Biochemical Pharmacology, Vol. 36, No. 7, pp. 1153–1161, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

EVALUATION OF MEMBRANE FLUIDITY EFFECTS AND ENZYME ACTIVITIES ALTERATIONS IN ADRIAMYCIN NEUROTOXICITY

GEORGE DELICONSTANTINOS, LIUDMILA KOPEIKINA-TSIBOUKIDOU and VASSILIKI VILLIOTOU

Department of Experimental Physiology, University of Athens Medical School, GR-115 27 Athens, Greece

(*Received* 19 *August* 1986; *accepted* 16 *October* 1986)

Abstract—Adriamycin (ADR) increased the lipid fluidity of dog brain synaptosomal plasma membranes (SPM) labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH), as indicated by the steady-state fluorescence anisotropy $[(r_o/r)-1]^{-1}$. Arrhenius-type plots of $[(r_o/r)-1]^{-1}$ indicated that the lipid phase separation of the membrane at 23.3 ± 1.2° was perturbed by ADR such that the temperature was reduced to 16.2 ± 1.1°. Arrhenius plots of $(Na^+ + K^+)$-stimulated ATPase activity exhibited a break point at 22.8 ± 1.1° in control SPM which was reduced to 15.8 ± 1.0° in ADR treated SPM, suggesting differences in the interaction of $(Na^+ + K^+)$-stimulated ATPase with lipids between ADR treated and untreated SPM. $(Na^+ + K^+)$-stimulated ATPase and Ca^{2+}-stimulated ATPase activities were increased at a concentration range 10^{-18}–10^{-15} M of ADR; higher concentrations (up to 10^{-7} M), however, led to a progressive inhibition of the enzyme activities. The allosteric properties of SPM-bound $(Na^+ + K^+)$-stimulated ATPase by fluoride (F^-) (as reflected by changes in the Hill coefficient) were modulated by ADR whereas those of SPM-bound acetylcholinesterase remained unaffected. We propose that ADR achieves these effects through asymmetric perturbations of the membrane lipid structure and that changes in membrane fluidity may be an early key event in ADR induced neurotoxicity.

Adriamycin (doxorubicin, ADR) is an anthracycline antibiotic with antineoplastic activity against a wide variety of tumours. Unfortunately, the development of acute and chronic cardiac damage often interferes with the full therapeutic potential of the drug (for reivew see ref. 1). Studies on the distribution of the drug using isotope labelling and fluorimetric techniques, showed long-lasting levels of adriamycin in the brain, quantitatively comparable to the plasma levels [2, 3]. Administration of the drug by direct intrathecal injection or after disruption of the blood–brain barrier, induced detectable cell changes in the brain [4]. Neurotoxic effects of ADR injected i.v. occur in the mouse, in regions outside the blood–brain barrier and can be expected in situations where the barrier is damaged. Furthermore, retrograde axonal transport of ADR may be used as a model for experimental motor neuron disease [5–7]. ADR has been shown to influence many properties of cellular membranes and model membrane systems. These include: transport of ions [8], membrane fluidity [9], organization of lipids [10] and membrane enzyme activities [11]. The importance of these membrane changes to the cytotoxic action of ADR compared to its ability to bind to DNA and inhibit nucleic acid synthesis has not been clearly established. However, it has been previously reported that change in membrane fluidity is the earliest measurable alteration occuring in cells following exposure to the drug [12]. Possible modulations of brain synaptosomal plasma membrane (SPM) fluidity, with consequent effects on the expression of membrane-bound enzymes, may initiate the series of events leading to brain cytotoxicity.

Our experiments were designed to explore the relationship between brain synaptosomal plasma membrane fluidity and the mechanism of the cytotoxic action of ADR in the brain. In our previous studies we reported on the effect of charged drugs or negatively charged phospholipids on the activity of some membrane-bound enzymes [13–15], suggesting that these compounds can alter the membrane fluidity, causing functional changes in the allosteric properties of integral enzymes. This report focuses on the modulations of lipid dynamics and lipid–protein interactions in dog brain SPM, induced by ADR using as functional parameters the activities of the integral enzymes $(Na^+ + K^+)$-stimulated ATPase, Ca^{2+}-stimulated ATPase and acetylcholinesterase. Even if the plasma membrane is not the primary target for the cytotoxic effects of ADR, it is clear that interaction at this level is still required for entry into the cell. Consequently, it is reasonable to assume that the structure of the cell membrane may be an important determinant of the cytotoxicity of ADR, regardless of the ultimate cellular target of the drug. The experiments below demonstrate that ADR increases the SPM fluidity with subsequent decrease of the $(Na^+ + K^+)$-stimulated ATPase activity and increase of the Ca^{2+}-stimulated ATPase activity. These effects could be related to the cytotoxic effects of the drug in the brain.

A preliminary report of these results has been presented [16].

MATERIALS AND METHODS

Materials. The following materials were obtained from the sources indicated: ^{45}Ca (spec. radioactiv.

2 mCi/116 μg Ca^{2+}) (Radiochemical Centre, Amersham, U.K.). Adriamycin; 1,6-diphenyl-1,3,5-hexatriene (DPH) (Sigma Chem. Co., St Louis, MO). Ethylene glycol bis (2-aminoethyl-ether) *N-N'*-tetraacetic acid (EGTA); calcium chloride; ATP (Serva, F.R.G.). All other chemicals were of the highest purity commercially available.

Synaptosomal plasma membranes (SPM) from dog brain were prepared from Ficoll-purified and subsequently osmotically soaked synaptosomes by ascending fractionation through a discontinuous sucrose density gradient, as previously described [17]. Compared with the original homogenate from which they were prepared, they showed a 7–10-fold increase in the ($Na^+ + K^+$)-stimulated ATPase activity (EC 3.6.1.3). The protein content was determined by the Lowry method as described by Miller [18].

($Na^+ + K^+$)-stimulated ATPase assay. ($Na^+ + K^+$)-stimulated ATPase activity of SPM was assayed in an incubation medium consisting of 40 mM Tris·HCl pH 7.4; 5 mM $MgCl_2$; 3 mM disodium ATP; 80 mM NaCl; 20 mM KCl; 1 mM ouabain and 0.1 mg of SPM protein, in a final volume of 1.0 ml. Incubations were performed in the reaction medium for 30 min at 37°. The reaction was started by the addition of ATP and stopped with 0.2 ml of 50% trichloroacetic acid. The liberated inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow [19]. ($Na^+ + K^+$)-stimulated ATPase activity was defined as the difference between the Pi liberated during incubation in the presence and absence of ouabain. The ouabain-inhibitable component of ATPase activity is the activity that is lost when Na^+ or K^+ is omitted from the reaction mixture. Arrhenius plots of the enzyme activity at temperatures of 5–42° at 3–4° intervals, were analysed using a least squares minimalisation process to determine the break points. In every case initial rates were determined under maximal velocity conditions and constant pH to preclude artifactual breaks in slope. For the assay of the inhibition by fluoride (F^-) of the ($Na^+ + K^+$)-stimulated ATPase the reaction mixture contained increasing amounts of NaF, as indicated in Fig. 6(A).

