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DESULFATION OF SULFOGLYCOLIPIDS BY ANCHIMERIC ASSISTED SOLVOLYSIS*

CLIFFORD LINGWOOD†, DARINKA SAKAC, AND GEORGE J. VELLA*
Department of Biochemistry, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8 (Canada)
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ABSTRACT

A mild method for acid-catalyzed desulfation of sulfoglycolipids in acetone is described. No ancillary degradation was observed. The method is suitable for glyceroglycolipids that might be deacylated under acid conditions. It is proposed that the formation of an acetone adduct during the reaction anchimerically assists the removal of the sulfate group.

INTRODUCTION

Sulfoglycolipids are widely distributed in eukaryotic cells[1]. Their role in biological membranes is uncertain but they can account for a considerable portion of the cell-surface negative charge. During a study of the biosynthesis of one such sulfoglycolipid, 3-*O*-(β-D-galactopyranosyl-3-sulfate)-2-*O*-hexadecanoyl-1-*O*-hexadecyl-D-glycerol (acylalkylsulfatogalactosylglycerol, SGG, **1**), a mild method of solvolysis was developed that gave 100% desulfation of GG (**2**) without detectable deacylation. It is suggested that the reaction proceeds *via* the formation of two intermediate isopropylidene derivatives. Desulfation occurs from the first intermediate, and a reaction mechanism is proposed.

EXPERIMENTAL

Materials. — Compound **1** was purified from bovine testes as previously described[2]. ^{35}S-Labelled **1** was similarly isolated, from adult Sprague Dawley rat testes, 24 h after intraperitoneal injection of 74 MBq of $H_2{}^{35}SO_4$ (New England Nuclear, Boston, MA 02118; 1.59 MBq/μg). ^{14}C-Labelled **1** was isolated 24 h after intratesticular injection of 37 MBq of D-[U-^{14}C]galactose (New England Nuclear; 263 MBq/nmol). Cerebroside sulfate (**3**) was obtained from Supelco, Inc. (Bellefonte, PA 16823).

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†To whom reprint requests should be addressed.

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Solvolysis. — Samples of **1** were treated with acetone (reagent grade, kept over molecular sieves) (1–2 mg/mL) containing 10mM hydrogen chloride (diluted from 0.1M hydrogen chloride in methanol) at 37°. (It was subsequently found that methanol was not essential for the reaction.) Aliquots of equal volume were periodically removed and, without further treatment, separated by t.l.c. in 65:25:4 (v/v) chloroform–methanol–water. After 2 h, the mixture was evaporated under nitrogen, and treated for a further 10 min with methanol containing 10mM hydrogen chloride at 37°. The mixture was analyzed by t.l.c., and the carbohydrate-containing lipids were detected by the orcinol spray. Radiolabeled species were detected by autoradiography, scraped, resuspended in ACS liquid-scintillation solution (Amersham Corp., IL 60005), and counted in a liquid-scintillation spectrometer. The reaction intermediates were isolated by preparative t.l.c.

RESULTS

Fig. 1 shows the t.l.c. pattern of aliquots taken from the reaction mixture during the solvolysis of ^{35}S-labeled **1** after being sprayed with orcinol (a) and by autoradiography (b). After 1.5 h, virtually all **1** had been converted into **2** plus a faster migrating, orcinol-positive component (*B*), which was not ^{35}S-labeled. On treatment with methanolic hydrogen chloride, compound *B* was converted into **2**. Another compound (*A*) was also formed during the reaction; it was orcinol positive and contained a sulfate ester. It appeared early during the reaction (maximum at 4 min), and decreased later ($t_{0.5}$ 20 min). The release of free sulfate correlates temporally with the appearance of **2** and *B*. The reaction was repeated with metabolically labeled **1** using D-[^{14}C]galactose under similar conditions (1 μmol in 800 μL

