Biochimica et Biophysica Acta, 423 (1976) 382–397

BBA 47055

INACTIVATION AND REACTIVATION OF MITOCHONDRIAL RESPIRATION BY CHARGED DETERGENTS

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(Received July 7th, 1975)

SUMMARY

Respiration of submitochondrial preparations can be inhibited by the cationic detergent cetyl trimethyl ammonium bromide and the anionic detergent sodium dodecyl sulfate in the range of 0.3–2 μmol of detergent per mg of mitochondrial membrane protein depending on the substrate and detergent used. This inhibition can be rapidly reversed by neutralizing a given detergent by the detergent of the opposite charge. At higher levels of the inhibiting detergent, no such reactivation was observed. Spin labeling assays of membrane structure were used to correlate structural effects with the loss and recovery of respiratory functions.

Because the detergents progressively disrupt membrane structure, mitochondria were cross-linked with bifunctional imidoesters to an extent that osmotic properties and detergent lysis were gone, but respiration remained. Such fixed respiring mitochondria also show inhibition reactivation phenomena.

INTRODUCTION

The effect of detergents on membrane structure has been investigated by numerous workers [1], often with regard to the problem of membrane reconstitution [2, 3]. In general, ionic detergents are highly effective solubilizers of membranes but they often lead to an irreversible loss of enzyme activities at low detergent levels and are therefore not favored for reconstitution studies. Detergents bind strongly to membranes and in the case of charged detergents, alter the surface charge of the membrane. Polycations and polyanions have also been used to interact with membranes [4]. It was found that polycations inhibited cytochrome oxidase activity of whole heart homogenates and that this inhibition could be reversed by the addition of polyanions. This inhibition of cytochrome oxidase activity seems to be due, at least in part, to a competition between polycations and cytochrome *c* for a negatively charged binding

Abbreviations: 5N9, 2,2-dimethyl-5,5-dibutyl-*N*-oxazolidinyloxyl; A12NS, 2(10-carboxyldecyl)-2-(hexyl)-4,4-dimethyl-N-oxazolidinyloxyl; CDTAB, 4-(cetyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide; Cet Me_3AmBr, cetyl trimethyl ammonium bromide.

site on the membrane [5, 6]. In this investigation, the interaction of two ionic detergents, sodium dodecyl sulfate and cetyl trimethyl ammonium bromide ($CetMe_3AmBr$), with submitochondrial preparations has been studied with the goal of elucidating a correlation between structural disruption and the loss of certain enzyme functions. It was discovered that charge neutralization of ionic detergents can reverse the loss of respiratory capacity of submitochondrial preparations with various substrates. This finding has been used to further clarify the nature of detergent-induced inactivation of membrane-related functions.

MATERIALS AND METHODS

Oxidation studies. Mitochondria were prepared as described by Stancliff, et al. [7]. The submitochondrial preparation was prepared by sonication as described by Kielley and Bronk [8].

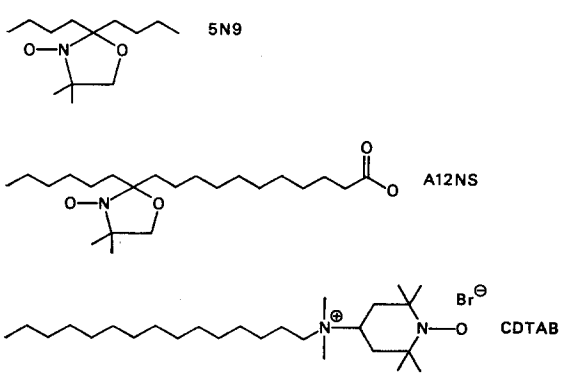
NADH, succinate and ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine oxidase were determined polarographically using 1 mM NADH, 5 mM succinate and ascorbate. *N,N,N',N'*-tetramethyl-*p*-phenylenediamine concentration was 0.30 mM. All reactions were at 25 °C in 250 mM sucrose with 50 mM Tris · HCl at pH 7.5.

The oxidation was initiated in the sample cell by addition of the submitochondrial preparation membrane. Small volumes of concentrated detergent (0.25–0.5 M) were added to yield the final concentration.

Spin labels. The hydrocarbon spin label, 2,2-dimethyl-5,5-dibutyl-*N*-oxazolidinyloxyl (5N9), was synthesized by the procedure of Keana et al. [9] and purified by solvent partitioning. The fatty acid derivative, 2-(10-carboxyldecyl)-2-(hexyl)-4,4-dimethyl-*N*-oxazolidinyloxyl (A12NS), was a gift of A. D. Keith. This spin probe was synthesized by the Keana procedure and purified by thin-layer chromatography.

The spin-labeled detergent, 4-(cetyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide (CDTAB), was synthesized from 4-(*N,N*-dimethylamino)-2,2,6,6-tetramethylpiperidine [10] and 1-bromo hexadecane. These two liquids were mixed at room temperature and subsequently held at 100 °C in the dark for 4 h. The solid white product was dissolved in water and extracted against diethyl ether. The aqueous fraction was oxidized using a solution of ethylenediamine tetraacetic

SPIN PROBES



acid, NaOH, Na_2WO_4 and H_2O_2 [10]. The product of this procedure was dissolved in *n*-butanol and extracted against a large excess of water. The butanol fraction was subsequently extracted with diethyl ether. The butanol was then removed under a stream of air and the product dissolved in water. This product was further purified by recrystallization from water. The concentration of CDTAB was determined by precipitation experiments against a sodium dodecyl sulfate solution of known molarity, as discussed in Results and also more crudely by an estimation of the spin concentration from electron spin resonance measurements. These estimates agreed to within a factor of 2.

