Biochimica et Biophysica Acta, 1103 (1992) 148–156

BBAMEM 75448

Liposomes as a model for the study of the mechanism of fish toxicity of sodium dodecyl sulfate in sea water

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(Received 15 July 1991)

Key words: Liposome; Lipid bilayer; Detergent; Fish toxicity; (Shark)

The mechanism underlying the shark repellency of SDS was studied by comparing it with the shark nonrepelling detergent, Triton X-100. The findings can be summarized as follows: (1) The effective concentration of SDS for termination of shark tonic immobility (an immediate and fast response) was close to its critical micellar concentration in sea water (70 μM). The fish lethal concentrations (LD_{50}) were far below the CMC value for SDS, and at CMC level for Triton X-100. (2) In sea water SDS possesses a strong affinity for lipid membranes, expressed in a lipid sea water partition coefficient (K_p) of about 3000. (3) In liposomal systems examined by assays of turbidity, fluorescence resonance energy transfer and kinetics of carboxyfluorescein (CF) release, the pattern of SDS induced changes in the phospholipid bilayer suggests: (a) absence of vesicle-vesicle fusion; (b) occurrence of vesicle size increase, and (c) nonlytic gradual release of CF above and below its CMC values. In contrast, Triton X-100 above its CMC induces membrane solubilization. (4) Assays coupling CF release from liposomes to potassium diffusion potential induced by valinomycin indicate that SDS related CF release can also be attributed to a specific mechanism such as cation pore formation and not only to membrane solubilization. The hypothesis of pore formation by SDS is discussed.

Introduction

Some marine organisms secrete amphipathic substances into their surroundings which serve as defensive agents [1,2]. These substances can be classified as (a) low molecular weight compounds resembling synthetic detergents such as Pahutoxin, secreted by box fishes [3,4] or Holothurin, secreted by sea cucumbers [1,2]; or (b) polypeptide substances such as grammistins and pardaxins derived from the skin secretion of soap fishes and flat fishes, respectively [5–8].

The common denominator of all these compounds is their ability to disrupt and change the barrier properties of cell membranes of the alien organism. Experiments have shown that the skin secretion of the flat fish, *Pardachirus marmoratus*, can repel sharks [9,10]. Based on the assumption that the biological effects of the skin secretion and its derived toxin Pardaxin are due to their amphipathic-surfactant properties, it has been suggested that commercial surfactants may also possess shark repellent abilities [11]. This hypothesis did not withstand experimental examination. Out of 15 different synthetic surfactants only the sodium and lithium salts of dodecyl sulfate (SDS, LDS) have demonstrated a clear and convincing repellent effect [12]. Two effects of SDS on the behavior of sharks have been demonstrated: (1) An ability to induce a fast turning and escape response in aggressive Lemon

Abbreviations: CF, 5,6-carboxyfluorescein; Chol, cholesterol; CMC, critical micelle concentration; DCP, dicetyl phosphate; DPH, 1,6-diphenylhexa-1,3,5-triene; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HC, 4-heptadecyl-7-hydroxycoumarin; N-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidylethanolamine; N-RH-PE, *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine; PC, phosphatidylcholine; RET, resonance energy transfer; SDS, sodium dodecyl sulfate; b.b.SPM, bovine brain sphingomyelin; SUV, small unilamellar vesicles; SW, sea water; TI, tonic immobility.

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sharks (*Negaprion brevirostris*) and blue sharks (*Prionace glauca*) while in the process of attacking ('feeding assay', [12–13]); and (2) an ability to rapidly terminate, in an excitatory manner, the tonic immobility of sharks which had been induced by restraining them in an inverted position [14]. In both assays the SDS solutions were introduced into the buccal cavity of the shark.

In a recent study of Kalmanzon et al. [15], SDS, when compared to a series of ionic and nonionic detergents, showed the greatest decrease in the CMC value in transition from distilled to sea water. What seems to be most significant (see Discussion), is the possession of negative micelle electrical surface potential of SDS (−47 mV) in sea water.

The aim of the present work was to study the possible relationship between the shark repelling capacity of SDS and its physicochemical mode of interaction with lipid bilayers in the natural shark habitat, sea water. For comparative purposes, another well-characterized detergent, Triton-X-100, was included in this study. Triton X-100 was previously shown to possess 20–30 times lower repellent activity toward sharks [12].