Ca^{2+}-stimulated ATPase assay. Ca^{2+}-stimulated ATPase activity of SPM was assayed in the following incubation medium; 40 mM Tris–HCl pH 7.4; 2 mM $MgCl_2$; 100 mM KCl; 100 μM $CaCl_2$, 100 μM EGTA; 2 mM dipotassium ATP and 0.25 mg of SPM protein in a final volume of 1.0 ml. Incubations were carried out at 37° for 45 min and stopped with 0.2 ml of 50% trichloroacetic acid. The liberated inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow [19]. Ca^{2+}-stimulated ATPase activity was defined as the difference between the Pi liberated during incubation in the presence and absence of calcium.

Assay of ^{45}Ca uptake. $^{45}Ca^{2+}$ uptake to SPM was measured at 37° in a shaking water bath using an adaptation of the Millipore-filtration technique as previously described [20], and was always done on freshly prepared SPM. The reaction mixture was similar to that used for estimating Ca^{2+}-stimulated ATPase activity. Samples (100 μl) taken from the mixtures were placed on pre-wetted HAWP Millipore filters (0.45 μm pore diameter) and filtered under suction by using a Millipore 3025 sampling manifold. The filters were washed three times by adding 3 ml of ice-cold, 0.25 M sucrose–5 mM Tris·HCl (pH 7.4) and transferred into counting vials. They were then shaken in 1 ml of 1% sodium dodecyl sulfate and mixed with 10 ml dioxane-based scintillation fluid [21]. Prior to use, all filters were washed with 10 ml of 5 mM Tris·HCl/100 μM EGTA pH 7.4 solution; this procedure was required for low background counts. Appropriate blanks (no protein added), controls, and aliquots of reaction mixtures from blank tubes were counted for each experiment. Radioactivity (c.p.m.) was converted into nmol by using the known specific radioactivity. The amount of radioactivity that was constantly absorbed on to SPM at zero time (instantaneous deposition of radioactivity) was substracted in every case from all samples. Uptake in the absence of ATP was complete by 2 min and was subtracted to give ATP-dependent accumulation values.

Acetylcholinesterase assay. Acetylcholinesterase activity was determined by measuring the hydrolysis of acetylthiocholine by the method of Ellman *et al.* [22]. The assay mixture (3 ml) contained 1 mM acetylthiocholine iodide, 0.125 mM 5,5'-dithionitrobenzoid acid, 100 mM NaCl, 0.24 M sucrose and 10 mM Tris·HCl pH 8.0. Protein concentration was 0.06 mg/3 ml incubation mixture. The reaction was followed spectrophotometrically by the increase of A_{412} by using a Beckman Acta MVI Spectrophotometer. For the assay of the inhibition by fluoride (F^-) of the acetylcholinesterase the reaction mixture contained increasing amounts of NaF as indicated in Fig. 6(B).

Malondialdehyde (MDA) assay. Malondialdehyde (MDA) was measured by the thiobarbituric acid colour reaction as described by Wilbur *et al.* [23] but with slight modification. One millilitre of incubation medium contained NaCl (150 mM), KCl (10 mM), $MgCl_2$ (5 mM) and 0.3–0.5 mg SPM protein in 50 mM Tris·HCl buffer pH 7.4 with or without 10^{-7} M ADR. Incubations were carried out at 25° for 1 hr under continuous magnetic stirring, and terminated by the addition of 1 ml of 20% trichloroacetic acid in 0.6 M hydrochloric acid. After centrifugation at 2000 *g* for 15 min in a refrigerated (4°) bench centrifuge, 1.5 ml of the supernatant was added to 0.3 ml of 0.12 M thiobarbituric acid solution, followed by 15 min incubation in a boiling water bath. After cooling slowly to room temperature the colour produced was read at 535 nm and the amount of MDA formed was determined by using appropriate reference samples and the molar extinction coefficient $E_{535} = 1.56 \times 10^{-5}$ [24]. A stock solution of 10 mM MDA was made by hydrolyzing 8.2 μl of malondialdehyde tetramethyl acetal (Eastman, Rochester, NY) with 3 or 4 drops of concentrated hydrochloric acid, and then dilution to a final volume of 5 ml with distilled water. For determination of recovery, a portion of the stock solution was diluted with saline to give 0.1 mM MDA, and known amounts were added to saline or SPM. The contents were decanted into trichloroacetic acid and treated as described above.

A solution of 0.12 M thiobarbiturate was prepared by dissolving thiobarbituric acid (Eastman) in 0.25 M Tris·HCl pH 7.0 and filtered. The MDA produced during 1 hr incubation in the presence and/or absence of ADR was expressed as nmol MDA per mg SPM protein.

Steady-state fluorescence polarization studies. Lipid fluidity was assessed by the steady-state fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) according to the method described by Shinitzky and Barenholz [25]. Measurements were made in an AMINCO SPF-500 Spectrofluorometer with polarization accessory for fluorescence polarization measurements at excitation wavelength 360 nm and emission wavelenth 430 nm. The polarization of fluorescence was expressed as the fluorescence anisotropy, r, and the anisotropy parameter $[(r_0/r) - 1]^{-1}$ was calculated using a value of $r_0 = 0.365$ for DPH [26]. The anisotropy parameter varies directly with the apparent rotational relaxation time of the probe and thus inversely with the fluidity. The fluorescence anisotropy, r, was calculated according to the equation $r = I_{VV} - I_{VH}/I_{VV} + 2I_{VH}$, where I_{VV} and I_{VH} are the intensities of the emitted light oriented, respectively, parallel and perpendicular to the plane of the exciting beam. Light scattering corrections were always <5% of the total signal and did not differ significantly in various preparations. Measurements were made at least in triplicate. The temperature dependence of $[(r_0/r) - 1]^{-1}$ was determined over the range of 4–40°. Membranes (approx. 50 μg) were warmed initially to 40° and the fluorescence polarization was estimated every 1–2° as the suspension cooled slowly to 4°. Plots of log $[(r_0/r) - 1]^{-1}$ versus $1/T$ were constructed to detect thermotropic transitions as previously described [26]. The movements of the DPH within the medium depend on both life-time (τ) and viscosity of the medium ($\bar{n}$). It is generally assumed that τ is constant at one temperature and thus fluorescence polarization is directly related to $\bar{n}$. However, the presence of a third compound can modify τ if this compound acts as a quencher. A direct measurement of τ in the presence or in the absence of quencher checks this possibility. An estimate can be obtained by measuring the intensity of total fluorescence $F = I_{VV} + 2I_{VH}$. The identical fluorescence intensities measured in presence and in absence of ADR (10^{-8} M) tested, allows us to conclude that ADR has no effect on τ and that consequently the observed variation in $[(r_0/r) - 1]^{-1}$ values represent fluidization of the lipid membrane bilayer.

