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The Use of Enzyme-Linked Immunosorbent Assays (ELISA) for the Determination of Triton X Nonionic Detergents

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An enzyme-linked immunosorbent assay (ELISA) for 4-t-octylphenyl ethoxylates such as Triton X-100 was developed. Both the 4-t-octylphenyl and the ethoxylate moiety were required for antibody recognition since members of the Triton N series showed low cross-reactivity, and polyethylene glycol polymers as well as a variety of other neutral and ionic surfactants and pesticides showed no cross-reactivity. The ELISA was sensitive in the low nanogram-per-milliliter range and was highly reproducible. It was shown to be capable of analyzing the active ingredients in vaginal contraceptives and Triton X-100 in the presence of proteins. Immunoassays thus offer advantages in the analysis of such complex mixtures.

Because of their unique and valuable properties, the Triton X nonionic detergents (4-t-octylphenoxy polyethoxyethanols, Fig. 1) are used extensively for a variety of purposes which may result in their introduction into the environment or in human exposure. Their use in industrial and household cleaners and as emulsifiers for agricultural chemicals is certainly major among the many roles for these important chemicals (1). Direct human exposure to these and related surfactants also occurs through their use in the cosmetic and pharmaceutical industries. For instance, Triton X and Triton N nonionic detergents (Fig. 1) and closely related compounds represent the active ingredients in the majority of vaginal contraceptives produced in the United States (2). In addition, numerous biological and biochemical investigations depend upon the use of nonionic detergents, particularly in the field of membrane biochemistry. It is often difficult to remove detergents from the macromolecules, and analysis of bound detergent in biochemical samples becomes important.

Quantification of these nonvolatile detergents is therefore a frequently encountered problem both for their residue analysis as well as for their presence in biochemical products. Most quantitative analytical procedures are based upon the reaction of the surfactants with a heteropolyacid or heteropolyacid derivative to form an insoluble complex, followed by volumetric, gravimetric, or colorimetric measurement of the surfactant present. Such methods include (i) a volumetric procedure using ferrocyanic acid (3); (ii) a gravimetric procedure employing phosphotungstic acid (4) and silicotungstic acid (5); and (iii) colorimetric procedures using ammonium cabaltothiocyanate (6) or phosphomolybdic acid (5) (see also included references for variations in procedures). Ultraviolet spectroscopy was also employed for the detection of microgram quantities of Triton X-100 (7). Recently, a one-dimensional thin-layer chromatographic separation method for the determination of nonionic detergents in the presence of common natural lipids has been described. Although the method is relatively simple, it lacks both sensitivity and ease of quantification (8). High-performance and open-column liquid chromatography have also been used for the separation and analysis of nonionic surfactants (9–11).

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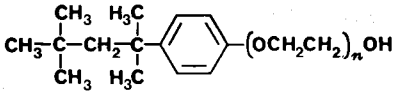

FIG. 1. Structure of Triton X. Most members of the Triton X series vary in the number of ethoxylate units (n) per 4-t-octylphenoxy moiety. The ethoxylate number varies in a normal distribution about the average value indicated for some members of the series in Table 2. Members of the Triton N nonionic detergent series have similar structures with a 4-nonyl substituent. Synonyms include octoxynol, α-[4-(1',1',3',3'-tetramethylbutyl)phenyl]-ω-hydroxy poly(oxy-1,2-ethanediyl) (9CI), and p-t-octylphenoxy polyethoxyethanol.

Most nonionic detergents present difficult analytical problems. Not only are they nonvolatile and lacking in a chromophore with a high extinction coefficient, but most such detergents consist of a complex mixture of closely related, rather unreactive compounds (Fig. 1). Due to their surfactant properties, extraction, workup, and concentration of these compounds are further complicated. Immunochemical analysis seemed especially suited to solving such a complex set of problems. Thus, the development of specific and sensitive immunoassays for these nonionic detergents was undertaken.

