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POLAROGRAPHIC STUDIES ON A SOLUBLE MEMBRANE ATPase AND AQUEOUS DISPERSIONS OF PHOSPHOLIPIDS

WILLIAM R. REDWOOD* AND WILHELM GODSCHALK

Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Va. 22901 (U.S.A.)

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SUMMARY

In a new approach to the study of lipid–protein interactions an investigation was made of the polarographic properties of mixtures of a water-soluble ATPase and aqueous dispersions of homogeneous phospholipid vesicles. The protein under study produced a characteristic d.c.-polarographic wave which was found to be perturbed when the phospholipid vesicles were added to the solution. A matrix rank analysis, performed on a phase-sensitive a.c. polarogram of this mixture, indicated that at least three components were present. These results suggest that an interaction occurs in solution between the artificial membrane system and the soluble ATPase, resulting in the formation of a vesicle–ATPase complex.

It was also found that random currents appeared at the polarized mercury–water interface as the size of the phospholipid dispersion particles was increased. These random interfacial currents, which occurred both in the presence and absence of the soluble ATPase, could be eliminated by low concentrations of detergents, alcohol and certain drugs.

INTRODUCTION

The recent report[1] that random currents appear at the polarized mercury–water interface in the presence of membrane fragments rich in mammalian Na^+–K^+-activated ATPase prompted similar studies to be undertaken in this laboratory. The protein of specific interest was the water-soluble, non-particulate, Mg^{2+}-activated ATPase derived from *Streptococcus faecalis* membrane ghosts. This enzyme can be prepared and purified by the recently developed method of Schnebli and Abrams[2]. The function of the ATPase in the intact organism is not clearly understood. It is thought, however, to be involved in the active transport of monovalent cations across the plasma membrane[2]. This was implied mainly by the observation of Harold and Baarda[3] that *N,N'*-dicyclohexylcarbodiimide inhibits the membrane-bound ATPase *in vitro* and also inhibits the uptake of K^+ *in vivo*[3]. It has also been reported

* Present address: Department of Biochemistry, College of Medicine and Dentistry of New Jersey at Newark.

by Abrams and Baron[4] that the solubilized ATPase can be reattached to the enzyme-depleted streptococcal membranes in the presence of Mg^{2+}. In a previous paper from this laboratory, it was reported that the soluble ATPase at concentrations above 10^{-9} M interacts with lipid bilayer membranes to produce a 10^2–10^4-fold increase in the electrical conductance of the bilayer[5]. The magnitude of the conductance increase is dependent on the presence of Mg^{2+} and upon the concentrations of both Na^+ and K^+ in the region of 10^{-2}–10^{-1} M. An additional tenfold increase in conductance is obtained when ATP is added to the aqueous phase surrounding the bilayer–ATPase interactant system. It was, therefore, suggested that the bilayer–ATPase interactant complex may be similar in structure and properties to the membrane–ATPase complex in the intact organism[5].

Recent studies using spectrophotometric titration and differential centrifugation techniques have shown that the soluble ATPase also interacts with the liposome systems which are formed by the dispersion of phospholipids in aqueous solution (ref. 6, and W. R. Redwood, D. C. Gibbes and T. E. Thompson, unpublished). The purpose of the present investigation was to determine whether any electrical changes occur during the interaction between the soluble ATPase and the liposome structures analogous to the effects observed with the planar bilayer system[5]. The polarographic technique was chosen to study the lipid–protein interaction, since the high interfacial tension of the mercury–water interface would tend to adsorb the membranous structures and any electrical changes could be quantitatively monitored at the electrode surface. Ion transport through lipid monolayers has already been investigated at the polarized mercury–water interface[7,8], and the polarographic technique has provided a convenient approach to the study of electrical conduction through biological material[1].

In the presence of low concentrations ($5 \cdot 10^{-10}$ M) of the purified ATPase intermittent conductance changes, of short duration, have been observed with the planar bilayer membrane[9]. The fluctuations in the bilayer conductance are limited to discrete increments of approx. 10^{-10} Ω^{-1}. These quasi-quantized conductance changes are reminiscent of the discrete conductance changes which occur when similar bilayer membranes are treated with low concentrations of excitability-inducing material (EIM)[10,11] and may correspond to the opening and closing of conducting channels in the bilayer. The polarographic studies of Blank and Britten[1] on the particulate Na^+–K^+-activated ATPase revealed that random currents occurred when the membrane fragments were adsorbed at the polarized mercury–water interface. It was, therefore, hoped that similar random currents could be generated at the polarized mercury–water interface in the presence of the liposome–ATPase interactant system. Although such currents might not correspond to the same molecular events which result in the appearance of the intermittent conductance changes in the bilayer–ATPase interactant system, this polarographic phenomenon would be interesting *per se*, and it might be possible by these studies to identify the type of ions which cause the random currents at the polarized mercury–water interface.

