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Sulfatide prolongs blood-coagulation time and bleeding time by forming a complex with fibrinogen*

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Sulfatides (galactosylceramide I^3-sulfate), which are found in serum lipoproteins of various mammals, effectively increased prothrombin time (anticoagulant effect) and also effectively prolonged bleeding time (anti-platelet effect). When equal volumes of a homogeneous micellar solution of sulfatide and fibrinogen in phosphate-buffered saline were mixed, an insoluble complex precipitated. Analysis of the precipitated complex showed that the molar ratio of sulfatide to fibrinogen was about 400:1. These results indicate that the sulfatide micelle binds tightly to fibrinogen and thereby interferes with both fibrin gel formation (anticoagulant activity) and platelet function.

Keywords: anticoagulant, fibrinogen, sulfatide, prothrombin time, thrombin, bleeding time

Abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered saline; PT, prothrombin time.

Introduction

Sulfatides, galactosylceramide I^3-sulfate, are found in various organs or body fluids of mammals and various physiological roles have been proposed [1]: as a constituent of myelin, involvement in salt transport in kidney, and a specific role in an opiate receptor, etc. It has been reported that sulfatide binds to the adhesive glycoproteins laminin, thrombospondin, and von Willebrand factor, suggesting involvement of sulfatide in cell adhesion [2]. Activation of the blood coagulation factor XII or XI with sulfatide has also been reported [3, 4]. The authors reported for the first time that sulfatides as a major glycosphingolipid are found in serum lipoproteins of various mammals [5]. A marked increase in sulfatide content was observed in both the serum lipoproteins and aorta of Watanabe hereditary hyperlipidaemic (WHHL) rabbits, the animal model of human familial hypercholesterolaemia [6, 7]. It was hypothesized that hyperlipidaemia might give rise to an elevated level of sulfatide in order to normalize the high blood coagulability. One of the physiological functions of sulfatide was thought to be as an anticoagulant in circulating blood [5, 8]. A pharmacological experiment showed that sulfatide is a powerful anticoagulant or antithrombotic agent in rabbits [9]. It was also shown that sulfatide inhibits neither thrombin nor blood coagulation factor Xa. Thus, the mechanism of the anticoagulant action of sulfatide remains to be clarified. In the present paper, we describe the anticoagulant activity of sulfatide in terms of prothrombin time, an assay for blood clotting activated via the extrinsic pathway. Previously we measured sulfatide activity by using activated partial thromboplastin time (aPTT), an assay for blood clotting activated via the intrinsic pathway. Prolongation of bleeding time by sulfatide was also studied to understand its effect on platelet function.

Materials and methods

Materials

Sulfatide (Na salt) was extracted and purified from pig spinal cord [10]. Thrombin from bovine plasma (T4265) and ovalbumin, grade V, were from Sigma Chemical Co., USA. Fibrinogen, plasminogen-free, from bovine plasma and *o*-phenylenediamine.2HCl were from Nacalai Tesque Co. Thromboplastin-C was the product of Dade Co., USA. Rabbit serum for prothrombin time was obtained from an ear artery of a Japanese White rabbit. Male mice (Std:ddY,

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8 weeks) for the assay of bleeding time were purchased from Japan SLC Co., Shizuoka. Immuno plates (Poly Sorp, Nunc, Denmark) were used for ELISA. Purified human fibrinogen was obtained from Protogen Ag Co., Switzerland. Horseradish peroxidase-conjugated goat IgG fraction, anti-human fibrinogen, was from Organon Teknika Co., USA. Sepharose CL-4B and a molecular weight calibration kit were from Pharmacia, Sweden.

Prothrombin time (PT)

Prothrombin time was measured by a routine method based on the method by Quick [11] as follows: 16 mg of sulfatide were suspended in 1 ml of phosphate-buffered saline, pH 7.6, (PBS) and dissolved at 75 °C for 3 min by swirling. Two-fold serial dilutions were prepared and used for inhibition of PT. In separate experiments, thromboplastin-C was diluted 10- and 100-fold with saline containing 25 mM $CaCl_2$ and BSA (1 mg ml^{-1}) and used for PT.

Effect of sulfatide on bleeding time [12, 13]

Sulfatide was dissolved in PBS at a concentration of 5 mg ml^{-1}. Mice were injected in a lateral vein with PBS (0.1 ml per 25 g body weight) or with sulfatide (500 μg per 25 g body weight). After 1, 5, 10, 30 or 60 min, or 5 h, 2 mm of tail tip was cut off with a scalpel. The tail was immediately placed in PBS at 37 °C and bleeding time was measured. Each mouse tail was cut just once without anaesthesia [12, 13]. The average bleeding time obtained from four to ten mice at each time point was calculated.