MATERIALS AND METHODS

Reagents. Alkaline phosphatase conjugated goat antirabbit immunoglobulin G (IgG)[1] was prepared as described by Engvall and Perlman (12) and also obtained from Miles Laboratory (Elkhart, Ind.). Goat antirabbit immunoglobulin and normal rabbit serum were obtained from Antibodies Incorporated (Davis, Calif.). Triton X-15, X-45, X-100, X-102, X-114, X-200, X-305, X-405; Triton N-57, N-101, N-150, N-401; and nonylphenol were gifts from Rohm and Haas Company (Philadelphia, Pa.). Ovalbumin (OA), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), and complete Freund's adjuvant (CFA) were purchased from Calbiochem (San Diego, Calif.); p-nitrophenyl phosphate, Sepharose 4B, and other detergents from Sigma Chemical Company (St. Louis, Mo.); tri-n-butylamine, isobutylchloroformate, and cyanogen bromide from Eastman-Kodak Company (Rochester, N. Y.); 4-n-octylphenol, 4-t-octylphenol, and diethanolamine from Aldrich Chemical Company (Milwaukee, Wisc.); and incomplete Freund's adjuvant (IFA) from GIBCO (Grand Island, N. Y.). Conceptrol and Ortho-Gynol (Ortho Pharmaceutical), Koromex[II] (Holland-Rantos Co., Piscataway, N. J.), and Emko Foam (Schering Corp., Kenilworth, N. J.) were purchased locally. Unfortunately, authentic standards of the active ingredients were not available from the manufacturers.

Preparation of Triton X-100–COOH. To a solution of 5 g of Triton X-100 (7.96 mM) in 25 ml of dry pyridine was added 3.88 g (39.8 mM) of succinic anhydride, and the combined solution was stirred at room temperature overnight. The solvent was then removed by flash evaporation at 50°C under partial vacuum. To eliminate most of the pyridine, the residue was dissolved in 70 ml of benzene and evaporated to dryness again. The residue was then dissolved in 60 ml of 1% H_2SO_4 and extracted with 75 ml $CHCl_3$ (three times). The chloroform layer was dried with $MgSO_4$ and evaporated to give a pale yellow oil in quantitative yield. The ir spectrum showed the absence of OH group and the presence of carbonyl peak at 1730 cm^{-1}. NMR ($CDCl_3$/TMS) showed the presence of succinic acid methylene protons at δ 2.61 and the carboxylic acid proton at δ 9.26 and indicated the presence of 1 succinate moiety for each phenyl moiety present. Under these conditions hemisuccinates of free phenols possibly present in the technical material are not formed.

Preparation of Triton X-100–protein con-

[1] Abbreviations used: IgG, immunoglobulin G; OA, ovalbumin; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; IFA, incomplete Freund's adjuvant; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PEG, polyethylene glycol.

jugates. Triton X-100–COOH (0.15 mM) was dissolved in 1 ml dry dioxane. To this solution was added tri-n-butylamine (0.185 mM) and then isobutylchloroformate (0.185 mM). The resulting mixture was stirred at room temperature for 30 min and then added dropwise to a solution of OA (200 mg), KLH (250 mg), or BSA (250 mg) in 40 ml of 0.25 M borate buffer, pH 9.0, and stirred at room temperature overnight. The conjugates were then dialyzed extensively in 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS). The protein concentration was estimated by Lowry assay (13), and the degree of substitution was calculated at 7–15 mol/100,000 daltons of protein by ELISA as described in the following section.

Immunization. Two New Zealand white female rabbits were immunized with Triton X-100–OA conjugates (2–4 mg) in PBS emulsified with an equal amount of CFA. A volume of 1 ml of antigen emulsion was injected at multiple intradermal and subcutaneous sites. Booster injections were then given at monthly intervals using the same antigen emulsified in IFA. Blood was obtained from the ear vein of rabbits 1 week after the injection, and the serum was separated by centrifugation and stored at −20°C. Although both rabbits produced high-titer anti-Triton X antibodies as detected by ELISA, only serum from one rabbit, which was obtained 7 days after the third immunization, was used throughout this study.

