Journal of Chromatography, 137 (1977) 397–403

CHROM. 9946

PREPARATION AND THIN-LAYER CHROMATOGRAPHY OF SULFATE CONJUGATES OF THE COMPONENT OLIGOMERS OF TRITON X-100

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(Received January 20th, 1977)

SUMMARY

The preparation and thin-layer chromatographic identification of sulfate conjugates of the individual oligomers of the surfactant Triton X-100 are described. The use of these compounds as standards in the characterization of radioactive substances formed upon incubation of Triton X-100 with tissue enzyme systems and radioactive sulfate is also presented.

INTRODUCTION

The non-ionic detergent Triton X-100 is widely used in biological studies, but little attention has been given to the possible biochemical reactions of its components or their metabolites. Oxidative biodegradation has been demonstrated in bacterial systems[1,2], but little is known about the metabolism of this surfactant in animal tissues.

In the course of an investigation of steroid sulfation in animal enzyme systems, Triton X-100 was one of a number of agents used for dispersion of lipid substrates[3]. Its presence greatly stimulated [^{35}S]sulfate incorporation into the lipid fraction in certain enzyme systems. Thin-layer chromatography (TLC) showed that the lipid extracts contained several radioactive compounds that were not formed in the absence of Triton X-100. The possibility that these substances might be sulfate conjugates of the individual components of the surfactant prompted the development of the methods presented in this report.

Triton X-100 is a mixture of *p-tert.*-octylphenoxypolyethoxyethanols, abbreviated here as *p-t*-OPE$_n$, where n is the number of oxyethylene units; the average chain length is 9 to 10 oxyethylene groups[4]. A recently reported liquid chromatographic method for separation and purification of the individual oligomers of Triton X-100

and similar surfactants was used to obtain several of the oligomers of Triton X-100[5].

This paper describes the preparation of sulfate conjugates of individual Triton X-100 oligomers and the characterization of these compounds by TLC. The use of these *p-t*-OPE_n sulfate conjugates as standards in the determination of the nature of the radioactive compounds formed upon incubation of tissue enzyme systems with Triton X-100 and radioactive sulfate is also presented.

EXPERIMENTAL

Materials

Triton X-100 was obtained from Rohm and Haas, Philadelphia, Pa., U.S.A.; cholesterol, A grade, was obtained from Calbiochem, San Diego, Calif., U.S.A.; dehydroepiandrosterone sulfate, pregnenolone, and lithocholic acid were obtained from Sigma, St. Louis, Mo., U.S.A. Carrier-free [^{35}S]sulfuric acid was purchased from New England Nuclear, Boston, Mass., U.S.A. Ethyl acetate (99% pure, from Ashland, Santa Fe Springs, Calif., U.S.A.), chloroform, methanol, 1-butanol, and acetic acid, all reagent grade, were redistilled before use.

Authentic *p-t*-OPE_5 for use as a standard was prepared by condensation of recrystallized *p-tert.*-octylphenol with tetraethylene glycol monochloride as described by Mansfield and Locke[6].

Individual standard oligomers of Triton X-100 (*p-t*-OPE_n), having n values of 3–17, were separated and purified on Porasil A(60), 37–75 μm (Waters Assoc., Milford, Mass., U.S.A.); a linear gradient of ethyl acetate–acetic acid–water (100:32:30) *vs.* ethyl acetate was used as eluent[5]. The n values of the oligomers were identified by comparing their R_F values with that of authentic *p-t*-OPE_5 in a TLC system (SS-1) described below.

Cholesterol and pregnenolone sulfates were synthesized by the method of Mumma[7]; lithocholic acid sulfate was prepared by the method of Palmer and Bolt[8].

Methods

Sulfation of Triton X-100 oligomers. Sulfate conjugates of several *p-t*-OPE_n oligomers were prepared according to a modification of the method of Fieser[9] reported by Palmer and Bolt[8]. One milliliter of pyridine (previously dried over sodium sulfate) was placed in a vial and chilled in an ice-bath. Chlorosulfonic acid (0.1 ml) was added dropwise, and the mixture was stirred with a glass rod. A solution of purified Triton oligomer in pyridine (50 mg/ml) was chilled and added to the pyridine sulfate suspension and rinsed in with an additional milliliter of pyridine. The resulting suspension was stirred well, warmed slightly in a water-bath until clear, and then allowed to stand at room temperature for at least three days, or until the reaction was 90% complete as demonstrated by TLC of the reaction mixture as described below.

