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ENZYMATIC SULFATION OF TRITON X-100

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Summary

Triton X-100 increased incorporation of [^{35}S]sulfate into lipid-soluble substances when it was incubated with the high-speed supernatant enzymes of bovine or sheep adrenal cortex or rat liver, kidney or brain. Thin-layer chromatographic comparison of these compounds with chemically synthesized standards indicated that sulfate derivatives of Triton X-100 oligomers were formed in the liver and adrenal supernatant enzyme preparations. Further evidence for Triton X-100 sulfation by these enzymes was obtained by the use of [^{3}H]Triton X-100 and purified Triton X-100 oligomers. No discernible sulfation of Triton X-100 occurred in kidney or brain high-speed supernatant enzyme preparations.

A series of [^{35}S]sulfate-containing substances, which closely resembled the homologous series of [^{35}S]sulfate-labeled Triton X-100 oligomers formed by adrenal cortex enzymes, were formed in detergent-free adrenal incubates. Their chromatographic behavior suggests that they are not steroid sulfates.

The kidney supernatant enzyme fraction was shown to contain steroid sulfotransferase activity; sulfation of cholesterol, dehydroepiandrosterone, pregnenolone, cholic acid and lithocholic acid was stimulated by the presence of Triton X-100.

Introduction

Nonionic detergents are used in biological systems, both in vivo and in vitro, to solubilize drugs, hormones and membranes. The usually unexpressed assumption in these studies is the metabolic inertness of the detergent. Since

Abbreviations: OPE, *p*-tert-octylphenoxypolyethoxyethanol (of which Triton X-100 is a mixture); PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyloxazolyl-2)-benzene.

metabolism of these substances may result in changes in their chemistry, and/or in consumption of some constituents of the biological system, information concerning their metabolism is of great importance in interpretation of the experiments in which they are used.

During the course of studies of steroid sulfation by enzymes from various tissues, a number of agents, including Triton X-100, were used to disperse the lipid substrates. A great increase in [^{35}S]sulfate incorporation into the lipid fraction was observed in the presence of Triton X-100. Thin-layer chromatography of the lipid extracts revealed several labeled substances which were not formed in the absence of this detergent. Methods for the characterization of these substances were developed [1]. This report presents the application of these procedures in studies which establish the sulfation of Triton X-100 and its isolated oligomers by enzymes obtained from rat liver and from beef and sh sheep adrenals. The effect of Triton X-100 on sulfation of various added steroids by rat kidney enzymes, which are ineffective in sulfating this detergent, is also reported.

Materials and Methods

Chemicals

Triton X-100 was obtained from Rohm and Haas, Philadelphia, PA. A portion of this material was labeled in the aromatic ring to a specific activity of 472 μCi/mg by the tritium gas exposure method of Wilzbach (New England Nuclear, Boston, MA). Brij 96 and Tween 80 were obtained from Atlas Chemical Industries, Inc., Wilmington, DE.

Cholesterol, A grade, was obtained from Calbiochem, San Diego, CA. Pregnenolone, lithocholic acid, dehydroepiandrosterone, and dehydroepiandrosterone sulfate were purchased from Sigma Chemical Co., St. Louis, MO. Ethyl acetate (99% pure, from Ashland, Santa Fee Springs, CA) and $CHCl_3$, CH_3OH, *n*-butanol, and acetic acid, all of reagent grade, were redistilled before use. Carrier-free [^{35}S]sulfuric acid and [^{3}H]cholesterol sulfate ammonium salt, were purchased from New England Nuclear, Boston, MA.

Sulfation of Individual Triton X-100 oligomers and sterols

Individual oligomers of Triton X-100, having *n*-values 3—17, were separated and purified by high-pressure liquid chromatography on Porasil A(60), 37—75 μm (Waters Assoc., Milford, MA) with a linear gradient of ethyl acetate/acetic acid/water (100 : 32 : 30) vs. ethyl acetate as eluent [2].

Sulfate conjugates of several oligomers were prepared by the method described in an earlier publication [1]. Cholesterol and pregnenolone sulfates were synthesized by the method of Mumma [3]; bile acid sulfates were prepared by the method of Palmer and Bolt [4].

Incubation procedures

Rat tissues were obtained from male Sprague-Dawley rats weighing 150—250 g which had been killed by decapitation. Bovine and sheep adrenal glands were collected at the slaughter house immediately after killing and were chilled as rapidly as possible. Cortices were separated from the medullas.

Tissues were minced and homogenized in a Potter-Elvehjem homogenizer (glass/Teflon) at 0—4°C with a solution containing 0.1 M Tris-HCl and 0.03 M sodium phosphate buffers (pH 7.4), 2.5 mM cysteine and 6 mM $MgCl_2$ (1 ml per g tissue). The homogenate was centrifuged at 2—4°C for 1 h at 105 000 × *g* in a Beckman model L-2 ultracentrifuge. The clear portion of the supernatant was used for sulfotransferase studies. The basic incubation system contained 0.2 ml supernatant, 2.0 mM ATP, 1.2 mM $MgCl_2$, and approx. 10 μCi [^{35}S]-sulfuric acid in a final volume of 1.0 ml. Control samples contained no added ATP. Samples were incubated for 3 h at 37°C.

Steroid substrates were dissolved in 0.1 ml acetone, and 0.1 g of Triton X-100 was added. The mixture was heated gently under N_2 to remove acetone, and then diluted with warm 0.05 M Tris-HCl buffer, pH 7.4, while mixing vigorously. A similar procedure was used for the alternate dispersants, Tween 80 or Brij 96. In some cases, the substrate, dissolved in ethanol, was first added to the incubation tube and the ethanol evaporated under N_2 before introducing the other components of the incubation.

Extraction of lipid sulfates

Method 1 ($CHCl_3/CH_3OH$ method) [5]. After incubation, the reaction was stopped by the addition of 19 vols. of $CHCl_3/CH_3OH$ (2 : 1). After standing for at least 1 h, the samples were washed free of inorganic [^{35}S]sulfate and water-soluble sulfate conjugates by partition against 4 vols. of 0.74% KCl, centrifugation and repeated washing of the lower phase (five times) with 5 vols. 'theoretical upper phase' ($CHCl_3/CH_3OH$/0.37% KCl, 3 : 48 : 47). The washed $CHCl_3/CH_3OH$ extract was filtered to remove coagulated protein and made up to 25 ml with CH_3OH.