Inhibition of fibrin gel formation by sulfatide [14, 15]

Sulfatide was dissolved at 125 μg ml^{-1} in 50 mM Tris-HCl, 7.5 mM Na_2EDTA, 175 mM NaCl, pH 8.4 and two-fold serial dilutions were prepared. To 40 μl of the sulfatide solution was added the same volume of buffer containing BSA (5 mg ml^{-1}) and the solution was kept at 37 °C for 3 min. After 20 μl (0.72 U) of thrombin in the same buffer were added to the mixture and incubated for 30 s, 300 μl of fibrinogen solution (3.75 mg ml^{-1} in 75 mM Tris-HCl, pH 7.15), preincubated at 37 °C, were added and the clotting time was measured at 37 °C.

Complex formation of fibrinogen with sulfatide

Sulfatide was dissolved in PBS at 4 mg ml^{-1} and two-fold serial dilutions were prepared. GM1 ganglioside solution (1 mg ml^{-1}) was also prepared and used as a control. One ml of fibrinogen solution (2 mg in PBS) was added to 1 ml of the sulfatide solution. The mixture was incubated at 37 °C for 30 min and became turbid due to formation of an insoluble complex. Binding of fibrinogen molecules to the surface of the sulfatide micelles can be expected to produce very large complexes or aggregates which are insoluble. Thus, the amounts of the insoluble complex were measured at 330 nm as turbidity.

In separate experiments, the micelles containing various amounts of galactosylceramide and sulfatide were prepared to see if galactosylceramide produces a complex with fibrinogen. Galactosylceramide has the same structure as sulfatide but it lacks the sulfate group and is thus too hydrophobic to be solubilized by itself in PBS or saline. To compensate for this, micelles composed of various amounts of galactosylceramide and sulfatide were prepared. Galactosylceramide and sulfatide were dissolved separately in chloroform:methanol (1:1 by vol) and mixed in different ratios, while the total amount of the glycosphingolipids in each test tube was kept constant (4 mg per tube) as follows: sulfatide:galactosylceramide (4:0, 3.6:0.4, 3.2:0.8, 2.8:1.2, 2.4:1.6, 2.0:2.0, 1.6:2.4, 1.2:2.8, 0.8:3.2, 0.4:3.6, 0:4 mg:mg, respectively). After the solvent was removed under a stream of nitrogen at 37 °C, the test tubes were kept over KOH under vacuum for 2 h to remove traces of organic solvent. Then 4 ml of PBS were added to disperse the residue. It was impossible to produce a solution of galactosylceramide by itself. One ml each of the lipid solutions was mixed with 1 ml of fibrinogen solution (2 mg) and incubated and analysed as above.

Enzyme-linked immunosorbent assay (ELISA)

Solutions of 10 μg of sulfatide, galactosylceramide, or GM1 ganglioside in 1 ml of MeOH were prepared and two-fold serially diluted. One-tenth ml of each solution was added to each well of the 96-well immuno plate and the solvent was evaporated to dryness by standing overnight at room temperature. The glycosphingolipid-coated plates were blocked for 1.5 h at room temperature with 5% ovalbumin in PBS. After three 250 μl washes with 0.5% ovalbumin in PBS, 0.1 ml of human fibrinogen solution in 0.5% ovalbumin in PBS (1 μg per 0.1 ml) was added to each well and the plates were incubated 1 h at 37 °C. The plates were washed as above, 0.1 ml of the horseradish peroxidase-conjugated goat anti-human fibrinogen in 0.5% ovalbumin in PBS was added, and the plates were incubated as above. After washing, 0.1 ml of the substrate solution (10 mg of *o*-phenylenediamine.2HCl, 25 ml of 0.1 M citrate-phosphate buffer, pH 5.0, and 25 μl of 30% hydrogen peroxide) was added and incubated for 30–45 min at 37 °C. The reaction was stopped by adding 0.1 ml of 4 N sulfuric acid to the well and the absorbance was measured with an ELISA reader (MTP-22, Corona, Japan) at dual wavelengths of 492/610 nm.

In a separate experiment, the wells were coated with 500 ng of sulfatide. After blocking and washing, 0.1 ml of fibrinogen solution (10 μg or two-fold serial dilutions) was added and incubated as above.