The samples were transferred to 100-ml round-bottom flasks with 15 ml of water and lyophilized to remove pyridine. This method was found preferable to evaporation in a rotary evaporator, since several samples could be handled simultaneously, and foaming did not occur at the low temperatures used. The sulfate conjugates were extracted three times with 2 volumes of 1-butanol. The combined butanol extracts were washed twice with 0.5 volume of water, made slightly alkaline

with NH_4OH and stored at —20°. Some solvolysis occurred during an 18 month storage period.

Biologically sulfated Triton X-100 oligomers or sterols were prepared by incubating the substrates with soluble high speed supernatants from tissue homogenates, [^{35}S]sulfuric acid, ATP, and Mg^{2+} as described elsewhere[10].

Thin-layer chromatography. TLC was carried out on precoated silica gel G plates (250 μm) (Merck, Darmstadt, G.F.R.). The following solvent systems were used: SS-1: ethyl acetate–acetic acid–water (140:32:30)[11]; SS-2: chloroform–methanol–NH_4OH (18:6:1); SS-3; acetone–benzene–water (50:50:1)[12]; and SS-4: 1-butanol–acetic acid–water (10:1:1).

Radioautography with Kodak No-Screen medical X-ray film was used for localization of radioactivity on the plates. Steroid sulfates and other lipids were visualized by spraying with 10% phosphomolybdic acid in ethanol and heating in an oven at 60°. Triton oligomers were visualized with a modified Dragendorff's reagent prepared as follows: solution A —barium chloride in distilled water, 20% (w/v); solution B— a solution of 1.7 g of basic bismuth nitrate in 100 ml of 20% aqueous acetic acid was mixed with a solution containing 40 g of potassium iodide in 10 ml of water; 200 ml of glacial acetic acid were added and the solution was diluted to 1000 ml with distilled water. Just prior to use, one volume of solution A was mixed with two volumes of solution B. Solutions A and B may be stored at room temperature for at least 6 months.

RESULTS AND DISCUSSION

Sulfate conjugates of the oligomers of Triton X-100 can be characterized by TLC using the solvent systems described above. SS-1 separates the entire range of *p-t*-OPE_n–sulfates; R_F values increase as n, the number of oxyethylene units, decreases (Figs. 1 and 2). A typical radioautogram of a chromatogram of biologically sulfated Triton X-100 in SS-1 is shown in Fig. 1. The series of radioactive compounds in samples 3 and 4 are the [^{35}S]sulfate conjugates of the components of Triton X-100 formed by enzymes from beef adrenal cortex and rat liver, respectively. The n value of each *p-t*-OPE_n sulfate conjugate may be identified by comparing its R_F with the R_F values of standard biosynthetic *p-t*-OPE_8 and *p-t*-OPE_{13} sulfates (samples 1 and 2). Oligomers with decreasing n values appeared in sequence with increasing R_F values. The spots with higher R_F values in sample 2 are uncharacterized water-soluble lipid sulfates formed by bovine adrenal cortex enzymes. They are separated from the *p-t*-OPE_n sulfate conjugates during the standard Folch procedure when the chloroform–methanol extract of the incubation mixture is washed with "theoretical upper phase"[13]. Spot A of sample 4 is cholesterol sulfate.

Chemically sulfated individual oligomers with n values from 3–17 are compared with biologically sulfated Triton X-100 in SS-1 (Fig. 2). The n values o the bio ogically sulfated oligomers, as identified by comparison of their R_F values with those of the chemically synthesized *p-t*-OPE_n sulfate conjugates, were identical to those determined by comparison with standard oligomers sulfated enzymatically (Fig. 1). Unsulfated Triton X-100 and several steroid sulfates are shown in Fig. 2 for comparison. The steroid sulfates are separated well from each other but their R_F values overlap those of the *p-t*-OPE_n sulfates.