Affinity chromatography. KLH was coupled to CNBr-activated Sepharose 4B at a concentration of 1 mg protein/ml Sepharose 4B (14). The immunosorbent was washed on a Büchner funnel with Na_2CO_3 solution, H_2O, and 0.2 M glycine–HCl buffer, pH 2.8, before equilibration with PBS. The serum was then passed through the immunosorbent, and the effluent and initial PBS wash were combined and concentrated by negative pressure to the initial volume and dialyzed against 7 mM phosphate buffer containing 0.15 M NaCl, pH 7.7.

Enzyme-linked immunosorbent assay (ELISA). ELISA was carried out according to Voller *et al.* (15) with slight modifications. All the reactions were performed in polystyrene plates (Dynatech or Gilford cuvettes). A volume of 200 μl was used in the Dynatech plates, whereas 250 μl was employed for Gilford plates throughout the study. The coating antigens were diluted in 0.1 M sodium carbonate buffer, pH 9.6, containing 0.02% NaN_3 and added to each well. The plates were then kept overnight at 4°C. The coated plates were washed three times with phosphate-buffered saline containing 0.05% Tween 20 and 0.02% NaN_3 (PBS–Tween). After addition of diluted antiserum in PBS–Tween, plates were incubated for 2 h at room temperature. After three further washes with PBS–Tween, goat antirabbit Ig–alkaline phosphatase conjugate diluted in PBS–Tween (1/2500) was added and allowed to incubate at room temperature for 2 h. After another three washes, phosphatase activity was measured following the addition of a 1-mg/ml solution of p-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8. The reaction was stopped 30–40 min later by addition of 50 μl of 3 N NaOH, and the color intensity was measured using a Gilford EIA reader at 405 nm.

The preparation of inhibition curves was as follows. One milliliter of the appropriately diluted antiserum was transferred to 13 × 100-mm culture tubes. To these antibody solutions were added different inhibitors dissolved in a maximum of 20 μl of acetonitrile or water with increasing hapten concentration. Alternatively, up to 0.9 ml of a biological sample was added to a tube followed by enough antibody in 10× PBS–Tween to give the usual antibody concentration and pH. After incubation at room temperature overnight, the serum was added to the Triton X–KLH-coated plates. The assays were then performed as described above. Maximum and background binding were assessed by adding solvent only, or no antibody, respectively, to the system. Unknowns were deter-

TABLE 1

THE SPECIFICITY OF TRITON X-100–KLH AS COATING ANTIGEN IN ELISA

Coating antigen	Concentration (μg/ml)	Relative titer[a]
Triton X-100	20	<100
KLH + Triton X-100–COOH[b]	20	100
KLH + Triton X-100[c]	20	100
Triton X-100–KLH	2	8100
OA	10	>24,300

[a] The cuvettes were incubated with different antigens in coating buffer, pH 9.6. Serially diluted sera were then added and incubated for 2 h. Subsequently, goat anti-rabbit Ig conjugated with alkaline phosphatase was added, and the enzymatic activity was demonstrated by the addition of 0.1% solution of *p*-nitrophenyl phosphate. An OD at 405 nm of greater than 0.3 was used as the positive reference.

[b] Prepared by mixing of KLH with Triton X–COOH in 0.1 M borate buffer, pH 9.0, and stirred at room temperature overnight.

[c] Prepared by the mixed anhydride procedure as described under Materials and Methods.

mined from standard curves generated with known Triton X-100 solutions, and the reproducibility of the assay within and between runs was determined.

Contraceptives were analyzed by dissolving them in ethanol–water solutions, using vigorous sonication. Small aliquots were diluted in PBS and then assayed by the ELISA procedure described above. In each case five dilutions of each material were analyzed such that at least 3 points fell on the linear region of the appropriate standard curve. Because octoxynol and nonoxynol are ethoxylated octyl and nonylphenols, Triton X-100 and Triton N-101 were used to generate the appropriate standard curve in lieu of authentic standards.