MATERIALS AND METHODS

The soluble ATPase was prepared from *S. faecalis* membrane ghosts by an extensive washing procedure[12]. The enzyme purification involves a heat treatment

followed by molecular sieve chromatography on Agarose and repeated column chromatography on DEAE-cellulose[2]. The enzyme assays were carried out according to the technique of Abrams[12], while the protein concentrations were determined by the method of Lowry *et al.*[13].

The liposomes were generated by the dispersion of chromatographically pure hen egg phosphatidyl choline[14] in aqueous solution. These phospholipid aggregates can be prepared in multi-lamellar myelinic figures or single-lamellar vesicles dependent upon the technique employed to disperse the dried phospholipid in the aqueous medium. The size and shape of the liposomes, and, therefore, the colloidal stability and optical clarity of the dispersion, depend mainly on the degree of mechanical agitation used in the preparation[6]. In the polarographic studies, the whole range of the liposome class of model membrane systems was investigated. The heterogeneous liposomes, ranging in size from mm down to μm and consisting of many lipid bilayers, each separated by an interspersed lamella of water, were prepared by hand shaking small amounts of phosphatidyl choline in water. The dispersion was quite turbid and settled on standing for a short time. A more stable and optically clearer dispersion was prepared by prolonged ultrasonication of lyophilized phosphatidyl choline under non-oxidizing conditions, whereupon most of the phospholipid appeared in the form of single bimolecular lamellar vesicles between 200–300 Å in diameter. A homogeneous vesicle preparation was then made by subjecting this dispersion, after ultracentrifugation, to molecular sieve chromatography on Sepharose 4B, according to the procedure of Huang[15]. A less homogeneous, electrically charged vesicle preparation was made by the addition of 10% by weight of stearylamine to the lyophilized phosphatidyl choline prior to sonication. The resulting dispersion was centrifuged and filtered through a 1000-Å Sartorius membrane filter. In addition, a heterogeneous liposome dispersion of pure dipalmitoyl phosphatidyl choline (Mann Chemicals, lot No. V-1534) and a briefly sonicated (2 min) dispersion of the washed *S. faecalis* membrane ghosts were prepared for study in the polarograph.

Doubly distilled water was used for all buffer solutions and the inorganic chemicals were Analytical Reagent grade. The ethanol used was USP 200 and the drugs were of the highest purity commercially available. The detergents, Triton X-100 and cetyldimethylbenzylammonium bromide were obtained from Beckman Instrument Co. and Winthrop-Stearns, Inc., respectively.

The polarographic apparatus employed was the Model 170 Electro-chemistry System from Princeton Applied Research, Inc. This equipment was found to be most satisfactory for the present studies and provided several unique features not standard on earlier polarographic instruments. The use of the sampling device greatly facilitated the detection of small random interfacial currents. The sensitive electrometer of the instrument permitted the measurement of currents as low as 10^{-9} A at the polarized mercury–water interface. Furthermore, it was possible to eliminate the effect of capacitive currents by the use of pulse polarography and phase-sensitive alternating current polarography, built-in features of the instrument.

RESULTS AND DISCUSSION

Polarographic properties of the soluble ATPase and homogeneous vesicles

The ordinary direct current polarogram of the buffer solution for the soluble ATPase is shown in Fig. 1a. The mercury drop time of 1 s was controlled mechanically

and the current was recorded continuously over the polarization range of −200 mV to −1.4 V with respect to the saturated calomel electrode. The current oscillations are smallest at the electrocapillary maximum which is located at approx. −560 mV on the polarogram. The buffer consisted of 10^{-2} M $MgCl_2$ and $2 \cdot 10^{-2}$ M succinate titrated with NaOH to pH 6.3. No characteristic polarographic waves were observed