Quantitative precipitation of fibrinogen by sulfatide

Four mg of sulfatide were dissolved in 1 ml of saline as above and serial dilutions were prepared. Then 1.25 mg of

fibrinogen was dissolved in 1 ml of saline or 2 mM $CaCl_2$-saline and the sulfatide solution (0.5 ml) and fibrinogen solution (0.5 ml) were mixed and incubated at 37 °C for 30 min. The reaction mixtures were centrifuged at 14 000 rpm for 30 min at 20 °C to obtain a firm precipitate. The amount of fibrinogen in the supernatant was determined by the method of Vera [16] as follows. Fifty μl of the supernatant were added to 450 μl of saline. To the solution, 250 μl each of 20% sodium dodecyl sulfate and 60% trichloroacetic acid were added. After 30 min at room temperature, the turbidity was measured at 340 nm.

UV spectra of fibrinogen incubated with sulfatide

Various amounts of sulfatide were dissolved in PBS (1, 0.8, 0.6, 0.4, 0.2, and 0 mg ml^{-1}). A solution of GM1 ganglioside was used as control (1 mg ml^{-1}). One ml of each sulfatide solution, ganglioside solution or PBS was incubated with 1 ml of fibrinogen solution (2 mg) for 30 min at 37 °C. UV spectra of the mixtures were obtained with a double beam spectrophotometer (UVIDEC-610C, Nippon Bunko Kogyo Co.).

Analysis of complexes formed with sulfatide and fibrinogen

Twenty-five ml of sulfatide solution (5 mg ml^{-1} in PBS) were incubated with the same volume of fibrinogen solution (2 mg ml^{-1} in PBS) at 37 °C for 30 min. The complex thus formed was collected by centrifugation at 2000 × **g** for 90 min and washed three times with PBS by resuspending and centrifuging to remove free fibrinogen or sulfatide. Sulfatide and fibrinogen in the complex were separated from each other by extraction of the lipid with hexane-isopropanol [17]. The two fractions thus obtained were dried thoroughly over KOH under vacuum until constant values were obtained.

Gel filtration of sulfatide

Four mg of sulfatide were dissolved in 2 ml of PBS and applied to a Sepharose CL-4B column (1.6 × 94 cm). Sulfatide was eluted with PBS at a flow rate of 12 ml h^{-1} at room temperature; fraction size was 3 ml. The content of sulfatide in each fraction was monitored with the anthrone-sulfuric acid method [18]. The elution volumes of catalase (232 kDa) and thyroglobulin (669 kDa) were used for molecular weight calibration.

Results

Anticoagulant and anti-platelet activities of sulfatide

Blood coagulation via the extrinsic pathway (PT) was effectively inhibited by sulfatide (Fig. 1). The amount of sulfatide required to prolong the clotting time to twice the normal level was 100 μg when the original tissue factor solution was used, whereas only 25 μg and 6 μg of sulfatide, respectively, were required when the tissue factor solutions diluted 10- and 100-fold were used. Thus, the concentration of sulfatide required for the prolongation of clotting time due to the extrinsic pathway is dependent on the concentration of the tissue factor present. The results indicate that sulfatide inhibits blood clotting via the extrinsic pathway, although its inhibition of blood clotting via the intrinsic pathway was reported previously [9].