Materials and Methods

Materials

SDS, Triton-X-100, valinomycin and the various buffers used were purchased from Sigma (USA). Radiolabeled dodecyl sodium [^{35}S]sulfate (specific activity 15.1 mCi/mmol) was purchased from Amersham (UK). 5,6-Carboxyfluorescein was purchased from Eastman Kodak (USA) and was further purified as previously described [16].

Test animals

Sharks of the species *Mustelus mosis* (Carcharinidae) (70–80 cm long) were caught in the Eilat bay, the Red Sea, and kept in a large container of circulating sea water. *Gambusia affinis* fishes (length 30–40 mm, 286 ± 31 mg; mean ± S.D., $n = 10$) were collected from local water ponds and kept in aerated containers of fresh water. The *Gambusia* fishes employed in experiments were acclimated for 3 days to 10% SW and displayed completely normal behavior.

Liposomes

Small unilamellar vesicles (SUV) were employed throughout the study. First, we prepared multilamellar large vesicles (MLV) of phospholipids and cholesterol by thin lipid hydration [17,18] in either sea water, sea water containing 0.1 M sodium CF at pH 8.0, or 0.01 M Hepes buffer solution (pH 8.0) containing 0.1 M of either potassium or sodium salts of 5,6-carboxyfluorescein. The MLV were then exposed to ultrasonic irradiation [17] using a Heat System 350 W Sonicator and 3/4 inch probe coated with sapphire (in order to reduce contamination with metal particles). The SUV were fractionated by differential centrifugation according to a previously described method [17,18]. In all measurements of CF release or leakage the pH of the external medium and the intraliposomal pH were identical (pH 8.0).

Three kinds of liposomes were used: (a) Neutral liposomes composed of (mol/mol) phospatidylcholine (70%), cholesterol (30%), (PC-Chol liposomes); (b) negatively charged liposomes composed of phosphatidylcholine (63%), cholesterol (30%) and dicetyl phosphate (7%) (PC-Chol-DCP liposomes), and (c) bovine brain sphingomyelin (b.b.SPM) liposomes. The SPM liposomes are highly resistant to fusion and to spontaneous leakage. Therefore, they were more convenient for the studies of CF release by potassium difusion potential [23].

Determination of the partition coefficient lipid / SW of SDS

The assay was based on the employment of a radioactive tracer ([^{35}S]SDS) in an equilibrium dialysis system using methodology modified from Sikaris et al. [19]. Both PC-Chol and PC-Chol-DCP liposomes were used.

Mixtures of radioactive and nonlabeled SDS of the final concentration range of 1–150 μM were introduced into sea water (final volume 4.5 ml) in which a dialysis bag, filled with 0.5 ml of 1 mM suspension of liposomes in sea water, was immersed. At various time intervals, aliquots of SDS solution were removed and counted for radioactivity. The time dependent reduction in the radioactivity of the medium due to diffusion into the dialysis bag (until equilibrium between the external medium and the dialysis bag solution was achieved at 16 h) was monitored. The concentration of SDS at equilibrium inside ($[SDS]_{in}$) and outside ($[SDS]_{out}$) the dialysis bag was determined. The excess SDS inside the dialysis bag was determined as:

$$[SDS]_L = [SDS]_{in} - [SDS]_{out}$$

where $[SDS]_L$ is the SDS concentration in liposomes. $[SDS]_L$ and $[SDS]_{out}$ values were used to calculate K_p, the liposome/sea water (SUV/SW) SDS partition coefficient [19]. Since Triton-X-100 is not well dialyzable, its K_p could not be determined in the above experimental system.

Temperature

The various assays with liposomes, CMC determinations and shark tonic immobility were performed at the temperature of the Red Sea water (22 ± 1°C). Some of the ionophore dependent CF release assay was performed at other temperatures as specified.

Determination of liposome fusion and/or size change

The method is based on release of resonance energy transfer (RET) between pairs of membranal fluorescent markers. N-NBD-PE served as the donor (excitation 470 nm, emission 545 nm), and N-RH-PE as acceptor (excitation 540 nm, emission 585 nm), with both present in the same membrane. In both molecules the fluorophore is covalently attached to the free amino group of the phosphatidylethanolamine and therefore is a part of the phospholipid head group [20,21]. The advantage of these molecules is that they do not diffuse between membranes [20]. The principle of RET is that its efficiency is dependent on the average distance between the acceptor and the donor fluorophores. The dilution of the two fluorophores by unlabelled molecules increases the average distance between donor and acceptor, thereby causing reduction of RET. Upon excitation of the N-NBD-PE (470 nm) [20,21], RET is expressed as the increase in fluorescence intensity of N-NBD-PE (545 nm) concomitant with the reduction in fluorescence intensity of N-RH-PE (585 nm).