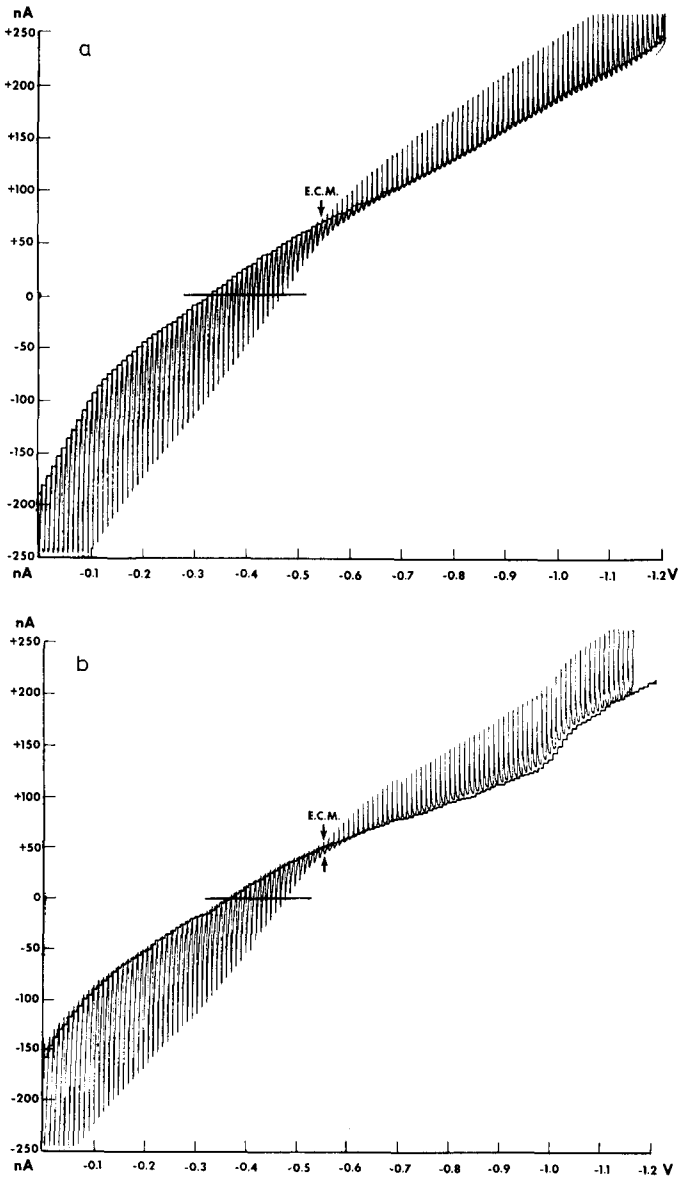

Fig. 1. (a) Polarogram of the succinate–$MgCl_2$ buffer used throughout this work. A continuous current scan shows the drop oscillations. A tracing of the current, sampled during 20 ms, at 1 s intervals, is also shown. (b) Polarogram of a typical ATPase preparation. Protein concentration, approx. 20 μg/ml. Both the continuous scan and the sampled current scan are shown. E.C.M. = electrocapillary maximum.

with this buffer system in the polarization range studied. Also shown in the diagram is the record of the current-sampling scan, in which the current-sampling gate is opened for only 20 ms near the end of each drop life. A typical d.c. polarogram of the ATPase solution, recorded continuously and in the current sampling mode is shown in Fig. 1b. The polarographic wave which appears around −1.1 V is a reproducible feature of the purified ATPase preparation and the magnitude of the wave is directly proportional to the protein concentration. Addition of the positively charged detergent cetyldimethylbenzylammonium bromide causes precipitation of the soluble ATPase and a concomitant elimination of this polarographic wave.

Fig. 2 illustrates the sampled current d.c. polarograms of the homogeneous phospholipid vesicles alone and in the presence of the soluble ATPase. The buffer polarogram is included as a control. The results indicate that the homogeneous vesicles of pure phospholipid unlike the soluble ATPase are not polarographically active. However, it should be noted that the addition of the homogeneous vesicles to the ATPase solution causes a substantial decrease in the polarographic current and almost eliminates the wave at −1.1 V. This suggests that there is an interaction occurring between the phospholipid vesicles and the soluble ATPase. Similar polarographic evidence was obtained for the interaction of the homogeneous vesicles with bovine serum albumin. It is possible that this technique could be employed to quantitatively study lipid–protein interactions. Thus, for example, the polarographic wave at −1.1 V could be continuously monitored to assay the concentration of the soluble ATPase, during a titration with the phospholipid vesicles, in an analogous way to the use of spectrophotometric titration methods[16].

The exact nature of the observed polarographic wave is not known with certainty. It has been shown that adenine and its nucleotides are polarographically active at low pH, with half-wave potentials of −1.09 V and −1.17 V, respectively[17].

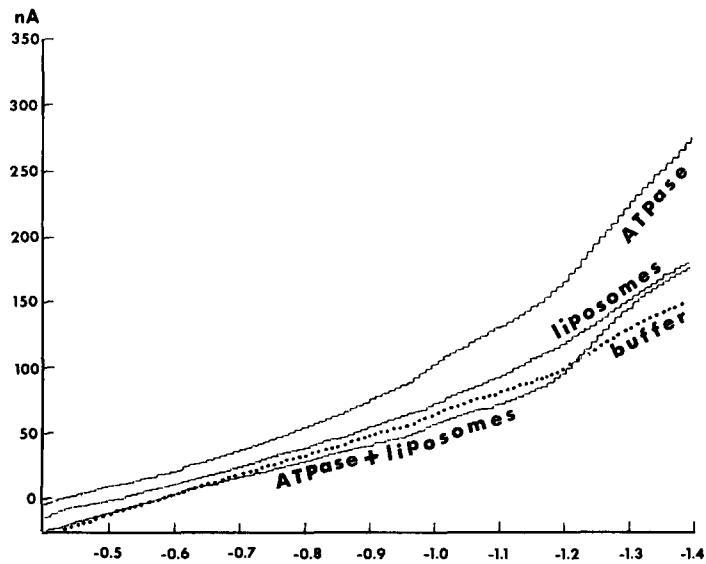

Fig. 2. Comparison of the sampled current d.c. polarograms of solutions containing 10 μg/ml ATPase, 50 μg/ml homogeneous phospholipid vesicles, and a combination of both. The buffer polarogram has been added to show the magnitude of the residual current.