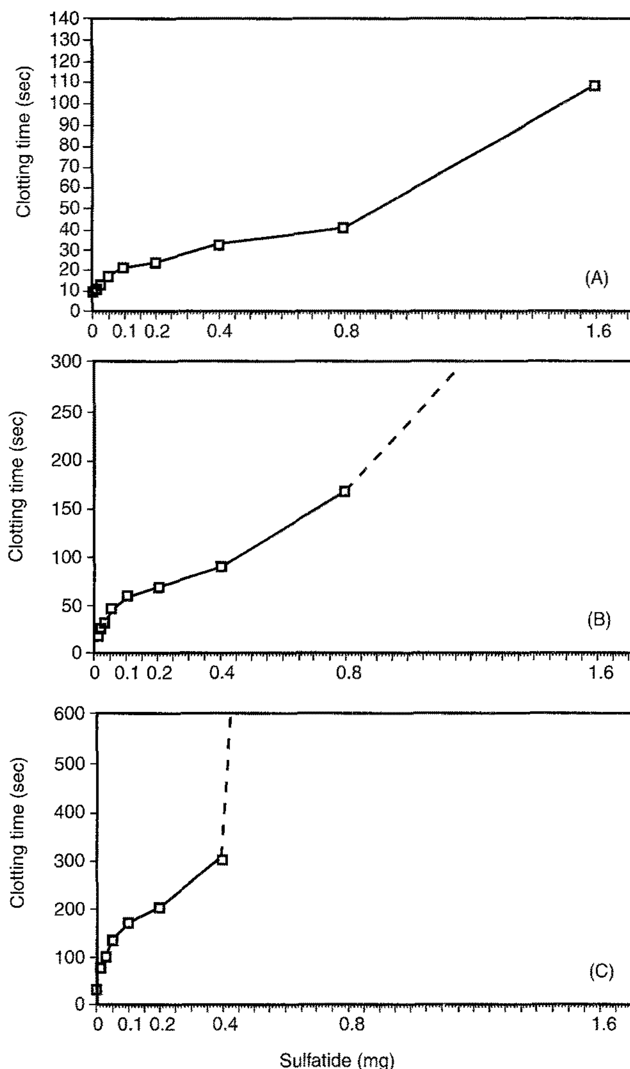

Figure 1. Anticoagulant activity of sulfatide in the PT test. The solution containing various amounts of sulfatide was preincubated with citrated plasma from rabbit. Tissue factor solution (thromboplastin-C) was added to the mixture and fibrin clotting time was measured. The difference between the three figures is in the concentration of tissue factor. (A) Original tissue factor solution (0.2 ml) was used to initiate clotting. (B) Original tissue factor solution was diluted to 1/10 with PBS containing Ca^{2+} and BSA and 0.2 ml of the solution was used to initiate the clotting. (C) 0.2 ml of the tissue factor solution diluted 1:100 was used to initiate clotting.

The test for bleeding time is well known to examine

platelet function, such as platelet adhesion and aggregation. Sulfatide effectively inhibited the bleeding time, as shown in Fig. 2. The maximum effect was observed 30 min after injection of sulfatide (almost 20 times normal). Thus, it is suggested that sulfatide is a physiological inhibitor of platelet function.

Interaction of sulfatide with fibrinogen

As the previous paper [9] showed that sulfatide inhibited neither thrombin nor factor Xa activity, we examined the action of sulfatide on fibrinogen, which is a major factor common to the intrinsic and extrinsic coagulation pathways and to platelet function. First, sulfatide effectively inhibited the formation of fibrin gel in a purified system in a dose-dependent manner (Fig. 3). The result indicated that sulfatide reacted with fibrinogen, because it had no inhibitory action on thrombin [9].

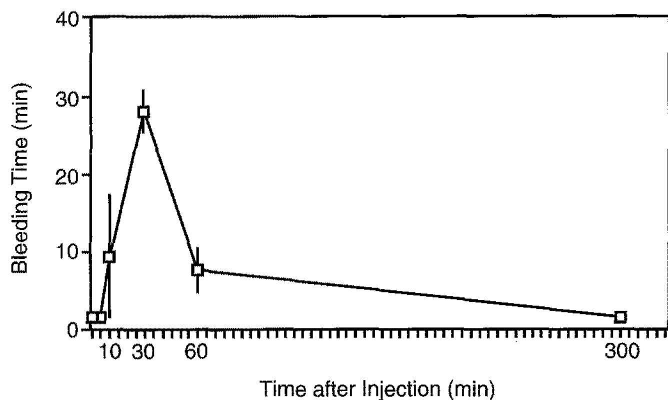

Figure 2. Effect of sulfatide on bleeding time. Mice were injected in a lateral vein with sulfatide solution (500 μg per 25 g body weight). A tail chip was cut and kept in PBS at 37 °C and the bleeding time was determined at various time points after the injection of sulfatide. The vertical line at each data point indicates the standard deviation.

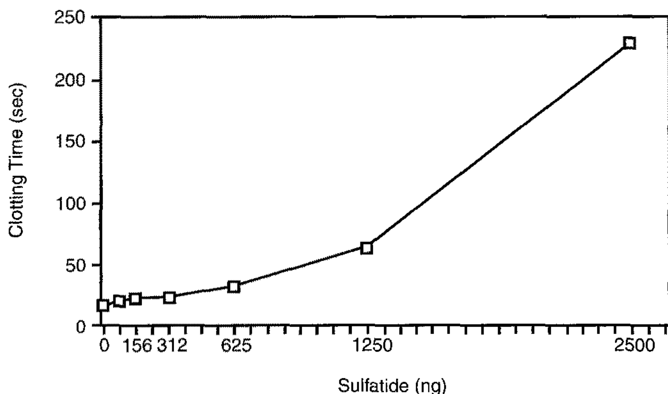

Figure 3. Inhibition of fibrin gel formation by sulfatide. The solution containing various amounts of sulfatide was added to the thrombin solution (0.72 U). Fibrinogen solution (1.25 mg in 0.3 ml) was added to the mixture and fibrin clotting time was determined.

Then, the solution containing various amounts of sulfatide was incubated with a constant amount of fibrinogen in the absence of thrombin. The incubation mixtures became turbid due to the formation of an insoluble complex. Measurement of the turbidity (Fig. 4) showed that the complex of sulfatide and fibrinogen was formed in a dose-dependent manner; it precipitated by standing overnight. GM1 ganglioside, an acidic glycolipid, did not form an insoluble complex.