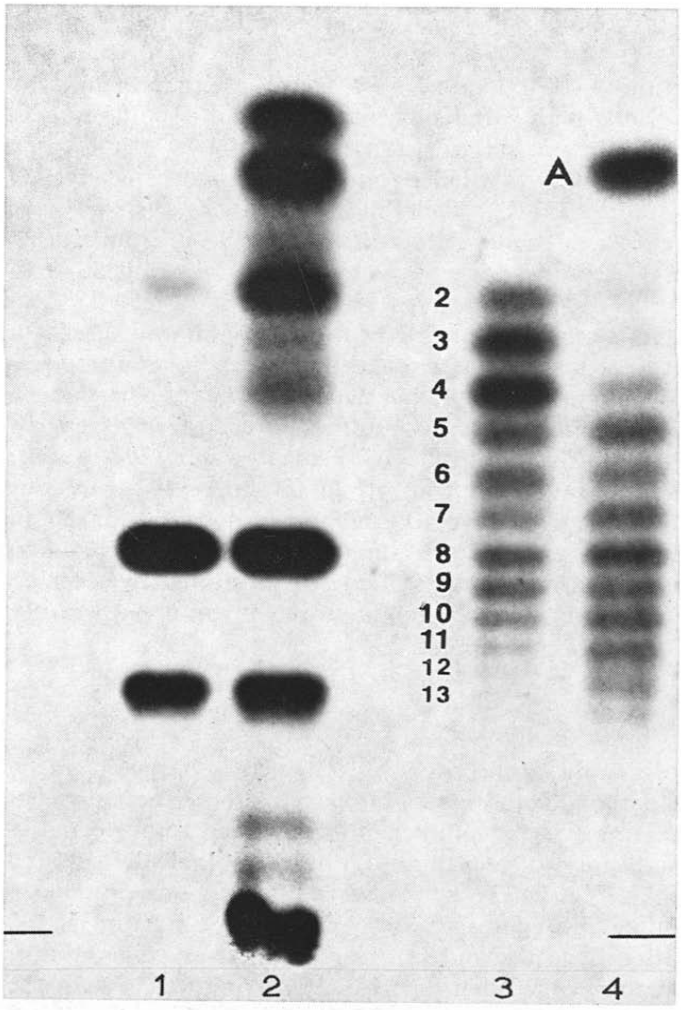

Fig. 1. Radioautogram of a thin-layer chromatogram of biosynthetic [^{35}S]sulfate conjugates of Triton X-100 and its $n = 8$ and $n = 13$ oligomers, developed in SS-1, ethyl acetate–acetic acid–water (140:32:30). Soluble supernatants (100,000 *g*) of homogenates of animal tissues were incubated with [^{35}S]sulfuric acid and Triton X-100 or its purified oligomers as described elsewhere[10]. Chloroform–methanol (2:1) extracts were washed with "theoretical upper phase" containing 0.74% KCl to remove water-soluble ^{35}S-labeled substances[13]. (1) Bovine adrenal cortex + *p-t*-OPE$_8$ and *p-t*-OPE$_{13}$; (2) same, extract not washed; (3) bovine adrenal cortex + Triton X-100; (4) rat liver + Triton X-100. Numbers adjacent to spots in sample 3 indicate the number of oxyethylene units per oligomer.

The relative migration of *p-t*-OPE$_n$ sulfates in SS-2, with respect to *n*, was the reverse of that observed in SS-1. In SS-2, the R_F increased with increasing chain length up to an *n* value of 7, after which only a very gradual increase was perceptible (Fig. 3). Unsulfated Triton oligomers did not separate in this system but moved with an R_F of approximately 0.85. Lithocholic acid sulfate could be distinguished from the other steroid sulfates which did not separate appreciably from each other.

