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INHIBITION OF SPECIFIC OPIATE BINDING TO SYNAPTIC MEMBRANE BY CEREBROSIDE SULFATASE

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Abstract—The role of cerebroside sulfate in the specific binding of opiates to the synaptic membrane was investigated by the use of the enzyme, sulfatase A (cerebroside sulfatase, cerebroside-3-sulfate-3-sulphohydrolyase, EC 3.1.6.8). It was observed that the hydrolysis of sulfatides in the synaptic membrane by this enzyme was dependent on the presence of a heat stable protein molecule. When [^{3}H] naloxone binding to the enzyme-treated membrane was determined, specific binding was attenuated. This inhibition of specific binding was dependent on the concentration of the activator molecule and could not be prevented by the addition of bovine serum albumin in the binding assay mixtures. The enzymatic pH optimum for inhibiting the specific opiate binding was between pH 4.7 and 5.0. This correlated well with the pH optimum for the sulfatase A to hydrolyze free cerebroside sulfate molecules. Scatchard analysis indicated a significant decrease in the number of binding sites with no statistically significant alteration in binding affinity of the remaining sites.

There is a large body of evidence which implicates the involvement of the acidic lipid, cerebroside sulfate (CS), in the pharmacological action of the opiates. In a heptane:water partition system, this sulfatide has high affinity for the opiates and the affinities correlate well with the analgesic potencies of the drugs [1–3]. The partially purified receptor reported by Lowney *et al.* [4] has been shown to be essentially cerebroside sulfate [1]. The dye, Azure A, which has high affinity for sulfatides, decreases the analgesic potency (AD_{50}) of morphine and inhibits opiate binding to the synaptic membrane [5]. The AD_{50} value of morphine in the Jimpy mouse, a genetic mutant which has a lower cerebroside sulfate content in the brain than its littermate control, is significantly increased, while the opiate binding to the synaptic membrane is decreased, when compared to the littermate control [5]. All these observations indirectly suggest that CS may be a component present in the opiate receptor complex.

On the other hand, other membranous acidic lipids have also been implicated as being involved in the opiate binding. Abood *et al.* [6, 7] have demonstrated the binding of opiates to phosphatidylserine (PS) in an organic solvent:water partition system and an increase in the specific opiate binding to synaptic membrane after PS is incorporated. Although there is no correlation between the *in vivo* potency and the affinity of the opiates for PS, the ability of phospholipase A and to some extent phospholipase C [8, 9] to inhibit the opiate specific binding lends stronger supportive evidence for PS being involved in opiate binding than it does for CS. Previous efforts to determine the effect of hydrolysis of cerebroside sulfate by the specific enzyme sulfatase A (EC 3.1.6) on opiate binding have been fruitless. Two possible explanations could be the inability of this enzyme to degrade the sulfatides in the synaptic membrane or simply that CS is not involved in the binding of opiates. Although sulfatase A hydrolyzes the artificial substrate (nitrocatechol sulfate) rapidly, the enzymatic hydrolysis of cerebroside sulfate is observed only in the presence of taurodeoxycholate and $MnCl_2$ [10], or at low ionic strength (buffer concentration less than 10 mM) [10], or in the presence of a physiological protein activator [11]. In the present studies, the enzymatic action of cerebroside sulfatase (EC 3.1.6.8) on opiate specific binding is re-examined in the presence of the protein activator.

MATERIALS AND METHODS

Synaptic membranes were prepared from adult male Sprague–Dawley rats (200–250 g) according to the method of Gray and Whittaker [12], as modified by Terenius [13]. The membranes obtained after the purification of the P_2 fraction with a discontinuous sucrose gradient (0.6 M and 1.0 M sucrose buffered with 5 mM HEPES* at pH 7.6) were stored at −20° until use. No appreciable loss in the binding capacity was observed during the course of the experiments (8 weeks). Sucrose was removed from the membrane preparations before each experiment by diluting the membrane suspension with 10 vol. of 25 mM HEPES buffer (pH 7.6) and centrifuging at 25,000 *g* for 20 min.