Treatment of synaptosomal plasma membranes (SPM) with adriamycin. Preincubations of SPM with ADR ($10^{-18} - 10^{-7}$ M) were carried out for 1 hr at 25° in an incubation mixture of 20 mM Tris·HCl/20 mM KCl pH 7.4, containing 0.2 mg of SPM protein in a final volume of 1 ml with continuous stirring with a magnetic bar. $(Na^+ + K^+)$-stimulated ATPase activity, Ca^{2+}-stimulated ATPase activity, Ca^{2+} binding, acetylcholinesterase activity and steady-state fluorescence polarization were estimated in portions of the membranous suspensions as described above. Following incubation of SPM with ADR the bound and free ADR were separated by centrifugation at 100,000 g for 1 hr. The bound and free ADR fractions were pooled separately, extracted and analyzed by thin layer chromatography as described by Johnson *et al.* [27] in a solvent system chloroform:methanol: acetic acid:water (40:10:3:1). The extracts of membrane-bound and free ADR moved identically with those of unincubated ADR, suggesting that no significant metabolic conversion of membrane-bound and free ADR occurred under the present incubation conditions.

Statistical analysis. All experiments were performed in duplicate or triplicate. Computer analysis for curve fitting was performed with a programmable Hewlett-Packard HP-97 calculator. The correlation coefficients (r) for the straight lines were above 0.95. The mean value and SD were calculated. Comparisons between two groups were performed by the use of a paired standard Students t-test. A P value of less than 0.05 was considered significant. When two different curves were made with the same membrane preparation under the same experimental conditions the individual points and the slopes obtained showed a maximum variability of 5%. At different membrane preparations the maximum variability was less than 10%.

RESULTS

Changes of SPM-bound $(Na^+ + K^+)$-stimulated ATPase activity at different concentrations of ADR are illustrated in Fig. 1. A considerable increase of the enzyme activity (~48%) was appeared at approx. 10^{-15} M ADR. Higher concentrations of the ADR, however, led to a progressive inhibition of the enzyme activity (~35%) with respect to the control SPM (untreated with ADR). Mg^{2+}-stimulated ATPase activity was not changed at different concentrations of ADR. The curve representing the changes of Ca^{2+}-stimulated ATPase activity at different concentrations of ADR has biphasic character (Fig 2A). Up to a concentration of 10^{-15} M of ADR a dramatic increase in the specific activity of the enzyme was induced, with maximal effect of approx. 75% above its original value. Further increases in ADR concentration above this point, however, led to inhibition of the enzyme activity with respect to the maximal percentage of stimulation. The activity of the SPM-bound acetylcholinesterase, showed a slight but not statistically significant increase ($P > 0.05$) at different ADR concentrations (Fig. 2A). Data from ^{45}Ca uptake experiments indicate that the SPM uptake system requires ATP (Fig. 2B). Ca^{2+} uptake was measured over a range of Ca^{2+} free concentrations, from 0.1 to 10 μM, as calculated by Ca^{2+}-EGTA buffers [28]. As illustrated by these experiments, a significant increase (~3-fold) is seen in Ca^{2+} uptake when ATP is added to the incubation media containing Ca^{2+}. The ATP-dependent Ca^{2+} uptake is 3.8 nmol/mg SPM protein and represents the difference between Ca^{2+} binding to the SPM surface prior to uptake and the amount of total Ca^{2+} accumulation. We have used Ca^{2+} uptake as an operational term to include all the processes whereby $^{45}Ca^{2+}$ becomes associated with membrane vesicles. We think the term binding should be used for chemical association between Ca^{2+} and a membrane site. Given this distribution of Ca^{2+} at the SPM, it is clear

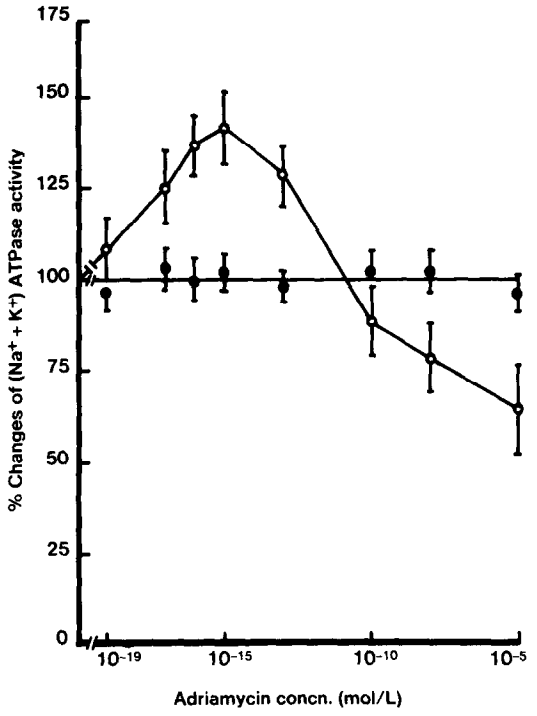

Fig. 1. Effect of Adriamycin (ADR) on dog brain synaptosomal plasma membrane (SPM)-bound ($Na^+ + K^+$)-stimulated ATPase activity (○——○). Aqueous solutions of ADR were preincubated with SPM (0.2 mg/ml) in a medium consisting of 20 mM Tris·HCl/80 mM NaCl (pH 7.4) for 1 hr at 25°. Enzyme activity was estimated as described in Materials and Methods sections. The specific activity of the enzyme in control SPM was 12.1 μmol of Pi/hr per mg of SPM protein. The straight line represents the Mg^{2+}-ATPase activity (●——●). Values are means ± SD of three different experiments.