Very small traces of compounds of this type could be responsible for the observed wave. On the other hand, traces of metal ions are certainly present; typical heavy metal contents of commercial analytical grade chemicals are in the neighborhood of 0.0005%. Using the well-known Ilkovič equation, we arrive at polarographic currents of the order of 1 nA for a buffer of low ionic strength. At short drop times, this figure will be slightly higher, because of increased convective flow. Although the instrument was easily capable of measuring these small currents, we routinely employed a somewhat lower sensitivity, so that the normal trace contaminations of the buffer would not show up (see Fig. 1a). The most commonly occurring metals, displaying waves in the vicinity of −1.1 V, are zinc, nickel and cobalt. Half-wave potentials of these metals in the $MgCl_2$–succinate buffer we used are not known, but the values in pH 4.7 acetate are −1.07 V, −1.13 V, and 1.18 V, respectively[18]. During the course of its isolation, the enzyme could have bound and accumulated metal ions from the chemicals used in the buffer solutions. The wave height, which is of the order of 50 nA in Fig. 1b, was found to be variable among different enzyme preparations. No direct correlation between the height of the polarographic wave and the ATPase activity could be demonstrated. The presence of zinc in a sample of precipitated protein was confirmed by emission spectrographic analysis. Appreciable quantities of iron were also found by this technique, and indeed, many of our polarograms reveal the presence of Fe^{2+}, which has a half-wave potential of approx. −1.4 V in most supporting electrolytes.

The vesicle–ATPase system was also investigated using the phase-sensitive a.c. polarography technique, whereby a signal of 10 mV (peak to peak) of frequency 225 Hz was imposed on the regular d.c. ramp. Fig. 3 is a representative a.c. polarogram of this system with the phase angle set in phase with the a.c. voltage. At this phase angle there is no contribution to the sampled current from the electrical double layer capacity of the polarized mercury–water interface; therefore, the currents measured are purely faradaic. The a.c. polarogram of the mixture of the homogeneous vesicles and the soluble ATPase is not a linear combination of the polarograms of

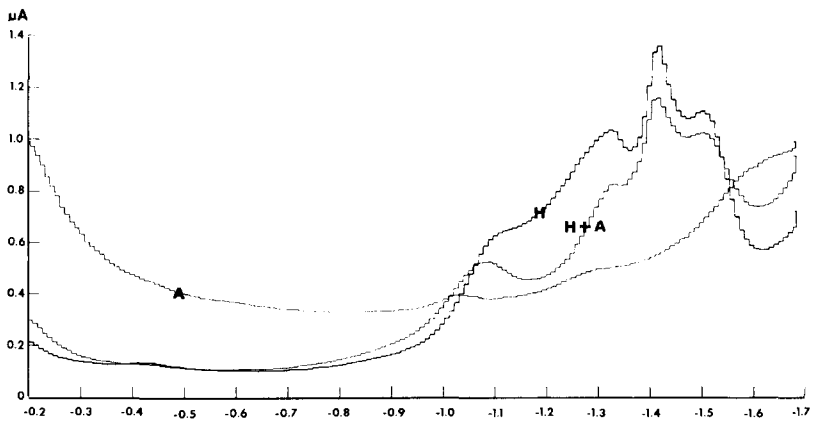

Fig. 3. Phase-sensitive a.c. polarograms (Faradaic component) of the same three solutions as in Fig. 2. a.c. signal: 225 Hz, 10 mV (peak to peak). Phase angle, 0°. Trace A, ATPase; Trace H, liposomes; Trace H + A, mixture of both.

the components, as can be shown by a matrix rank analysis[19]. The three a.c. polarograms shown in Fig. 3, representing the ATPase alone, phospholipid vesicles alone, and a combination of both, were sampled at ten different potentials. The sampling points were chosen so that all the characteristic features of the three curves were represented. A 10 × 3 matrix of the current values was set up and subjected to the rank analysis. After two reduction steps, all three rows of the matrix were still clearly significant, meaning that at least three polarographically distinguishable species were present in solution. This strongly suggests that a vesicle–ATPase complex exists in the mixture, a conclusion which could also be drawn directly upon close inspection of Fig. 3. The a.c. polarogram of the combined solution displays a maximum at −1.07 V. Immediately above this potential, this trace has a negative slope which could not possibly result from any linear combination of the traces of the separate components, both of which have positive slopes in that region. Further studies are currently in progress to investigate the binding of the soluble ATPase to the liposome system. These include spectrophotometric titration, spectropolarimetry, molecular sieve chromatography and analytical ultracentrifugation (W. R. Redwood, unpublished).