Cerebroside sulfatases were prepared from human liver according to the method of Stinshoff and Jatzkewitz [10], which included steps of ammonium sulfate precipitation, gel filtration, electrofocusing and ion exchange chromatography. The 20,000-fold enriched enzyme preparation has a specific activity of 30 units/mg of protein, as determined by the method of Baum *et al.* [14]. One unit of enzymatic activity is defined as the amount of enzyme required to degrade 1 μmole of nitrocatechnol sulfate/min at 37°. When this enzyme preparation was subjected to disc gel electrophoresis, one major protein band was observed. The physiological protein activator for this enzyme was prepared from human liver by acetone precipitation, heat treatment,

electrofocusing and gel filtration, as described previously [11].

In determining the ability of cerebroside sulfatase to hydrolyze sulfatides in the synaptic membrane, tritiated sulfatide (5×10^5 cpm in less than 0.1 nmole) in chloroform–methanol (2:1) were evaporated to dryness in the incubating vessels (1.5 ml Eppendorf tubes) and then sonicated in the presence of various amounts of synaptic membrane (containing 0.55 nmole of sulfatide/μg of protein) or 20 nmoles of unlabeled sulfatides in 50 μl of 0.1 M potassium acetate buffer at pH 4.8. After addition of 0.1 units of cerebroside sulfatase and various amounts of activator, the mixtures were incubated in a total volume of 100 μl of acetate buffer (pH 4.8) at 39° for 2 hr. Activity of the enzyme was determined by the analysis of the incubation mixtures with radio thin-layer chromatography as described earlier [15].

In the experiments which determined the effects of sulfatase on opiate binding, 1.2 ml of the synaptic membrane (2.2 mg protein/ml) was washed with 25 mM HEPES buffer (pH 7.6) and resuspended in 50 mM potassium acetate buffer (pH 5.0). Incubations with the enzyme were initiated by adding 0.1 units of sulfatase and various concentrations of the activator. Final incubation mixtures (total volume = 2.0 ml) were placed in a shaking water bath at 37° for various time intervals up to 2 hr. The mixtures were vortexed periodically to ensure a uniform suspension of the membranes during the course of incubation. The reactions were terminated by the addition of 20 ml of HEPES buffer at 4° and the membranes were pelleted by centrifuging at 25,000 *g* for 20 min. The pellets were resuspended by homogenization in 1.2 ml of 25 mM HEPES buffer (pH 7.6). Opiate receptor binding was iniated by the addition of 0.2 ml of the membrane to a mixture containing a final concentration of 10 nm [^{3}H]naloxone (New England Nuclear, Dreieich, West Germany, 20 Ci/m.mole) + 5 μM levorphanol in a total volume of 1.0 ml. Incubations were carried out at 0° for 2 hr. Afterwards, the membranes were collected on Whatman GF-C filters and the filters where washed with 2 × 5 ml of HEPES buffer at 0°. Specific is defined as the difference in the amount of [^{3}H]naloxone bound to the membranes in the presence or absence of 5 μM levorphanol.

RESULTS

Hydrolysis of membranes sulfatides by cerebroside sulfatase. Though cerebroside sulfatase can hydrolyze the cerebroside sulfate vesicles in the presence of the activator, the ability of the enzyme to catalyze the removal of the sulfate groups from the sulfatides in the synaptic membrane matrix has not been established. As shown in Fig. 1A, with a fixed amount of activator (33 μg) in the incubation mixtures, a catalytic rate for the sulfatase could be observed to be dependent on the synaptic membrane concentration. The amount of product formed (cerebroside) reached a plateau when 45 μg or more of membrane protein was present in the mixture. However, the amount of product formed is also dependent on the activator concentration (Fig. 1B). Thus, the ability of this enzyme to hydrolyze CS molecules incorporated into the synaptic membrane is analogous to its ability to hydrolyze free CS molecules.

Effects of preincubation at various pH levels on opiate binding. Since the enzymatic pH optimum in hydrolyzing the cerebroside sulfate molecule is 4.5 [10] and that for the specific opiate binding to the synaptic membrane is 7.5 [16], an irreversible denaturation of the opiate receptor might might occur after prolonged incubation at acidic pH. However, this was demonstrated not to be the case. When the synaptic membranes were resuspended in 25 mM HEPES buffer at pH 7.6, there was no observable difference in the