Method 2 [6]. The reaction was stopped by the addition of 1 ml of 2 N NH_3 saturated with $(NH_4)_2SO_4$. The mixture was extracted three times with ethyl acetate or, where indicated, with ethanol. The combined extracts were washed with 0.5 ml of 7/8 saturated $(NH_4)_2SO_4$. The volume of the final extract was adjusted to 10 ml with the extracting solvent.

Aliquots of the extracts were dried in counting vials and 10 ml of scintillation fluid (2 ml CH_3OH/50 mg PPO/1 mg POPOP/8 ml toluene) were added; radioactivity was counted in a Beckman liquid scintillation counter.

Thin-layer chromatography

Thin-layer chromatography was done on precoated silica gel G plates (0.2 mm) without fluorescent indicator (E. Merck, Darmstadt, F.R.G.). The following solvent systems were used: solvent system I, $CHCl_3/CH_3OH/NH_4OH$ (18 : 6 : 1); solvent system II, ethyl acetate/acetic acid/water (140 : 32 : 30) [7]. Steroid sulfates and other lipids were visualized by spraying with 10% phosphomolybdic acid in ethanol. Glycols were detected with Dragendorff's reagent [1]. Radioactive spots on the plates were located by radioautography with Kodak No-Screen X-ray film or, later, with DuPont Cronex Medical X-ray film. Following radioautography, radioactive spots were removed from the plate by loosening the powder and sucking it into a glass wool-plugged Pasteur pipette attached to a vacuum outlet. The radioactive material was then eluted by washing the pipette several times with ethyl acetate or with $CHCl_3/CH_3OH$ (2 : 1).

Results

Lipid sulfation in the absence and presence of Triton X-100

Triton X-100 increased [^{35}S]sulfate incorporation into the lipid fraction when it was incubated with the supernatant enzymes of bovine or sheep adrenal cortex or of rat liver, kidney or brain (Table I). This increase was greatest in the adrenal cortex (10-fold or more), somewhat less in the liver (2- to 3-fold), and lowest in the brain and kidney.

^{35}S-labeled lipids present in $CHCl_3/CH_3OH$ extracts of rat liver, kidney and brain supernatants incubated without added Triton X-100, or other added substrates, were found to contain only one major radioactive component (Fig. 1, No. 1, rat liver). The substance in liver and kidney lipid extract was identified in an earlier study as cholesterol sulfate [8]. The sulfated lipid formed in the brain supernatant was recrystallized with authentic cholesterol sulfate (Table II), indicating that brain also contains a cholesterol sulfotransferase.

The sulfotransferase activity of bovine adrenal cortex control samples was variable. Approximately half of the pooled batches of tissue incorporated [^{35}S]sulfate into five or more as-yet-unidentified compounds (Fig. 1, No. 5); the remainder incorporated only small amounts of isotope into these com-

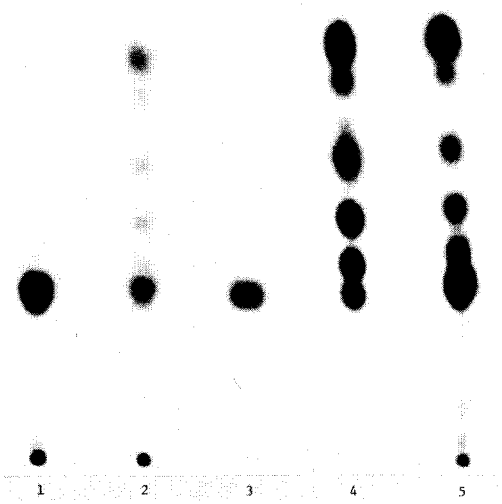


Fig. 1. Radioautogram of a thin-layer chromatogram of lipids of soluble supernatants of rat liver and bovine adrenal cortex following incubation with [^{35}S]sulfate in the presence and absence of Triton X-100. Incubation conditions were as described in Table I. Lipids were extracted by the $CHCl_3/CH_3OH$ method. Solvent system I. Samples; 1, rat liver control; 2, rat liver + Triton X-100; 3, [^{3}H]cholesterol sulfate; 4, bovine adrenal cortex + Triton X-100; 5, bovine adrenal cortex control.

TABLE I

INFLUENCE OF DETERGENTS ON [^{35}S]SULFATE INCORPORATION INTO LIPIDS OF BOVINE AND SHEEP ADRENAL CORTEX AND RAT LIVER, KIDNEY AND BRAIN

Each incubation sample contained 0.20 ml high-speed supernatant from 50% tissue homogenate; [^{35}S]sulfuric acid, carrier-free, $1.27 \cdot 10^7$ cpm; 0.05 M Tris-HCl buffer, pH 7.4; 6.0 mM sodium phosphate buffer, pH 7.4; 0.50 mM cysteine; 2.0 mM ATP; 1.2 mM $MgCl_2$, in a final volume of 1.0 ml. Samples were incubated for 3 h at 37°C. Lipid extraction was done by the $CHCl_3/CH_3OH$ method or, where indicated, by Method 2.

Addition	Radioactivity incorporated (cpm)						
	Adrenal cortex			Rat liver	Rat kidney		Rat brain
	Bovine		Sheep *		Expt. 1	Expt. 2 **	
	Expt. 1	Expt. 2					
None (ATP omitted)	2070	6360	25 200	6300	3460	8700	1130
None	22 900	25 500	103 000	68 800	38 000	65 200	6600
Cholesterol, 0.60 mM	—	—	108 000	—	—	65 600	—
Dehydroepiandrosterone, 0.60 mM	—	—	398 000	—	—	—	—
Triton X-100, 0.2%	1 110 000	677 000	1 105 000	191 000	58 900	94 200	15 600
Triton X-100 + cholesterol	841 000	794 000	1 070 000	522 000	267 000	155 000	10 100
Triton X-100 + dehydroepiandrosterone	—	—	373 000	—	—	—	—
Brij 96, 0.2%	2 840 000	—	1 840 000	704 000	—	—	—
Brij 96 + cholesterol	2 100 000	—	—	1 040 000	—	—	—
Tween 80, 0.2%	17 700	23 700	50 500	24 200	11 500	—	3470
Tween 80 + cholesterol	23 800	33 000	—	214 000	73 200	—	8100

* Lipids were extracted with ethyl acetate.

** Lipids were extracted with ethanol.

pounds, and the major portion into a conjugate having a R_F value indistinguishable from that of cholesterol sulfate. Attempts to crystallize this material with authentic cholesterol sulfate showed that only a small percentage, if any, of the radioactive material was cholesterol [^{35}S]sulfate (Table II).

