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DETERMINATION OF TRITON X-100 BINDING TO MEMBRANE PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

The molecular weight of proteins in protein-detergent complexes can be determined from ultracentrifugation experiments if the amount of bound detergent is known. A new sensitive method to measure the binding of the nonionic detergent Triton X-100 to proteins has been developed. For the membrane proteins studied, less than 50 μg of protein was required to achieve an accuracy of 10 % in the determination of the detergent-protein weight ratio.

The proteins were equilibrated with the detergent by electrophoresis into polyacrylamide gels containing radioactively labelled Triton X-100. The gels were then sliced and the amount of bound detergent calculated from the increase in radioactivity in the slices containing the protein zone. The amounts of protein were determined by amino acid analysis of identical protein zones cut from gels running parallel.

INTRODUCTION

Ionic detergents, such as dodecyl sulphate, bind to most proteins and cause disruption of subunit structures and drastic changes in the native conformation of the proteins [1]. In contrast, nonionic detergents (and bile salts) bind only to lipophilic proteins [2, 3] usually without causing denaturation. Therefore, these detergents are very useful for the solubilization of biologically active proteins from membranes. (For a review see ref. 4.)

Membrane proteins requiring detergents for their solubilization have been shown to bind up to several times their own weight of the nonionic detergent Triton X-100 [4, 5]. The determination of the molecular weights of such proteins, or protein complexes, in Triton X-100 solutions can be made only when the extent of the binding is known [6].

In this paper, a new method for the determination of the amount of Triton X-100 bound to proteins is described. Among previously used methods for this

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purpose have been gel filtration [2], ion exchange chromatography [7], equilibrium dialysis [5], and centrifugation in sucrose density gradient [3]. The main advantage of the present method is the low amount of protein required. A similar technique has been used previously to study the binding of steroid hormones to proteins [8].

MATERIALS AND EXPERIMENTAL PROCEDURES

Chemicals. Triton X-100 (polyoxyethylene *p*-tert-octylphenol) was obtained from Rohm and Haas. The ring ^{3}H-labelled Triton X-100 ($0.46 \cdot 10^9$ dpm/g) was a gift from Dr. K. Simons. Thin-layer chromatography of these two Triton X-100 preparations gave identical patterns [7]. The absorbances at 275–276 nm of aliquots diluted to submicellar concentrations in 50 mM phosphate buffer (pH 7.5) gave an extinction coefficient of $2.19 \pm 0.02\ mg^{-1} \cdot ml^{-1} \cdot cm$ within 5 % of previously reported values [5, 9]. All Triton X-100 concentrations were subsequently determined by absorbance measurements using this value. For the binding experiments, labelled Triton X-100 was diluted with an equal amount of unlabelled Triton X-100. Recrystallized [10] acrylamide and bisacrylamide (Eastman Kodak Co.) were used for all electrophoreses. Buffer salts (Merck) were of analytical grade. Tris buffer was found unsuitable since it caused variation of the Triton X-100 concentration upon electrophoresis.

Proteins. Membrane penicillinase from *Bacillus licheniformis* 749/C [11] was a gift from Dr. K. Simons. The membrane flavo-protein of *Acholeplasma laidlawii* [12] and glycophorin [13] were gifts from Dr. K.-E. Johansson and Dr. L. Liljas, respectively. The protein concentration of these samples was 0.4–0.6 mg/ml. Lyophilized human growth hormone, purified according to Roos et al. [14] was a gift from Dr. P. Roos. Human serum albumin was obtained from Kabi AB, Stockholm, Sweden, and crystallized ovalbumin from Sigma. These last three proteins were dissolved in buffer at concentrations of 4–6 mg/ml.

Preparation of gels and electrophoresis. Four 100 mm long polyacrylamide gels, containing ^{3}H-labelled Triton X-100, were cast in quartz tubes (length 130 mm, internal diameter 5 mm). The tubes were mounted in the electrophoresis apparatus so that the whole length of the gel was immersed in the lower electrode solution. The samples, containing 60 mg/ml sucrose, were layered on top of the gels using calibrated polythylene tubing (internal diameter 0.2 mm) attached to a glass syringe. To minimize pH and temperature differences due to the electrophoresis, a peristaltic pump was used to circulate the buffer between the electrode vessels.