Observation of random interfacial currents

Although no random interfacial currents, analogous to those reported by Blank and Britten[1] were observed with solutions containing either the soluble ATPase or homogeneous phospholipid vesicles, it was possible to obtain these effects in solutions containing either multi-lamellar phospholipid dispersions or dispersed

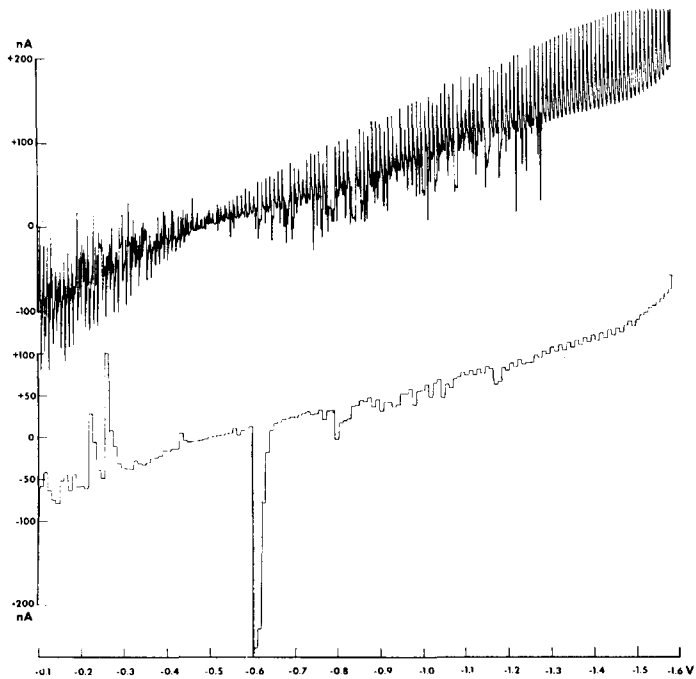

Fig. 4. Continuous (upper curve), and sampled current polarogram (lower curve) of a 150 μg/ml suspension of egg lecithin.

fragments from *S. faecalis* membrane ghosts. Fig. 4 illustrates the complete d.c. polarogram, recorded continuously and in the current-sampling mode, for a heterogeneous egg lecithin liposome dispersion prepared in the succinate–magnesium buffer. In both polarograms the random currents can clearly be seen. Below the electrocapillary maximum they are positive; above the electrocapillary maximum they are negative. These fluctuation phenomena can best be seen in the current-sampling mode and are exemplified by the records in Fig. 5 in which two time sweeps were recorded at constant voltages of −200 mV and −1 V, respectively. It was also possible

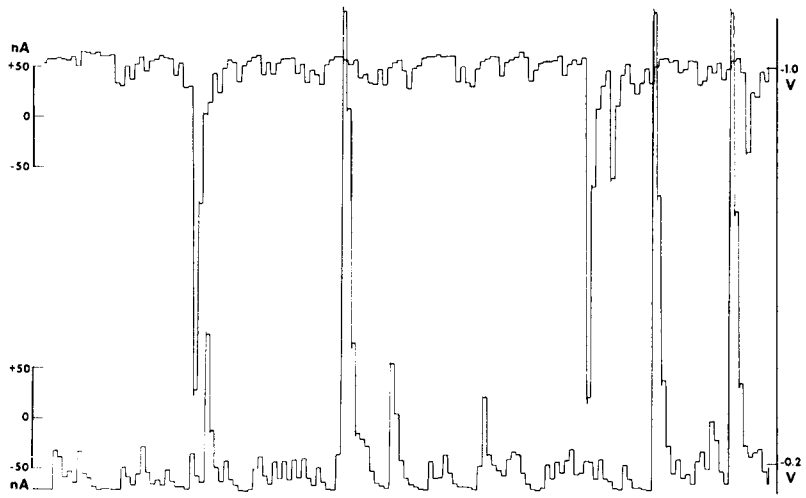

Fig. 5. Constant potential scans at —0.2 V and —1.0 V for the egg lecithin suspension of Fig. 4. Total recording time for each scan, 150 s. The instrument was in the current sampling mode.

to generate random currents at the polarized mercury–water interface in the presence of a heterogeneous liposome dispersion of pure dipalmitoyllecithin. The results are illustrated in Fig. 6, where one time sweep was recorded continuously (A) and a second one in the current-sampling mode (B). In both scans, the potential was programmed to switch from −200 mV to −1 V and back again with a period of 50 s. The random currents visible in these records are similar to those obtained with the egg lecithin liposomes but are of smaller magnitude, probably because the dipalmitoyl lecithin dispersion settles more rapidly on standing in the polarographic cell. It is possible, however, to completely eliminate the random currents from both systems by the addition of low concentrations of Triton X-100. The inhibitory effect of the detergent is illustrated in Fig. 6 (C and D).