Electron spin resonance measurements. Electron spin resonance were obtained with a Varian E3 spectrometer at room temperature. Power settings were generally 8 mW or less.

Electron spin resonance spectral analysis. Spin label mobility was expressed in terms of the parameter

$$\tau_0 = 6.5 \cdot 10^{10} \cdot W_0 \, [(h_0/h_1)^{\frac{1}{2}} - 1]$$

where W_0 and h_0 are the peak to peak width and height of the mid field line and h_1 is the height of the high field line. For fast isotropic tumbling, this parameter corresponds to the correlation time [11], but more generally it can be used as a mobility index for comparative purposes, e.g. for the spin probe A12NS which is expected to exhibit anisotropic motion in membranes [12].

X-ray studies. Aqueous detergent samples were packed into thin-walled glass capillaries and exposed to X-rays for 1.5 h. These experiments were carried out for us by Dr. K. Palmer of the U.S.D.A. Laboratories with an X-ray camera at room temperature and at atmospheric pressure.

Cross-linking of mitochondria. Stock solutions of suberimidate were prepared by dissolving the HCl salt in 0.133 M triethanolamine · HCl and 0.33 M sucrose (pH 9.0) immediately prior to use. Amidination was carried out by incubating mitochondria (1 mg/ml) with 10 mM suberimidate in 0.05 M triethanolamine · HCl and 0.25 M sucrose (pH 8.5) for 30 min at room temperature (24–26 °C). Following incubation, the reaction mixture was diluted 4-fold with ice-cold 0.25 M sucrose to quench the reaction, and the membranes were reisolated by centrifugation (10000 rev./min, 10 min, 4 °C, Sorvall SS34 rotor). The pellet and material on the sides of the centrifuge tube were resuspended in 0.25 M sucrose. Mitochondria fixed under these conditions did not respond to osmotic gradients as assayed by light scattering. Additional experiments were carried out with mitochondria fixed as above with 50 mM suberimidate.

RESULTS

Respiration

Inhibition. The oxidation activity of submitochondrial preparations was monitored continuously with an oxygen polarograph. Three substrates: NADH, succinate and ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine were used to study different components of the mitochondrial electron transport chain. The data for inhibition of respiration by $CetMe_3AmBr$ and sodium dodecyl sulfate are given in Fig. 1. NADH oxidation involves three "coupling sites", and this activity is most readily inhibited.