Slicing. When the optical scanning (for details see ref. 15) indicated that the Triton X-100 concentrations (absorbances) on both sides of the protein zone were the same (Fig. 1), the electrophoresis was stopped and one gel at a time was removed by rimming. The gel was dropped onto a sheet of Parafilm and excess water was removed with filter paper. The gel was then sliced using a razor blade and a Perspex block with a longitudinal trough, 5 mm wide and 8 mm deep, and transverse slits, 2 mm apart. The gel slices were transferred into preweighed glass scintillation vials and their weight determined. To minimize evaporation, the Perspex block was kept wrapped in polyethylene film while each slice was weighed.

Radioactivity measurement. The gel slices were incubated in tightly capped vials containing 250 μl of a 30 % hydrogen peroxide solution. The vials were tilted so that

the slices were maximally immersed in the hydrogen peroxide. After 10–12 h at 60–70 °C, during which the gel structure disappeared, 10 ml scintillation liquid (666 ml toluene, 333 ml Triton X-100, 100 ml water, 5.5 g PPO and 0.1 g POPOP) was added, and the samples were counted after allowing 3 h for equilibration in the counter.

Quantitative amino acid analysis. The section of a gel running parallel, containing the protein zone, as indicated by a scan tracing, was cut out and hydrolysed in 2.0 ml 6 M HCl for 24 h at 110 °C. After the evaporation of HCl the residue was dissolved in 1.5 ml buffer (pH 2.2), the solution was cleared by centrifugation and 1 ml applied to the column. The analysis was done using a Beckman 120 B amino acid analyzer with a 9 × 500 mm bed of Beckman AA-15 resin as described [16]. The recorder was adapted to give full scale deflection at an absorbance of 0.1. The hydrolyzate was cleared by centrifugation and 1.0 ml applied to the column. Because of the large amount of ammonia in the hydrolyzate [17] a double regeneration time was used.

Calculation of amount of bound Triton X-100 and protein. The radioactivity of each vial, a, was divided by the weight of the corresponding gel slice, w, and the values were plotted (see Figs. 1–3). The amount of bound Triton X-100, W_{TX}, was calculated from the equation:

$$W_{TX} = \frac{1}{b} \cdot \frac{\rho}{c} \cdot \Sigma(a_p - b \cdot w_p)$$

where b is the mean of the a/w values of at least 5 vials in front of the protein zone, ρ is the density of the gel, c is the concentration of Triton X-100, and a_p and w_p are the activities and weights, respectively, of the slices containing the protein zone. The summation was done over the slices p.

Only the most abundant amino acids could be measured with sufficient accuracy when less than 20 μg protein were analyzed. Therefore, the amount of a protein was determined indirectly by making use of the known amino acid composition. From the determined amounts of Asp, Glu, and Leu three values for the corresponding amount of protein were calculated. The mean of these was taken as the amount of the protein in the gel.

RESULTS

Table I shows the results of the binding determination for the proteins studied. Of these, the Triton X-100 binding has previously been determined for albumin and glycophorin by centrifugation in sucrose density gradients [3]. Within the inherent experimental errors the values obtained in the present study agree with those obtained by centrifugation. Table II shows the results of some experiments conducted to study the influence of temperature and Triton X-100 concentration on the binding of Triton X-100 to pencillinase. Most published molecular weight determinations of proteins in Triton X-100 solutions (and corresponding binding ratios) have been made at the arbitrarily chosen Triton X-100 concentration of 0.50 mg/ml [2, 3, 7], a concentration which is three-times higher than the critical micelle concentration [5, 7, 9]. In these experiments, it has been assumed that the proteins would be saturated with Triton X-100 so that variations in the Triton X-100 concentration would have little effect on the binding. The results in Table II show that for penicillinase at 26±1 °C the binding at 0.50 and 0.80 mg/ml Triton X-100 was the same. However, at 8±2 °C a significantly lower binding was attained at 0.50 than at 0.80 mg/ml.

TABLE I

BINDING OF TRITON X-100 TO DIFFERENT PROTEINS

The proteins were equilibrated with 0.50 mg/ml ^{3}H-labelled Triton X-100 by electrophoresis in polyacrylamide gels (see Figs. 1–3). The amount of bound Triton X-100 was determined from radioactivity measurements and the corresponding amount of protein from amino acid analyses as described in the text. The buffer used was 50 mM sodium phosphate (pH 7.8). The temperature was 26±1 °C, as measured with a thermistor placed on the surface of an electrophoresis tube. The voltage across the tubes was 44–48 V and the current 4 mA per tube; according to ref. 19 the radial temperature difference in the gel is then less than 0.2 °C. For further details see Figs. 1–3.