In the presence of Triton X-100, five or more [^{35}S]sulfate conjugates were formed by the soluble enzymes of rat liver and bovine adrenal cortex (Fig. 1, Nos. 2 and 4). These compound had R_F values almost identical to those of the compounds formed in the adrenal cortex control samples (Fig. 1, No. 5). These radioactive conjugates were not detected in incubates of kidney or brain enzymes with Triton X-100.

Mechanism of effect of Triton X-100

Two possible explanations for the effect on [^{35}S] sulfate incorporation by Triton X-100 were considered: (1) the detergent may stimulate sulfation of endogenous lipids through an influence on the physicochemical state of either the sulfotransferase(s) or the substrates; or (2) the component oligomers of the detergent themselves may be sulfated by enzyme(s) present in the tissue supernatants. These possibilities were tested by the following experiments.

(a) Sulfation of [^{3}H]Triton X-100. Adrenal cortex supernatant enzymes were incubated with tritium-labeled Triton X-100 or with unlabeled detergent and [^{35}S]sulfate, and $CHCl_3/CH_3OH$ lipid extracts were chromatographed in solvent system I. Tritium-labeled compounds were formed which had the same mobilities as did the [^{35}S]sulfate conjugates (Fig. 2, Nos. 3 and 4), indicating that the radioactive detergent had been sulfated.

(b) Characterization of biosynthetic [^{35}S]sulfate conjugates. The apparent enzymatic sulfation of [^{3}H]Triton X-100 was substantiated by chromatographic comparison of the ^{35}S-labeled compounds formed by rat liver and bovine adrenal cortex enzymes in the presence of Triton X-100 with standard synthetic sulfate conjugates of individual Triton X-100 oligomers. The radioactive compounds present in $CHCl_3/CH_3OH$ extracts of both tissues had TLC mobilities in solvent system I and solvent system II which were identical with those of standard sulfate conjugates of Triton X-100 oligomers (liver conjugates are shown in Figs. 3 and 4). Similar results were obtained with both tissues,

TABLE II

CRYSTALLIZATION OF [^{35}S]LIPID OF RAT BRAIN AND BOVINE ADRENAL CORTEX WITH POTASSIUM CHOLESTEROL SULFATE

Incubation conditions were the same as described in Table I. Lipids were extracted by the $CHCl_3/CH_3OH$ method. CH_3OH was used for crystallization.

Purification step	cpm per mg cholesterol	
	Brain	Adrenal
Starting material	1230	120 000
First recrystallization	782	2660
Second recrystallization	794	613
Third recrystallization	801	344
Fourth recrystallization	—	367

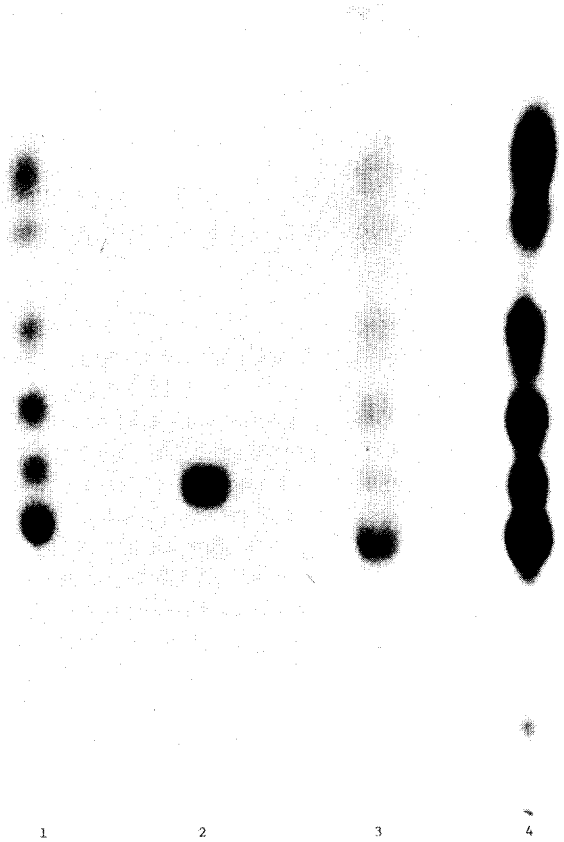


Fig. 2. Radioautogram of a thin-layer chromatogram of lipids of bovine adrenal cortex supernatant following incubation with [^{3}H]Triton X-100 and with unlabeled Triton X-100 and [^{35}S]sulfate. Incubation conditions were as described in Table I. Lipids were extracted by the $CHCl_3/CH_3OH$ method. Solvent System I. Samples: 1, adrenal + [^{3}H]Triton X-100 + [^{35}S]sulfate; 2, [^{3}H]cholesterol sulfate; 3, adrenal + [^{3}H]Triton X-100; 4, adrenal + [^{35}S]sulfate + unlabeled-Triton X-100.

except that there were differences in the relative amounts of sulfate conjugates of Triton X-100 oligomer formed, and that rat liver lipid extracts contained, in addition, cholesterol [^{35}S]sulfate (Fig. 3, spot F; Fig. 4 spot CS).

Solvent system II, which fractionates most of the sulfated oligomers of Triton X-100 [1], was used to further fractionate and identify the radioactive materials eluted from a chromatogram in solvent system I. The fastest moving spot in solvent system I separated into several spots, which correspond to the sulfate conjugates of OPE_7 to OPE_{13} (Fig. 4, No. 1). The slowest-moving spot was a mixture of the sulfate conjugate of OPE_2 and cholesterol sulfate, with a trace of OPE_1 sulfate (Fig. 4, No. 6). The intermediate radioactive compounds had R_F values corresponding to the sulfate conjugates of the oligomers with 3, 4, 5 and 6 ethoxy units.