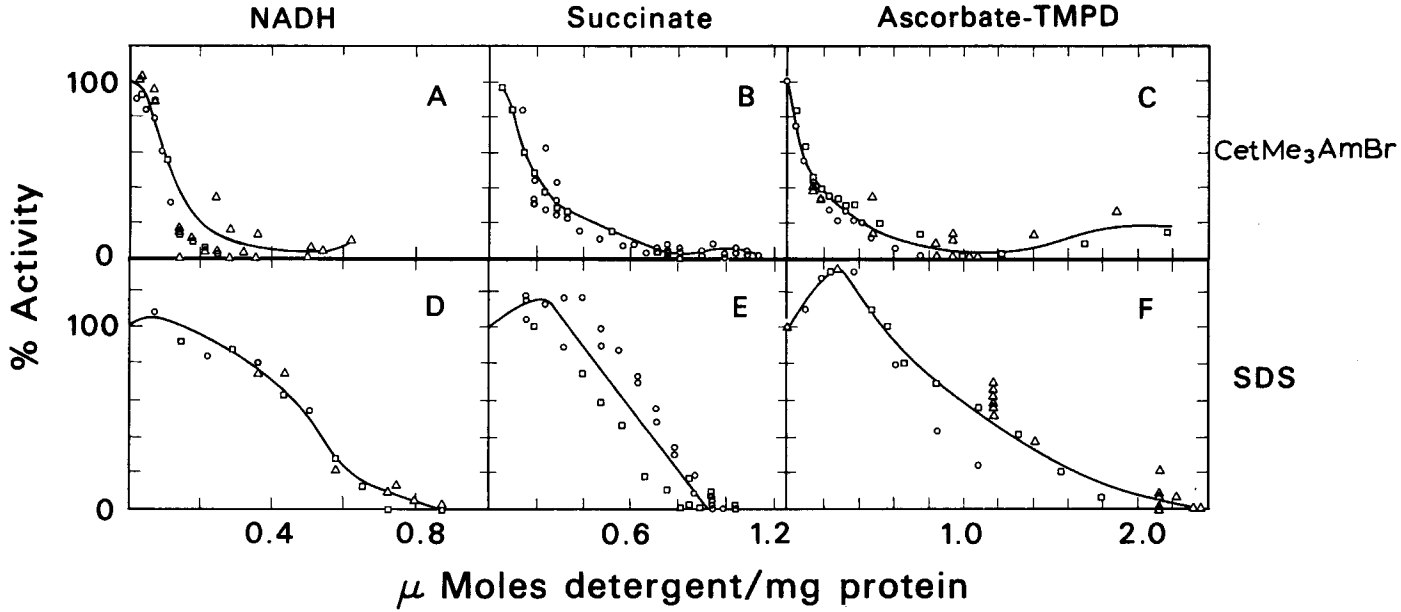


Fig. 1. Inactivation of submitochondrial respiration by sodium dodecyl sulfate (SDS) and $CetMe_3AmBr$. The protein concentration was 0.5 mg in a 1500 ml cuvette. (A) Inactivation of NADH oxidation by submitochondrial preparations with $CetMe_3AmBr$. Several different preparations are represented by Δ while two representative single experiments are denoted by ○, □. (B) Inactivation of succinate oxidation by submitochondrial preparations with $CetMe_3AmBr$. A representative experiment is denoted by □ while five separate experiments are included under ○. (C) Inactivation of ascorbate-*N,N,N′,N′*-tetramethyl-*P*-phenylenediamine oxidation by submitochondrial preparations with $CetMe_3AmBr$. Different experiments are denoted by □, ○, and △. (D) Inactivation of NADH oxidation by submitochondrial particles with sodium dodecyl sulfate. Different submitochondrial preparations are denoted by □, △, and ○. (E) Inactivation of succinate oxidation by submitochondrial preparations with sodium dodecyl sulfate. Two experiments representing different preparations are denoted by ○ and □. (F) Inactivation of succinate oxidation by submitochondrial preparations with sodium dodecyl sulfate. Representative experiments are given by ○ and □, while six separate experiments are pooled under △.

Succinate, having two "coupling sites" is less sensitive to detergent inhibition. Finally, the oxidation of ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine, which involves only a single "coupling site", is most resistant to the effect of the detergent.

It is noteworthy that low levels of sodium dodecyl sulfate stimulate respiration to the same extent as common uncouplers. This was shown with the uncoupler trifluoromethoxycarbonyl cyanide which produced about the same maximum stimulation of respiration as sodium dodecyl sulfate.

It was also noted that inactivation by $CetMe_3AmBr$ did not occur progressively with increasing $CetMe_3AmBr$ levels. Instead, (see Figs. 1a–1e), there is a concentration range in which increasing levels of $CetMe_3AmBr$ produce a slight stimulation of the respiratory rate.

Reactivation. Inactivation by $CetMe_3AmBr$ or sodium dodecyl sulfate can be reversed by adding detergent of the opposite charge. A representative polarographic trace showing the rapid inactivation and reactivation kinetics is shown in Fig. 2. Table I summarizes the data on reactivation. For a given activity with an inhibiting detergent maximum reactivation rates follow the order ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine > succinate > NADH. In general, an excess of sodium dodecyl sulfate over $CetMe_3AmBr$ is required for optimal reactivation.

Maximal reactivation of all respiratory functions is highest for small levels of the inactivating detergent and progressively decreases as inactivation proceeds until at some level of the inactivating detergent, reactivation is no longer possible. The minimum activity of 0.0 in Table I refers to the onset of complete inactivation, where reactivation is possible.

The reactivation found in these experiments was inhibited by 3 mM KCN. Also, NaBr produced no detectable inactivation at the levels used for the reactivation studies. Exogenous cytochrome *c* further stimulated the respiratory rate of reactivated submitochondrial preparations. Also, cytochrome *c* in itself could be used to reactivate sodium dodecyl sulfate-treated submitochondrial preparations to some extent.

It is noteworthy that the respiratory rates of some reactivated submitochondrial preparations are substantially higher than the control values.

Prolonged incubation of the submitochondrial preparations with an inactivating detergent leads to only a slight loss of the reactivation potential. For example,

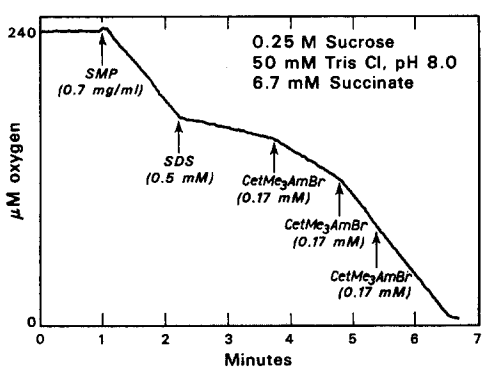


Fig. 2. Example of an O_2 polarographic trace showing the rapid kinetics of inactivation and reactivation. The suspension medium contained 0.25 M sucrose, 50 mM Tris · HCl and 6.7 mM succinate.

TABLE I

REACTIVATED OXIDATION RATES OF SUBMITOCHONDRIAL PREPARATIONS

Respiratory activity of reactivated submitochondrial particles for various levels of detergent inactivation. The numbers in parentheses refer to the number of experiments which were averaged to deduce the quoted errors. The notation SDS → $CetMe_3AmBr$ means that sodium dodecyl sulfate-inactivated submitochondrial particles were reactivated with $CetMe_3AmBr$. The optimum reactivation data for one of the succinate experiments was not determined because of technical difficulties.

	NADH			Succinate			Ascorbate-TMPD		
	Minimum activity (%)	Reactivated activity (%)	mol SDS / mol $CetMe_3AmBr$	Minimum activity (%)	Reactivated activity (%)	mol SDS / mol $CetMe_3AmBr$	Minimum activity (%)	Reactivated activity (%)	mol SDS / mol $CetMe_3AmBr$
	74	129	3.50	48	>146	?	37	84	1.20
SDS	17	38	1.71	17	117	1.25	5	72	1.10
→	2	8	1.55	1.9	92	1.38	0	41	1.23
$CetMe_3AmBr$	±1(3)	±2	±0.20	±3.3(3)	±20	±0.17		±11	±0.10
$CetMe_3AmBr$	40	69	1.33	40	179	1.56	36	129	1.75
→				±6(3)	±37	±0.10	±4(3)	±6	±0.08
SDS	13	44	1.05	13	233	1.20	18	133	1.50
	0.2	13	1.32	2.5	150	1.41	0.0	101	1.24
	±0.5(5)	±4	±0.16	±1.7(6)	±22	±0.14	(3)	±5	±0.06

SDS, sodium dodecyl sulfate; $CetMe_3AmBr$, cetyl trimethyl ammonium bromide; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

submitochondrial preparations were treated with enough sodium dodecyl sulfate to reduce oxidation of succinate to < 1.0 % of the control value and held at room temperature for 15 min. The activity of these submitochondrial preparations after the addition $CetMe_3AmBr$ was about 95 % of the reactivated rate of control submitochondrial preparations which had been treated with sodium dodecyl sulfate for less than 1 min (Tinberg, H. M., personal communication).