Protein	Weight Triton X-100 per weight protein*		Amount of protein per gel (μg)	Amino acid composition
Penicillinase	2.0±0.1	(6,2)	14	[20]
Membrane protein from *A. laidlawii*	1.5±0.1	(3,1)	8	[12]
Glycophorin	1.3±0.2	(7,2)	20,40	[21]
Albumin	0.030±0.002	(3,1)	520	[22]
Growth hormone	0.008±0.001	(3,1)	525	[23]
Ovalbumin	0.008	(1,0)**	308	

* The first and second numbers in parentheses are the numbers of gels taken for radioactivity and amino acid analysis, respectively. The variability of the measured values is given as ±S.E.
** The amount of protein was determined by optical scanning [18].

The ionic conditions at which the bindings were determined were checked by measuring variations in the conductivity and pH through the gels (Fig. 1). The conductivity in the middle of the gel was not significantly different from that of the buffer and the variations in pH (not shown) was less than 0.02 units.

The variation in the radioactivity of the gel slices due to experimental error sets a limit to the sensitivity of the method. The errors given in Tables I and II indicate a precision of ±1 μg Triton X-100, for 3 gels or more. For growth hormone a binding of 4 μg was found. Too little is known yet about the binding of Triton X-100 to

TABLE II

EFFECT OF TRITON X-100 AND TEMPERATURE ON THE BINDING OF TRITON X-100 TO PENICILLINASE

Penicillinase was equilibrated with ^{3}H-labelled Triton X-100 by electrophoresis in polyacrylamide gels. Except for temperature and Triton X-100 concentration the conditions were as described in Table I and Fig. 1.

Triton X-100 conc. (mg/ml)	Temp. (°C)	Weight Triton X-100 per weight protein*
0.50	26±1	2.0±0.1 (7,2)
0.80	26±1	2.0±0.1 (3,1)
0.50	8±2	1.5±0.2 (7,2)
0.80	8±2	2.0±0.1 (3,1)

* The first and second numbers given in parentheses are the numbers of the gels used for radioactivity and amino acid determinations, respectively. The variability of the measured values is given as ±S.E.