Similar effects were observed with a briefly sonicated dispersion of *S. faecalis* membrane ghosts which were depleted of the soluble ATPase. In Fig. 7, obtained with the natural membrane fragments, the upper wave was continuously recorded, while the lower (irregular) curve was recorded in the current-sampling mode. In both time scans, positive spikes can be seen at −200 mV, and negative spikes at −1 V. The lower smooth curve was obtained after the addition of ouabain at 10^{-4} M to the membrane ghost preparation in the polarographic cell. These observations provide direct confirmation of the report by Blank and Britten[1] that random currents appear

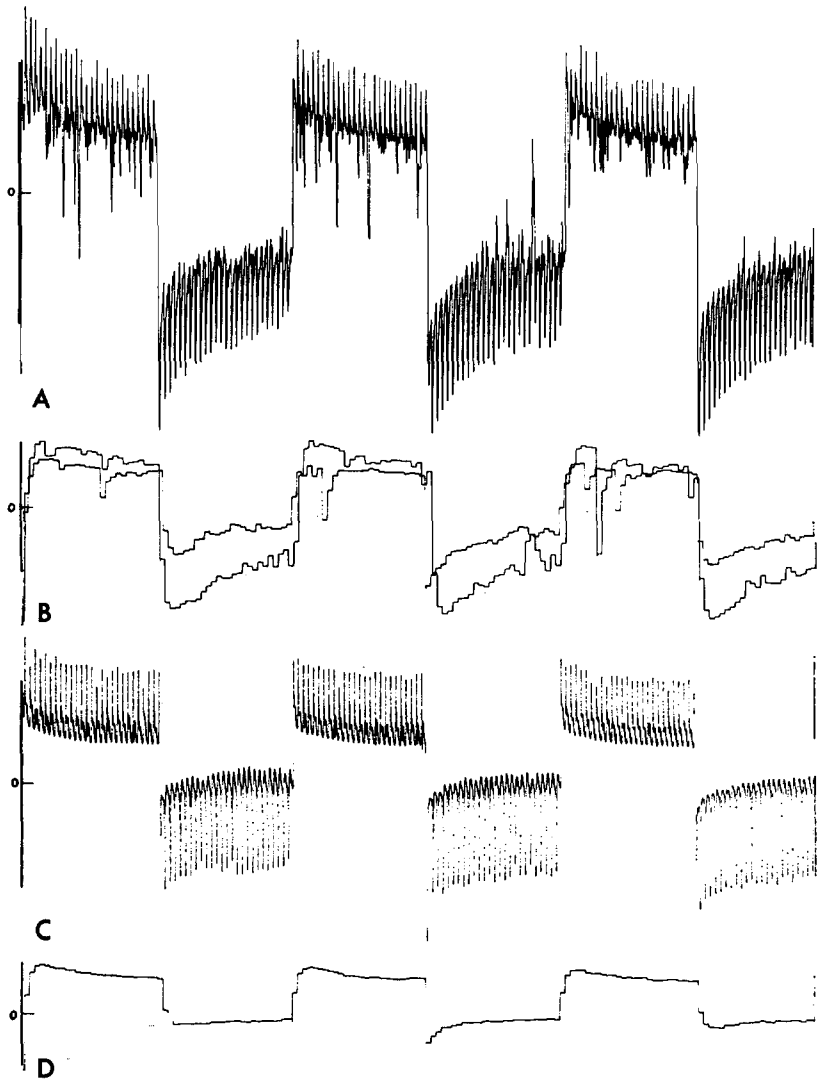

Fig. 6. Constant potential scans for a suspension of 150 μg/ml pure dipalmitoyl lecithin. The applied voltage is switched between —0.2 and —1.0 V with a period of 50 s. A, continuous scan, showing both drop oscillations and random currents. B, sampled current scans. The outer curve (highest amplitude) represents the dipalmitoyl lecithin alone; the inner curve was obtained after addition of Triton X-100 to a concentration of 0.25 mg/ml. A slight inhibition of the random currents can be observed. C, continuous scan, after addition of more Triton X-100, bringing its concentration up to 0.75 mg/ml. The random currents have disappeared. D, sampled current scan of the same solution. Comparison with B shows the effect of the detergent.

at the polarized mercury–water interface in the presence of membranous structures. Furthermore, it has been confirmed that these random currents can be eliminated by the action of detergents, alcohol and certain drugs. The inhibitors found in the present study are listed in Table I.