Then, a potential effect of galactosylceramide on the formation of the insoluble complex of sulfatide and fibrinogen was examined (Fig. 5). Increasing amounts of galactosylceramide abolished the ability of the micelles to form the insoluble complex with fibrinogen. Thus, it is

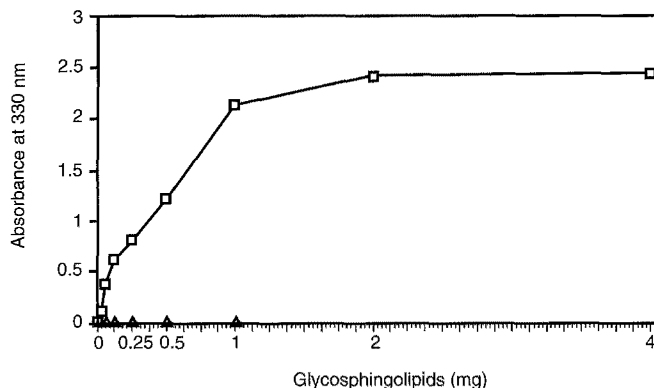

Figure 4. Complex formation of fibrinogen with sulfatide. One ml of solution containing various amounts of sulfatide (□) or GM1 ganglioside (△) was mixed with 1 ml of fibrinogen solution (2 mg) and the reaction mixture was incubated for 30 min at 37 °C. Turbidity caused by formation of the fibrinogen-sulfatide complex was measured at 330 nm.

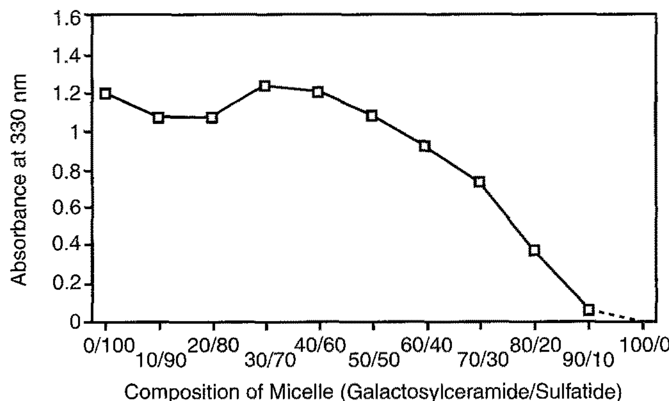

Figure 5. Effect of galactosylceramide on the formation of the fibrinogen-sulfatide complex. One ml of glycosphingolipid solution (4 mg) containing different ratios of galactosylceramide:sulfatide was mixed with 1 ml (2 mg) of fibrinogen solution. The mixture was incubated for 30 min at 37 °C and the resulting turbidity was measured at 330 nm. The X-axis shows the composition of the lipid as %.

suggested that the precipitation interaction is specific to sulfatide.

The enzyme-linked immunosorbent assay revealed that fibrinogen specifically bound to immobilized sulfatide on a plastic plate (Fig. 6). In contrast, neither galactosylceramide nor ganglioside bound fibrinogen. In another experiment, various amounts of fibrinogen added to a fixed amount of immobilized sulfatide were dose-dependently bound to the sulfatide (Fig. 7).

Furthermore, since the sulfatides in blood are found in the serum lipoproteins [6], their interaction with fibrinogen was quantitatively simulated with micellar sulfatide to observe the interaction under more physiological conditions. Figure 8 shows that the amount of fibrinogen in the precipitates was directly proportional to the amount of sulfatide added in the range of 0 to 0.5 mg. One mg of sulfatide completely precipitated all of the fibrinogen in the solution (625 μg) – not even a trace amount of fibrinogen could be detected in the supernatant. The presence of 1 mM calcium ion had no effect.