The simultaneous addition of sodium dodecyl sulfate and $CetMe_3AmBr$ to submitochondrial preparations leads to about the same rate of oxidation as the sequential addition of two detergents. This experiment shows that mixing of submitochondrial preparations and the detergent complex does occur. It also suggests that reactivation is not due to separation of detergents from the membranes.

Spin labeling

CDTAB resembles $CetMe_3AmBr$ structurally. A spin-labeled analogue of $CetMe_3AmBr$ designated as CDTAB was used to study the interaction of detergent molecules with submitochondrial preparation membranes. A spectrum of this label at 5 mM is shown in Fig. 3. The sharp three line component in this spectrum is due to spin labels tumbling as monomers in dilute solution while the broad underlying single line component arises from exchange narrowed label molecules in micelles. A plot of the normalized line height arising from the sharp three line spectrum as a function of CDTAB concentration is given in Fig. 3. The break in the plot represents a transition from a mixed population of monomers and micelles to monomers and is identified as the critical micelle concentration for CDTAB. This is about two times smaller than the critical micelle concentration for $CetMe_3AmBr$ and shows that the spin-labeled molecule behaves similarly to $CetMe_3AmBr$.

It is well known that spin labels are reduced and oxidized by mitochondria and chloroplasts [13–15] so the spin-labeled CDTAB cannot be used to study the disruptive effects of detergent on submitochondrial preparation oxidative functions without

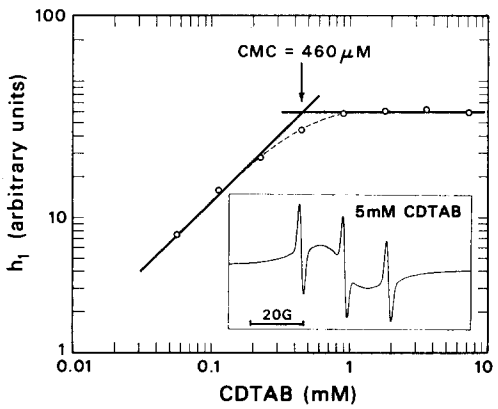


Fig. 3. A plot of the low field line height, h_1, against CDTAB concentration in distilled water. The break in the curve is interpreted as the critical micelle concentration (CMC) of CDTAB. Insert: An electron spin resonance spectrum of 5mM CDTAB in water. The three narrow lines arise from non-interacting molecules whereas the broad single line is due to molecules which interact with each other in micelles. Magnetic field strength increases towards the right; the scale is given in gauss.

the added complication of modified electron transport involving CDTAB. However, the data on the critical micelle concentration of CDTAB taken together with the structural similarity of this spin label to $CetMe_3AmBr$ suggest that studies with CDTAB can be used to analyze the effects of $CetMe_3AmBr$ on membrane structure.

CDTAB binding to submitochondrial membranes

The spin-labeled detergent CDTAB was used to show that dilution of the membranes in the suspension medium leads to a progressive loss of detergent molecules from the membranes to the aqueous phase. A spectrum of CDTAB in a submitochondrial suspension is shown in Fig. 4. The narrow lines arising from the aqueous phase are readily distinguished from the broad spectrum of the membrane-bound label. The line heights of the aqueous components provide a convenient measure of the concentration of this fraction of molecules. Actual concentrations were obtained from spectra of CDTAB in membrane free suspension medium at concentrations below the critical micelle concentration.

Upon dilution of CDTAB-labeled mitochondria, the broad spectral component diminishes more rapidly than the narrow aqueous spectrum. This is due to an increase in the aqueous fraction of detergent molecules as the relative volume of the aqueous phase increases. Using the known total concentration of CDTAB, the bound concentration was obtained from a knowledge of the aqueous component. These data are plotted in Fig. 4.

The bulk of the CDTAB molecules is bound to the membranes at all concentrations considered in Fig. 4. An arrow denotes concentrations of submitochondrial protein that were used in the respiration studies. On the other hand, spin label studies

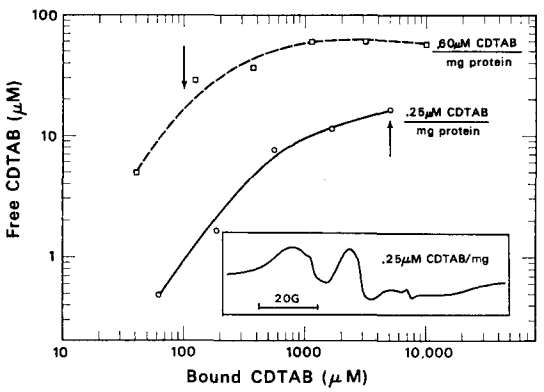


Fig. 4. A plot of free versus bound CDTAB as a function of dilution of the submitochondrial preparations in buffer. The free CDTAB concentration was estimated from the line heights of the high field narrow line aqueous component, h_{-1}, while the bound component was estimated from the dilution factor and subtraction of the free CDTAB concentration. The dashed line refers to submitochondrial preparations initially labeled with 0.60 μM CDTAB per mg protein, while the solid line refers to an initial label concentration of 0.25 μM CDTAB per mg protein. The downward arrow refers to spin labeling conditions used to obtain the data of Fig. 9. Insert: An electron spin resonance spectrum of 0.25 μmol CDTAB per mg of protein in submitochondrial preparations. The small narrow line contribution which is most clearly resolved in the third line represents free label, while the remaining broad spectral components are due to concentration broadened, immobilized membrane-bound CDTAB.