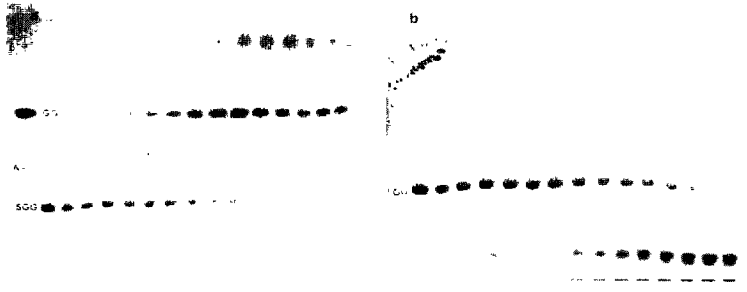
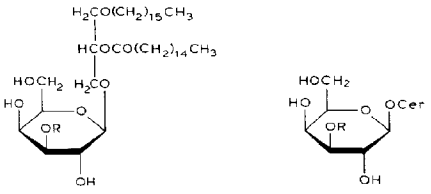


Fig. 1. Time course of solvolysis of ^{35}S-labeled **1** in acetone solution. Compound **1** (1.3 μmol) was treated at 37° with acetone (360 μL), methanol (40 μL), and M hydrogen chloride (4 μL). Aliquots (5 μL) were withdrawn at 0, 2, 4, 6, 8, 10, 20, 30, 40, 50, 60, 90, 120, and 150 min, and separated by t.l.c. in 65:25:4 (v/v) chloroform–methanol–water. Glycolipids were detected by orcinol (a) and radiolabeled bands by autoradiography (b). SGG, **1**; GG, **2**.



of acidic acetone). Autoradiography showed both compounds *A* and *B* to be ^{14}C-labeled. The radioactive compounds were eluted and counted, and reactions (using [^{35}S]- and D-[^{14}C]galactose-labeled **1**) are represented graphically in Fig. 2.

Compounds *A* and *B* were isolated from a similar reaction mixture at 10 and 60 min, respectively. The compounds were stable in the absence of acid. The purified intermediates were further subjected to the conditions described in the legend to Fig. 3 and analyzed by t.l.c. On continued solvolysis in acidic acetone, *A* was converted to *B* (and then **2**), and *B* was converted to **2** (Fig. 3, Lanes 5 and 8). The latter reaction is reversible since **2** was converted to *B* under these conditions (Fig. 3, Lane 9). In the absence of acetone, however, the reaction is irreversible,

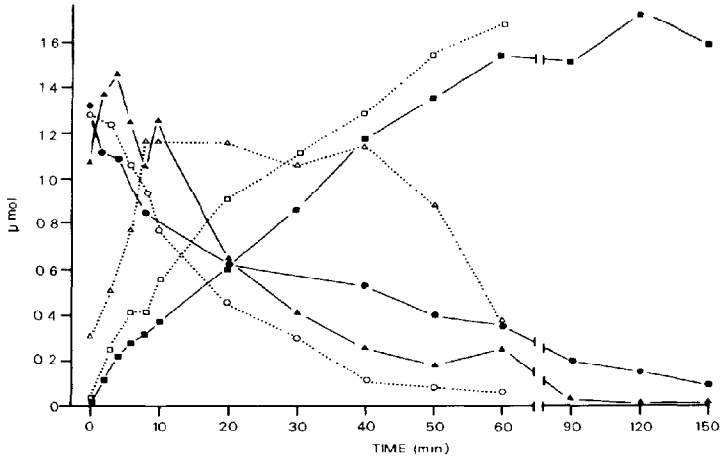


Fig. 2. Radiolabeled bands from t.l.c. separations of experiments with ^{35}S- and ^{14}C-labeled **1** were scraped and counted. The concentration of each species was calculated from the original specific activity of **1**: (●——●) ^{35}S-labeled **1**; (▲——▲) ^{35}S-labeled compound *A* (× 10); (■——■) $^{35}SO_4^{2-}$ released; (○ . . . . ○) ^{14}C-labeled **1**; (△ . . . . △) ^{14}C-labeled compound *B* (×5); and (□ . . . . □) ^{14}C-labeled **2**.