Other solvent mixtures have been observed to separate the oligomers of Triton

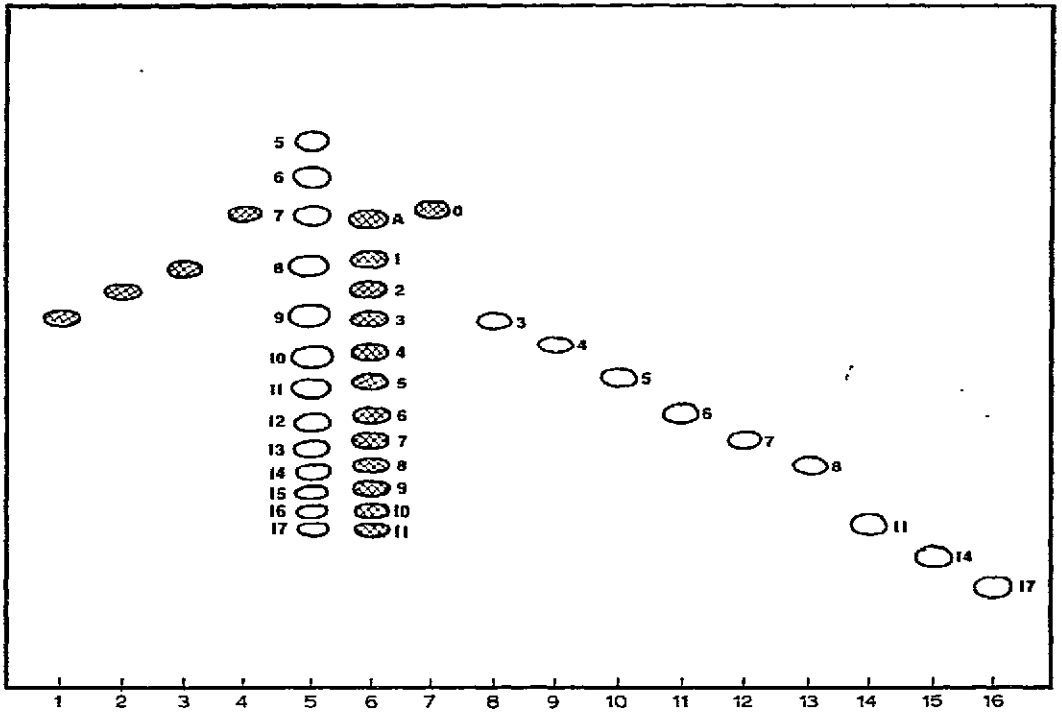

Fig. 2. Thin-layer chromatogram comparing biosynthetic [^{35}S]sulfate conjugates of Triton X-100 with its chemically sulfated individual oligomers and with biosynthetic steroid [^{35}S]sulfates and octylphenoxy [^{35}S]sulfate. Solvent was SS-1, ethyl acetate–acetic acid–water (140:32:30). (1) Dehydroepiandrosterone [^{35}S]sulfate; (2) pregnenolone [^{35}S]sulfate; (3) lithocholic acid [^{35}S]sulfate; (4) cholesterol [^{35}S]sulfate; (5) Triton X-100; (6) Triton X-100 [^{35}S]sulfate formed by rat liver supernatant; (7) octylphenoxy [^{35}S]sulfate; (8) through (16) chemically sulfated *p-t*-OPE$_n$ oligomers with n values of 3, 4, 5, 6, 7, 8, 11, 14, and 17, respectively. The plate was radioautographed prior to staining with Dragendorff's reagent. Spots with cross-hatching are radioactive. Numbers adjacent to spots indicate the number of oxyethylene units per oligomer.

X-100 in the same order as SS-1, *i.e.*, with R_F increasing as n decreases. Two systems which were tested were acetone–benzene–water (50:50:1) (SS-3) and 1-butanol–acetic acid–water (10:1:1) (SS-4). The former solvent separated the unsulfated Triton oligomers with n values of 1–13, but did not move the sulfate conjugates away from the origin. The 1-butanol–acetic acid–water system separated the sulfateconjugates with n values of 1 through 10 but was considerably slower than SS-1. The effect of modifying the solvent ratios in SS-2 and SS-4 was not explored, since the resolution obtained with ethyl acetate–acetic acid–water (140:32:30) (SS-1) was adequate for the requirements of these studies.