RESULTS AND DISCUSSION

Triton X is a mixture of 4-t-octylphenoxy polyethoxy oligomers, and it has been shown to contain more than 16 individual oligomers (9–11) (Fig. 1). Because our goal was to produce anti-Triton X antibody to the class rather than the individual oligomers, technical Triton X-100 was therefore chosen for the present study. Triton X-100 is a small molecule, which is not antigenic by itself, and it must be attached to an antigenic carrier for the antibody production. Therefore, Triton X-100 was first converted to a carboxylic acid derivative by reaction with succinic anhydride and then reacted with isobutylchloroformate to give the active intermediate which is capable of binding covalently to carrier protein molecules.

Two months after immunization of the rabbits with two different preparations of Triton X-100–OA, anti-Triton X antibodies with high titers (>25,000) were obtained for both rabbits using Triton X–KLH as the coating antigen in ELISA, whereas normal rabbit serum gave a relative titer of only 100. However, when KLH alone was used as the coating antigen, a titer of 400 was observed. To reduce the nonspecific binding of the antisera to KLH in the ELISA, they were first passed through a Sepharose 4B–KLH immunosorbent, and the resulting serum was then used in the subsequent study. The specificity of the immunosorbent purified serum was further demonstrated by using (i) Triton X-100 alone; (ii) a mixture of KLH and Triton X-100–COOH, and (iii) Triton X-100 + KLH reacted with isobutylchloroformate using conditions similar to those used in the preparation of covalently conjugated Triton X–KLH. There was some question that Triton X could either bind directly to the plastic cuvettes or noncovalently to protein. However, titers of ≤100 were observed for the above three coating antigens as shown in Table 1. On the other hand, a titer of 8100 was found when Triton–KLH was employed as the coating antigen, clearly indicating that classical coupling procedures must be used when dealing with this detergent. The anti-OA titer was found to be >24,300 when OA alone was used as coating antigen. However, all attempts to generate precipitin bands by Ouchterlony double-dif-

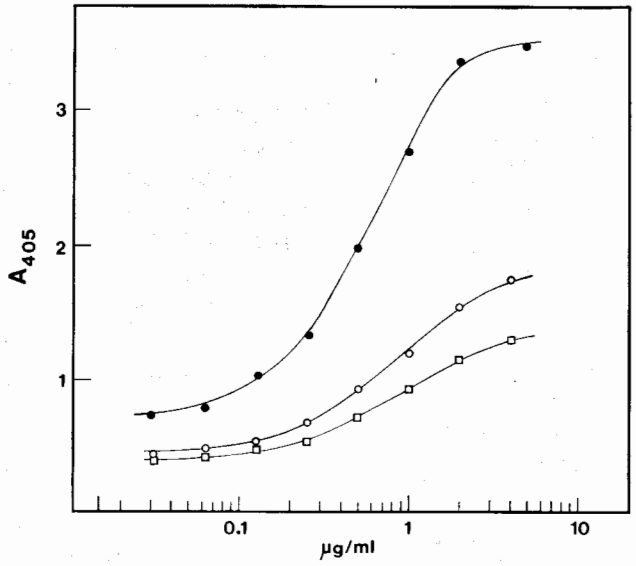

FIG. 2. The binding of a constant amount of rabbit anti-Triton X serum (●, 1/2000; ○, 1/4000; and □, 1/8000) to an increasing concentration of Triton X–KLH conjugate as coating antigen. Subsequently, goat antirabbit Ig conjugated with alkaline phosphatase (1/2500 dilution) was added, and the enzymatic activity was demonstrated by the addition of 0.1% solution of *p*-nitrophenyl phosphate. The reaction was stopped with 3 N NaOH 15 min later.

fusion techniques using Triton conjugated to BSA or KLH were unsuccessful.

