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# Detection in extracts of bovine brain of lipophilic complexes of sulfate esters of cholesterol and β-sitosterol

**(sulfolipids/cholesteryl methyl ether)**

VEERAMAC V. K. PRASAD*, EZZAT EL-MARAGHY*, LAURA PONTICORVO*, AND SEYMOUR LIEBERMAN*

*Departments of Biochemistry and Molecular Biophysics and of Obstetrics and Gynecology and the International Institute for the Study of Human Reproduction, College of Physicians and Surgeons, Columbia University, 630 West 168 St., New York, NY 10032

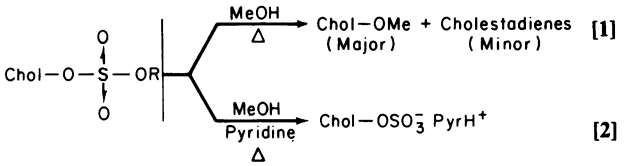
*Contributed by Seymour Lieberman, December 26, 1984*

**ABSTRACT** **Evidence indicating that there exist in bovine brains hitherto-unrecognized lipophilic conjugates of sterol sulfates is presented. These conjugates are soluble in nonpolar solvents and, when heated in methanol containing pyridine, yield polar sterol conjugates. These polar substances have the chromatographic mobility of sterol sulfates and are cleaved to free sterols when subjected to a solvolytic process known to be specific for sulfate esters. The brain sterols that have been identified in this way are cholesterol and β-sitosterol.**

In a period spanning some 15 years Oertel and his co-workers published several papers describing the occurrence, composition, and metabolic properties of lipophilic conjugates of steroid sulfates (1–4). These conjugates, detected in various mammalian tissues, were said to have the general formula, R-O-$SO_2$-OR′, where R is pregnenolone, dehydroisoandrosterone, or another $C_{21}$ or $C_{19}$ steroid and R′ is a diacylglyceryl moiety. By analogy to the phosphatides the name "sulfatide" was given to these compounds. The steroid sulfatides are unstable in the presence both of acid and of alkali and in some of the conventional chromatographic systems used for separation of the conjugates (2). This extreme lability is probably one reason why our early attempts, as well as those of other investigators, to confirm the existence of such derivatives of steroid sulfates were unsuccessful. Because these complexes could be important for a proper understanding of steroid biochemistry, we have explored the subject further.

The isolation of highly lipophilic sulfate conjugates from lipid extracts of tissues as intact entities is difficult and hence we chose to attempt indirect approaches to confirm their existence. Our attention was directed to lipophilic derivatives of cholesteryl sulfate rather than to the $C_{19}$- and $C_{21}$-steroid sulfate derivatives detected by Oertel. Furthermore, we examined brain for the presence of $C_{27}$-sterol sulfolipids since this tissue is known to have a high $C_{27}$-sterol content.

Our experimental approach was derived from a preliminary study of the reactions of two dialkyl sulfates, cholesteryl methyl sulfate and cholesteryl 1,2-dipalmitoylglyceryl sulfate (cholesterol sulfatide). The latter compound was prepared by the procedure described by Oertel (4). Its reactions, we have observed, are similar to those of cholesteryl methyl sulfate. This dialkyl sulfate, for example, has been shown by McKenna and Norymberski (5) to be converted into cholesteryl methyl ether when heated in methanol (Eq. **1**). Other products to be expected are isomeric cholestadienes. Since the procedures usually used for extraction of tissues involve the use of alcohol, sometimes hot alcohol, it is reasonable to suppose that sulfolipids of the kind suggested by Oertel, if they occur naturally, would be altered during extraction in such a way that their existence would not be detected easily. When treated in methanol containing pyridine, dialkyl sulfates, like cholesteryl methyl sulfate, are converted into their stable pyridinium sulfate salts (Eq. **2**) (5).




Chol, cholesteryl; R, methyl or diacylglyceryl

The procedures used in this study to obtain evidence for the existence of lipophilic complexes of sterol sulfates are based on the reactions described by Eqs. **1** and **2**. In one procedure, a lipophilic extract of bovine brains was boiled in methanol and the reaction mixture was then analyzed for any sterol methyl ethers and polyunsaturated sterols that might have been formed (Eq. **1**). In the second procedure, the lipophilic extract of bovine brains was heated in methanol containing pyridine; then the reaction products were analyzed for pyridinium sulfate salts of sterols (Eq. **2**). The latter procedure is outlined in Fig. 1. Although the first procedure, heating the methanol extract of bovine brain, did not give rise to methyl ethers, it did lead to the detection by their mass spectra of cholestadienes and cholestatrienes, suggesting that labile olefin-forming derivatives of sterols occur in the extract. Heating the extract in methanol containing pyridine did however lead to the formation of pyridinium sulfate salts of two sterols that were identified by the second procedure as cholesterol and β-sitosterol. Thus, even though the lipophilic substances themselves have not been isolated in this study, the detection of sterols by this process constitutes proof of the existence of hitherto-unrecognized lipophilic complexes of sterol sulfates.