of membrane disruption were carried out near the upper concentration limit of 20 mg protein/ml. The data of Fig. 4 shows that for both experiments most of the label is bound to the membrane so it is possible to make a direct comparison of the experiments on respiratory function and those on lipid structure on the basis of detergent to protein ratios.

Effects of sodium dodecyl sulfate and $CetMe_3AmBr$ on membrane structure

The two spin labels, CDTAB and A12NS were used to study the disruptive effects of the two charged detergents on submitochondrial membrane structure. The nitroxide group of CDTAB is expected to be located at the aqueous interface of the membrane, while that of A12NS is expected to reside in the hydrophobic interior. The results of these studies were compiled in terms of the mobility parameter, τ_0 and are presented in Fig. 5. For a given experiment, submitochondrial preparations were spin labeled to make a stock solution and a detergent solution was labeled separately. Then the two solutions were mixed to achieve the desired concentration ratio of detergent to submitochondrial protein. For the conditions of these experiments, spin reduction was negligible over a period of 1 h.

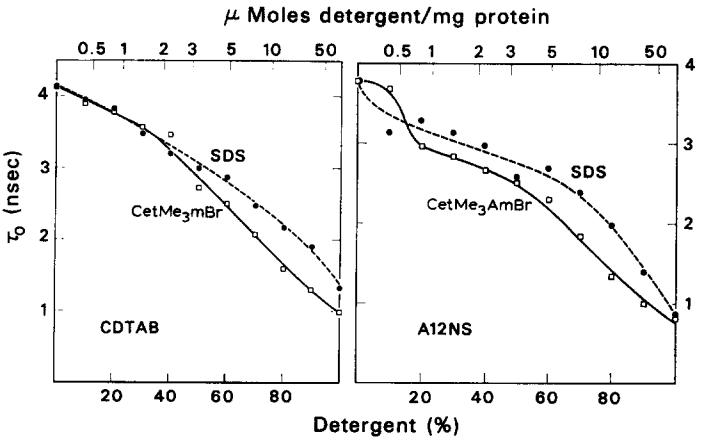


Fig. 5. (a) The mobility of CDTAB in submitochondrial preparations, expressed as the rotational correlation time, as a function of detergent concentration. The pure detergent solution represented 50 mM of detergent, corresponding to 14.4 mg/ml of sodium dodecyl sulfate and 18.2 mg/ml of $CetMe_3AmBr$. The pure submitochondrial preparation represented 15.4 mg/ml of protein. The spin-labeled submitochondrial suspension was mixed with a spin-labeled detergent suspension to achieve the given percentage of detergent, e.g. the 50 % detergent mixture represents 25 mM detergent and 7.7 mg of submitochondrial protein. The spin label concentration was 200 μM. The dotted line refers to sodium dodecyl sulfate, the solid line to $CetMe_3AmBr$. (b) The mobility of A12NS in submitochondrial preparations. Details of the procedure are given above. The rotational correlation time for sodium dodecyl sulfate additions was difficult to estimate because an aqueous spectral component was present.

Studies with detergents in the absence of membranes

Properties of the detergent mixture. When $CetMe_3AmBr$ is added to an equimolar solution of sodium dodecyl sulfate, a turbid product forms. Physical studies of this mixture were carried out to clarify the structural aspects of reactivation.

TABLE II

X-RAY SPACINGS OF 250 mM SODIUM DODECYL SULFATE 250 mM $CetMe_3AmBr$ MIXTURE

X-ray spacings of a 250 mM sodium dodecyl sulfate 250 mM $CetMe_3AmBr$ mixture. Intensities were estimated visually, and are intended to provide only a progression of strong to weak scattering peaks. Data for the fatty acids, denoted by *, were taken from a handbook (cf. ref. 16).

Intensity	Spacing in Å	Palmitic Acid*	Lauric Acid*	Sodium stearate*
70	3.81	70, 3.73	80, 3.77	20, 3.90
100	4.16	100, 4.17	100, 4.15	100, 4.18
50	4.64	40, 4.40	70, 4.43	20, 4.59
40	4.89	—	—	—
10	5.93	—	—	—
5	9.01	—	—	—

High angle X-ray studies revealed that a 0.5 M solution of either $CetMe_3AmBr$ or of sodium dodecyl sulfate did not produce any discrete scattering angles but that a mixture of the two detergents gave rise to a number of sharp diffraction rings. These were measured and converted to lattice spacings. Table II gives the observed Bragg spacings together with data on two fatty acids and a fatty acid salt [16]. This shows that the detergent mixture forms a well-ordered crystal with the same lattice parameters as a solid fatty acid salt. Intensities were estimated visually and are given only for comparative purposes.