Generally, there are four reasons for dilution related RET reduction in the system in which fluorescent labelled liposomes, unlabelled liposomes and detergent molecules coexist:

(i) Fusion of labelled and unlabelled liposomes.
(ii) Solubilization of labelled liposomes by detergent to form mixed micelles.
(iii) Intervesicular transfer of the fluorophore molecules to unlabelled liposomes or to detergent micelles.
(iv) Partition of detergent molecules into the labelled vesicle bilayer without bilayer solubilization.

All the above four reasons cause surface dilution and therefore reduction in RET. It is noteworthy that dequenching due to reduction in RET does not occur upon vesicle aggregation, since aggregation does not affect surface concentration of donors and acceptors.

Each measurement was repeated three times with very good reproducibilty.

The effect of SDS and Triton-X-100 on the turbidity of liposome dispersion

Increase in turbidity of liposome suspensions occurs upon liposome aggregation and/or size increase. On the other hand, liposome solubilization is followed by major turbidity reduction. Therefore, change in the specific turbidity (turbidity M^{-1}) was used as a sensitive parameter to study detergent dependent size changes in liposomes. The change in turbidity was measured as follows: 3 ml suspension of 1 mM PC-Chol liposomes in sea water was placed in a spectrophotometer. The absorbance at 400 nm was recorded as base line, then the desired amount of either SDS or Triton-X-100 in minimal volume was added. The dispersions were mixed and their turbidity was followed with time. Measurements were carried out at detergent concentrations below and above CMC.

Estimation of detergent induced release of liposomal content

Small unilamellar vesicles were prepared in the presence of 100 mM potassium or sodium salt of 5,6-carboxyfluorescein (CF) in 10 mM Hepes buffer pH 8.0 as described above, and were kept in the dark at 4°C. Prior to the experiment, the unencapsulated CF was removed by gel exclusion chromatography using mini columns of Sephadex G-50 medium (Pharmacia, Sweden) equilibrated with isoosmotic solution of 10 mM Hepes buffer pH 8.0 containing sodium chloride [21]. At 100 mM CF, the fluorescence intensity is almost completely quenched [22]. Monitoring of CF release was done by adding SUV made of 25 nmol phospholipids loaded with CF to 2 ml final volume of thermally equilibrated 10 mM isoosmotic Na-Hepes buffer pH 8.0 containing NaCl in a Perkin Elmer LS-5 spectrofluorometer, at the desired temperature. Fluorescence intensity was recorded to obtain baseline CF release data. Then either detergent or valinomycin was added at the desired concentration. The effect of the added agent was calculated from the change in slope of the time dependent fluorescence intensity. Major CF dilution occurs upon its release from the aqueous compartment of the liposomes to the external medium, leading to a fluorescence dequenching to the range in which fluorescence (emission 520 nm; excitation 490 nm) is directly proportional to CF concentration. 100% release was obtained by complete solubilization of the vesicle using high Triton-X-100 concentration [22]. Fluorescence intensity (F) is temperature (T) dependent, therefore quantitation was corrected according to an experimentally obtained calibration curve described by the following linear equation:

$$F = 100.49 - 0.712 \cdot T(°C)$$

Determination of the shark effective concentrations of SDS

The test system was essentially based on the shark tonic immobility (TI) assay described by Gruber and Zlotkin [14] employing *M. mosis* sharks. The sharks were restrained in an inverted position for 5–10 s followed by a quiescent state of immobility of at least 10 minutes duration (Figs. 6, 7). The device for the determination of the threshold effective concentration of SDS, is presented and described in Fig. 6; for details see legends to Fig. 6 and Fig. 7.

Determination of fish lethality

Standard LD_{50} determinations were performed using *Gambusia* fishes. Groups of five fishes were used

for each detergent concentration. Each group was placed in a separate glass beaker in a volume of 100 ml of 10% SW. Death, expressed in the arrest of opercular movements, was monitored after 24 h. The kinetics of detergent induced fish death was determined as described in the legend to Fig. 8.