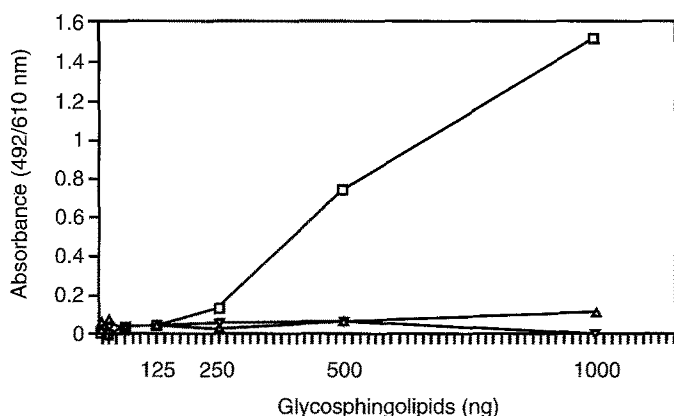

Figure 6. Binding of fibrinogen to sulfatide (ELISA). Various amounts of sulfatide (□), galactosylceramide (▽), or ganglioside (△) were coated on each well of a 96-well immuno plate. After blocking the well with 5% ovalbumin in PBS, we added 0.1 ml of human fibrinogen (1 μg) and incubated it for 1 h at 37 °C. Then 0.1 ml of horseradish peroxidase-conjugated goat anti-human fibrinogen was added to the well and incubated for a further 1 h. Finally, the substrate solution (*o*-phenylenediamine and hydrogen peroxide in citrate/phosphate, pH 5.0) was added and the mixture was incubated 30–45 min at 37 °C. After the reaction was stopped with acid, the absorbance was read at 492 and 610 nm.

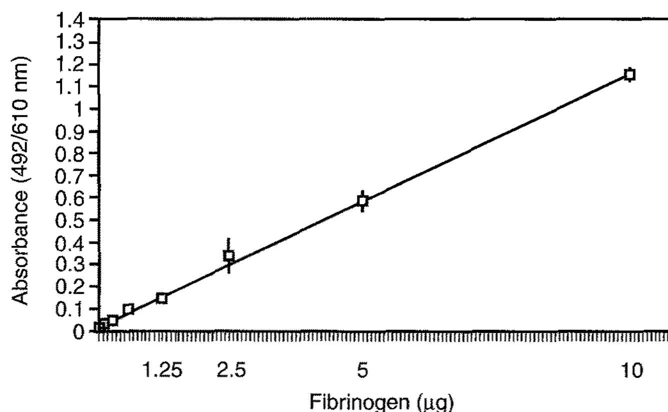

Figure 7. Binding of fibrinogen to immobilized sulfatide (ELISA). Each well of the immuno plate was coated with 500 ng of sulfatide. Various amounts of fibrinogen were added to the wells. The fibrinogen bound to the fixed sulfatide was measured as in Fig. 6.

In a larger-scale experiment, the composition of the complex was determined. Thirty-nine mg of sulfatide and 34 mg of fibrinogen were obtained from the well-washed complex. This result, together with those in Fig. 8, indicates that sulfatide can precipitate approximately an equal weight of fibrinogen. The molar ratio of sulfatide to fibrinogen in the precipitated complex was calculated to be 425:1.

The interaction of sulfatide and fibrinogen was also studied by analysis of ultraviolet spectra of the interaction mixture (Fig. 9). The absorption peak of fibrinogen (spectrum 6 in Fig. 9) gradually shifted from 280.0 nm to 274.6 nm by incubation with increasing amounts of sulfatide (spectra 5, 4, 3, and 2). No additional shift was observed at a higher concentration of the lipid (spectrum 1). The absorption spectrum of fibrinogen was not affected by ganglioside (Fig. 9). This result also supports the specific nature of the interaction between sulfatide and fibrinogen.

Since sulfatide is an amphiphilic molecule, and appears to react with fibrinogen in the form of micelles, evidence for micelle formation was sought by gel filtration chromatography of sulfatide (Fig. 10). The lipid eluted

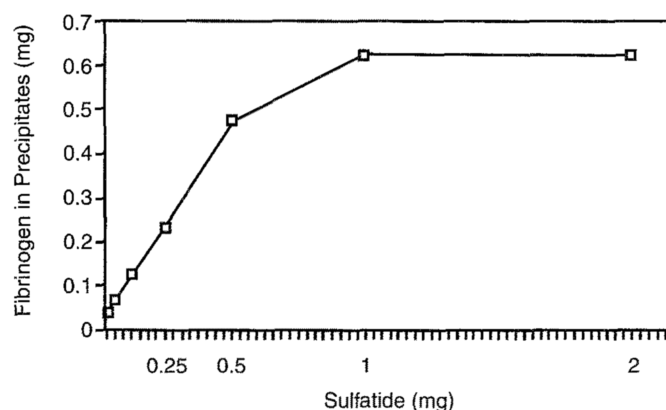

Figure 8. Quantitative precipitation of fibrinogen with sulfatide. Four mg of sulfatide in 1 ml of saline and its two-fold serial dilutions were prepared. Then 1.25 mg of fibrinogen was dissolved in 1 ml of saline and 0.5 ml each of the sulfatide and fibrinogen solutions were mixed and incubated 30 min. After centrifugation of the reaction mixture the content of fibrinogen in the supernatant was determined. The amount of fibrinogen in the precipitate was calculated by subtracting the fibrinogen content in the supernatant from the total amount of fibrinogen in the test tube.