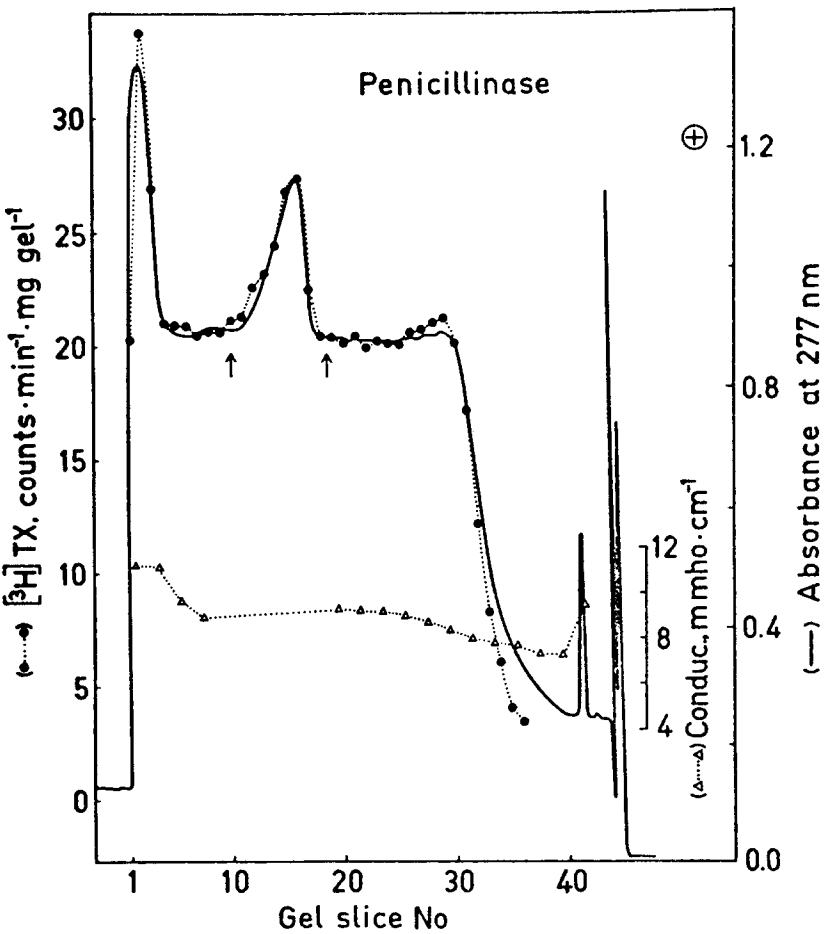

Fig. 1. Optical scanning, radioactivity, and conductivity determinations of 5×100 mm cylindrical polyacrylamide gels containing 0.50 mg/ml ^{3}H-labelled Triton X-100 ([^{3}H]TX), after electrophoresis of 14 μg penicillinase for 30 h. The gels were made with 40 mg/ml monomer and 1.6 mg/ml bisacrylamide. Other conditions were as described in Table I. The sharp absorption peaks to the very right were due to light refraction at the end of the electrophoresis tube. The sharp peak to their left was due to a mark in the tube. The two arrows indicate the portion of a gel running parallel taken for quantitative amino acid analysis. The rest of this gel was cut into slices and the conductivity was determined.

proteins at this low level to validate an interpretation. For a single gel of ovalbumin (Fig. 3), the calculated "binding" was 2 μg. This was probably due to an experimental error since Makino et al. [24], using a more sensitive equilibrium dialysis method, found no binding to ovalbumin.

Amino acid analysis of stained proteins zones from polyacrylamide gels has previously been reported to give reliable results [17]. In the present study ten-times smaller amounts of protein were analyzed.

A comparison of the resultant amino acid composition of two identical samples

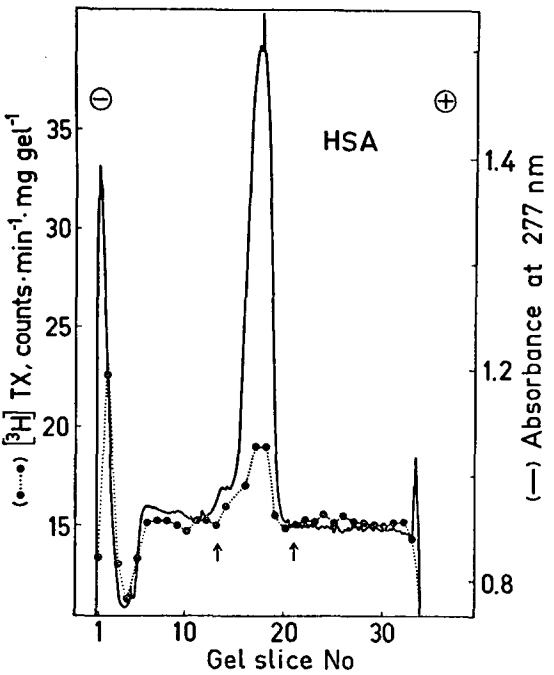

Fig. 2. Optical scanning and radioactivity determination of a polyacrylamide gel containing 0.50 mg/ml ^{3}H-labelled Triton X-100 ([^{3}H]TX) after electrophoresis of 520 μg serum albumin for 6 h. Only the upper part of the scanning record is shown (cf. Fig. 1). The gel was made with 50 mg/ml monomer and 1.5 mg/ml bisacrylamide. Other conditions were as described in Table I. The two arrows show the portion of an identical gel taken for amino acid analysis. HSA, human serum albumin.

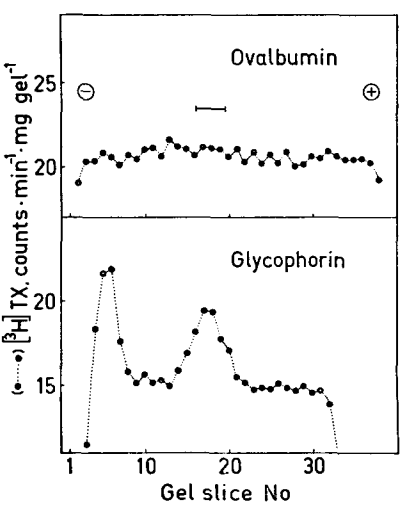

Fig. 3. Radioactivity determinations of polyacrylamide gels containing 0.50 mg/ml ^{3}H-labelled Triton X-100 ([^{3}H]TX) after electrophoresis of 308 μg ovalbumin and 20 μg glycophorin for 6 and 12 h, respectively. The horizontal bar shows the position of the ovalbumin peak as determined by scanning at 277 nm (cf. Fig. 2). The gels were made with 50 mg/ml monomer and 1.5 mg/ml bisacrylamide. Other conditions were as described in Table I.