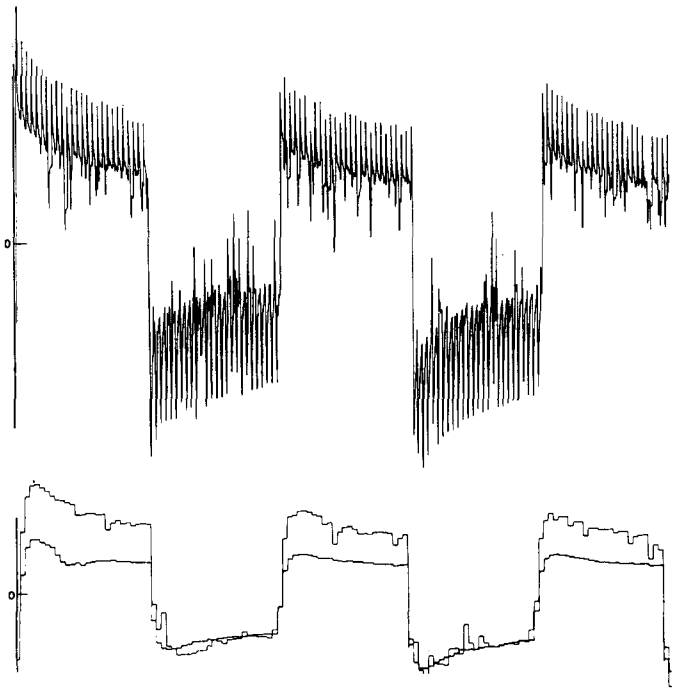

Fig. 7. Constant potential scans, switched between —0.2 and —1.0 V. Upper trace, continuous scan of a dispersion of *S. faecalis* membrane fragments, exhibiting random currents. Lower trace, the outer curve shows a sampled current scan of the same sample. The inner curve was obtained after adding ouabain up to a concentration of 10^{-4} M.

TABLE I

INHIBITION OF RANDOM CURRENTS BY DRUGS AND DETERGENTS

Compound	*Inhibition*
Ouabain	Active
Dicumarol	Active
Ethanol	Moderately active
Chlorhexidine acetate	Inactive
Triton X-100	Active
Cetyldimethylbenzylammonium bromide	Active

Interaction of the soluble ATPase with a charged vesicle preparation

From previous studies, it is known that the soluble ATPase interacts with a positively charged liposome preparation at pH 7.5 to form large lipid–protein aggregates which settle on standing. The mechanism of the interaction probably involves an initial electrostatic attraction between the oppositely charged macromolecules, but the binding of the protein to the liposomes may involve hydrophobic bonding[20]. The polarographic properties of this interactant system are shown in Fig. 8. The lower curve illustrates the sampled-current record of a dispersion of phosphatidylcholine vesicles containing 10 % by weight of stearylamine prepared in a buffer solution containing 10^{-2} M $MgCl_2$ and $2 \cdot 10^{-2}$ M Tris–HCl. The dispersion,

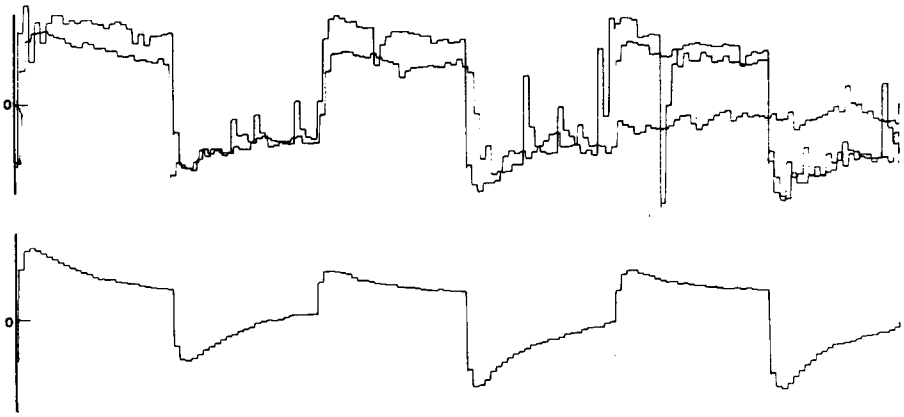

Fig. 8. Constant potential scans, switched between —0.2 V and —1.0 V. All curves were recorded in the current sampling mode. Upper traces, Dispersion of phosphatidylcholine vesicles (approx. 150 μg/ml) containing 10% of stearylamine, after addition of soluble ATPase (10 μg/ml). The inner curve, part of which has been recorded twice, was obtained after addition of chlorhexidine acetate ($4 \cdot 10^{-5}$ M) to the mixture. This inhibitor does not eliminate the random currents. Lower trace, The stearylamine–phosphatidylcholine vesicles alone, without the ATPase. The upper size limit was 1000 Å, and random currents were not observed.

which is optically clear and colloidally stable, produces no random currents at the polarized mercury–water interface. However, when the soluble ATPase is added to this dispersion in the polarographic cell, the solution becomes turbid and random currents soon appear, as seen in the upper traces. It was not possible to eliminate these interfacial currents with the specific enzyme inhibitor chlorhexidine[21], the inner curve of Fig. 8 (upper traces). This result suggests that the random currents were not related to the enzyme activity.