Results

The partition coefficient of SDS between sea water and liposomes

When a detergent is added to a liposome suspension it partitions into three phases: (a) as monomers in the aqueous solution; (b) as micelles (pure detergent micelles or detergent-lipid mixed micelles) and (c) as a bilayer component. The determination of the monomer concentration is achieved through the determination of the CMC. The partitioning of the detergent into the liposomes is measured using an equilibrium dialysis assay as presented in Materials and Methods and exemplified in Fig. 1. This figure presents the time course of the detergent diffusion into the dialysis bag. The plateau value of [^{35}S] radioactivity inside and outside the dialysis bag was used to calculate $[SDS]_{in}$ and $[SDS]_{out}$, from which the K_p was calculated as described in Materials and Methods. The average K_p values were obtained from experiments employing SDS concentrations in the range of 10–150 μM in sea water, where $K_p = 3084 \pm 435$ (mean $\pm$ S.D.; $n = 4$) for the negatively charged PC-Chol-DCP liposomes and $K_p = 3469 \pm 578$ (mean $\pm$ S.D.; $n = 4$) for the neutral PC-Chol liposomes. These data indicate that: (1) the high K_p value represents a strong affinity of SDS to lipid bilayers; (2) the K_p value is independent of the CMC value since detergent concentrations above and below CMC yielded the same partition coefficient; and (3) in sea water the detergent-lipid affinity is only slightly reduced by the presence of negatively charged phospholipid in the bilayer, possibly due to the large reduction in surface potential at such high ionic strength [15].

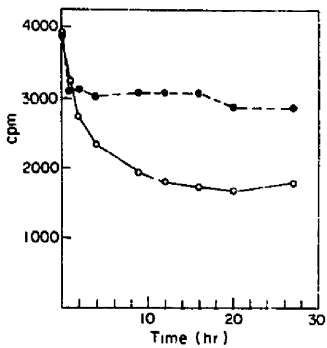

Fig. 1. A typical assay of equilibrium dialysis demonstrating the binding of SDS (80 μM) to the liposomal phospholipid. ○——○, Radioactivity in the external medium with liposomes inside the dialysis bag. ●— — —●, Radioactivity in the control assay (no liposomes present in the dialysis bag) (for details see Materials and Methods).

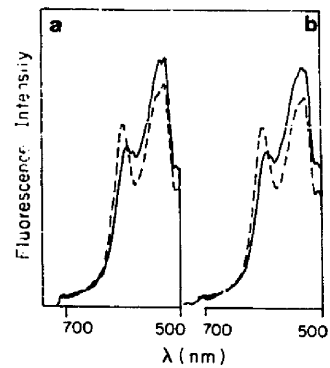

Fig. 2. Measurement of change in RET. Indication of SDS dependent size increase of liposomes labeled by the fluorescent markers N-NBD-PE (donors) and N-RH-PE (acceptors) in sea water (see Materials and Methods): The measurement of reduction in RET is performed in two parallel systems: (a) system to follow fusion which includes labeled liposomes, nonlabeled liposomes and the detergent, and (b) control system to follow solubilization which includes labeled liposomes and detergent only. Both fluorescent markers (N-NBD-PE and N-RH-PE) were present in the same PC-Chol SUV at 1% mol% each. SDS concentration was 100 μM. ——, the spectrum obtained before SDS addition. - - - - - -, the spectrum obtained after SDS addition.

Liposome size increase

The data presented in Fig. 2 show that SDS caused similar fluorescence dequenching of N-NBD-PE fluorescence and parallel decrease in the acceptor N-RH-PE fluorescence intensity in the presence (Fig. 2a) as well as absence (Fig. 2b) of unlabeled liposomes. Therefore the above changes in fluorescence cannot result from vesicle fusion or dilution by other lipid molecules due to intervesicular transfer. Changes in fluorescence should be attributed to an increase in liposome size due to incorporation of detergent molecules into the lipid bilayer. Turbidity measurement (see Materials and Methods) showed that SDS induced a transient decrease in turbidity within 1 min followed by a gradual increase in turbidity during the subsequent 30 min of observation (data not shown). This may indicate an increase of liposome size. On the other hand, Triton-X-100 at the concentration tested caused an obvious decrease in the turbidity (data not shown) probably due to a lytic affect leading to mixed micelle formation.