that ADR acts to increase ATP-dependent Ca^{2+} uptake into SPM. Following ADR treatment, the Ca^{2+} uptake by SPM is 5.1 nmol/mg protein and 7.8 nmol/mg protein, respectively at 10^{-15} and 10^{-8} M of ADR. Repeated (thrice) washing of ADR-treated SPM with 20 ml of ice-cold 5 mM Tris·HCl buffer pH 7.4 by means of centrifugation at 4° for 45 min at 100,000 *g* is found (Table 1) to restore ($Na^+ + K^+$)-stimulated ATPase and Ca^{2+}-stimulated ATPase by approx. 90%. These results suggest that ADR exerts its action not directly on the protein molecules, but rather through its association with the lipid matrix of the membrane bilayer. To assess the ability of ADR to form lipid peroxides from peroxidation of membrane fatty acids, the presence of malondialdehyde (MDA) was measured with the use of a modification of the thiobarbituric acid reaction method as described in the Experimental Section. One disadvantage of the spectrophotometric determination of MDA is that other substances may be extracted which have absorption in the visible range. We overcome this problem by using appropriate controls as reference samples. A second problem is that compounds other than MDA are known to react with thiobarbituric acid to form colored pigments. We know of no such pigment with an absorption maximum at 535 nm which is why we scanned between 450 and 550 nm to ensure that a distinct peak occurred at this wavelength. MDA production during 1 hr incubation of SPM in the presence of 10^{-8} M ADR is shown in Table 2. A statistically significant increase ($P < 0.05$) approx. 25% of MDA production was observed in ADR treated SPM as compared to untreated SPM.

The temperature dependence and the Arrhenius activation energy of SPM-bound ($Na^+ + K^+$)-stimulated ATPase activity in the absence and in the presence of 10^{-8} M ADR is shown in Fig. 3 and in Table 2. Arrhenius plots of the ($Na^+ + K^+$)-stimulated ATPase showed a transition temperature at 22.8 ± 1.1°. The depression of the transition temperature to 15.8 ± 1.0° in the presence of ADR may be due to modulations in the physical state of the membrane lipids. The Arrhenius activation energies (E_a) for the ($Na^+ + K^+$)-stimulated ATPase above and below the break point was increased from 14.5 ± 0.7 kJ/mol and 79.3 ± 6.9 kJ/mol for the controls (SPM non-treated with ADR) to 39.8 ± 4.1 kJ/mol and 269.4 ± 15.9 kJ/mol for the ADR treated SPM, respectively. Presumably, the lipid microenvironment around the particular integral enzyme and the nature of the specific lipid–protein interaction influence the transition temperature and the Arrhenius activation energy [29]. These results are indicative of microscopic changes in the relationship between membrane-bound ($Na^+ + K^+$)-stimulated ATPase and its associated lipids, induced by ADR. Incubation of SPM for 1 hr at 25° with various concentrations of ADR resulted in a linear increase of lipid fluidity estimated by the fluorescence anisotrophy parameter, $[(r_0/r) - 1]^{-1}$, of DPH as shown in Fig. 4.

The effects of temperature on the fluorescence anisotropy parameter, $[(r_0/r) - 1]^{-1}$, of DPH in SPM are illustrated by representative Arrhenius plots in Fig. 5. The increase of the temperature produces concominant diminution in the $[(r_0/r) - 1]^{-1}$ values, which means an increase of the membrane fluidity. However, the evolution of the fluidity was not linear; a thermotropic transition temperature was indeed observed at 23.3 ± 1.2° in untreated SPM, which separates two domains where the evolution of the fluidity is directly proportional to the variation of the temperature. Treatment of SPM with ADR exhibit a statistically significant ($P < 0.01$) decrease in $[(r_0/r) - 1]^{-1}$ compared to untreated (control) SPM. It must be realised that the corresponding increase in fluidity is constant over the full range of temperatures studied. Furthermore, the thermotropic transition temperature was depressed to 16.2 ± 1.1°. What is particularly significant in our present study is that the observed transition temperatures for ($Na^+ + K^+$)-stimulated ATPase correspond closely to the thermotropic transitions of the lipid-phase separations (Table 3).

Further evidence for the increase in the SPM fluidity induced by ADR was obtained by the allosteric inhibition of ($Na^+ + K^+$)-stimulated ATPase activity by fluoride (F^-). Figure 6(A) shows the curves obtained when the relative rates of the enzymatic activity were plotted against different concentrations of F^- in control SPM and in those preincubated with 10^{-8} ADR for 1 hr at 25°. The Hill coefficient (slopes)