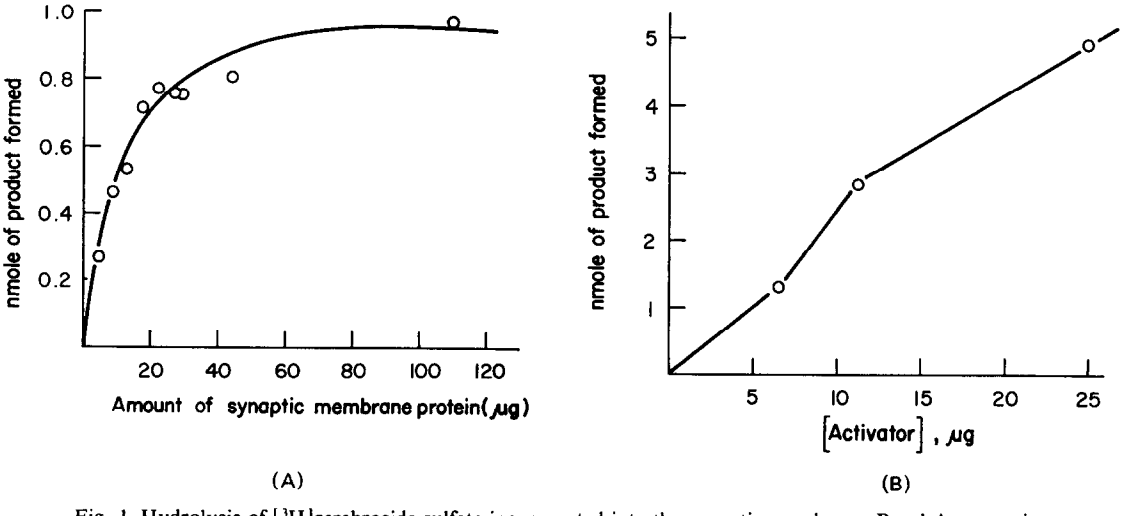

Fig. 1. Hydrolysis of [^{3}H]cerebroside sulfate incorporated into the synaptic membrane. Panel A: synaptic membrane concentration dependence of the product (cerebroside) formation. Incorporation of [^{3}H]CS and reaction conditions were as described in the text. The amount of the product formed after 2 hr of incubation at 37° was determined by the radiothin-layer chromatography procedures described previously [15]. Panel B: Activator concentration dependence of [^{3}H]CS hydrolysis. Various amounts of activator protein were added to incubation mixtures containing 0.1 units of sulfatase and 20 nmoles of sulfatides. The reactions were carried out at 37° for 1 hr. Each point represents the average of two separate incubations.

Table 1. Effect of the pH of the preincubation medium on the specific [^{3}H]naloxone binding to the synaptic membrane*

pH of Preincubation medium	Specific [^{3}H]naloxone binding (fmoles/mg protein ±S.E.)	Ratio of $pH_x/pH_{7.6}$
7.6	264.9 ± 29.9	1.00
6.0	254.1 ± 25.8	0.96 ± 0.10
5.2	269.5 ± 18.1	1.02 ± 0.07
4.7	240.2 ± 9.6	0.91 ± 0.04
4.0	248.8 ± 19.7	0.95 ± 0.07

*In each experiment, 2.64 mg of synaptic membrane was suspended in 5.0 ml of 50 mM potassium acetate buffer at the desired pH level and incubated at 37° for 2 hr. Potassium phosphate (50 mM) buffer was used for the pH 7.6 incubation. Incubations were terminated by the addition of 40 ml of 25 mM HEPES buffer (pH 7.6) at 0°C. After centrifugation at 25,000 *g* for 20 min, the membrane was resuspended in 2.4 ml of 25 mM HEPES buffer (pH 7.6). The membrane solution (0.4 ml) was added to each incubation mixture for opiate binding determination as outlined in the text. The final concentration of [^{3}H]naloxone (sp. act. = 18 Ci/m-mole) used was 20 nM.

amount of [^{3}H]naloxone specifically bound to the membranes preincubated at pH 7.6, 6.0, 5.2, 4.7 and 4.0 (Table 1). Even at the lowest pH tested, 4.0, when the membranes were observed to be aggregated due to the acidic pH, the opiate binding capacity was not affected by the 2 hr of incubation.