The above data suggest that the increase in vesicle size, which occurs upon SDS addition, is not related to fusion or inter vesicular lipid transfer, but rather to the partitioning of SDS into the vesicles.

Detergent induced leakage

The data presented in Fig. 3 demonstrate the effect of SDS and Triton-X-100 on the release of CF from neutral PC-Chol and negatively charged PC-Chol-DCP liposomes in sea water. Triton-X-100 in concentrations close to its CMC (80 μM, Fig. 3C, D) induced a fast and complete release of CF in both kinds of liposomes. On the other hand, SDS, even in concentrations of about seven times its CMC (Fig. 3A, B), induced only a gradual time dependent release in both kinds of liposomes. Both detergents were more effective on the negatively charged liposomes. This may be related to charge-reduced reduction in packing density at the head group region which increases the rate of detergent incorporation into the vesicle bilayer. Fig. 4 demonstrates the concentration dependence of the rate of SDS induced CF release, using the time required to obtain 50% release (data were taken from Fig. 3B). This indicates a strong concentration dependency, only below the CMC of SDS.

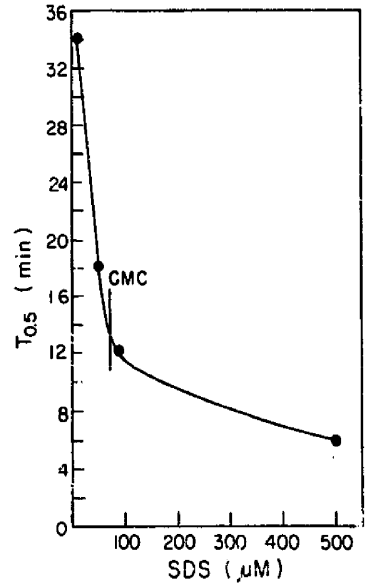

Fig. 4. SDS concentration dependent rate of CF release. The time corresponding to 50% of maximal release of CF ($t_{1/2}$) from the negatively charged liposomes (PC-CHOL-DCP) is presented. Data taken from Fig. 3B.

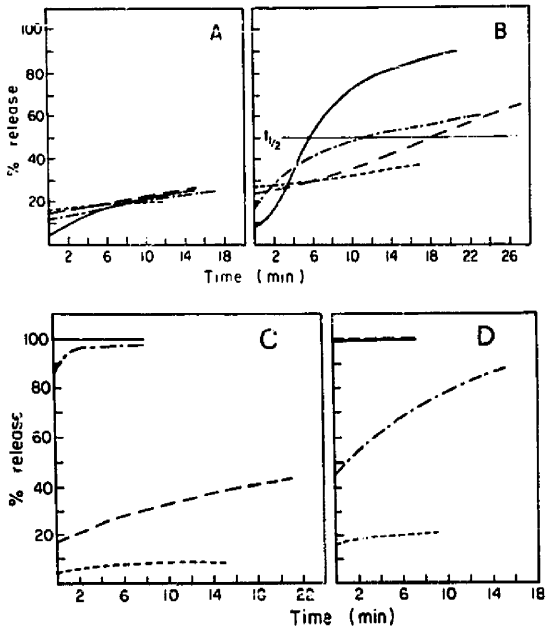

Fig. 3. Release of CF from liposomes. The liposomes were prepared in sea water (pH 8.0) containing 0.1 M sodium carboxy fluorescein. (A) SDS with neutral liposomes. (B) SDS with negatively charged liposomes. (C) Triton-X-100 with neutral liposomes. (D) Triton-X-100 with negatively charged liposomes (for more details see Materials and Methods). The following concentrations of SDS were used: ·····, 10 μM; — — —, 50 μM; -·-·-, 90 μM; ———, 500 μM. The following concentrations of Triton-X-100 were used: ·····, 10 μM; — — —, 40 μM; -·-·-, 80 μM; ———, 400 μM.

