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Interaction of adriamycin with human erythrocyte membranes. Role of the negatively charged phospholipids

Arlette Garnier-Suillerot and Liliane Gattegno

Laboratoire de Chimie Bioinorganique (UA CNRS 198) and Laboratoire de Biologie Cellulaire, UFR de Santé, Médecine et Biologie Humaine, Université Paris-Nord, Bobigny (France)

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The interaction of the antitumor compound adriamycin with human erythrocyte membranes, used as models of target cell membranes, has been studied using circular dichroism measurements. In order to elucidate the nature of the sites involved in the electrostatic interaction between adriamycin and erythrocyte membranes, its interaction with the following macromolecular systems was studied: phosphatidylserine-containing small unilamellar vesicles (SUV), prepared from total lipid extracts of erythrocytes, sialic acid-depleted erythrocyte ghosts and mucopolysaccharides. We have shown that the interaction between adriamycin and carboxylate groups is very weak and that negatively charged phosphate groups, in the case of membranes, or sulfate groups, in the case of mucopolysaccharides, are responsible for the prime interaction of adriamycin with these macromolecular systems.

Introduction

The anthracycline antibiotic, adriamycin, is an important antitumor agent with marked activity against a wide variety of human neoplasms [1,2]. The nature of the cellular locus of action of this drug is controversial as it is capable of physical interaction with both DNA and membranes [3,4]. From studies on the mechanism of anthracyclines action in vivo, nuclear DNA has usually been considered the prime target for this antibiotic's antineoplactic action [3]. However, powerful evidence that its cytotoxicity is expressed through a membrane-mediated effect comes from studies using polymer-immobilized adriamycin which does not enter the cell [5].

In any case, the interaction of adriamycin with cell membranes is of prime importance, either because membranes are the targets for the cytotoxic action of the drug, or because adriamycin must cross the membrane in order to reach the target, e.g., DNA, inside the cell.

The interaction of adriamycin with DNA has been largely studied. It has been shown that at least two types of binding occur between this drug and DNA. In one type, the drug molecule intercalates between the base pairs of DNA with the ammonium group of the sugar moiety involved in an electrostatic interaction and H-binding interaction with a neighboring phosphate group [6]. This occurs at a 'high' nucleotide-to-drug molar ratio,

Abbreviations: SUV, small unilamellar vesicles; CD, circular dichroism; RBC, red blood cells; PBS, phosphate-buffered saline; PS, phosphatidylserine; PC, phosphatidylcholine.

Correspondence: A. Garnier-Suillerot, Laboratoire de Chimie Bioinorganique (UA CNRS 198), UFR de Santé, Médecine et Biologie Humaine, Université Paris-Nord, 74, rue Marcel Cachin, 93012 Bobigny Cedex, France.

i.e., more than 6. The other type of binding appears at 'low' nucleotide-to-drug molar ratios once the primary intercalative binding sites have been saturated; the molecule of drug binds to DNA by electrostatic interactions involving the DNA phosphate group and the amino sugar of the drug [7,8].

The interaction of adriamycin with membranes appears to be more complex. The interaction of adriamycin with cell plasma membranes and with membranes of intracellular components has been reviewed [9]. Recently, the study of the adriamycin interaction with negatively charged model membranes has revealed the presence of two different binding sites (I and II). The negative charges were provided by cardiolipin, phosphatidic acid and/or or phosphatidylglycerol [10,11]. In site I, the amino sugar of adriamycin is bound to the ionized phosphate and the dihydroxyanthraquinone lies outside the bilayer. In site II, the amino sugar is still bound to the phosphate, but the dihydroxyanthraquinone lies outside the bilayer. A recent study by Griffin et al. [12] has corroborated the existence of at least two binding sites. In any case, it appears that, as in the case of DNA, the first type of interaction is an electrostatic one.

The interaction of adriamycin with natural cell membranes is much more complex, as negative charges, responsible for the electrostatic interaction with adriamycin, could be provided not only by ionized phosphate groups of phospholipids, but also by carboxylate groups from sialic acid and from amino acids. It has thus been proposed that the interaction of adriamycin with erythrocyte membranes would occur at the level of spectrin [13].