(c) Sulfation of purified OPE_8 and OPE_{13}. Further evidence that Triton X-100 is a substrate for rat liver and bovine adrenal cortex sulfotransferase(s)

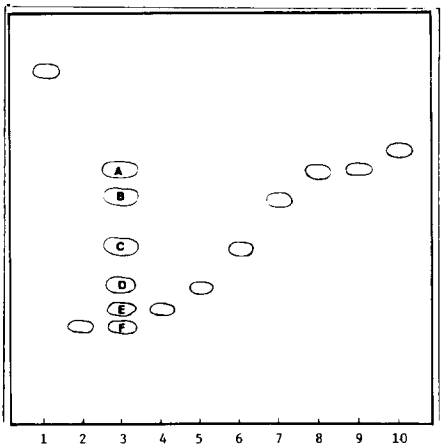


Fig. 3. Thin-layer chromatogram in solvent system I comparing biosynthetic [^{35}S]sulfate conjugates formed in the presence of Triton X-100 with chemically sulfated individual oligomers of Triton X-100. Samples: 1, Triton X-100; 2, potassium cholesterol sulfate; 3, $CHCl_3/CH_3OH$ extract of liver enzymes following incubation with [^{35}S]sulfate and Triton X-100; 4—10, chemically sulfated OPE_n oligomers with n values of 3, 4, 5, 6, 7, 8 and 14, respectively. The plate was radioautographed to locate the spots in sample 3. Samples 1 and 2 were located with phosphomolybdic acid and samples 4—10 with Dragendorff's reagent.

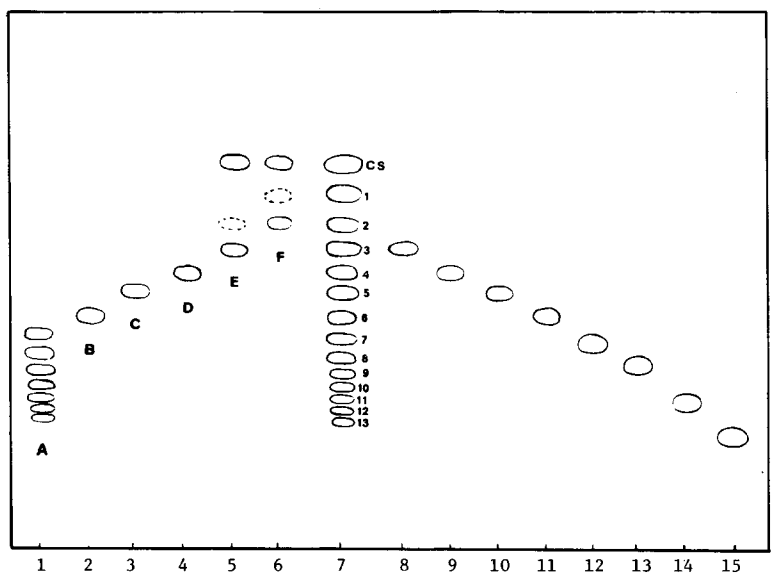


Fig. 4. Thin-layer chromatogram in Solvent system II comparing biosynthetic [^{35}S]sulfate conjugates formed in the presence of Triton X-100 with chemically sulfated individual oligomers of Triton X-100. Samples: 1—6, spots A, B, C, D, E, and F, respectively, eluted from a TLC in solvent system I (see Fig. 3, No. 3); 7, $CHCl_3/CH_3OH$ extract of liver enzymes following incubation with [^{35}S]sulfate and Triton X-100 (numbers indicate n value of labeled oligomer); 8—15, chemically sulfated OPE_n oligomers with n values of 3, 4, 5, 6, 7, 8, 11, and 14, respectively. The plate was radioautographed to locate spots in samples 1—7 prior to staining with Dragendorff's reagent to visualize spots in 8—15.

was provided by incubation of two purified oligomers, OPE_8 and OPE_{13}, with the enzyme preparations. Both oligomers stimulated [^{35}S]sulfate uptake in the adrenal cortex supernatant, and OPE_8 stimulated sulfation in the liver supernatant (Table III).

Thin-layer chromatography showed that in both tissues radioactive compounds were formed with R_F values corresponding to those of the sulfate conjugates of the 8- and 13-ethoxy oligomers (Figs. 5a and 5b). An additional sulfate conjugate was formed in the liver supernatant when OPE_8 was the substrate; it was observed with both solvent systems (Fig. 5a, No. 3, spot 8a; Fig. 5b, No. 3, spot 8a). A second conjugate for OPE_{13} was formed in both tissues but was distinguished from the major conjugate only in solvent system I (Fig. 5a, Nos. 4 and 9, spot 13a); its relative concentration was greater in the adrenal cortex than in the liver. In addition, a third conjugate was seen faintly in the liver extract in solvent system I after incubation with OPE_{13} (Fig. 5a, No. 4, spot 13b). The identity of these additional sulfate conjugates is unknown at this time.

When both of the purified oligomers were combined as a substrate, the 8-ethoxy oligomer was conjugated in preference to the 13-ethoxy oligomer. Further studies of the effect of substrate concentration will be necessary before the reason for this effect can be interpreted. The preference for the 8-ethoxy oligomer could be due to its higher concentration, or to enzyme specificity.

(d) [^{35}S]Sulfate conjugates in adrenal controls. ^{35}S-labeled compounds formed in adrenal cortex supernatant control samples (examples are shown in Fig. 1, No. 5) were compared with the [^{35}S]sulfate conjugates formed in the presence of Triton X-100. The radioactive compounds were eluted from a thin-layer chromatogram in solvent system I (Fig. 6a, Nos. 3 and 4), and pairs of compounds with similar R_F values were rechromatographed side-by-side in solvent system II (Fig. 6b). In each pair, the 'control' [^{35}S]sulfate conjugate had a slightly higher R_F value than did the Triton X-100 oligomer conjugate. Fraction A from both the 'control' and the Triton X-100 samples separated into several components in solvent system II (Fig. 6b, Nos. 1 and 2; Fig. 6c, Nos. 2 and 3).

TABLE III

EFFECT OF TRITON X-100 AND ITS OLIGOMERS OPE_8 AND OPE_{13} ON [^{35}S]SULFATE INCORPORATION IN SOLUBLE ENZYME SYSTEMS OF RAT LIVER AND BOVINE ADRENAL CORTEX

Incubation conditions were the same as those described in Table I. Each sample contained $8.6 \cdot 10^6$ cpm of [^{35}S]sulfuric acid. Lipids were extracted by the $CHCl_3/CH_3OH$ method.