containing 25 μg penicillinase, one hydrolyzed with and one without a gel segment, showed that the values of the 8 most abundant neutral or acidic amino acids agreed within ±7 %, except those for Gly which yielded a value 18 % higher in the presence of the gel.

As shown by Figs. 1–3, some protein samples seemed to contain slowly migrating components which bound large amounts of Triton X-100. Staining of these gels with Coomassie brilliant blue indicated that protein was not responsible for the binding. Fig. 1 also shows that Triton X-100 migrated slowly towards the cathode. This may be due to the binding of hydronium ions to the polyoxyethylene chains, as has been proposed [25].

DISCUSSION

Using the method described in this paper, it has been possible to determine the binding ratio of Triton X-100 to membrane proteins with an accuracy better than 10 % and using less than 50 μg protein. This error gives an uncertainty of less than 2 % for the molecular weight calculated from ultracentrifugation data [6]. The accuracy of the binding method can be considered sufficient since the other experimental parameters are difficult to determine with an error of less than 10 % [6].

In polyacrylamide gel electrophoresis in dodecyl sulphate the membrane penicillinase behaves as a protein of molecular weight $33 \cdot 10^3$ [11] and in Triton X-100 solution it probably occurs as a monomer (Simons, K., unpublished). These data and the binding ratio of 2.0±0.1 (Table I) imply that the penicillinase molecule in Triton X-100 solution is associated with 104±5 molecules of Triton X-100, assuming a molecular weight of 636 for Triton X-100 [24]. Molar ratios of about 100 have previously been measured for two other monomeric membrane proteins [5, 26], and it has been suggested that Triton X-100 in these cases may be bound in the form of a micelle [5]; in its free form the micelle consists of about 130 monomers [27, 28]. The membrane protein from *A. laidlawii* (Table I), with an apparent molecular weight of $34 \cdot 10^3$ [29], was found to have a molar ratio of 80±4, however, its state of aggregation is not known. The glycophorin-detergent complex contained only 57±8 Triton X-100 molecules, possibly because the dimeric structure of the protein [3] hinders the formation of a Triton X-100 micelle.

The results of the Triton X-100 binding to the proteins used in this work are in accordance with those of earlier studies [2, 3], and indicate that Triton X-100 binds in large amounts only to those proteins natively associated with lipids by means of hydrophobic interaction. For membrane proteins, the binding seems to be confined to the part of the polypeptide previously immersed in the lipid bilayer [5, 30]. The capacity of a membrane protein to bind Triton X-100 can therefore be used as a criterion for association with a membrane by means of a hydrophobic segment. The method described should prove useful for such determinations.

Little data exist on the influence of Triton X-100 concentration on the binding of Triton X-100 to membrane proteins. For cytochrome b_5 a 50 % higher binding was measured at 0.8 than at 0.5 mg/ml [5], and for rhodopsin the binding was 28 % higher at 3.0 than at 0.5 mg/ml [26]. For penicillinase no significant difference was found at 0.8 and 0.5 mg/ml Triton X-100 at a comparable temperature (see Table II). Whether these different binding characteristics are due to differences in the hydro-

phobic segments or to different experimental conditions (or both) remains to be seen.

No previous study has been done on the influence of temperature on the binding of Triton X-100 to membrane proteins. Penicillinase was found to have a 33 % lower binding at 8±2 °C than at 26±1 °C at a Triton X-100 concentration of 0.5 mg/ml. This observation indicates that it is essential that the different characteristics of the detergent-protein complex, necessary for a calculation of the molecular weight [6], are determined at the same temperature.

In the present work optical scanning has been used as a practical means of determining suitable separation times and locating the protein zones. However, availability of a scanning device is not an absolute necessity for the described method, since essentially the same information can be obtained by protein staining or from the radioactivity determinations.

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