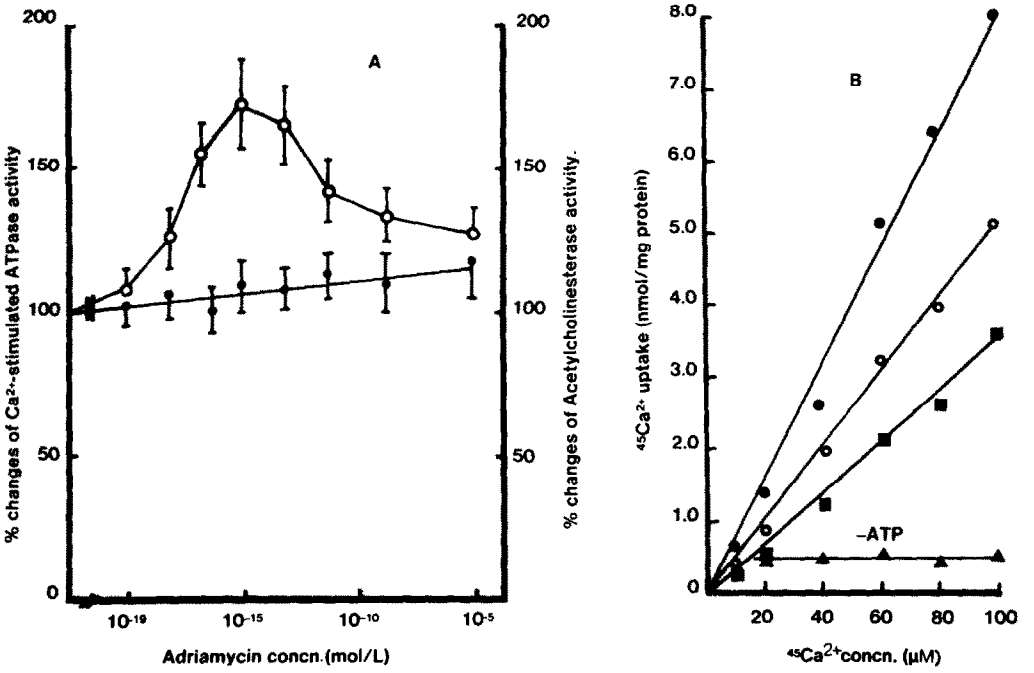

Fig. 2. (A) Effect of ADR on SPM-bound Ca^{2+}-stimulated ATPase (○——○) and acetylcholinesterase (●——●) activities. Ca^{2+}-stimulated ATPase activity was estimated at approx. 10 μM Ca^{2+}, and the specific activity of the enzyme in control SPM was 4.2 μmol of Pi/hr per mg of SPM protein. The value of the acetylcholinesterase activity estimated at 20° and pH 8 was 0.52 ± 0.02 ΔA_{412}/min per mg of SPM protein. For experimental details see the Materials and Methods section. Values are means ± SD of three different experiments. (B) Effect of ADR, 10^{-8} M (●——●) and 10^{-15} M (○——○) on ATP-dependent Ca^{2+} uptake by SPM, in the presence of 100 μM EGTA and Ca^{2+} concentrations varying between 10 and 100 μM. Control (untreated) SPM (■——■) and Ca^{2+} uptake by SPM in the absence of ATP (▲——▲). The straight lines were fitted by the method of least squares. Points in the drawn curves are mean values of duplicate determinations from a typical experiment, which was repeated three times.

Table 1. Effect of repeated washing of ADR (10^{-15} M) treated SPM on the recovery of ($Na^+ + K^+$)-stimulated ATPase and Ca^{2+}-stimulated ATPase activities

	($Na^+ + K^+$)-stimulated ATPase	Ca^{2+}-stimulated ATPase
Control SPM		
Before washing	12.4 ± 1.8	4.4 ± 0.8
After washing	10.1 ± 1.5	4.1 ± 0.7
ADR treated SPM		
Before washing	18.3 ± 2.7	7.1 ± 1.6
After washing	13.1 ± 1.9	4.7 ± 0.9

Each value represents the mean ± SD of three different experiments. Specific activity of the enzymes was expressed as μmol of Pi/hr per mg of SPM protein.

Table 2. Effect of ADR (10^{-8} M) on the malondialdehyde (MDA) production, transition temperature and Arrhenius activation energies of SPM-bound ($Na^+ + K^+$)-stimulated ATPase activity

	MDA produced (nmol/mg protein/hr)	Transition temperature (°)	Activation energy (Ea) (kJ/mol)	
			Above break	Below break
Control SPM	18.1 ± 1.8	22.8 ± 1.1	14.5 ± 0.7	79.3 ± 6.9
ADR treated SPM	22.5 ± 2.1*	15.8 ± 1.0*	39.8 ± 4.1*	269.4 ± 15.9*

Values represent means ± SD of three different experiments.
* Statistically significant compared with control ($P < 0.01$).

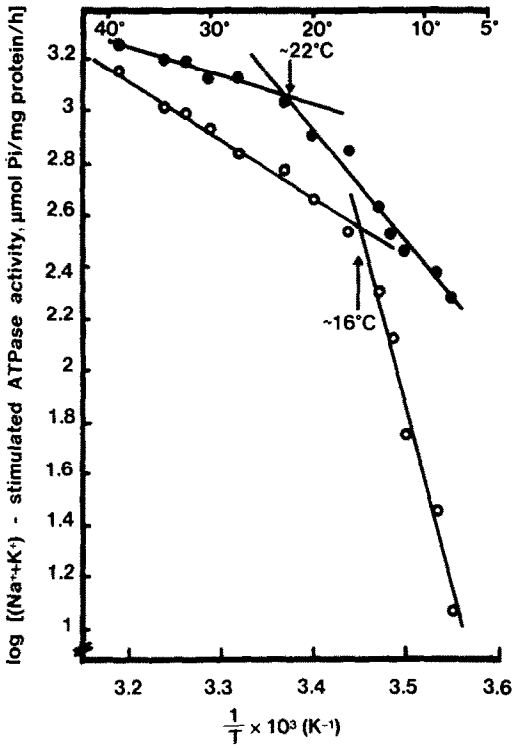

Fig. 3. Effect of temperature on the activity of SPM-bound ($Na^+ + K^+$)-stimulated ATPase in control (●——●) and ADR (10^{-8}) treated SPM (○——○). Each point represents the average value of duplicate determinations from a typical experiment which was repeated three times. The straight lines were fitted by the method of least squares.

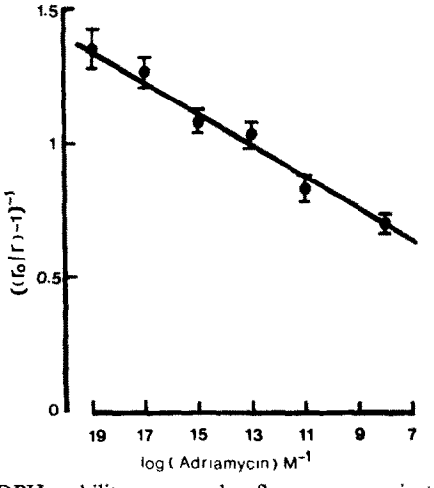

Fig. 4. DPH mobility expressed as fluorescence anisotropy $[(r_0/r) - 1]^{-1}$ in synaptosomal plasma membranes as a function of Adriamycin concentration, at 25°. Measurements were performed in duplicate for each sample. Decrease in fluorescence anisotropy reflects an increase in membrane fluidity. Each point represents the mean (±SD) of three membrane preparations.

for the control (non treated SPM) was $h = 2.15 \pm 0.3$, indicating the presence of cooper[illegible] which was reduced to $h = 1.38 \pm 0.1$ in SPM [illegible]ated with ADR. In contrast, there was no statistically significant difference ($P > 0.05$) in Hill coefficients of SPM-bound acetylcholinesterase activity assayed

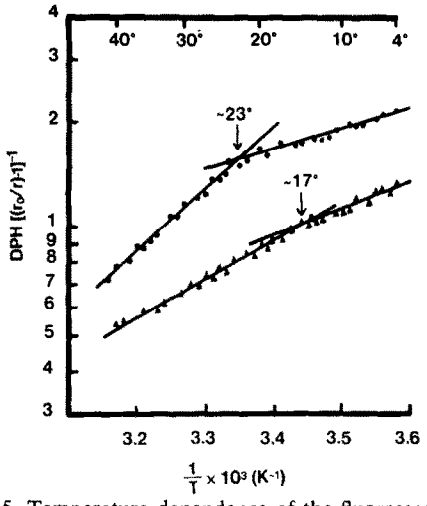

Fig. 5. Temperature dependence of the fluorescence anisotropy of DPH in control SPM (●——●) and ADR (10^{-8} M) treated SPM (▲——▲). The ordinate is the fluorescence anisotropy and the abscissa is the reciprocal of the absolute temperature. Experimental details are given in the test. This experiment is representative of three that were performed. The straight lines were fitted by the method of least squares.