The conclusion drawn from these studies on the phenomenon of the random currents is that their appearance depends critically on the size of the membranous particles being adsorbed onto the polarized mercury–water interface. Thus, phospholipid vesicles smaller than 1000 Å do not produce these effects, but larger particles, in the form of multi-lamellar myelinic figures or charged vesicle–ATPase complexes are capable of generating these currents on contact with the polarized mercury–water interface. The mechanism of formation of these random currents is not known, but is worthy of further investigation. It is probable that the sole criterion for the production of these effects is the statistical fluctuation in the frequency at which the particles reach the mercury–water interface. The inhibitory effect of detergent, alcohol, and certain drugs, on the interfacial currents suggests a common mode of action, which possibly entails the disruption of the large multi-lamellar structures of the colloidal particles. Alternatively, preferential adsorption of the smaller surface active molecules at the mercury–water interface could suppress the current fluctuations.

In general, the random currents flow in a direction opposite that of the capacitance current. Nevertheless, they do not represent random suppressions of the charging current. This is illustrated in Fig. 9, where a normal d.c. polarogram of a suspension of *S. faecalis* membrane fragments is compared with a pulse polarogram run on the same sample. In pulse polarography, the linear d.c. ramp of ordinary

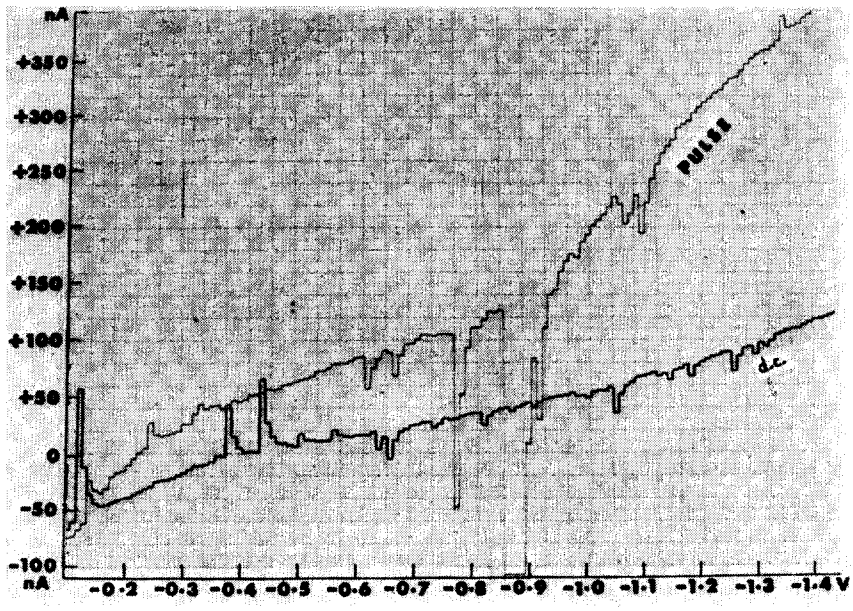

Fig. 9. Comparison of a d.c. polarogram (sampled current scan) and a pulse polarogram of a dispersion of *S. faecalis* membrane fragments. Both techniques show pronounced random current spikes.

polarography is replaced by a series of constant voltage pulses of increasing height, at the rate of one pulse per drop of mercury, near the end of the drop life. Between pulses, the applied potential returns to the starting value. The pulses generated by the Princeton instrument have a duration of 48 ms. A capacitance current will flow at the onset of each pulse, but will decay rapidly, as the surface area of the mercury drop is practically constant during the pulse. Current is sampled during the last 8 ms only, when the capacitance current has decayed to virtually zero. Pure faradaic currents are thus obtained. These currents are generally larger than the diffusion currents of ordinary polarography, because a steady state is established with a non-zero concentration of the reducible compound at the electrode interface. On the basis of this information, a comparison of the two curves in Fig. 9 bears out that the random current spikes figure quite prominently in the pulse polarogram. The direction of the current spikes usually reverses beyond -1.3 V, which leads one to conclude that they must be, at least in part, of a faradaic nature. Phase-selective a.c. polarography, in which the capacitance and faradaic currents can be separated, shows that random current spikes (of random sign) are observed in both.

Although a well-found explanation for the random currents cannot be given at this time, all these phenomena will probably be found to have a common explanation in the general theory of the electrical double layer at the mercury–water interface. It is quite reasonable to assume that the currents observed are capacitance discharge currents, caused by adsorption of uncharged surface-active particles hitting the electrode at random*. This process would give rise to the release or displacement of

* Thanks are due to an unidentified reviewer of this paper for pointing this out.

ions previously present in the double layer, causing a current to flow. The effect would also be noticeable in pulse polarography, where the double layer has already been charged up completely at the time of current sampling. For alternate explanations, one could invoke streaming phenomena around the electrode. Supporting data for these theories are not available at the present time, and an exhaustive study of these electrokinetic phenomena is beyond the scope of this paper.

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