The hydrocarbon spin label, 5N9, is useful for water-membrane partitioning studies at lipid concentrations of the order of 10 mg/ml. This spin label gives rise to symmetric spectra in solutions of sodium dodecyl sulfate and $CetMe_3AmBr$ as seen in

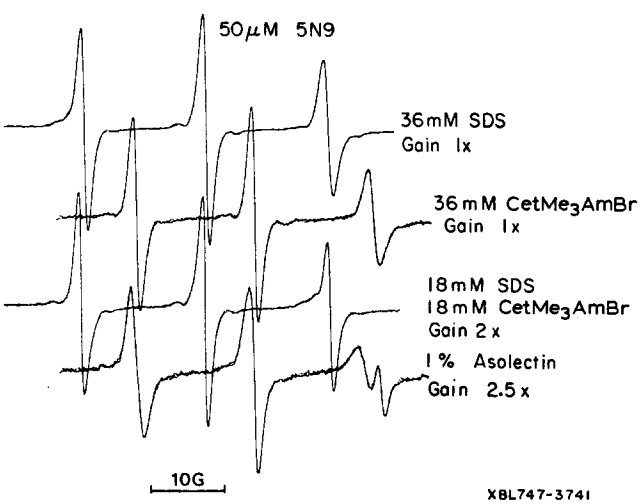


Fig. 6. A comparison of electron spin resonance spectra of 5N9 in detergents and asolectin under comparable weight to volume ratios. This spin label partitions under these conditions to give rise to both aqueous and hydrocarbon signals as resolved in the third line. The signal in an equimolar mixture of sodium dodecyl sulfate (SDS) and $CetMe_3AmBr$ also exhibits partitioning, but the hydrocarbon component is considerably smaller. No partitioning is observed for the pure detergents.

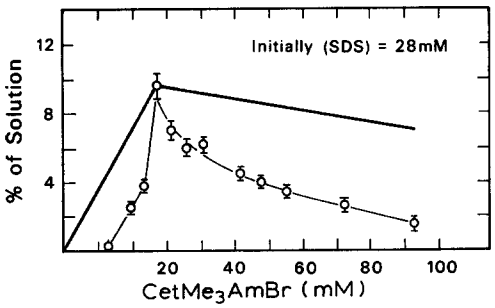


Fig. 7. Centrifugation study of detergent mixtures. Aliquots of $CetMe_3AmBr$ were added to a 28 mM solution of sodium dodecyl sulfate in water. The theoretical curve was obtained by assuming complete precipitation of the equimolar sodium dodecyl sulfate and $CetMe_3AmBr$ mixture normalized to the maximum value of the observed packed volume.

Fig. 6. However, an equimolar mixture of these two detergents produces a spectrum with aqueous and hydrophobic components. For comparison, a spectrum of 5N9 in an asolectin vesicle suspension at the same concentration is also presented [17].

Different mixtures of sodium dodecyl sulfate and $CetMe_3AmBr$ were spun down in a microhematocrit centrifuge to study the mixing characteristics of these detergents. The data are shown in Fig. 7 and compared with a theoretical curve computed on the assumption that uncharged equimolar mixtures of the detergents would spin down while excess charged detergent would remain in solution. Since the data do not fit this model, some interaction between the charged monomers and the uncharged aggregates must occur. Maximal crystal formation in this case occurs for an equimolar mixture of sodium dodecyl sulfate and $CetMe_3AmBr$.

An equimolar mixture of sodium dodecyl sulfate and CDTAB gives rise to a spectrum with one broad line, indicating that virtually all the CDTAB molecules are complexed with sodium dodecyl sulfate and undergo electron exchange. This is shown in Fig. 8 where about 99 % of the CDTAB molecules solubilized as monomers are removed from solution by the addition of sodium dodecyl sulfate as judged from the line heights of the sharp aqueous components.

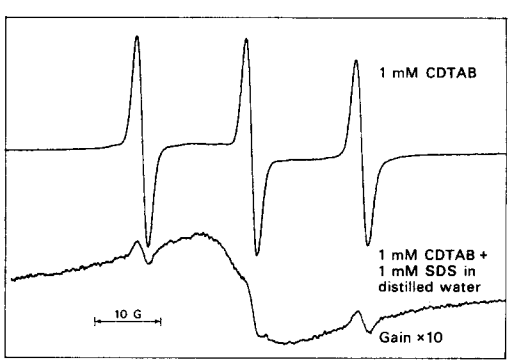


Fig. 8. Demonstration that sodium dodecyl sulfate complexes CDTAB. The upper trace corresponds to 1 mM $CetMe_3AmBr$ in water. The addition of an equimolar concentration of sodium dodecyl sulfate (SDS) reduces the concentration of free aqueous CDTAB by a factor of 100 at these conditions. The gain of the lower spectrum was increased 10 times relative to the upper spectrum.

Structural effects attending reactivation

Submitochondrial preparations were treated with $CetMe_3AmBr$ levels that were sufficient to bring about partial inactivation. The membrane viscosity was assayed with the two spin labels: CDTAB and A12NS. Then sodium dodecyl sulfate was added in a step-wise manner to study the effect of neutralizing the detergent charge on the correlation times of the spin label. The data of this study are summarized in Fig. 9. Under the conditions of these experiments, A12NS carries a charge of -1, so this label may itself act to alter membrane structure as sodium dodecyl sulfate does.

Two studies with CDTAB were used to show that the binary sodium dodecyl sulfate $CetMe_3AmBr$ mixture does not crystallize, but rather remains randomly dispersed in the submitochondrial membranes. In the first study, a sufficient amount of CDTAB was added to partially inactivate oxidative functions. This produced an exchange broadened signal with three lines which was readily distinguished from the lower spectrum of Fig. 8. Then an equimolar amount of sodium dodecyl sulfate was added. The electron spin resonance spectrum did not change appreciably, indicating that the type of crystal formation seen in Fig. 8 did not occur.