Effect of cerebroside sulfatase on opiate binding. When synaptic membranes were incubated with the enzyme cerebroside sulfatase at pH 5.0 for 2 hr, no significant decrease in the specific naloxone binding was observed (Fig. 2A). However, with the addition of the physiological activator to the incubation mixtures, the specific opiate binding of the enzyme-treated membranes decreased 50 per cent when compared to the control membranes. This inhibition by the sulfatase in the presence of the activator was time dependent (Fig. 2A) and was not due to the action of the activator alone (data not shown). Inhibition of [^{3}H]naloxone binding was observed only when the enzyme and activator were present in the incubation mixtures concomitantly. Maximal inhibition (50 per cent) was attained after 60 min of incubation at 37°. A further increase in the inhibition of opiate binding was not observed with an increase in enzyme concentration or prolongation of the incubation time beyond 90 min. Thus, in the subsequent experiments, incubations of the synaptic membranes with sulfatase (0.1 units) were carried out for 90 min at 37°.

The requirement for activating protein in the inhibition of opiate binding by cerebroside sulfatase was further demonstrated by the effects of various concentrations of activator on the magnitude of inhibition. As indicated by Fig. 2B, the ratio of the concentration of activator to that of synaptic membrane was crucial to the enzymatic hydrolysis of CS. Maximal inhibition was observed when the incubations with the enzyme were carried out at an activator to membrane protein ratio of 0.1 or above. A relatively high concentration of activator was necessary [activator vs enzyme (w/w) = 8] because of the impurity present in the synaptic membrane preparation and activator preparation. The

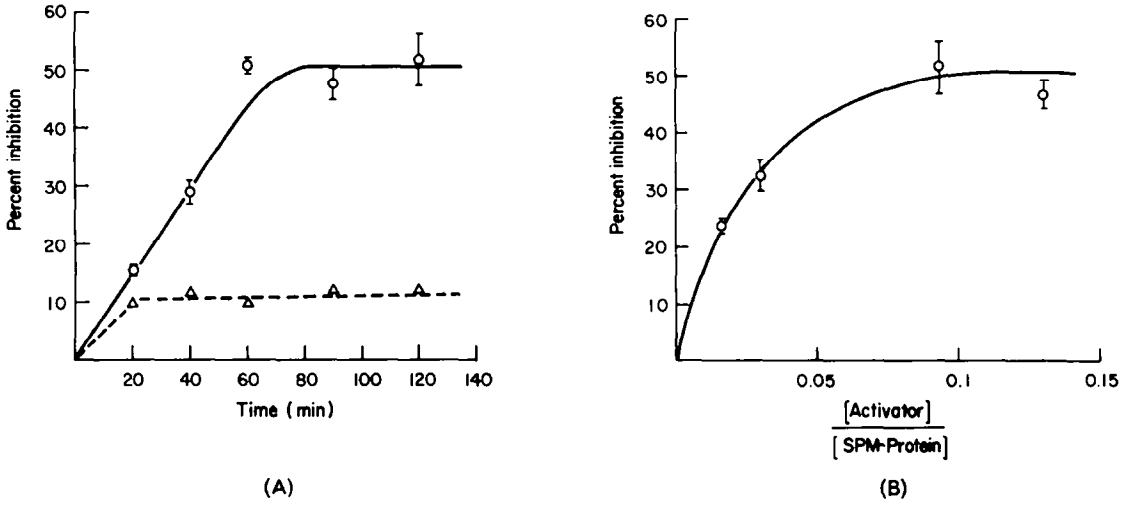

Fig. 2. Inhibition of specific [^{3}H]naloxone binding to synaptic membrane after treatment with cerebroside sulfatase. Panel A: time course of the inhibition. Synaptic membrane (13.2 mg) was incubated with 0.5 units of sulfatase and 1.3 mg of activator protein in 10 ml of 50 mM potassium phosphate buffer (pH 5.0) at 37°. At various time intervals, 2 ml of the incubation mixtures was removed and added to 30 ml of cold HEPES buffer at pH 7.6. A parallel incubation was carried out with the sulfatase alone. After centrifuging at 25,000 *g* for 20 min and resuspending the membrane pellets in 1.2 ml of HEPES buffer, opiate binding was determined on membrane treated with both the enzyme and the activator (O——O) and membrane treated with the enzyme alone (Δ- - - Δ). These values were compared with those obtained with membrane incubated at pH 5.0 and 37° in the absence of both enzyme and activator. Panel B: synaptic membrane activator dependence of the inhibition. Incubations of the membrane with various concentrations of activator were carried out at 37° for 90 min. The amount of membrane used in each determination was 2.64 mg. Per cent inhibition values were obtained by comparing the specific binding of [^{3}H]naloxone to treated membrane vs the control membrane.