Addition	Radioactivity incorporated (cpm)	
	Rat liver	Bovine adrenal cortex
None (ATP omitted)	20 300	6230
None	91 700	14 100
Triton X-100, 3.18 mM	162 000	530 000
OPE_8, 3.57 mM	202 000	388 000
OPE_{13}, 2.52 mM	90 400	213 000
OPE_8, 1.79 mM + OPE_{13}, 1.26 mM	165 000	335 000

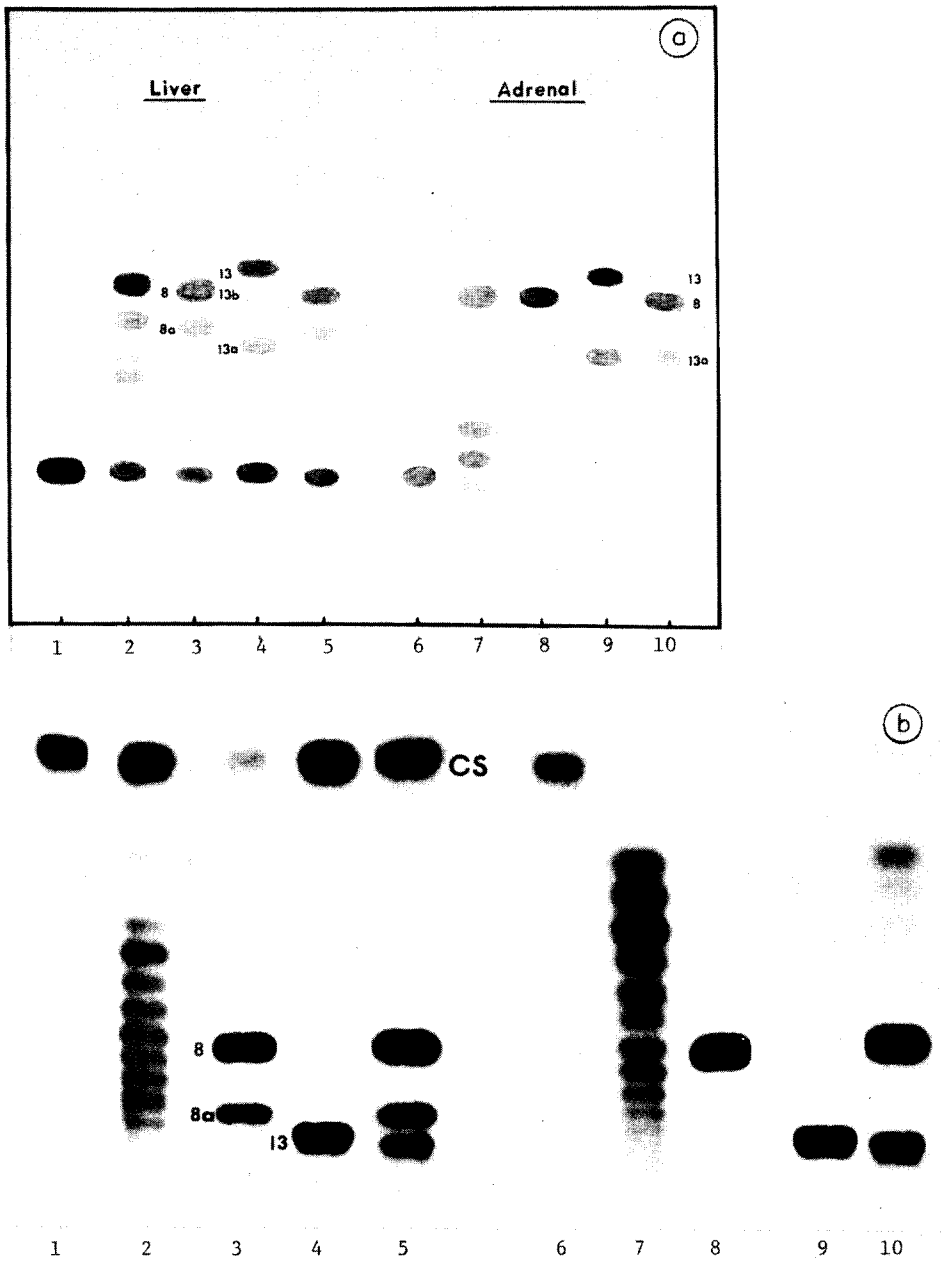


Fig. 5. a. Enzymatic sulfate conjugation of purified Triton X-100 oligomers, OPE_8 and OPE_{13}. Composite drawing of radioautograms of thin-layer chromatograms of lipid extracts of rat liver and bovine adrenal cortex enzymes after incubation with [^{35}S]sulfate and Triton X-100 or its purified oligomers. Developed in solvent system I. Samples 1—5: Rat liver supernatant incubated with (1) control; (2) Triton X-100, 3.18 mM; (3) OPE_8, 3.57 mM; (4) OPE_{13}, 2.52 mM; (5) OPE_8, 1.79 mM + OPE_{13}, 1.26 mM. Samples 6—10: Bovine adrenal cortex supernatant incubated with same substrates as samples 1—5. Incubation conditions are described in Table I. Lipids were extracted with $CHCl_3/CH_3OH$. b. Enzymatic sulfate conjugation of purified Triton X-100 oligomers. OPE_8 and OPE_{13}. Composite photograph of radioautograms of thin-layer chromatograms of the same samples shown in a, but using solvent system II.

These experiments indicate that Triton X-100 increases [^{35}S]sulfate incorporation in both liver and adrenal cortex supernatant enzyme systems by serving as a substrate for sulfotransferase(s) rather than through enhancement of sulfation of endogenous substrates. The radioactive compounds formed in the presence of Triton X-100, or its component oligomers, are identical in chromatographic behavior to synthetic sulfate conjugates prepared from purified Triton X-100 oligomers. Furthermore, they differ perceptibly from the sulfate conjugates of endogenous substrates, when chromatographed in solvent system II.

It is an interesting coincidental observation that the adrenal supernatant contains a family of compounds which yield sulfated derivatives that behave similarly, on thin-layer chromatography, to the sulfated oligomers of Triton X-100. It must be noted, however, that this series of sulfate conjugates represents only a portion of the total [^{35}S]sulfate incorporated into the lipid-soluble fraction of adrenal control samples. Ethyl acetate extracts contain several other ^{35}S-labeled compounds which are removed from the $CHCl_3$/CH_3OH extracts during the washing process (Fig. 6a, compare Nos. 1 and 4). The solubility of these conjugates in 'Folch upper layer' indicates that they are more polar than cholesterol sulfate and are probably conjugates of adrenal sterols.