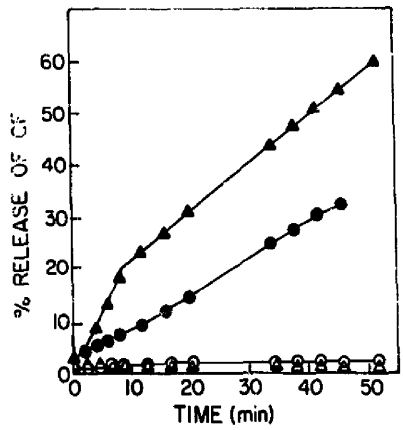

Fig. 5. Potassium diffusion potential dependent CF release. Release of CF was determined as described in Materials and Methods. SPM-SUV were used. Filled symbols describe the SPM-SUV loaded with K-CF, pH 8.0 (10 mM K-Hepes buffer) and medium containing NaCl at pH 8.0 (10 mM Na-Hepes buffer) (conditions to create potassium diffusion potential) either at 5°C (●) or at 14°C (▲). Empty symbols describe SPM-SUV loaded with Na-CF, pM 8.0 (10 mM Na-Hepes buffer in both intraliposomal and the external medium) in medium containing NaCl; conditions of no potassium diffusion potential; at 5°C (○) and 14°C (△). The reaction was carried out in the thermally equilibrated cuvette inside the spectrofluorometer. 25 nmol of SPM liposomes loaded with 100 mM CF were added. After recording background release rates, 0.5 nmol of valinomycin was added in DMSO (final DMSO concentration was below 0.5%) and increase in fluorescence intensity (520 nm) was followed with time (for more details see Materials and Methods).

The slow and noncomplete SDS induced CF release suggests that the process is nonlytic. It was important to demonstrate that nonlytic release can be driven by a specific change such as membrane potential and that it is not necessarily a reflection of a nonspecific structural membrane damage. This was demonstrated by the coupling between the CF release and potassium diffusion potential induced by a specific transport of this ion by the ionophore Valinomycin. The data presented in Fig. 5 demonstrate that leakiness of an anionic form of CF is coupled to the Valinomycin stimulated efflux of potassium ions [23]. The specificity of the above process was demonstrated by the obligatory requirement for the potassium diffusion potential. No release occurred when sodium-containing liposomes were used (Fig. 5). This suggests that CF may be released from an intact liposome through being coupled to a carrier mediated cation release.

Termination of tonic immobility

The concentration of SDS inducing the termination of *Mustelus mosis* tonic immobility, determined as described in Ref. 30 (Figs. 6 and 7), is 130 ± 37 μM (mean ± S.D.; $n = 4$). The response is almost instantaneous. This result closely resembles the data obtained with Lemon sharks in a similar experiment (124 ± 12 μM, mean ± S.D.; $n = 4$ [12]). As with the Lemon sharks, the termination of the tonic immobility was always preceded by deeper accelerated respiratory movement and contractions of the buccal cavity.

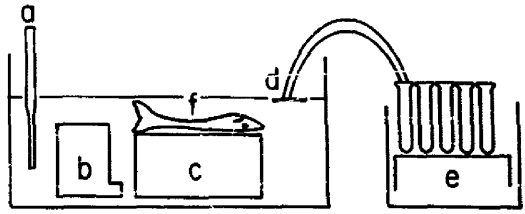

Fig. 6. The experimental system for the determination of the threshold effective concentration of SDS to sharks. A rectangular container filled with 70 l of SW included a fixed pipette (a) for the addition of the test substance; (b) an immersible pump (output 140 l/min) for the distribution of the test substance; (c) a support unit on which the tonically immobilized shark was placed (Fig. 7); (d) a siphon arrangement of tubing which continuously collected water in the vicinity of the shark's head and transferred it at a flow rate of 3–5 ml/s into a fraction collector, (e) which collected samples at 2-s intervals. The sample corresponding to the shark's rightening response was processed for the chemical determination of SDS concentration [30]. When selecting the samples, the volume of the siphon tubing was taken into consideration.

Lethality to Gambusia fishes

Lethality was followed for 24 h of exposure to the detergent in 10% sea water. The LD_{50} data correspond to 66.7 and 62.5 μM for SDS and Triton-X-100, respectively, while CMC of the two detergents are 270 and 65 μM, respectively [15]. The kinetics of response to Triton-X-100 shows a sharp transition between lethality and survival at concentrations close to the Triton CMC (Fig. 8). The data presented in Fig. 8 indicate that at concentrations close to the CMC (see below and Ref. 15) there is an exponential prolonga-

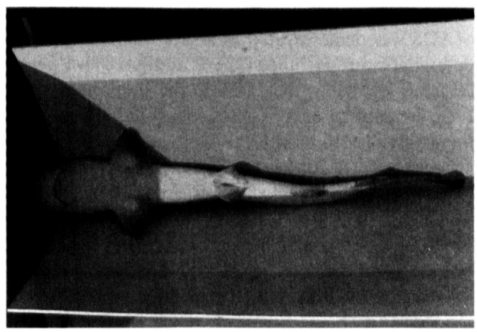

Fig. 7. Shark tonic immobility. The shark *Iago omanensis* placed in an inverted position on the support (Fig. 6) located in the test container (Fig. 6), displaying a typical posture of tonic immobility.