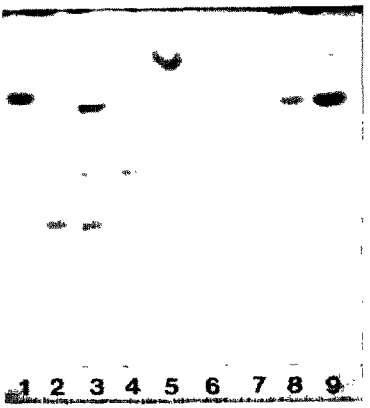


Fig. 3. T l c analysis of reaction components. (1) Standard **2**; (2) standard **1**; (3) solvolysis reaction mixture; (4) isolated compound *A*; (5) compound *A* treated with acidic acetone for 60 min at 37°. (6) isolated compound *B*, (7) compound *B* treated with acidic methanol (10mM hydrogen chloride) for 10 min at 37° (equally effective at room temperature); (8) compound *B* treated with acidic acetone for 60 min at 37°, and (9) compound **2** treated with acidic acetone for 60 min at 37°. Samples were separated by t l c in 65 :25 :4 (v/v) chloroform–methanol–water and detected by orcinol spray.

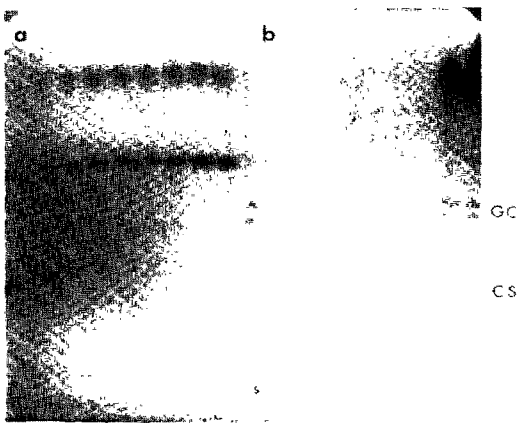


Fig. 4. Comparison of desulfation of **1** (a) and **3** (b) in acidic acetone. The reaction conditions were as described in legend to Fig. 1 (1 μmol in 800 μL of acidic acetone). Samples were withdrawn at 2, 4, 6, 8, 10, 20, 30, 60, and 90 min, separated by t l c and detected by orcinol. S = standards [**1** and **2** in (a), **3** (CS) and **4** (GC) in (b)].

and *B* was quantitatively converted into **2** (Fig. 3, Lane 7). When the solvolysis reaction was carried out with **1** in the presence of toluene instead of acetone, desulfation was greatly reduced and significant deacylation of both **1** and **2** occurred.

The desulfations of **1** and the corresponding sphingolipid, cerebroside sulfate (CS, **3**) in acidic acetone were compared (see Fig. 4). Under these conditions, **3** was also desulfated to give an intermediate compound that migrated faster than the desulfated product was formed (GC, **4**). In this case, the fast-migrating band was resolved into a doublet as was the final product, galactosylcerebroside (**4**), a result probably due to the fatty acid composition. On treatment of the products of the reaction at 30 min with D-galactose oxidase, the faster-migrating intermediate was not modified, whereas the final product was oxidized.

DISCUSSION

The procedure of solvolysis in acidic acetone gave quantitative conversion of **1** into its biological precursor **2**. It is a useful, mild method for the desulfation of glycolipids that are susceptible to mild acid degradation. Previous chemical methods for the desulfation of sulfoglycolipids include solvolysis in 1,4-dioxane[3,4], dimethyl sulfoxide[5], or mild acidic hydrolysis in methanol[1]. The reaction in 1,4-dioxane is, however, highly dependent on the water content of the solution, and sulfate removal is unpredictable, and dimethyl sulfoxide is tedious to remove. In the case of acid hydrolysis, some deacylation of glycoglycerolipids has been observed[2]. Enzymic removal of sulfate is the third alternative[6,7]. The present method is simple and reliable, and does not result in detectable deacylation. In order to study the mechanism of the reaction, the desulfation was performed at 37° so that the formation of intermediates could be monitored. However, the reaction is complete within 10 min at 60° without secondary effect (not shown).

The mechanism of the reaction appears to involve two separate intermediates, compounds *A* and *B* (Fig. 1). The reaction did not occur in the absence of acetone or in the absence of acid. The significant deacylation observed when acetone is replaced with toluene suggests that the acid is sequestered in the presence of acetone, and the reactive species may be protonated acetone. On treatment with acidic acetone, *A* was converted into *B* (Fig. 3), which suggests that *A* may be a sulfated isopropylidene derivative of **1** and *B* a nonsulfated isopropylidene derivative of **2**. The formation of **2** from *B* was reversible (Fig. 3, Lane 9) in the presence of acetone, and *B* was completely hydrolyzed to give **2** in acidic methanol (Fig. 3, Lane 7). In the reaction, *A* was formed slightly before the appearance of **2** , *B*, and the release of free sulfate (Fig. 1), which suggests that the isopropylidene derivative and not **1** is desulfated, to give the isopropylidene derivative of **2**, which is further hydrolyzed into **2**.