## EXPERIMENTAL PROCEDURES

Bovine brains were obtained from a local abattoir. Radioactive tracers were purchased from New England Nuclear. Cholesteryl methyl sulfate was prepared by treatment of pyridinium cholesteryl sulfate with diazomethane (5). For reversed-phase chromatography, Celite 535 was packed with 0.3 ml of hexane/toluene (3:1)/g (stationary phase), which had been equilibrated with 75% aqueous methanol (mobile phase) (6). Further purification of cholesteryl sulfate as its ammonium salt was achieved by partition chromatography on Celite using isooctane/ethyl acetate/*t*-butyl alcohol/methanol/1 M $NH_4OH$ (4:2:1.2:2:3) (system S52) as described (7). Silica gel for adsorption chromatography was obtained from Tridom Chemical, (Haupauge, NY). Gas chromatography/mass spectrometry was done on a DuPont 21-492B mass spectrometer system interfaced to a Perkin-Elmer 3920 gas chromatograph fitted with a glass column (1.6

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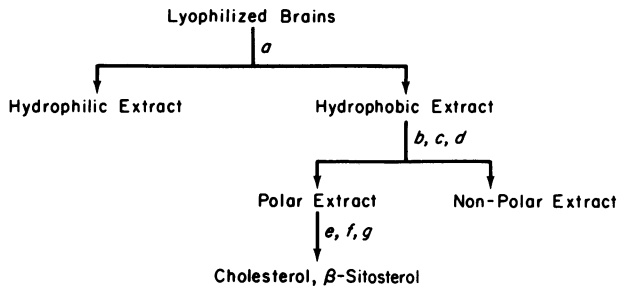


FIG. 1. (*a*) Extract with methanol, dilute with water, and partition with an equal volume of hexane/benzene (4:1). (*b*) Wash to eliminate sterol sulfates (step 1). (*c*) Heat with methanol/pyridine (step 2). (*d*) Residue partitioned between 80% aqueous methanol and hexane/benzene (4:1). (*e*) Evaporate methanol, extract aqueous solution with chloroform, and chromatograph chloroform-soluble material on Celite (step 3). (*f*) Solvolysis (tetrahydrofuran/$H^+$). (*g*) Chromatography (silica gel and HPLC) and GC/MS.

m × 2 mm) filled with 3% SP-2100 on 100/120 Supelcoport (Supelco, Bellefonte, PA) and to a Hewlett–Packard 21-094 data acquisition system. Helium at a flow rate of 30 ml/min was the carrier gas. The electron ionization energy was 70 eV. When analyzed under these conditions using a temperature program 220/260 at 2°C/min cholesteryl methyl ether and *i*-cholesteryl methyl ether had retention times of 19.1 and 14.4 min, respectively. The retention times of cholesterol and sitosterol trimethylsilyl derivatives using a temperature program 250/260 at 1°C/min were 9.9 and 15.3 min. The data were quantified by triangulation of the gas chromatographic tracing.

Bovine brains were reduced to a powder by lyophilization. The dried tissue (20 g) was stirred for 2 min in a Waring Blendor with methanol (15 ml/g). The insoluble material was removed by filtration. The filtrate was discarded because the presence of large amounts of intractable lipoidal constituents made the work-up of this fraction unmanageable. The insoluble residue from this first extract was rehomogenized with methanol (15 ml/g). Again the insoluble material was removed by filtration. (A control experiment in which synthetic tritium-labeled cholesterol sulfatide was added to lyophilized brains and the mixture was extracted twice with methanol showed that 25% of the radioactivity was present in the second methanol extract.) The second methanolic filtrate was diluted with water to give an 80% methanol solution and this mixture (500 ml) was then extracted with an equal volume of hexane/benzene (4:1). The latter phase containing hydrophobic material was separated and worked up as described below.

**Step 1. Separation into Hydrophilic and Hydrophobic Materials (Fig. 1): Elimination of Cholesteryl Sulfate from the Hydrophobic Material.** Sodium [$^3$H]cholesteryl sulfate (4 × $10^6$ cpm, 60 ng) was added to the hydrophobic extract, which was then washed with a solution of 80% methanol in water. Ninety-five percent of the tritiated sulfate was found in the aqueous methanol layer. This was separated and discarded. Another aliquot of [$^3$H]cholesteryl sulfate was added to the hexane/benzene layer and the washing with aqueous methanol was repeated. Again 95% of the tritiated sulfate added to the hydrophobic extract was eliminated. The process of adding tracer to the hexane/benzene layer and partitioning with aqueous methanol was repeated twice more to yield a hydrophobic extract essentially free of cholesteryl sulfate.