The checkerboard titration of both antigen and antiserum (15) resulted in determination of the optimal coating concentration for Triton X–KLH conjugates as shown in Fig. 2. Positive reactions were detected with a concentration of coating antigen as low as 125 ng/ml, and the plateau was reached at a concentration of ~2 μg/ml for the different antiserum concentrations used. Finally, a solution of 2 μg/ml of Triton X–KLH and a serum dilution of 1/3200 for Rabbit 10 was used throughout the study. Inhibition studies were then carried out to investigate the ability of various haptens to prevent the binding of anti-Triton X antibody to the Triton X–KLH-coated plates. The results in Fig. 3 are expressed as inhibition percentage of a standard amount of antibody plotted against a variable concentration of hapten. All members of the homologous Triton X series exhibited strong inhibition, and 50% inhibition points varied at the concentrations between 14 and 56 ng/ml; ultimately, 85% inhibition could be obtained (Table 2). The inhibition curves were linear over a wide range of hapten concentrations, thus allowing between 2 and 100 ng/ml of Triton X to be readily quantified. This sensitivity, which is defined as the smallest detectable amount of Triton X corresponding to 10% inhibition, was considered very satisfactory for the residue analysis of Triton X since it reaches the low ppb level. Sensitivity could be further increased by sample extraction and concentration and/or by the use of a fluorescent substrate in the ELISA (16). The standard deviations of the experimentally determined concentrations of unknown samples or points on the standard curve were shown to be less than 5% within a single run. The coefficients of variation for the same unknown analyzed on separate days were less than 4%.

As shown in Table 3, several Triton X detergents lacking the 4-t-phenoxyethoxylate moiety had negligible inhibitory activ-

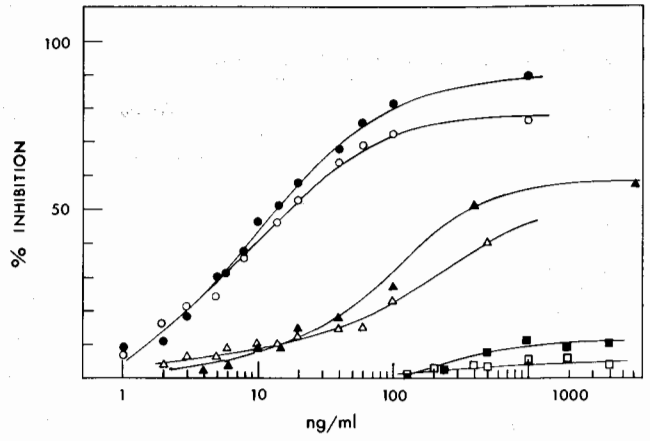

FIG. 3. Inhibition of binding of anti-Triton X antibody by various haptens. The Triton–KLH was absorbed to the solid phase. Various concentrations of haptens were reacted with the anti-Triton X antiserum (1/3200) and then used in the ELISA. Inhibitors shown include (●) Triton X-45, (○) Triton X-100, (▲) Triton N-101, (△) Triton N-57, (■) PEG_{6800}; and (□) PEG_{200}, PEG_{1000}, and PEG_{3400}.

ity. Separate experiments have shown that still other anionic and cationic surfactants have minimal cross-reactivity. All members of the Triton N series appeared to have much less inhibitory effect (50% inhibition at 400–1500 ng/ml) as compared to Triton X. However, the resulting assay could be used to analyze nonylphenoxyethoxylates with reduced sensitivity. The ethylene oxide polymer by itself did not bind to the anti-Triton X antibodies since all the polyethylene glycols (PEG) tested (M_r 200, 1000, 3400, and 6800) failed to inhibit the reaction (Table 3). The 4-t-octylphenol and nonylphenols used in the Triton X and Triton N series, respectively, gave only weak inhibition at high hapten concentrations (>1 μg/ml); the 4-n-octylphenol also failed to demonstrate any significant binding activity. Other detergents such as Brij 35, Lubrol WX, and Lubrol PX (ethoxylates lacking the alkylaryl moiety) did not bind to the anti-Triton X antibodies as shown in Table 3. From these results, it is apparent that anti-Triton X antibodies preferentially recognize the 4-t-octylphenol moiety of the molecule, and because TritonX-15 was shown to be a weaker inhibitor than Triton X-100, it seems that at least 1 or 2 ethoxylate units must be present to improve complementary fix between the antibody and its antigen. It is evident from Table 2 that with the exception of Triton X-15 the amount needed (in nanograms per milliliter) to inhibit 50% of the reaction increases slightly with the average number of ethoxylates present in the molecule. These data also support the hypothesis that com-