The results of kinetic calculations based on *Eq. 1*, where $[S]$ is the concentration of free sulfate groups, $[X]$ the acetone concentration, and $[Y]$ the water con-

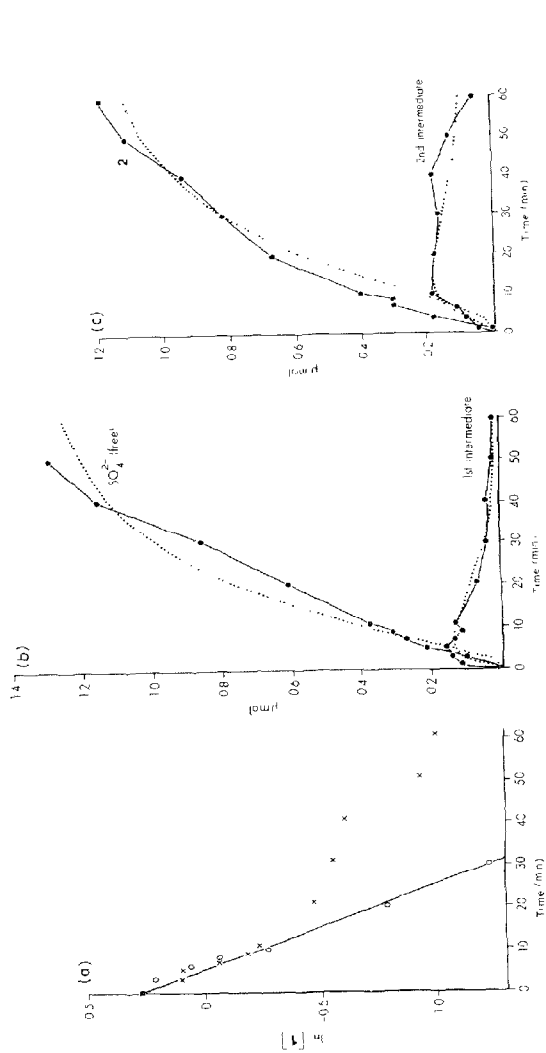


Fig. 5. Comparison of observed data and theoretical reaction-kinetics for a sequential mechanism of desulfation in acidic acetone. (a) $\ln([1]/[1]_0)$ vs time for (x) ^{35}S-labeled **1**, (o) ^{14}C-labeled **1**, slope: $k_1[X] = 50$ nmol min^{-1}. (b) 1st intermediate (compound *A*) and release of free sulfate, and (c) 2nd intermediate (compound *B*) and formation of **2** (●——●) Observed data (a 1-min time delay was included to allow for sample application to t.l.c. during the original experiment). (- - -) theoretical curve. Velocity constants used to generate theoretical curves: $k_1[X] = 50$ nmol min^{-1}, $k_2[Y] = 0$, $k_3[Y] = 360$ nmol min^{-1}, $k_4[Y] = 250$ nmol min^{-1}, and $k_5[X] = 20$ nmol min^{-1}.

centration, were compared to the data observed (Fig. 2). The rate equations for *Eq. 1* were *Eqs. 2, 3, 4, 5,* and *6.*

$$\mathbf{1} \underset{k_{-1}[Y]}{\overset{k_1[X]}{\rightleftharpoons}} A \xrightarrow[[S]]{k_2[Y]} B \underset{k_{-3}[X]}{\overset{k_3[Y]}{\rightleftharpoons}} \mathbf{2} \qquad (1)$$

$$\frac{d[\mathbf{1}]}{dt} = k_{-1}[Y][A] - k_1[X]\,\mathbf{1} = -k_1[X][\mathbf{1}]$$
(assuming $k_1[X] \gg k_{-1}[Y]$, see Fig. 3, Lane 5) (2)