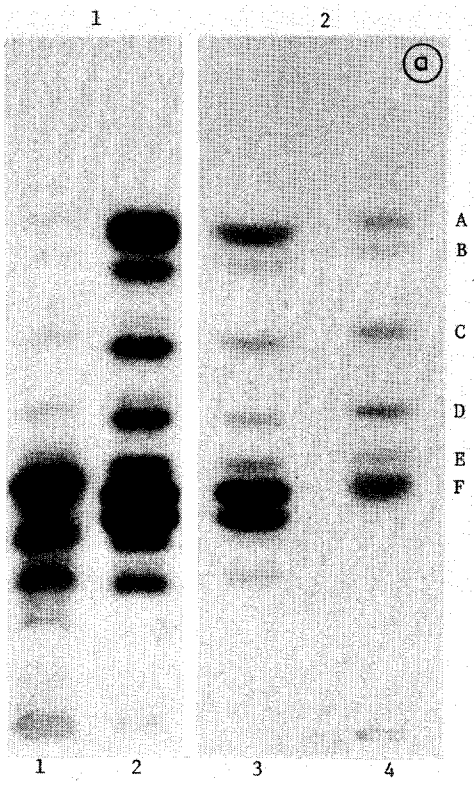
The sulfotransferases of rat liver and beef adrenal cortex have different specificities toward the component oligomers of Triton X-100. The liver supernatant enzymes sulfate primarily the oligomers with 5—11 ethoxy units, with approximately equal sulfation over this range (Fig. 5b, No. 2). This sulfation pattern conforms roughly to the relative concentration of the oligomers in the detergent mixture, which has been shown to be a Poisson distribution with an average n value of 9.5 [9]. In contrast, bovine adrenal cortex enzymes favor oligomers with n-values of 2—5, with a lesser incorporation of radioactivity into the 6- to 9-ethoxy range and a rapid decrease in sulfation as the value of n increases further.

Steroid sulfation in rat kidney and sheep adrenal supernatants

The sulfation of Triton X-100 itself, in the soluble enzyme systems of liver and adrenal cortex, complicated efforts to determine whether the detergent influences steroid sulfation in these particular systems. However, since Triton X-100 was not sulfated measurably by enzymes of rat kidney supernatants, this system was suitable for testing possible effects of the detergent on steroid sulfation.

Triton X-100 significantly stimulated the sulfation of cholesterol, pregnenolone, dehydroepiandrosterone, cholic acid and lithocholic acid in rat kidney supernatant (Table IV). Thin-layer chromatography showed that the only radioactive compounds present in the lipid extracts in significant amounts were the [^{35}S]sulfate conjugates of the added steroid substrates.

Dehydroepiandrosterone addition increased [^{35}S]sulfate incorporation in supernatant preparations from sheep adrenal cortex (Table I) and in rat kidney (Table IV). In the adrenal, dehydroepiandrosterone was conjugated in preference to Triton X-100 (Fig. 7, No. 9). The major radioactive compound formed in the presence of dehydroepiandrosterone, which co-chromatographs with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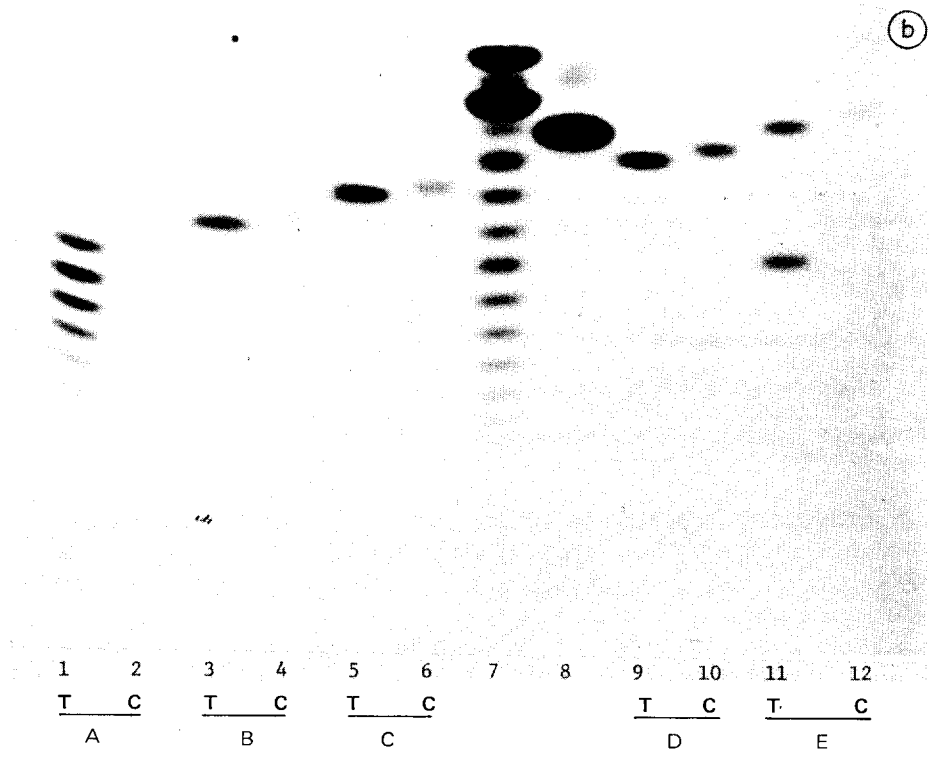
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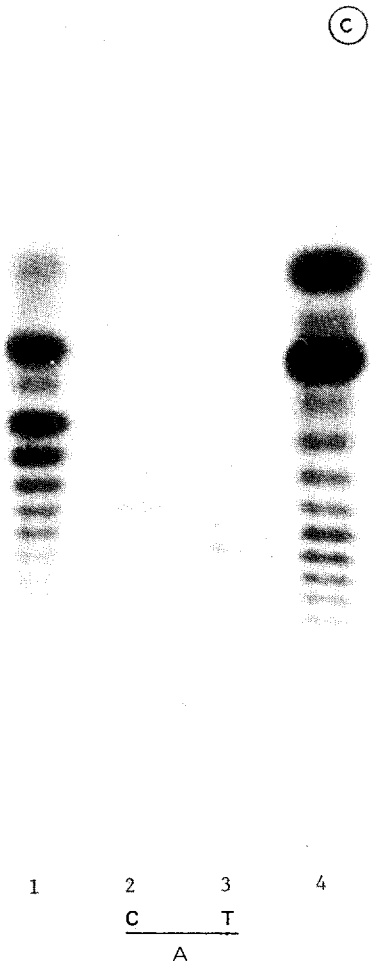