Table 2. Effect of bovine serum albumin on the inhibitory action of cerebroside sulfatase*

	Specific [^{3}H]naloxone binding (fmoles/mg protein ± S.E.)	Per cent of control
(A) pH 5.0 control	117.8 ± 2.4	100.0
Sulfatase + BSA	111.6 ± 5.3	94.8 ± 4.0
(B) pH 5.0 control	83.2 ± 7.9	100.0
Sulfatase + activator	36.9 ± 2.3	44.5 ± 2.8
pH 5.0 control + 1% BSA in assay mixture	54.7 ± 7.9	65.6 ± 9.5 (100.0)
Sulfatase + activator + 1% BSA in assay mixture	25.2 ± 1.0	30.1 ± 1.2 (45.8 ± 2.0)

*Incubations with cerebroside sulfatase were carried out in 50 mM potassium acetate buffer (pH 5.0) at 37° for 90 min as described in the text. In part A, the BSA (100 μg) used was recrystallized fraction V (Sigma Chemical Co., St. Louis, MO) and was added to the incubation mixture containing 0.1 unit of the enzyme. In part B, the BSA used was the fatty acid free grade (Sigma). The control and sulfatase-treated membranes were divided into two identical halves. To one half, 1% BSA was added to the assaying mixture. Specific binding was carried out at 0° for 2 hr as described in the text. The final [^{3}H]naloxone concentration was 10 nM.

method used in the present studies could only produce preparations of synaptic membranes of 50–60 per cent purity [12]. Nevertheless, the requirement for the physiological activator protein of cerebroside sulfatase suggested that the hydrolysis of CS was the cause for the inhibition of the specific opiate binding. Other proteins, such as bovine serum albumin, did not activate the enzyme and did not produce any inhibition of opiate binding to the membrane (Table 2).

If the observed inhibition was indeed a result of the hydrolysis of CS molecules in the synptic membrane, then the enzymatic catalyzed inhibition should have similar properties to the enzymatic catalyzed hydrolysis of free CS molecules. When the pH optimum for the inhibition of opiate binding was compared with that of the hydrolysis of CS, a similar optimal pH was observed. maximal inhibition of opiate binding was decreased significantly when the pH of the incubation mixture was raised above 5.0 (Fig. 3). This parallels the pH optimim (pH 4.5) for the enzymatic hydrolysis of CS. No inhibition of opiate binding was observed when the incubation with the enzyme was carried out at a pH above 6.0. This strongly suggests that the observed inhibition was not due to the presence of contaminating enzymes, such as phospholipases or proteases, in the enzymatic preparation.

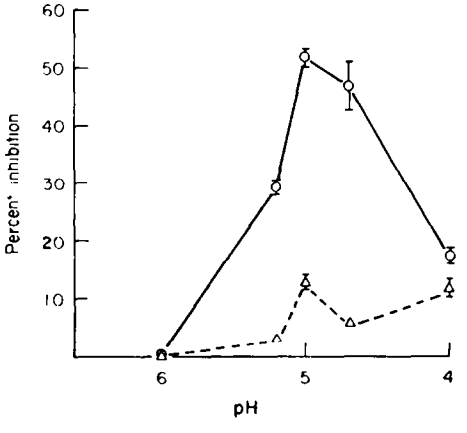

Fig. 3. pH Profile of the inhibition of opiate binding by cerebroside sulfatase treatment. Incubations at various pH levels were carried out in potassium acetate buffer (50 mM). Potassium phosphate buffer was used for the incubation at pH 7.6. key: (O——O) represents the membrane treated with both the sulfatase and the activator; (△- -△) represents membrane treated with the enzyme only. Per cent inhibition values were obtained by comparing the [^{3}H]naloxone specific binding of the treated membrane vs the membrane incubated at the specific pH for 90 min at 37°.