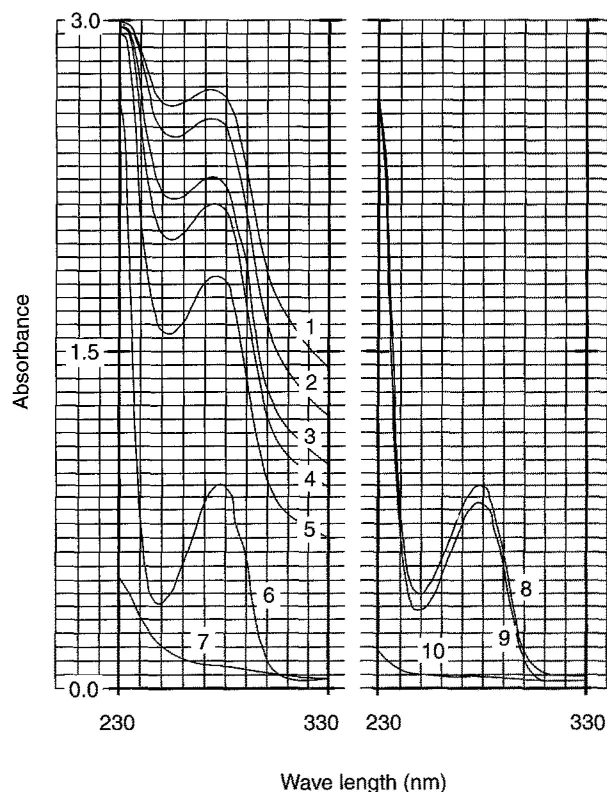

Figure 9. Ultraviolet spectra of fibrinogen incubated with sulfatide. Sulfatide or ganglioside was dissolved in PBS and a 1 ml solution was incubated with 1 ml of fibrinogen (2 mg) solution for 30 min at 37 °C. Ultraviolet spectra of the solutions are shown for mixtures containing the following amounts of sulfatide (mg): curve 1, 1; curve 2, 0.8; curve 3, 0.6; curve 4, 0.4; curve 5, 0.2; curve 6, zero. Curve 7 contains 1 mg sulfatide alone in PBS. Curve 8 contains 1 mg of ganglioside + fibrinogen. Curve 9 is the same as 6. Curve 10, 1 mg of ganglioside alone in PBS. The peaks of spectra 1 to 6, 8, and 9 were at 274.6, 274.6, 274.8, 276.4, 278.2, 280.0, 280.0, and 280.0 nm respectively.

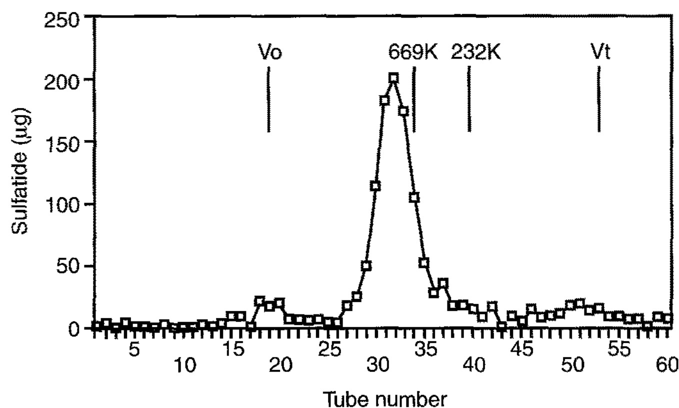

Figure 10. Gel filtration column chromatogram of sulfatide solution. Two ml of a solution containing 4 mg of sulfatide were applied to a Sepharose CL-4B column. Sulfatide concentration in each 3 ml fraction ($\mu g\, ml^{-1}$) was determined with anthrone [18]. The elution volumes of thyroglobulin (669 kDa) and catalase (232 kDa) are indicated for reference. V_o and V_t indicate void and total volumes.

as a symmetrical single peak centred at tube number 32. Negligible peaks were also visible at the void volume (very large micelles or aggregated forms) and at total volume (single molecules or very small micelles). The molecular mass of the primary sulfatide micelle was calculated from the elution volumes of thyroglobulin and catalase to be 1000 kDa. Thus, the sulfatide involved in the inhibition reaction of blood coagulation (PT), in the assay of bleeding time, and in the interaction between sulfatide and fibrinogen appears to be in the form of micelles.