Fig. 6. Thin-layer chromatographic comparison of ^{35}S-labeled compounds formed in sheep adrenal cortex supernatants in the absence and presence of Triton X-100. (a) Composite radioautogram. [^{35}S]Sulfate incorporation in the absence and in the presence of Triton X-100. Solvent system I. Samples in chromatogram 1: 1, adrenal control; 2, adrenal + Triton X-100. Samples in chromatogram 2: 3, adrenal + Triton X-100; 4, adrenal control. All samples extracted with ethyl acetate except No. 4 which was extracted with $CHCl_3/CH_3OH$. (b) Rechromatography of samples eluted from chromatograph shown in Fig. 6a (Nos. 3 and 4, spots A—E). Solvent system II, two cycles. Samples: 1, Spot A, Triton; 2, Spot A, control; 3, Spot B, Triton; 4, Spot B, control; 5, Spot C, Triton; 6, Spot C, control; 7, sheep adrenal supernatant + Triton X-100; 8, sheep adrenal supernatant + dehydroepiandrosterone; 9, Spot D, Triton; 10, Spot D, control; 11, Spot E, Triton; 12, Spot E, control. (c) Comparison of the fastest material in solvent system I (Spot A) and the whole lipid extract, in the absence and presence of Triton X-100. Solvent system II. Samples: 1, adrenal control; 2, spot A from control; 3, spot A from sample incubated with Triton X-100; 4, adrenal + Triton X-100.

standard dehydroepiandrosterone sulfate, was not present to any extent in control samples (Fig. 7, Nos. 1 and 8). This indicates that dehydroepiandrosterone sulfate is not a major product of [^{35}S]sulfate conjugation in this adrenal enzyme system.

When cholesterol was added to sheep adrenal supernatant preparations, in the absence or presence of Triton X-100, no change in the chromatographic

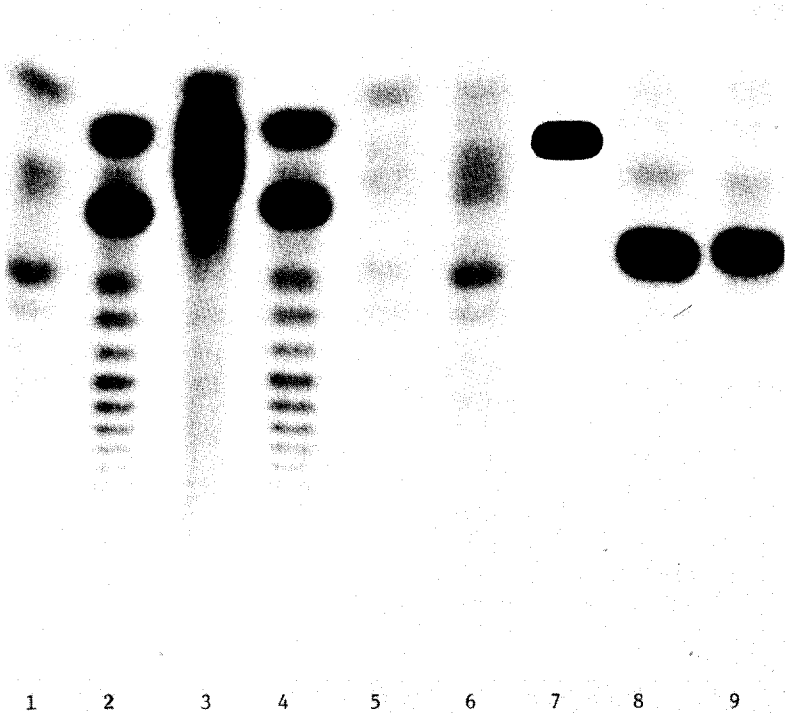

Fig. 7. Radioautogram of a thin-layer chromatogram of ^{35}S-labeled compounds formed in sheep adrenal supernatants in the presence of the detergents Triton X-100, Brij 96, and Tween 80. Solvent system II. Samples: 1, control; 2, Triton X-100 + cholesterol; 3, Brij 96; 4, Triton X-100; 5, Tween 80; 6, cholesterol; 7, potassium cholesterol sulfate standard; 8, dehydroepiandrosterone; 9, dehydroepiandrosterone + Triton X-100. Substrate concentrations as indicated in Table I. Lipids were extracted with ethyl acetate. Spot 7 is a chromatographic reference visualized by staining with phosphomolybdic acid after radioautography of the thin-layer plate.

pattern was observed (Fig. 7, Nos. 2 and 6). Cholesterol was not sulfated in these preparations and did not affect the sulfation of endogenous acceptors or of added Triton X-100.

Effect of other detergents on lipid sulfation

The detergent Brij 96 also increases [^{35}S]sulfate incorporation into the lipid fraction of rat liver and bovine and sheep adrenal cortex supernatants (Table I). Thin-layer chromatography of the ethyl acetate lipid extract showed one major radioactive component and a series of conjugates similar to those formed in the present of Triton X-100 (Fig. 7, No. 3); these latter bands are obscured in the photograph by the tailing from the principal component. Thus, it appears that this increase in lipid sulfation by Brij 96 is also due to sulfate conjugation of the detergent itself. Further work will be required to confirm this interpretation.

The detergent Tween 80 reduces the incorporation of [^{35}S]sulfate into the lipids of bovine and sheep adrenal cortex and rat liver, kidney and brain incubates (Table I). Thin-layer chromatography of the ethyl acetate lipid extract of sheep adrenal supernatant showed a relatively uniform reduction in

TABLE IV

EFFECT OF TRITON X-100 ON SULFATION OF STEROLS BY RAT KIDNEY SOLUBLE-ENZYMES

Incubation conditions were the same as those described in Table I. Sterol substrates (0.60 mM were added to sample tubes dissolved in ethanol; the solvent was evaporated under N_2. 3.18 mM Triton X-100, was added following addition of other incubation components. Lipids were extracted by Method 2 using ethanol.

Addition	Radioactivity incorporated (cpm)
None (ATP omitted)	8700
None	65 200
Triton X-100	94 200
Cholesterol	65 600
Cholesterol + Triton X-100	155 000
Dehydroepiandrosterone	152 000
Dehydroepiandrosterone + Triton X-100	248 000
Pregnenolone	172 000
Pregnenolone + Triton X-100	308 000
Cholic acid	124 000
Cholic acid + Triton X-100	182 000
Lithocholic acid	171 000
Lithocholic acid + Triton X-100	276 000

the amount of radioactivity in the observed components (Fig. 7, No. 5). The absence of any major new sulfated compound(s) indicates that Tween 80 itself is not sulfated in this system.