in the absence ($h = 1.75 \pm 0.15$) or in the presence ($h = 1.67 \pm 0.14$) of ADR (Fig. 6(B)). These results may suggest that ADR increases the fluidity of the inner leaflet of the membrane and subsequently changes the allosteric behaviour of the SPM-bound ($Na^+ + K^+$)-stimulated ATPase, whereas it does not modulate the fluidity of the outer leaflet of the membrane within which the acetylcholinesterase resides [15].

DISCUSSION

From studies on the mechanism of action of the anthracycline antibiotic adriamycin (ADR), nuclear DNA has usually been considered the prime target for this antibiotic's antineoplastic action [30]. However, recent studies have suggested that ADR may be cytotoxic primarily as a result of its interactions with cell plasma membranes (for a review, see ref. 31). The most serious side effect of adriamycin is a dose-related, cumulative, myocardial toxicity which appears when the total dose exceeds 550 mg/m^2 and which may lead to congestive heart failure [32]. Experimental studies have shown that ADR injected i.v. in the rat may induce changes in the peripheral ganglia, regions which are devoid of the vascular barrier [33]. Signs of cerebral injury have also been reported when the blood–brain barrier was circumvented by intrathecal injection of the drug [34] or when the barrier was disrupted by intracarotid injection of mannitol [4]. Progressive ataxia and anesthesia were reported in rats given a single i.v. injection of adriamycin in a dose corresponding to that used for patients [35].

The ability of ADR to interact with SPM isolated from dog brain, causing significant changes of the SPM-bound ($Na^+ + K^+$)-stimulated ATPase and Ca^{2+}-stimulated ATPase activities by altering the

Table 3. Effect of ADR (10^{-8} M) on the fluorescence anisotropy of DPH in SPM

	Diphenylhexatriene (DPH) $[(r_0/r) - 1]^{-1}$ at 25°	Thermotropic transition temperature (°)
Control SPM	1.43 ± 0.05	23.3 ± 1.2
ADR treated SPM	0.75 ± 0.03*	16.2 ± 1.1*

Values represent means ± SD of three different experiments.

Thermotropic transition temperatures were determined from Arrhenius plots illustrated in Fig. 5.

* Statistically significant compared with control ($P < 0.01$).

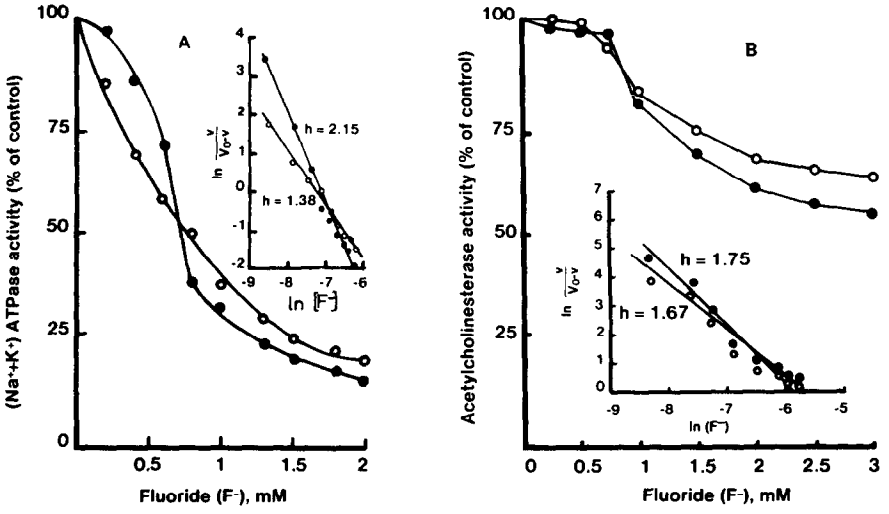

Fig. 6. Effect of F^- on the reaction rate of the SPM-bound ($Na^+ + K^+$)-stimulated ATPase (A) and effect of F^- on the reaction rate of SPM-bound acetylcholinesterase for ADR (10^{-8} M) treated (○——○) and untreated SPM (control; ●——●) (B). The inserts show Hill plots of the same data. Corresponding Hill coefficients (h) are as indicated. The correlation coefficients (r^2) for the straight lines in the inserts are >0.95. v is the reaction velocity, and V_0 is the rate of the reaction in the absence of F^-. Points in the curves drawn are mean values of duplicate determinations from a typical experiment which was repeated three times.

membrane fluidity, was investigated in the present study. The evidence presented here, derived both from measurement of calcium transport and enzyme activity, demonstrates that ADR increased the Ca^{2+}-stimulated ATPase of the SPM. The increase of Ca^{2+}-stimulated ATPase activity was observed with pharmacologically relevant concentrations of the drug, as the concentrations of ADR (10^{-15} M) which produced significant effects are associated with moderate to severe intoxications *in vivo* [31, 34]. The increase in Ca^{2+}-stimulated ATPase activity amounted to approx. 75% with a calcium concentration of ~10 μM (Fig. 2A). The calcium dependence of the effect indicated, however, that the enhancement is greater at lower concentrations of calcium. Because intracellular concentrations of free calcium are between 1 and 0.01 μM *in vivo* [36], it is likely that the effects of the drug are even greater under these conditions. A close relationship between increase of SPM Ca^{2+} uptake and enhance of SPM-bound Ca^{2+}-stimulated ATPase activities appears to exist (Fig. 2B). Since Ca^{2+} uptake is coupled to Ca^{2+}-stimulated ATPase activity which serves to transport Ca^{2+} at the expence of ATP hydrolysis, the present results suggest that ADR increase the capacity of SPM to retain Ca^{2+} by increasing the activity of the Ca^{2+}-stimulated ATPase. The curve representing the changes of ($Na^+ + K^+$)-stimulated ATPase activity at different concentrations of ADR has biphasic character (Fig. 1). Low concentrations of the drug progressively activated the ($Na^+ + K^+$)-stimulated ATPase activity; increasing the concentration above that maximally stimulating the enzyme activity caused a progressive inhibition with respect to the control value.

