) Methods Enzymol 58:537-47.
18 Bishayee S, Bachhawat BK (1975) Biochim Biophys Acta 334:378-88.
19 Baum H, Dodgson KS, Spencer B (1959) Clin Chim Acta 4:453-55.
20 Poulos A, Beckman K (1978) Clin Chim Acta 89:417-20.
21 Bradford MM (1976) Anal Biochem 72:248-54.
22 Fischer G, Jatzkewitz H (1975) Hoppe-Seylers Z Physiol Chem 356:605-13.

23 Inui K, Wenger DA (1982) Biochem Biophys Res Commun 105:745-51.
24 Dewji N, Wenger DA, Fujibayashi S, Donoviel M, Esch F, Hill F, O'Brien JS (1986) Biochem Biophys Res Commun 134:989-94.
25 Fischer G, Jatzkewitz H (1977) Biochim Biophys Acta 481:561-72.
26 Li Y-T, Li S-C (1984) in Lysosomes in Biology and Pathology, Vol 7, eds. Dingle JT, Dean RT, Sly W, Elsevier, Amsterdam, p 99-117.
27 Stevens RL, Fluharty AL, Kihara H, Kaback MM, Shapiro LJ, Marsh B, Sandhoff K, Fischer G (1981) Am J Hum Genet 33:900-6.
28 Inui K, Emmett M, Wenger DA (1983) Proc Natl Acad Sci USA 80:3074-77.
29 Wynn CH (1986) Biochem J 240:921-24.
30 Wynn CH (1986) J Theor Biol 123:221-30.
31 Wynn KH, Marsden A, Robson B (1986) Biochem Soc Trans 14:707-8.