Since the end products of phospholipolysis, fatty acids and lysophosphatides, have been shown to inhibit opiate binding to synaptic membranes [9], the observed inhibition of opiate binding by sulfatase could be the result of the action of one of the products, cerebroside or sulfate. Hence, the effect of these two products of sulfatase action on [^{3}H]naloxone binding was determined. In these experiments, 55–550 nmoles of the products were added to the incubation mixtures containing [^{3}H]naloxone and synaptic membranes. These concentrations of cerebroside and sulfate were used because when synaptic membranes were incubated with the enzyme sulftase, 10 nmoles of product were hydrolyzed from 1 mg of membrane (Fig. 1A). Thus, the concentrations of products added to the assay mixtures were considerably higher than those observed with the enzymatic hydrolysis. As summarized in Table 3, addition of cerebroside or sulfate to the incubation mixtures did not inhibit the specific opiate binding to the synaptic membranes. Moreover, a significant sulfate concentration-dependent increase in the [^{3}H]naloxone binding was observed. This increase of specific binding by sulfate was not reported by Pasternak *et al.* [17] when a crude brain homogenate preparation was used in the binding assays. It has been reported by Lin and Simon [9] that the action of phospholipase A on specific opiate binding could be prevented by the addition of bovine serum albumin (BSA) in the opiate binding assay mixtures. When fatty acid free BSA was added to the assay

Table 3. Effect of cerebroside and sulfate on [^{3}H]naloxone specific binding synaptic membrane*

	Amount of [^{3}H]naloxone bound (fmoles/mg ± S.E.)	Per cent of control
Control	121.5 ± 5.7	100
+Cerebroside		
550 nmoles	119.7 ± 7.7	98.5 ± 6.3
140 nmoles	105.3 ± 2.6	86.7 ± 2.1
55 nmoles	137.3 ± 6.0	113.0 ± 4.9
+Sodium sulfate		
550 nmoles	169 ± 54.	139.4 ± 4.4
140 nmoles	155.9 ± 6.3	128.3 ± 5.2
55 nmoles	148.8 ± 7.1	122.5 ± 5.8

*After removing the solvent with N_2, 5.5 μmoles cerebroside was dissolved in chloroform–methanol (2:1). The solvent was removed by blowing with N_2 and the cerebroside was resuspended in 2.0 ml of 25 mM HEPES (pH 7.6) + 100 mM Na^+ by sonicating with a Bronson sonicator fitted with a micro-tip for 10 min at full power. Then various amounts of lipid were added to the incubation mixtures containing 0.34 mg of synaptic membranes, 100 nM [^{3}H]-naloxone and 100 mM Na^+ in 25 nM HEPES (pH 7.6). Opiate binding was carried out at 4° for 2 hr as described in Materials and Methods. Similar incubation conditions were used to determine the effect of the anion, SO_4^{2-}, on naloxone binding.

mixtures containing the control membrane, a significant decrease (35 per cent) in the total amount of [^{3}H] naloxone specifically bound was observed (Table 2). However, when BSA was added to the assay mixtures containing membranes treated with the enzyme and activator, no reversal of the inhibition by the sulfatase was observed (Table 2).

The method of the sulfatase inhibition of opiate binding was determined by the Scatchard analysis of [^{3}H]naloxone binding to control and enzyme-treated membranes. As shown in Fig. 4, the number of binding sites decreased 47 per cent from 579 fmoles/mg of protein in the control membranes to 307 fmoles/mg of protein in the sulfatase-treated membranes. Linear regression analysis of these two straight lines in Fig. 4 yielded correlation coefficients of 0.999 and 0.949 for control and sulfatase-treated membrane respectively. Thus, the decrease in number of binding sites in the enzyme-treated membranes is highly significant ($P < 0.05$). The apparent increase in affinity for the remaining sites in the treated membranes, however, was not statistically significant. The K_{diss} values for [^{3}H]naloxone binding to control and sulfatase-treated membranes with 95 per cent confidence limits were 28.9 nM (27.4–30.7 nM) and 16.1 nM (11.0–30.6 nM) respectively.

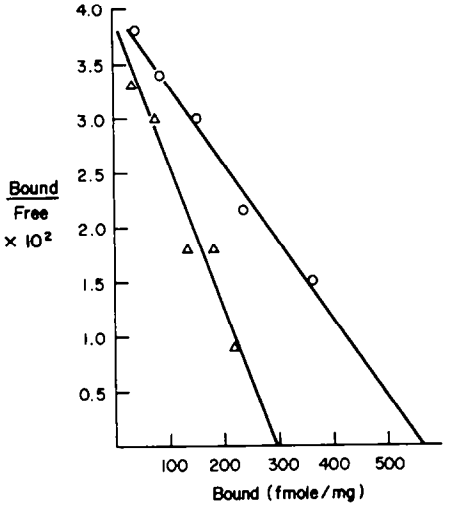

Fig. 4. Scatchard analysis inhibition of the [^{3}H]naloxone specific binding induced by sulfatase treatment. Incubation with the enzyme and activator (△——△) was carried out at pH 5.0 for 90 min at 37° as described in the text. Membrane incubated in the identical conditions in the absence of the enzyme and activator was used as control (○——○).