To investigate whether the effect of ADR on ($Na^+ + K^+$)-stimulated ATPase and Ca^{2+}-stimulated ATPase activities is consistent with an effect on the physicochemical characteristics of the membrane, and to discount the possibility that this drug interacts directly with the protein molecules to cause an irreversible change in their activities, the drug was removed from the membrane after washing of the drug-loaded SPM. The functional effects were fully reversible. Therefore the drug does not irreversibly change the ($Na^+ + K^+$)-stimulated ATPase and Ca^{2+}-stimulated ATPase activities and its presence in the membrane causes a modulation of the enzyme activities. The membrane functional effects of ADR appear to be mediated through changes in

the membrane fluidity, and the stimulation of the enzyme activities that ensued was presumably due to an increase in the conformational flexibility of the enzymes achieved by a relief of a physical constraint imposed by the bilayer on the protein molecules. At the ADR concentration of 10^{-8} M the decrease in the activity of the Ca^{2+}-stimulated ATPase with respect to the maximal stimulation and the decrease of the $(Na^+ + K^+)$-stimulated ATPase with respect to the original value, is probably due to fluidizing effects of ADR on bulk lipids of SPM or to displacement of annular lipids from around the enzyme molecules (see, e.g. ref. 37).

Acetylcholinesterase is an integral enzyme with its active site exposed at the external cell surface, and it may be a biologically significant component of the membrane, contributing to its integrity and to permeability changes occurring during transmission and conduction (for review, see ref. 38). It has been suggested that charged drugs may act preferentially at one or the other side of the plasma membrane, as the negatively charged (acidic) phospholipids predominate at the cytosol-facing surface [39]. Consistent with such a suggestion is that the negative charged phenobarbital does stimulate the acetylcholinesterase activity of SPM while the positive charged imipramine does not [15]. In the present study the lack of effect of the positive charged ADR on acetylcholinesterase activity (Fig. 2) reveals that ADR acts preferentially on the negative charged inner leaflet of the membrane. It was recently reported that ADR forms a specific complex with negatively charged phospholipids. The association constants were calculated to be $1.6 \times 10^6\,M^{-1}$ for cardiolipin and $1.8 \times 10^4\,M^{-1}$ for phosphatidylserine. Indeed, *N*-acetyladriamycin (uncharged) does not interact with cardiolipin or other phospholipids, but adriamycin does not interact with neutral phospholipids [40]. Since the outer membrane of the cells apparently does not represent a barrier to ADR, the latter has direct access to the cytosolic side of the inner membrane. The inhibitory effect of ADR on the SPM-bound $(Na^+ + K^+)$-stimulated ATPase activity may also be due to ADR–phosphatidylserine complex, since phosphatidylserine is essential for the enzyme to be active [41].

Further evidence for the preferential action of ADR on the inner half of the membrane was obtained from the alterations in the cooperative behaviour of the SPM-bound $(Na^+ + K^+)$-stimulated ATPase and acetylcholinesterase. As shown in Fig. 6(A, B), the value of the Hill coefficient, h, for the inhibition of $(Na^+ + K^+)$-stimulated ATPase by F^- was decreased by the positive charged ADR, indicating a loss of the co-operativity of the enzyme, while the Hill coefficient, h, for the inhibition of acetylcholinesterase by F^- was not changed by ADR suggesting that this drug influences the fluidity of the inner leaflet of the membrane bilayer [15, 42].

The observation that ADR can yield free radical species in plasma membranes is of special importance, since it has been reported an oxidative destruction of erythrocytes ghost membranes catalyzed by an adriamycin–iron complex [43]. Our *in vitro* studies showed a small (approx. 25%) but statistically significant increase of malondialdehyde (MDA), which is marker of lipid peroxidation, in response to *in vitro* incubation of SPM with ADR (Table 2). Gilbert and Savvas [44] have shown that MDA inhibits the $(Na^+ + K^+)$-stimulated ATPase activity of brain synaptosomes. It seems likely that the inhibitory effect of high amounts of ADR on the $(Na^+ + K^+)$-stimulated ATPase activity, shown in the present study, is due to radical damage of unsaturated fatty acids in membrane phospholipids which are essential for $(Na^+ + K^+)$-stimulated ATPase activity. Although MDA formation increases the molecular order of biomembranes (e.g. decreases the lipid fluidity) [45], in the present study, apparently, ADR overrides the MDA-dependent lipid ordering such that the bilayer becomes more fluid than untreated membranes.

In vitro exposure to ADR decreased the fluorescence anisotropy of DPH incorporated into SPM membranes. DPH is a nonpolar molecule which distributes throughout the hydrophobic core of the membrane bilayer [25]. The fluorescence anisotropy of DPH or, more rigorously, the rotational relaxation time, indicates the mobility of the probe and thus reflects the ordering of the membrane core. The decrease in fluorescence anisotropy observed in this study indicates that ADR disorders or fluidizes the SPM. In SPM a thermotropic lipid phase separation occurring at 23.3° can be detected by the DPH fluorescence anisotropy (Fig. 5). ADR decreased the temperature of the lipid phase separation from 23.3° to 16°, and this is matched by a similar effect on the $(Na^+ + K^+)$-stimulated ATPase activity (Fig. 3). Such a depression in the temperature presumably reflects the interaction of the positive charged ADR with acidic phospholipids in the internal half of the membrane, and would be in accord with the effects of ADR on the transition temperatures of model lipid membranes [46]. It is, therefore, probable that, under *in vivo* conditions, ADR will interact with the internal surface of the plasma membrane, lowering the temperature of the lipid phase separation to around 16°. This would imply that a variety of cell surface phenomena sensitive to the membrane lipid structure will be regulated by the binding of ADR to the inner leaflet of the plasma membrane.

It is then of particular interest that ADR can inhibit $(Na^+ + K^+)$-stimulated ATPase and increase Ca^{2+}-stimulated ATPase activities of brain synaptosomal plasma membranes. Ca^{2+}-stimulated ATPase has a pivotal role in regulating the neurotransmitter synthesizing enzymes, and the phosphorylation of synaptic membrane and vesicle protein [47]. A constant intracellular concentration must be maintained in order to have a dynamic equilibrium of all these processes. Any disturbance in the cellular calcium homeostasis may lead to neuronal dysfunction. The basis for these observations would appear to be related to our observation that ADR is able to increase the bilayer fluidity and also to induce lipid peroxidation in cell plasma membranes *in vitro*. However, the activity of a variety of other plasma membrane enzymes, binding of neurotransmitters, conformation of receptors, may be affected by alterations in membrane lipid fluidity. It may be therefore, that the increase in lipid fluidity is an early key event in brain cytotoxicity induced by ADR.