In another experiment, an equimolar mixture of CDTAB and sodium dodecyl sulfate having a spectrum as shown in Fig. 8, was progressively diluted with a suspension of submitochondrial preparations. The spectral changes were those expected for a dilution of spin labels and did not differ significantly from a dilution of CDTAB in submitochondrial preparations. This experiment as well as the previous one, does not, however, exclude the possibility that sodium dodecyl sulfate and $CetMe_3AmBr$ form dimers in the membrane.

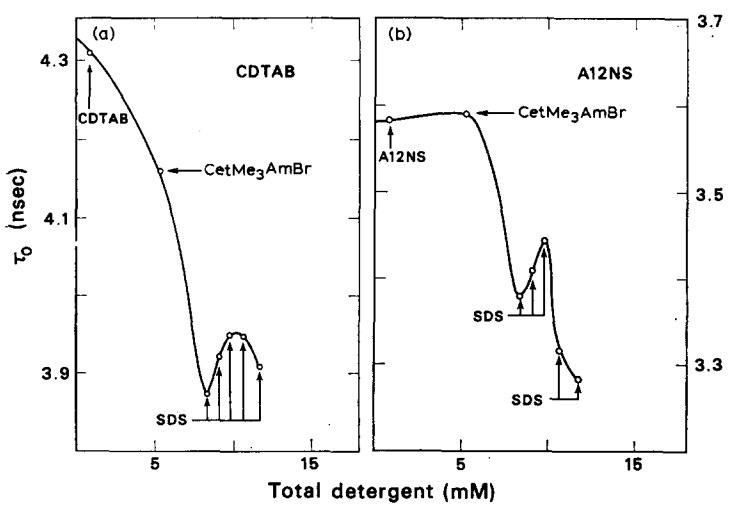


Fig. 9. (a) The mobility of CDTAB, expressed as the rotational correlation time, as sodium dodecyl sulfate (SDS) is added to $CetMe_3AmBr$-treated submitochondrial preparations. The electron spin resonance sample tube contained to 50 λ of spin-labeled submitochondrial preparations at a concentration of 15 mg/ml of protein and a spin concentration of 500 μM. The downward arrow refers to equimolar concentrations of $CetMe_3AmBr$ and sodium dodecyl sulfate. (b) The mobility of A12NS as sodium dodecyl sulfate is added to $CetMe_3AmBr$-treated submitochondrial preparations.

Studies with fixed mitochondria

Mitochondria were treated with 10 mM dimethyl suberimidate, i.e. to an extent which prevented osmotic swelling or contraction as assayed with light scattering (see procedure). Detergent studies of these fixed mitochondria revealed that inactivation of the ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine-linked respiration occurred at about the same detergent levels (μmol/mg protein) as was found for control mitochondria. However, the succinate activity was considerably more sensitive to detergents, about one-fifth the concentration of both sodium dodecyl sulfate and $CetMe_3AmBr$ was required to bring about a loss of respiration relative to the control preparations.

The reactivation of fixed mitochondria was comparable to that found for the unfixed submitochondrial preparations in terms of minimum and maximum activities. However, for succinate the net detergent levels were one-fifth of those used to bring about a reactivation of unfixed submitochondrial preparations.

In contrast to fixation with 10 mM suberimidate, when mitochondria were fixed with 50 mM suberimidate, no light scattering changes were produced by detergents at levels sufficient to abolish respiratory activities. However, at higher detergent levels light scattering changes were observed, suggesting that under these fixation conditions the membranes were still partially susceptible to detergent solubilization.

DISCUSSION

The disruption of membranes by detergents of known structure can provide a powerful tool for studying the relationship between structural integrity and the maintenance of membrane-related functions.

In regard to the inhibition of enzymatic oxidation in a submitochondrial membrane, detergent interaction was shown to be readily reversible. Previously, others [2] have shown reversibility of detergent solubilization by dialysing the detergent; our method can restore multi-enzyme complexes to activity after almost complete detergent inhibition. The exact nature of the sodium dodecyl sulfate/$CetMe_3$-AmBr association with the membrane is not known at this time, however, sodium dodecyl sulfate is a strong detergent that can break some proteins into subunits. We have not determined extensively the integrity of our solubilized membrane.

The disruption of the submitochondrial oxidizing membrane by low levels of detergent often shows an increase in rate similar to addition of an uncoupler. It seems that low levels of detergent may uncouple oxidative phosphorylation, as they do perturb the membrane, and this is a good way to uncouple a submitochondrial preparation. One puzzling aspect is: Why would the submitochondrial membrane in a reactivated state sometimes show a marked increase over the initial activity? We have no data to elucidate this question.

Spin labeling provides a convenient method for studying the disposition of molecules in membrane suspensions because spectra arising from different environments can be differentiated to some extent without a requirement for the physical separation of membrane components.

The spin-labeled detergent CDTAB was used to show that most of this detergent is bound to submitochondrial membranes over a wide range of membrane concentrations including concentrations used for studies of oxidation of substrates.

This spin label closely resembles $CetMe_3AmBr$ in structure and also has a comparable critical micelle concentration. Therefore, it seems safe to conclude that the bulk of $CetMe_3AmBr$ is also bound to the membranes under the conditions of the oxidative studies.