DISCUSSION

In the present paper the ability of cerebroside sulftase, an enzyme which has high specificity for sulfatides, to inhibit [^{3}H]naloxone binding to synaptic membrane is demonstrated clearly. The requirement for the physiological activator and the pH profile of opiate binding inhibition by sulfatase suggests that the inhibitory action is not due to the presence of contaminating proteases or lipases in the enzymatic preparation. More specifically, this inhibition of [^{3}H]naloxone binding by sulfatase is mediated via the hydrolysis of less than 2 per cent of the total sulfatide moieties in the synaptic membrane (Fig. 1A). Obviously, this inhibition of naloxone binding could result from the indirect effect of the removal of sulfatide molecules. The hydrolysis of sulfatides in the synaptic membranes could, in turn, cause a reduction in membrane stability and, thus, the inability of the glass fiber filters to retain the altered membranes. However, with no detectable reduction in the amount of [^{3}H]naloxone non-specifically bound to the enzymatic-treated membrane, such a possibility is unlikely. Thus, it seems that the sulfatides which are hydrolyzed by the cerebroside sulfatase in the presence of the physiological activator are closely associated with the opiate receptor complex in the synaptic membrane. Whether the cerebroside sulfate or other sulfatides hydrolyzed are components of the receptor cannot be distinguished in the present studies. Although Scatchard analysis indicated a decrease in the number of binding sites which would favor the sulfatides as a component of the receptor (possibly the anionic site), the responsiveness of the opiate binding to other enzymes such as phospholipases suggests otherwise. Possibly the removal of the sulfate moiety would cause a destabilization of the active conformation of the receptor. In this respect, the action of sulfatase would be similar to that of phospholipases. It has been suggested that the inhibitory action of phospholipases on the action of Na^+–K^+-ATPse, the transport of Ca^{2+}, and the uptake of neurotransmitter [18] is due to the disruption of the protein–membrane interaction by the product of the enzymes, lysophosphatides [19]. Since lysolecithin and detergents [8, 20] inhibit opiate binding, the surrounding membrane environment must have an important role in the opiate receptor action. Nevertheless, the inability of 1% BSA to prevent the sulfatase inhibition

suggests that this destabilization is distinct from the exhibited by phospholipases [8, 9].

It is of interest to note that 100 per cent inhibition of opiate binding by cerebroside sulfatase was not obtained in the present experiments. This could be due to (a) an insufficient amount of activator used or (b) another class of receptor which is not sensitive to sulfatide hydrolysis. As shown in Fig. 2B, an increase in the ratio of activator vs synaptic membrane protein concentration greater than 0.1 did not increase the magnitude of the maximal inhibition. Although the presence of excess synaptic membranes and other acidic lipids, such as phosphatidylinositol, can inhibit the sulfatase activity [10], the use of a relatively high ratio of activator vs enzyme (w/w = 8) in the present studies overcomes this inhibitory effect on the enzyme. The inability of the cerebroside sulfatase to inhibit [^{3}H]naloxone binding to the membranes completely was not caused by insufficient activator in the incubation mixtures.

When Scatchard analysis was used to determine the affinities of the opiate receptor sites which were not affected by the sulfatase, a statistically insignificant increase in the binding affinity was observed (Fig. 4). This apparent increase in binding affinity after the removal of sulfatides was also observed with the synaptic membranes prepared from Jimpy mutant mice [5]. Thus, it is tempting to postulate that there are at least two populations of receptor present in the synaptic membranes. One would possess CS as a component or stabilizer of the receptor complex and the other population might utilize acidic lipids in place of CS. Hence, it is not surprising for Miller *et al.* [21] to observe the non-correlation between the opiate receptor and cerebroside sulfate concentrations in various cell lines. In essence, Miller *et al.* [21] substantiated the observations with sulfatide and opiate receptor densities in the brain. Nevertheless, the presence of opiate receptor in cell lines which have no measurable cerebroside sulfate further suggests the multiple receptor hypothesis as postulated by Martin *et al.* [22].

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