TABLE 2

INHIBITION OF ELISA BY 4-t-OCTYLPHENYL ETHOXYLATES

Inhibitor	Average ethoxylate number	Amount required for 50% inhibition	
		ng/ml	pmol/ml[a]
Triton X-15	1	56.0	224
Triton X-45	5	14.0	32.9
Triton X-114	7–8	17.5	32.6
Triton X-100	9–10	17.0	27.1
Triton X-102	12–13	16.0	21.1
Triton X-305[b]	30	27.0	25.2
Triton X-405[b]	40	38.0	27.6
Triton X-100–COOH	9–10	15.0	20.2

[a] Calculated based upon the average molecular weight provided by Rohm and Haas (19).

[b] Nanograms per milliliter based on actual assay; picomoles per milliliter based upon the technical material containing 30% water.

TABLE 3

INHIBITION OF ELISA BY A VARIETY OF SURFACTANTS, CHEMICAL INTERMEDIATES, AND PESTICIDES

Inhibitor	Amount required for 50% inhibition (ng/ml)	Inhibitor	Amount required for 50% inhibition (ng/ml)
Triton X-200[a]	>2,000	Brij 35	>16,000
Triton X-202[a]	>2,000	Lubrol WX	>8,000
Triton X-301[a]	>4,000	Lubrol PX	>2,500
Triton X-155[a]	>16,000	PEG 200	>16,000
Triton N-57	1500	PEG 1000	>16,000
Triton N-101	400	PEG 3400	>16,000
Triton N-150	660	PEG 6800	>16,000
Triton N-401	1250	Paraquat	>35,000
Triton WR-1339	500	TH-6043[c]	>25,000
4-n-Octylphenol[b]	>4000	TH-6044[c]	>25,000
4-t-Octylphenol[b]	>4000	Glyphosate	>35,000
Nonylphenol[b]	>4000	Aldrin	>35,000

[a] Although termed Triton X, these are not 4-t-octylphenyl ethoxylates. Triton X-200 and X-301 are sodium alkylaryl polyether sulfonates with a maximum inhibition of about 45% at 1 μg/ml, while Triton X-155 is an alkylaryl polyether alcohol of undisclosed structure giving a maximum of 22% inhibition at 16 μg/ml.

[b] At a concentration of 4 μg/ml the inhibition percentage of 4-n-octylphenol, 4-t-octylphenol, and nonylphenol was <10, 44.3, and 18%, respectively.

[c] TH-6043 is *N*-[[[4-(trifluoromethyl)phenyl]amino]carbonyl]-2-chlorobenzamide and TH-6044 is *N*-[[[4-(trifluoromethyl)phenyl]amino]carbonyl]-2,6-difluorobenzamide.

plementary fit is largely dependent upon the 4-t-octylphenol moiety and several ethoxylate units since the inhibition based on molecular weight is rather constant.

The specificity of the antiserum used for this work is optimal for many studies involving the mammalian metabolism or environmental degradation of complex mixtures such as Triton X-100. The high specificity for the Triton X series over the very closely related Triton N and Brij series minimizes interference from other detergents in environmental samples. The polyethylene glycol and 4-t-octylphenol moieties which could result from Triton X degradation also cause minimal interference. In spite of this high specificity, the ELISA system detects all 4-t-octylphenyl ethoxylates with similar sensitivity, thus allowing one to measure the total surfactant present without the baffling complexity of a variety of homologs. Should additional data be needed on specific homolog composition, the ELISA can be used as a highly sensitive detector for thin-layer or liquid chromatographic systems capable of separating homologs in the Triton X series (9–11).