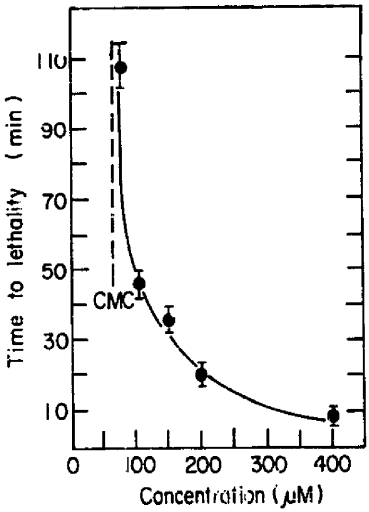

Fig. 8. The time course of *Gambusia* lethality as a function of Triton-X-100 concentration. The experiment was set up as described in Materials and Methods. Various concentrations ranging from 10 to 400 μM Triton-X-100 were applied to three *Gambusia* fish at each detergent concentration and the time to lethality was recorded. Average time to death is shown on the y axis, bars represent variability among the three fishes. No lethality was observed below 60 μM Triton-X-100 during 24 h of contact with the detergent (see Results).

tion of time to lethality, and complete survival at doses below CMC. (This range is not shown in Fig. 8). These data are in agreement with previously reported lethality assays performed in sea water with killifish (*Floridictiyes carpio*) [12]. There, SDS in 100% sea water induced lethality far below its CMC (LD_{50} 10.4 μM and CMC 70 μm) but the Triton-X-100 LD_{50} value (55.6 μM) closely corresponded to its CMC.

Discussion

The unique and exceptional shark repellent potency of SDS was demonstrated in assays where 15 different detergents were included [12]. Triton-X-100 was 30 and 20 times less active than SDS in the shark feeding and tonic immobility assays, respectively [12]. The aim of this study was to clarify the large difference in shark repellency between these two detergents and especially in respect to their activity as membrane solubilizers. The choice of Triton-X-100 for comparison to SDS was based on three considerations: (1) it is a well characterized, effective and broadly used detergent [24,25]; (2) it is significantly less effective than SDS as shark repeller [12]; and (3) in sea water it was shown to possess an equal CMC to SDS, and therefore both detergents resemble each other in their free energy of micellization [15]. Recently we proposed structure–function relationships for surfactants acting as shark repellents [15]. Such surfactants in sea-water should reveal a combination of negative electrical surface potential and high partition coefficient into lipid bilayers as prerequisites to obtain repellency. This work was aimed to further confirm these relationships.

Detergents form micellar aggregates above their critical micellar concentration (CMC). The CMC varies for different detergents in given medium conditions and varies for the same detergent in different medium conditions (26). The CMC is considered to be a thermodynamic characteristic property. CMC is affected by medium and temperature for all detergents, and by medium, pH, ionic strength and ionic composition mainly for ionic detergents [26,27]. The solubilizing capacity of detergent is achieved in its micellar form [18]. The detergent-membrane interaction is dependent on detergent concentration and on the mole ratio of detergent to membrane lipid [18,24]. In general, the membrane-detergent phase diagram can be divided into three main regions: (1) the lamellar (bilayer) zone at low detergent to membrane lipid mole ratio; (2) the mixed micelles region which is a result of membrane solubilization, and (3) the region in which lamellar and micellar phases coexist. In the lamellar zone, the detergent partitions into the bilayer, leading to its expansion and to an increase in membrane permeability by pore formation without grossly affecting membrane integrity [28,29]. The actual meaning of these pores is not yet known and may be related to effects such as increase in membrane 'fluidity', reduction in the order of membrane phospholipid acyl chains, increase in the rotational motion of the lipid molecules and alteration of the lateral distribution of membrane proteins and lipids [18]. These pores are responsible for the increase in membrane leakiness. The incorporation of a detergent into the lipid bilayer below its CMC indicates that the free energy (ΔF) required for the detergent to interact with the lipid bilayer is lower than the free energy of micellization. This is defined as:

$$\Delta F = RT \times \mathrm{Ln}CMC$$

(R = gas constant, T = absolute temperature). Our work with the sharks and the model membranes deals with the zone of the phase diagram in which the detergent and membrane lipid coexist in the bilayer.