Discussion

These studies demonstrate the sulfate conjugation of Triton X-100 and its isolated component oligomers by soluble enzyme preparations from liver and adrenal cortex. The ability of the liver enzymes to catalyze sulfation of this detergent is not particularly surprising, since sulfation is one of the principal mechanisms by which liver biotransforms many foreign chemicals. The capacity of adrenal cortex enzymes to promote this reaction is less easily understood, although this tissue has also been shown to be capable of drug biotransformation and steroid sulfation [10—13]. The failure of kidney supernatant enzymes to carry out this sulfation, in spite of the ability of this fraction to sulfate many steroid substrates, is somewhat unexpected, in view of the observed liver and adrenal cortex supernatant enzyme activity.

The demonstrated existence of Triton X-100 sulfotransferase(s) in the high-speed supernatant fraction of adrenal cortex and liver raises the question whether enzymes with similar activity occur in other tissues and/or in other subcellular fractions, particularly in those which have been shown to contain other sulfotransferases, such as brain [14,15], ovary [16], testes [17,18] and mammary gland [19]. The possibility of Triton X-100 sulfation should be kept in mind whenever this detergent, or similar ones, are used for solubilization of

either enzymes or substrates. This is especially true if the reaction system contains 3′-phosphoadenylyl sulfate or might be contaminated with soluble supernatant fractions which, in the presence of ATP and Mg^{2+}, may be able to activate sulfate.

It should also be recognized that sulfate conjugation of Triton X-100, a nonionic detergent, results in the formation of an anionic derivative which may have quite different effects from those of its precursor. Sohn and Marinetti [20] compared the effects of Alipal, a sulfated alkyl phenoxy polyethoxyethanol, and Triton X-100 on the activity of several enzymes of rat liver plasma membranes, and found several differences in the actions of the two detergents. Thus, measurements made in systems where sulfation is occurring may be the result of the mixed action of the uncharged detergent and of its anionic derivative.

The Triton X-100 sulfotransferases of rat liver and beef adrenal cortex appear to have different specificities. The major sulfated oligomers formed in the presence of the liver enzyme(s) have 5—11 oxyethylene units while in the case of the adrenal cortex enzyme(s), the predominant products have in the range of 2—5 oxyethylene units. This difference in specificity possibly reflects a difference in the nature of their normal endogenous substrate(s). Purification and determination of the properties of these enzymes should permit an assessment of their normal role.

Stimulation by Triton X-100 of steroid sulfation in the rat kidney enzyme preparation may involve either solubilization of membrane-bound enzyme, through the formation of protein-detergent complexes [21], or the solubilization of the substrate through the incorporation of suspended steroid particles into mixed micelles in which the steroid substrate is more readily accessible for interaction with the sulfotransferase. The latter function for the detergent would be similar to that reported by Dennis and Owens [22] who found that interaction of Triton X-100 with phosphatidyl choline bilayers resulted in the formation of mixed micelles of detergent and phospholipid which increased the accessibility of phosphatidyl choline to phospholipase A. Although both functions may play a role in the present system, it seems likely that solubilization of the substrate rather than solubilization of the enzyme is of major importance, particularly since most of the cell membrane material is removed from the kidney homogenate by prolonged high-speed centrifugation.

The increase of [^{35}S]sulfate incorporation into the lipid fraction in rat liver, and bovine and sheep adrenal cortex enzyme preparations by Brij 96, appears to have been due largely to sulfate conjugation of the detergent. As was observed with Triton X-100, new sulfated compounds were formed in the presence of Brij 96. The enzyme(s) which transfers sulfate to Triton X-100 was probably also involved in Brij 96 sulfation, since these detergents have similar hydrophilic moieties, polyethoxyethanol polar groups with an average of 9—10 ethoxy units. The different hydrophobic moieties, an oleyl group in Brij 96 and an octylphenoxy group in Triton X-100, were probably responsible for the differences observed in the activity of the sulfotransferase(s) and in the chromatographic patterns of the sulfated oligomers present in the lipid extracts.

Addition of Tween 80 to rat liver and bovine adrenal cortex enzyme prep-

arations resulted in a decrease in [^{35}S]sulfate incorporation into the lipid fraction with no change in the TLC pattern. This observation indicates that, in addition to the obvious negative effect that Tween 80 has on some component of the system which adds sulfate to endogenous acceptor(s), it is not itself appreciably sulfated in these enzyme preparations. Perhaps sulfation is prevented because the rather bulky polyethoxysorbitol 'head' group of Tween 80 blocks its access to the active site of the Triton X-100 sulfotransferase.

The series of sulfate conjugates present in washed $CHCl_3/CH_3OH$ extracts of adrenal cortex control samples do not correspond, to our knowledge, to any previously reported family of compounds. The regularity of their chromatographic separation pattern in the ethyl acetate/acetic acid/water solvent system is similar to that of the sulfated oligomers of Triton X-100 which differ from each other by only an oxyethylene unit. Thus, these substances may be a family of natural compounds found in the adrenal cortex which have a regular difference in their structure comparable to that in Triton X-100 oligomers.

The sulfation of Triton X-100 and related detergents, shown to occur in in vitro systems when the appropriate transfer enzymes and a sulfate activating system are present, may also occur in vivo. The use of such detergents for food and drug processing, cosmetics and other applications could lead to their ingestion. The demonstrated ability of some tissues to form sulfate derivatives of Triton X-100 may explain, in part, why Triton X-100 is more toxic than is Tween 80, which is not sulfated [23]. The latter detergent is among a relatively small group of synthetic detergents which can be added, in limited quantities, directly to food products [24].

Triton X-100 is occasionally used in studies with intact cells and tissues [25,26]. The observed effect on these cells and tissues is presently considered to be primarily the result of membrane modification by this nonionic detergent. The results herein reported indicate that Triton X-100 sulfation must be taken into account in the interpretation of these studies. Depletion of 3'-phosphoadenylyl sulfate and the resulting reduction of other intracellular sulfated compounds, and interaction of sulfated Triton X-100 with cellular constituents are among the additional possible causes of gross changes in intact cells and tissues exposed to Triton X-100. The observations reported above may require, in some instances, a reevaluation of the results of these studies. Future experiments which involve the use of this or similar detergents must be designed with the recognition that sulfation of Triton X-100 can occur in some biological systems.

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