Since Triton X-100 and other commercial detergents are frequently used as solubilizing agents, it is important to recognize that these surfactants may be metabolized in some biological systems. In cases where the enzyme studies might be influenced by metabolic conversion of the detergent, the reaction products should be examined for the possible presence of metabolites of the detergent itself. The method given above will be helpful in detecting the formation of sulfate conjugates of Triton

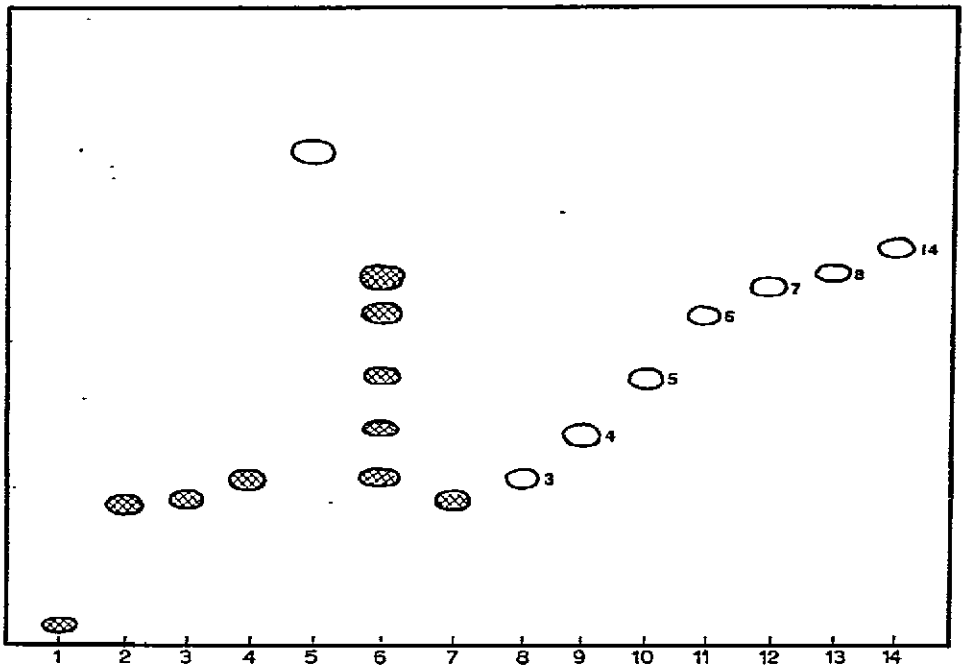

Fig. 3. Thin-layer chromatogram comparing biosynthetic [^{35}S]sulfate conjugates of Triton X-100 with its chemically sulfated individual oligomers and with biosynthetic steroid [^{35}S]sulfates and octylphenoxy [^{35}S]sulfate. Solvent was SS-2, chloroform–methanol–ammonium hydroxide (18:6:1). (1) lithocholic acid [^{35}S]sulfate; (2) dehydroepiandrosterone [^{35}S]sulfate; (3) pregnenolone [^{35}S]sulfate; (4) cholesterol [^{35}S]sulfate; (5) Triton X-100; (6) Triton X-100 [^{35}S]sulfate formed by rat liver supernatant; (7) octylphenoxy [^{35}S]sulfate; (8) through (14) chemically sulfated *p-t*-OPE$_n$ oligomers of Triton X-100 with *n* values of 3, 4, 5, 6, 7, 8, and 14, respectively. The plate was radioautographed prior to staining with Dragendorff's reagent. Spots with cross-hatching are radioactive. Numbers adjacent to spots 8 through 14 indicate the number of oxyethylene units per oligomer.

X-100 and should aid in interpreting experimental data obtained when this surfactan is used for solubilization of enzymes or substrates.

ACKNOWLEDGEMENT

This work was supported by the Medical Research Service of the Veterans Administration, by research grant No. 451 from the Los Angeles County Heart Association, Los Angeles, Calif., and grant R01-A104788 from the Institute of Allergy and Infectious Diseases, National Institutes of Health, U.S. Public Health Service.

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