The similarity between the concentration of SDS required to terminate tonic immobility in Lemon or Mustelus sharks, and its CMC in sea water, suggests that its shark repellence may result from general detergent solubilizing capacity. This conclusion is strongly contrasted by the following considerations and data supplied by the present and previous [15] studies:

(a) The LD_{50} concentrations of SDS to the killifish (in SW, LD_{50} 10.4 μM [12], CMC 70 μM [15]) and the

present *Gambusia* fishes (in 10% SW, LD_{50} 66.7 μM, CMC 270 μM [15]) is definitely far below its CMC value. On the other hand, the lethal effect of Triton-X-100 (the LD_{50} to killifish and *Gambusia* is 55.6 and 62.5 μM, respectively), closely corresponds to its CMC values in the above media (60 and 65 μM in SW and 10% SW, respectively. This suggests the toxicity induced by the two detergents is related to two different mechanisms: solubilization for Triton-X-100 and another mechanism for SDS.

(b) Triton-X-100, in contrast to its minimal effect on sharks [12] has demonstrated immediate liposome lysis above its CMC. This effect was demonstrated by (1) fast and almost complete release of CF from liposomes (Fig. 3) and (2) a decrease in the turbidity of liposomal suspension (see Results).

(c) SDS, on the other hand, has revealed only a moderate effect on liposomes leakiness, demonstrated by a slow, progressive and time dependent CF release. This was the case even above its CMC. The rate of leakage described by the $t_{1/2}$ value was strongly dependent on SDS concentration below the CMC and concentration independent above the CMC (Fig. 4). Therefore, the SDS induced leakage is different from that obtained by Triton-X-100 under our experimental conditions. Also, SDS does not induce reduction in turbidity. The accordance between the data concerning liposomal leakage, liposome solubilization and fish toxicity (see (a)) is obvious. In both cases, SDS, in contrast to Triton-X-100, was effective in concentrations below its CMC value.

(d) One explanation for this difference is that the SDS effect is related to its ability to form pores in lipid bilayers. The possible relation between the SDS induced CF release and pore formation is supported by the data presented in Fig. 5. They indicate that CF release is not necessarily a consequence of membrane destruction but may also result from a specific process such as the coupling to a net cation efflux. This hypothesis is supported by the consideration that SDS in the medium of sea water exhibits two properties, the combination of which may enable it to form cation channels or act as a cation carrier: firstly, its high affinity to the lipid bilayer which is not affected by its CMC, and secondly its negative charge on the micellar surface [15]. The leaking of cations may thus induce CF release. It is noteworthy that channel formation by detergents has been previously proposed by Schlieper and Robertis [29].

(e) The above concept of pore formation is further supported by present data indicating the incorporation of SDS molecules into vesicle bilayer. The latter is suggested by the (i) increase in spatial distance between the phospholipid molecules as assessed from the resonance energy transfer release between fluorophores in the bilayer (Fig. 2) which is not due to fusion, intravesicular phospholipid transfer or vesicle aggregegation, and (ii) the increase of vesicle specific turbidity induced by SDS concentrations below and above CMC which is a measure of vesicle size growth.

It may be thus proposed that the exceptional and unique shark repellent potency of SDS is not simply a consequence of its detergent-solubilizing properties, but rather represents specific interactions with biological systems at high ionic strength presumably through a pore forming process. We suggest that SDS forms negatively charged pores in the lipid bilayer which resemble inverted micelles. These pores can serve as cation 'channels' and thus induce disturbances in externally exposed shark sensory-neuronal tissues ('pain production'). This hypothesis may explain the effectiveness of SDS as a broad spectrum shark repellent over anionic detergents such as Triton-X-100, positively charged detergents such as lauryl trimethyl ammonium bromide or negatively-charged detergents such as cholic acid salts which are chargeless in SW [15]. The above hypothesis will serve as a target for future studies.

Acknowledgement

This work was supported in part by grant PHS-NIH HL17576.

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