$$\frac{d[A]}{dt} = k_1[X][\mathbf{1}] - (k_2 + k_{-1})[Y][A] \qquad (3)$$

$$\frac{d[S]}{dt} = k_2[Y][A] \qquad (4)$$

$$\frac{d[\mathbf{2}]}{dt} = k_3[Y][B] - k_{-3}[X][\mathbf{2}] \qquad (5)$$

$$[S] = [B] + [\mathbf{2}] \qquad (6)$$

From *Eq. 1*, the velocity constant $k_1[X]$ was obtained by plotting ln [**1**] *vs.* time (see Fig. 5a both for $^{35}SO_4^{2-}$ and D-[^{14}C]galactose-labeled **1**). The value (0.05 μmol min^{-1}, obtained from the initial rates) was used to calculate the theoretical reaction curves for [*A*] and [*S*] for various values of $k_{-1}[Y]$ and $k_2[Y]$. These curves were compared for best fit to the [^{35}S]sulfate data (Fig. 5b). The values of $k_{-1}[Y]$ and $k_2[Y]$ (0 and 0.36 μmol min^{-1}, respectively) that gave the best agreement were used to generate theoretical reaction curves for [*B*] and [**2**] for various values of $k_3[Y]$ and $k_{-3}[X]$. These curves were compared for best fit to the data obtained for D-[^{14}C]galactose-labeled **1** (Fig. 5c). The values of $k_3[Y]$ and $k_{-3}[X]$ that best fitted the experimental data were 250 and 20 nmol min^{-1}, respectively. Under these conditions, a reasonable fit for all four variables (*A*, *S*, *B*, and **2**) was obtained. The slight diversion from theoretical values for *B* during the later stages (Fig. 5c) may result from increased hydrolysis of *B* owing to the release of sulfuric acid during the reaction. Therefore, we suggest that desulfation occurs as described in Scheme 1.

$$\mathbf{1} + \text{acetone} + H^+ \xrightarrow[\searrow H_2O]{50\ \text{nmol min}^{-1}} A \xrightarrow[\nearrow H_2O \quad \searrow SO_4^{2-}]{360\ \text{nmol min}^{-1}} B \underset{20\ \text{nmol min}^{-1}}{\overset{H_2O\ 250\ \text{nmol min}^{-1}}{\rightleftharpoons}} \mathbf{2} + \text{acetone} + H^+$$

Scheme 1.

Compounds *A* and *B* are the isopropylidene derivatives of **1** and **2**, respectively. CPK models of **1** and the possible isopropylidene derivatives suggest a 4′,6′-*O*-isopropylidene derivative for **1**. Rearrangement of this derivative to the more thermodynamically stable 3′,4′-*O*-isopropylidene derivative may anchimerically assist the removal of SO_3^--3′ of the D-galactosyl residue. Alternatively, formation of the 4′,6′-*O*-isopropylidene derivative may facilitate acid hydrolysis of the sulfate ester directly. In an attempt to differentiate between these two possible pathways, C-6 of the D-galactosyl residue was oxidized selectively with D-galactose oxidase. If C-6 hydroxyl is blocked by an isopropylidene group, the D-galactosyl residue is not expected to be a substrate for the enzyme. The D-galactose oxidase–sodium borotritide procedure[8] was used to label the products of desulfation of **3** with acidic acetone (glyceroglycolipids are not substrates for D-galactose oxidase[8]). Whereas the final product (galactosyl ceramide, **4**) was susceptible to D-galactose oxidase-dependent labeling, the faster-migrating intermediate was not, indicating that the D-galactosyl residue of the intermediate was no longer a substrate for D-galactose oxidase, and was most probably an isopropylidene derivative. However, this does not conclusively identify the position of the *O*-isopropylidene group because a 3′,4′-*O*-isopropylidene group may also inhibit the action of D-galactose oxidase at C-6. The second intermediate in the desulfation of **1** (compound *B*) was also not an acceptor for the testicular sulfotransferase[9] that transfers a sulfate group to C-3′ of the D-galactosyl residue of **2**. However, again interpretation is limited because substitution of the D-galactosyl residue at either O-4′,6′ or -3′,4′ may prevent enzymic addition of a sulfate group.

The present method for the removal of sulfate from sulfate-containing glycolipids is reliable, rapid, and quantitative. The mild reaction conditions are particularly suited to acid-labile glyceroglycolipids. Thus, it is possible to obtain suitable substrates for the study of galactolipid sulfotransferases *in vitro*.

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