**Step 2. Treatment with Methanol/Pyridine: Separation into Polar and Nonpolar Extracts.** The hexane/benzene extract from above was taken to dryness by rotary evaporation at <45°C. The residue thus obtained was dissolved in methanol (25 ml/g of residue) containing 10% pyridine, and the solution was heated to gentle reflux for 1 hr. The solvents were evaporated *in vacuo* and the residual pyridine was eliminated by azeotropic distillation with isooctane. The residue was then partitioned between 80% methanol and hexane/benzene (4:1) as before to yield a polar and nonpolar extract.

**Step 3. Elimination of Endogenous Cholesterol from the Polar Extract.** The polar extract was labeled with [$^3$H]cholesterol (4.2 × $10^6$ cpm) and washed with hexane/benzene (4:1). The hexane/benzene wash containing 92% of the radioactivity was discarded. Another sample of [$^3$H]cholesteryl was added to the polar extract and it was again washed with hexane/benzene (4:1) to reduce further the endogenous cholesterol content. The elimination process was repeated several times until it was estimated that no more than $10^{-5}$ percent of endogenous cholesterol (originally present in the entire tissue sample) remained in the polar extract. After the polar extract was concentrated on a rotary evaporator to remove most of the methanol, the resulting residue was diluted with 150 ml of water and extracted with an equal volume of chloroform [in which pyridinium salts of sterol sulfates are soluble (5)]. The residue obtained by evaporation of the chloroform was labeled with a tracer amount of sodium [$^3$H]cholesteryl sulfate and subjected to reversed-phase Celite chromatography as described (6). The material eluting in the first holdback volume with the tracer [$^3$H]cholesteryl sulfate was rechromatographed on 50 g of Celite using the sulfate system S52. In this system the [$^3$H]cholesteryl sulfate marker eluted in the fourth holdback volume. The material eluting with the marker was used as the source of the sterols later identified by mass spectroscopy.

**Isolation of Cholesterol and β-Sitosterol from Polar Material.** The polar material, presumably sulfate esters, obtained from the above-mentioned chromatogram, was solvolyzed in tetrahydrofuran using perchloric acid (0.5 ml/100 ml of tetrahydrofuran) at 50–60°C for 2 hr (8). The reaction mixture was then neutralized with an excess of $NH_4OH$ and evaporated to dryness. The residue was extracted into benzene and the benzene extract was washed with water. More than 95% of the $^3$H label (originating from the sodium [$^3$H]cholesteryl sulfate added in step 3) was found in the benzene extract. The radioactive material in the benzene was purified by chromatography on silica gel (eluted by 4% ethyl acetate in benzene). The materials eluting with the label were then subjected to GC/MS as their trimethylsilyl ethers. Peaks at 9.9 and 15.5 min were observed and by comparison with authentic standards they were identified as cholesterol and β-sitosterol, respectively. Their assignments were corroborated by their mass spectra, which were identical with those of authentic standards. Quantification by triangulation of the peaks observed in one experiment gave values of 3.9 μg/g (of lyophilized brain) and 0.5 μg/g for cholesterol and β-sitosterol, respectively. When the experiment was repeated with a second sample of brains, values of 2.1 and 2.9 μg/g were obtained for cholesterol and β-sitosterol, respectively.

**Treatment of Bovine Brains with Methanol.** Lyophilized brains were extracted twice with methanol as described above. After filtration to remove insoluble material, the methanol extracts were combined. The combined extract was divided into two aliquots and to one (control) 5 mg of the cholesterol sulfatide was added. Each aliquot was refluxed for 2 hr and then taken to dryness by rotary evaporation. To each residue was added a tracer amount of tritiated cholesteryl methyl ether and the mixtures were chromatographed on silica gel (30 g). Stepwise elution was carried out with hexane containing increasing amounts of benzene. The tracer was eluted with benzene. The radioactive fraction from each was then rechromatographed on silica gel and the radioactive eluates were analyzed by GC/MS as described above. In the control experiment, cholesteryl methyl ether, *i*-cholesteryl methyl ether, and cholestadienes were readily

detected and identified by GC/MS. In the other aliquot only cholestadienes were detected by GC/MS. The GC tracing gave no evidence for the presence of the anticipated methyl ethers of cholesterol.