These results are not directly applicable to sodium dodecyl sulfate because of the different hydrocarbon chain length and head group charge of this detergent. Probably the chain length is the principal factor in determining membrane binding, so a smaller fraction of sodium dodecyl sulfate molecules may interact with membranes than of $CetMe_3AmBr$. This could explain the greater efficacy of $CetMe_3AmBr$ in inhibiting oxidative functions.

As seen in Fig. 4, the bound spectrum of CDTAB at about 25 μmol/mg protein is very broad and represents highly immobilized nitroxide groups. It is expected that these molecules would be oriented with the quarternary nitrogen among the charged headgroups of the membrane lipids and perhaps proteins. This would lead to immobilized spectra of the type seen in the submitochondrial membranes. Very similar spectra are obtained for CDTAB in asolectin vesicles, suggesting that either CDTAB is localized among mitochondrial lipids, or, the interaction of CDTAB with proteins does not differ significantly from its interaction with lipids.

Studies on membrane disruption with CDTAB and A12NS showed that significant alterations in membrane structure attend the inactivation of oxidative functions by sodium dodecyl sulfate and $CetMe_3AmBr$ treatment. Fig. 5 contains data on the mobility of these two spin labels in pure detergent micelles as a standard of comparison to which the membrane data can be related. To a first approximation, the mobility of these spin labels at any detergent concentration can be inferred from a linear interpolation of the values in pure detergent and pure membranes. This suggests that the structure of detergent-treated membranes be viewed as intermediate between these two extremes as well.

The two spin labels probe different parts of the membrane: CDTAB reports on the aqueous interface, while A12NS gives information about the hydrophobic membrane interior. Both labels appear to reflect about the same degree of mobility increase with detergent treatment. Thus, the action of these ionic detergents is one of a general loosening up of the membrane structure which extends from the surface into the membrane interior.

Figs 1a and 1b show that inactivation occurs at about 0.3 μmol of $CetMe_3AmBr$ per mg protein. Although the proportion of $CetMe_3AmBr$ molecules interacting with the membrane is not known, we adopt 0.3 μmol/mg protein as representative of the levels required for inactivation at the membrane level. Assuming a lipid to protein ratio of 2 : 3 for inner mitochondrial membranes, this corresponds to about 1 mol of detergent per 2 mol of lipid. Spectra of CDTAB at this concentration in submitochondrial membranes are broadened by concentration effects. If there were a fraction of membrane-bound CDTAB molecules which was not concentration broadened, this would be discernible on the basis of its relatively narrow spectrum. Since we do not see any evidence of signal heterogeneity, we conclude that CDTAB is fairly randomly laterally dispersed in the membrane. Similar considerations apply to $CetMe_3AmBr$ and sodium dodecyl sulfate.

The spin label data on reactivation represented by Fig. 9, shows that reactivation, and hence inactivation, is not due to a membrane fluidity effect since the fluid-

izing effect exerted by one detergent is not greatly altered by the addition of the counterionic detergent. There is a slight arrest of the increasing mobility of the spin label upon neutralization of charge but this appears to be of a small magnitude relative to the overall mobility change relative to the untreated controls.

The data with pure detergents demonstrate that the uncharged binary crystalline mixture of CDTAB and sodium dodecyl sulfate gives rise to a characteristic electron spin resonance spectrum which differs from the spectrum of CDTAB in water and in membranes. This observation was used to test the hypothesis that the mixed detergents formed separate domains from membrane lipids, perhaps by a mechanism of lateral phase separation [18] such that crystalline and fluid domains are contiguous. This hypothesis could be ruled out from an analysis of the electron spin resonance spectra as described in Results.

The inactivation-reactivation phenomena observed in this study are analogous to the effect of polycations and polyanions on cytochrome *c* oxidase activity [4]. Thus, it might be argued that the charged detergents behave like polyions with high charge densities. This possibility is ruled out, however, from the behavior of CDTAB in submitochondrial membranes which differs markedly in terms of its electron spin resonance spectrum from CDTAB in micelles in aqueous solution. Upon interacting with submitochondrial membranes, the CDTAB molecules become dispersed and thus lose the polycationic character of micellar aggregates.

In conclusion, it appears that inactivation of oxidative functions of submitochondrial preparations by sodium dodecyl sulfate and $CetMe_3AmBr$ is due to a generalized alteration of membrane structure, principally as a result of a modification of the surface charge. The disruptive effects of the detergents on membrane fluidity, as assayed by spin labeling, seems to be of secondary importance, as far as respiratory functions are concerned.

The method described in this paper for inactivation and reactivation of oxidative functions is suitable for the controlled modification of membrane structure. For example, one detergent can be used to incorporate hydrophobic substances into the membranes to study how these affect oxidative functions. The counterionic detergent can then be added to minimize the disruptive effect of the solubilizing detergent. Preliminary studies in our laboratory with exogenous lecithin incorporation into submitochondrial preparations have demonstrated the feasibility of this technique.

Experiments with fixed mitochondria showed that detergent effects were similar to those found for control mitochondria even though the fixed membranes no longer responded to osmotic gradients. The observation that these fixed mitochondria could be inactivated and reactivated by detergents suggests that the detergent molecules can be inserted into the membrane. Hence it would seem that the membrane surface area can be changed in local domains even though the area of the entire membrane is fixed. However, these ideas must remain speculative until more is known about the effects of imidoester cross-linking.

ACKNOWLEDGMENTS

We wish to thank J. McGuire, J. Eliason and P. Chun for their excellent technical assistance and Dr. H. M. Tinberg for useful discussion of this work. This research was supported by the Energy Research and Development Administration.

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