Acknowledgements—This research was supported by grants from the Medical School, University of Athens (Grant Nos 69/1985 and 80/1985).

REFERENCES

1. G. Aubel-Sardon and D. Londos-Gagliardi, *Biochimie* **66**, 333 (1984).
2. N. R. Bachur, A. L. Moore, J. G. Bernstein and A. Liu, *Cancer Chemother.* **54**, 89 (1970).
3. P. M. Wilkinson and G. E. Mawer, *Br. J. clin. Pharmac.* **1**, 241 (1974).
4. E. A. Neuwelt, M. Pagel, P. Barnett, M. Glassberg and E. P. Frenkel, *Cancer Res.* **41**, 4466 (1981).
5. L. Bigott and Y. Olsson, *Acta Neurol. Scand.* **70** (suppl. 100), 55 (1984).
6. D. Van Derkooy, K. A. Zito and D. C. S. Roberts, *Neurosci. Lett.* **53**, 215 (1985).
7. R. J. Boegman, B. Scarth, L. Dragovic and D. M. Robertson, *Exp. Neurol.* **87**, 1 (1985).
8. F. Villani, E. Monti, F. Piccinity, L. Favalli, A. R. Dionigi and E. Lanza, *Biochem. Pharmac.* **35**, 1203 (1986).
9. S. A. Murphree, T. R. Tritton, P. L. Smith and A. C. Sartorelli, *Biochim. biophys. Acta* **649**, 317 (1981).
10. L. D. Mayer, M. B. Bally and P. R. Cullis, *Biochim. biophys. Acta* **857**, 123 (1986).
11. T. W. Robison and S. N. Giri, *Biochem. biophys. Res. Commun.* **136**, 745 (1986).
12. J. A. Siegfried, K. A. Kennedy, A. C. Sartorelli and T. R. Tritton, *J. biol. Chem.* **258**, 339 (1983).
13. G. Deliconstantinos, *Neurochem. Res.* **8**, 1143 (1983).
14. S. Tsakiris and G. Deliconstantinos, *Biochem. J.* **220**, 301 (1984).
15. G. Deliconstantinos and S. Tsakiris, *Biochem. J.* **229**, 81 (1985).
16. G. Deliconstantinos and L. Kopeikina-Tsiboukidou, First International Conference of Anticancer Research. Greece 26–30 October 1985. Abstr. 152, *Anticancer Res.* **5**, 587 (1985).
17. A. Papaphilis and G. Deliconstantinos, *Biochem. Pharmac.* **29**, 3325 (1980).
18. G. L. Miller, *Analyt. Chem.* **31**, 964 (1959).
19. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 375 (1925).
20. G. Deliconstantinos, K. Anastasopoulou and P. Karayiannakos, *Biochem. Pharmac.* **32**, 1309 (1983).
21. G. A. Bray, *Analyt. Biochem.* **1**, 279 (1960).
22. G. L. Ellman, D. Courtney, V. Andres and R. M. Featherstone, *Biochem. Pharmac.* **7**, 88 (1961).
23. K. M. Wilbur, F. Bernheim and O. W. Shapiro, *Archs Biochem.* **24**, 305 (1949).
24. Z. A. Placer, L. L. Cushman and B. C. Johnson, *Analyt. Biochem.* **16**, 359 (1966).
25. M. Shinitzky and Y. Barenholz, *Biochim. biophys. Acta* **515**, 367 (1978).
26. G. Deliconstantinos, L. Kopeikina-Tsiboukidou and S. Tsakiris, *Biochem. Pharmac.* **35**, 1633 (1986).
27. B. A. Johnson, M. S. Cheang and G. J. Goldenberg, *Cancer Res.* **46**, 218 (1986).
28. T. Bartfai, *Adv. Cycl. Nucleotide Res.* **10**, 219 (1979).
29. E. J. McMurchie and J. K. Raison, *Biochim. biophys. Acta* **554**, 364 (1979).
30. A. DiMarco, *Cancer Chemother. Rep. Part 1* **6**, 91 (1975).
31. E. Goormaghtigh and J. M. Ruysschaert, *Biochim. biophys. Acta* **779**, 271 (1984).
32. E. A. Lefrak, J. Pitha, S. Rosenheim and J. A. Gottlieb, *Cancer* **32**, 302 (1973).
33. B. S. Jortner and E. S. Cho, *Cancer Treat. Rep.* **64**, 257 (1980).
34. P. C. Merker, L. R. Lewis, M. D. Walker and E. P. Richardson, Jr., *Toxic. appl. Pharmac.* **44**, 191 (1978).
35. F. A. Philips. A. Gilladoga, H. Marquardt, S. S. Sternberg and P. M. Vidal, *Cancer Chemother.* **6**, 177 (1975).
36. C. F. McGraw, D. A. Nachshen and M. P. Blaustein, in *Calcium and Cell Function,* Vol. II (Ed. W. Y. Cheung), p. 81, Academic Press, New York (1982).
37. M. D. Houslay, I. Dipple and L. M. Gordon, *Biochem. J.* **197**, 675 (1981).
38. P. Ott, *Biochim. biophys. Acta* **822**, 375 (1985).
39. J. E. Rothman and J. Lenard, *Science* **195**, 743 (1977).
40. E. Goormaghtigh, P. Chatelain, J. Caspers and J. M. Ruysschaert, *Biochim. biophys. Acta* **597**, 1 (1980).
41. P. Chatelein, N. Reckinger and R. Roncucci, *Biochem. Pharmac.* **38**, 3677 (1979).
42. I. Dipple, L. M. Gordon and M. D. Houslay, *J. biol. Chem.* **257**, 1811 (1982).
43. C. E. Myers, W. P. McGuire, R. H. Liss, I. Ifrim, K. Grotzinger and R. C. Young, *Science* **197**, 165 (1977).
44. J. C. Gilbert and A. H. Savas, *Archs Int. Pharmac.* **263**, 189 (1983).
45. M. T. Curtis, D. Gilfor, J. L. Farber, *Archs Biochem. Biophys.* **235**, 644 (1984).
46. T. R. Tritton, S. A. Murphree and A. C. Sartorelli, *Biochem. biophys. Res. Comm.* **84**, 802 (1978).
47. H. Schulman and P. Greengard, *Nature, Lond* **271**, 478 (1978).