Because alkylaryl phenyl ethoxylates are the most common active ingredients in vaginal contraceptives, the Triton X ELISA was used to analyze several commercial products. Using the Triton X-100 standard curve, Koromex[II] was found to contain 0.81% Triton X-100 equivalents (advertised as 1%) and Ortho-Gynol was found to contain 1.28% Triton X-100 equivalents (advertised as 1%). Using the Triton N-101 standard curve, Conceptrol was found to contain 5.6% (advertised as 5%), and Emko 8.2% (advertised as 8%) Triton N-101 equivalents. When several dilutions of the contraceptives were assayed, they yielded inhibition curves parallel to those of the standards used in the assay. Thus, the ELISAs clearly are sufficiently sensitive to monitor active ingredients in formulated products and probably are adequate to monitor their persistence and distribution.

Because 30–50 million pounds of surfac-

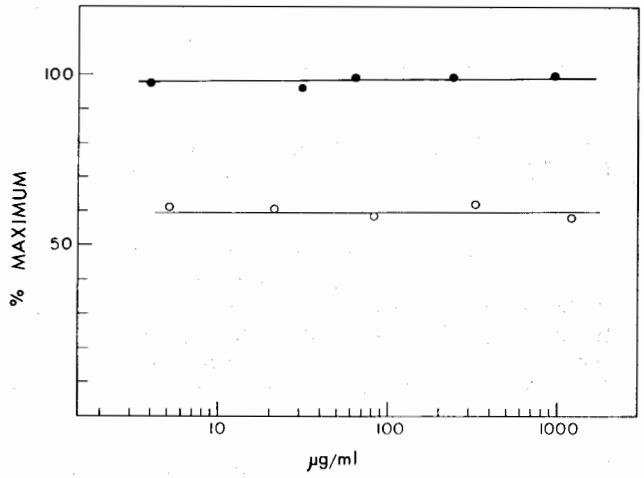

FIG. 4. The effect of increasing BSA concentration on the binding anti-Triton X antibody (1/3200) to Triton X-KLH-coated plate in ELISA. ●, No Triton X-100; ○, with 10 ng/ml Triton X-100.

tants are probably sprayed annually in pesticide formulations, a possible use of immunoassays could be analysis of environmental samples. Thus, a number of pesticides were also tested as inhibitors of the binding of anti-Triton X antibodies, some of which are indicated in Table 3. In all cases they were found to be inactive even at very high concentrations. Therefore, this simple technique can be used in residue analysis of surfactant in samples obtained from the field in which a variety of pesticides may also be present. In fact, Triton X has been quantified by ELISA from foliage samples following actual pesticide application. The specificity of these anti-Triton X antibodies also demonstrated the power of immunoassay in the analysis of extremely low levels of antigen.

Studies have shown that membrane-bound proteins as well as globular proteins bind surfactant molecules (17,18). However, the ELISA procedure can also be used to quantify the amount of Triton X-100 in the presence of proteins such as BSA and HSA. For instance, when a standard amount of Triton X-100 was first incubated in the presence of varying amounts of BSA, the BSA caused practically no interference in the subsequent determination of Triton X-100 with ELISA (Fig. 4) even at a very low Triton X-100 concentration (10 ng/ml). This observation is probably due to the very strong binding between the antibody and its hapten, as seen in many hapten–antibody systems. These results also indicate that antibody affinity columns could be used to strip tightly bound detergent molecules from macromolecules. This high affinity of the antibodies for Triton X facilitates the analysis of Triton X in complex biological samples such as milk, probably by pulling the hapten out of lipid micelles and off of proteins by mass action. This property greatly speeds residue analysis by eliminating time-consuming extraction and concentrating procedures.

Thus, the ELISA system described in this paper promises to be very useful for investigating the presence and fate of Triton X nonionic detergents in a variety of systems. The successful immunochemical analysis of Triton X also indicates that this technology may be generally applied to the complex problems involved in surfactant, dispersant, and detergent analysis.

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