Discussion

It was shown that sulfatide inhibited blood coagulation via the extrinsic pathway and that the concentration required for the inhibition was dependent on the concentration of tissue factor. The anticoagulant activity of sulfatide was much more effective when a smaller amount of tissue factor was used in the test (Fig. 1). Even 6 μg of sulfatide is sufficient to prolong blood coagulation time to twice the normal level when tissue factor diluted 100-fold is used. Bleeding time, which reflects the platelet function (aggregation for haemostasis), was also effectively prolonged by injection of sulfatide *in vivo*. Thrombosis, enough to obstruct blood-flow, is caused by either superficial or deep injury of the intima and plaques [19, 20]. The amount of tissue factor exposed to the blood stream under these conditions may be very small, compared with that in our assay condition. Thus, a small but significant amount of sulfatide in serum lipoproteins of various mammals (0.15–15 $\mu g\, ml^{-1}$ of serum) [5] would seem to be effective for the prevention of thrombus formation in these situations. The level of sulfatide is easily supplemented by injection, so it may prove to play a prominent role as an antithrombotic drug in prevention of thrombosis. Identification of the *in vivo* complex between lipoprotein-bound sulfatide and fibrinogen remains to be studied.

Recently, two distinct mechanisms were proposed for platelet aggregation: (a) high shear stress causes the aggregation of platelets linked together with von Willebrand factors; and (b) low shear stress causes the aggregation of platelets linked together with fibrinogen [21]. Since it was shown that sulfatide definitely binds to fibrinogen as well as to von Willebrand factor [2], it is probable that sulfatide inhibits both types of platelet aggregation.

It is also suggested that sulfatide forms a micelle and binds to fibrinogen sufficiently to inhibit the formation of fibrin gel. The lipid may bind to the basically charged or hydrophobic amino acid residues [22]. It seems that sulfatide binds to fibrinogen (MW of bovine fibrinogen: 330 kDa) as a large micelle (1000 kDa), so the molecular ratio of the sulfatide micelle to that of fibrinogen is

~3:1. Thrombin may not bind to or cleave fibrinogen molecules due to the steric hindrance of the sulfatide micelle. If thrombin could cleave fibrinogen even under this condition, the anticoagulant action of sulfatide could be due to inhibition of the formation of a fibrin network.

As shown above, the finding that sulfatide quantitatively precipitated all the fibrinogen seems to be useful not only for the prolongation of both the blood coagulation time (aPTT and PT) and the bleeding time, but also for the removal of fibrinogen from the blood to reduce its level in some useful way, as in the case of heparin used for haemorheological treatment of acute stroke [23].

It has already been reported by the authors that sulfatide accumulates not only in serum lipoproteins but also in the atherosclerotic aortal plaques of the WHHL rabbit, an animal model of human familial hypercholesterolaemia [6, 7]. Sulfatide accumulated in the plaques may trap fibrinogen by its specific binding. The trapped fibrinogen may not be converted to fibrin by thrombin, as observed in Fig. 3, and it may thus remain as a complex with sulfatide in the plaque. Fibrinogen is indeed present in atherosclerotic plaques [24] and it has been reported to be a cardiovascular risk factor [25, 26]. Thus, the trapping of fibrinogen by sulfatide in the plaque may be regarded as one of the possible mechanisms of accumulation of fibrinogen in the tissue.

An interesting observation in a preliminary experiment was that fibrinogen solution containing a small amount of sulfatide (1/10 amount of fibrinogen by weight) never solidified at 4 °C ($OD_{280} = 2.11$). Fibrinogen solution containing no sulfatide was easily solidified at the same temperature even at $OD_{280} = 0.92$. Probably the physical properties of fibrinogen are changed by the interaction with sulfatide, which may also indicate a specific interaction between the two.

Sulfatide has been reported to be one of the activators of blood coagulation factor XII in the intrinsic pathway [3]. This activity was ascribed to the negatively charged surface of sulfatide. Heparin, a typical anticoagulant, also activates factor XII in the intrinsic pathway in the purified system [27]. Fatty acids also activate purified factor XII due to their negatively charged groups [28]. However, it is suggested that in plasma, heparin does not support the activation of factor XII, although it can activate purified factor XII in the presence of prekallikrein in the purified system [29]. Thus it is probable that sulfatide plays a physiological role as an anticoagulant in plasma, although it activates factor XII *in vitro*. This possibility may be supported by the powerful anticoagulant (but never procoagulant) activity of sulfatide, when injected into rabbits [9] or mice in the present study. Generally, blood coagulation via the extrinsic pathway is thought to be physiological, while blood coagulation via the intrinsic pathway is still obscure [30, 31]. Fibrinogen is the blood coagulation factor I common to both the intrinsic and extrinsic pathways. Sulfatide may exert its anticoagulant activity in addition to the inhibition of platelet function by binding with fibrinogen.

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