## DISCUSSION

Two aspects of the isolation procedure outlined in Fig. 1 were crucial for the proof of the existence in bovine brain of hitherto-unrecognized lipophilic complexes of sterol sulfates. The first was the elimination from the organic extract of lyophilized brains of endogenous sterol sulfates (step 1), one of which, cholesteryl sulfate, has previously been found to be present in rat brain (9). The removal of the sulfates of sterols originally present, as well as other possible polar conjugates (glycosides, glucuronides, etc.) of sterols from the organic extract was achieved by partitioning as described in step 1. The second crucial step was that which ensured the complete removal of free cholesterol from the polar material obtained by treatment with methanol containing pyridine (step 2). The polar extract was expected to contain the newly generated pyridinium salt of cholesteryl sulfate. Since our intent was to establish the presence of such a polar conjugate by converting it into cholesterol and then identifying the sterol as such, it was imperative that the polar extract be made completely free of cholesterol. This was achieved by the partition process described in step 2. The last hexane/benzene wash was analyzed by GC/MS for residual endogenous cholesterol. Since only 35 ng of cholesterol per g of lyophilized tissue was found in the organic phase, cholesterol if it were present in the polar aqueous methanol layer would have been present in still smaller amounts. At this stage of the isolation scheme, procedures were introduced to determine whether the polar derivatives in the aqueous extract had the characteristics of the anticipated pyridinium salts of the sterol sulfates. Since pyridinium sulfates of sterols can be extracted from aqueous solutions by chloroform (5), the polar fraction was extracted with this solvent. The chloroform-soluble material was further purified by chromatographic procedures that are applicable to sterol sulfates. The material so purified was ultimately solvolyzed with perchloric acid in tetrahydrofuran. This solvolysis procedure was chosen because it is, as far as it is known, specific for the hydrolysis of organic sulfates. Other polar derivatives of sterols such as phosphates and glucuronides are unaffected by this process. The sterols released were again purified by chromatography and then analyzed by GC/MS. Because of the specificity of the partition chromatogram for sulfate esters in system S52 and of the solvolysis procedure, it can be concluded that the sterols detected were derived from their sulfate esters.

The processes used in this study were designed on the assumption that the structure of the sterol-containing complex lipid occurring in bovine brain could be formulated as a disubstituted ester of sulfuric acid as originally proposed by Oertel and his colleagues. As expected, sterol sulfates were detected following the reaction with methanol and pyridine (Eq. **2**). This result is consistent with the existence of a dialkyl sulfate. However when treated with boiling methanol in the absence of pyridine, extracts of bovine brain did not lead to formation of the methyl ethers of cholesterol or of sitosterol as would be predicted from Eq. **1**. In a control experiment, the products anticipated from Eq. **1** were obtained. When a tritiated sample of the synthetic cholesterol sulfatide was added to a nonpolar extract of brain and the mixture was heated in methanol, formation of the methyl ether of cholesterol, *i*-cholesteryl methyl ether and small amounts of isomeric cholestadienes was readily confirmed. Thus even though sterol sulfates were detected after treatment with methanol/pyridine, the failure to form methyl ethers by reaction of brain extract with methanol alone appears to rule out the likelihood that the brain sulfolipids detected in this study have the dialkyl sulfate structure proposed by Oertel. The formation of dienes and trienes in refluxing methanol suggests the occurrence of labile derivatives of sterols, but as yet there is no evidence directly linking these labile substances with the progenitors of the sterol sulfates formed in methanol/pyridine. Not until the moiety (or moieties) that confers lipophilicity on the sterol sulfates is isolated and identified will it be possible to provide structures for these complex sterol-containing sulfolipids.

The results reported in this paper are important in a qualitative sense because they reveal the existence of lipophilic complexes of the sulfates of cholesterol and sitosterol. The true concentrations of these substances have not yet been determined; the values reported are at best minimal. Although the source of the derivatives described in this report is bovine brain, it is likely that such derivatives are also present in other mammalian tissues.

The significance of the sitosterol-containing sulfolipid(s) in bovine brain detected in this study is not apparent. The plant sterol has not previously been identified in this organ or in any other endocrine tissue. Why it should occur in brain as a sulfolipid remains to be determined. Analyses for both free sitosterol and the polar $\beta$-sitosteryl sulfate were also carried out in appropriate fractions but neither was found. In normal human subjects intestinal absorption of plant sterols is practically negligible (10) but it seems reasonable to suppose that the sitosterol detected in bovine brain originated in the animal feed.

The occurrence of a sulfolipid of cholesterol is also of unknown significance. It is not unreasonable to suggest that this form of the sterol has some special metabolic function. Cholesteryl sulfate has been found in various mammalian tissues (9, 11), but the results reported here raise the possibility that the sterol sulfate previously identified does not exist *in situ* as such but is actually derived by cleavage of complex sulfolipids during isolation. It seems evident that further research in the area is warranted, particularly because it is likely that analogous sulfolipids of steroids such as pregnenolone or dehydroisoandrosterone exist in mammalian tissues (1).

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