The aim of our work was to determine the nature of the sites involved in the electrostatic interaction between adriamycin and cell membrane. For this purpose, we used erythrocyte membrane, which has been well characterized, as a model of target cell membrane. We studied the interaction of adriamycin with either intact or modified red cell ghosts in order to remove some negatively charged groups. The interaction of adriamycin with individual components of ghost have also been studied. These interactions were monitored using absorption and circular dichroic (CD) spectroscopy. Thus, we show that the electrostatic interactions of adriamycin with this membrane occurs mostly at the level of the ionized phosphate groups and that the carboxylate groups are not, or only very slightly involved. We corroborated the lack of interaction, or at least the weakness of the electrostatic interaction between adriamycin and carboxylate groups, by studying the interaction of adriamycin with mucopolysaccharides, showing that this interaction only occurs at the level of ionized sulfate groups.

This study could be relevant to two recent observations: first, in most cases, the cell's resistance adriamycin can be correlated with the phosphorylation of a membrane protein [14], second, chemotherapeutic agents, such as anthracyclines, which commonly produce drug resistance inhibit the activity of protein kinase C and, furthermore, the mode of inhibition appears to be through interference with the activation of the enzyme by phosphatidylserine [15].

Materials and Methods

Purified adriamycin was kindly provided by Laboratoire Roger Bellon (France). Concentrations were determined by diluting aqueous stock solutions to approx. 10^{-5} M and using $\epsilon_{480} = 11\,500\ M^{-1} \cdot cm^{-1}$ [16]. As anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just before use. L-α-Phosphatidyl-L-serine (PS) from bovine brain, L-α-phosphatidylcholine (PC) from egg yolk type V-E and trypsin were purchased from Sigma Chemical Co, *Vibrio cholerae* neuraminidase (VCN) was from Behringwerke and pronase was from Calbiochem Heparin sodium salt and chondroitin sulfate were purchased from Sigma and Merck, respectively. Their S content was analyzed as a percentage of the dry matter; values of S equal to 10.7 for heparin and 5.5 for chondroitin sulfate were obtained. This yield a mean value of 0.90 and 2.05 sulfonic residues per subunit for chondroitin sulfate and heparin, respectively. All other reagents were of the highest quality available, and deionized, double-distilled water was used throughout the experiments. Unless otherwise stated, buffer solutions were 0.01 M Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) and 0.1 M KCl (pH 7.2).

Absorption spectra were recorded on a Cary 219 spectrophotometer and circular dichroism spectra were recorded on a Jobin Yvon Mark V dichrograph. In the figures, $\Delta\epsilon$ is the differential molar dichroic absorption coefficient ($cm^{-1} \cdot M^{-1}$). The spectra of the suspensions of vesicles without adriamycin were subtracted from those with adriamycin. They consisted of a plain curve of increasing $\Delta\epsilon$, with decreasing wavelengths, originating from the light scattering of vesicles. We checked that the light scattering membrane background between 200–800 nm, was not modified by the addition of adriamycin. The absence of artefact due to light diffusion in the CD study was ascertained by checking that the CD spectral pattern depended neither on the cuvette pathlength (varied for this control between 0.02 cm and 0.2 cm) nor on the distance between the sample cell and the detector (varied between 0 and 10 cm from the end-window of the photomultiplier tube).

Unilamellar phospholipid vesicles. Small unilamellar vesicles (SUV) were prepared according to Newman and Huang [17]. Sonication was performed at room temperature and under nitrogen. SUV were also prepared from total lipids extracted from membranes by a mixture of chloroform and methanol.

Erythrocyte ghosts. Erythrocytes (RBC), drawn from human healthy donors at the Seine Saint Denis blood bank and collected on adenosine citrate dextrose, were washed three times using $1000 \times g$ centrifugation in phosphate buffer saline and the buffy coat was discarded after each centrifugation. Packed erythrocytes were hemolyzed in a 9-fold volume of 10 mM Tris/plus 0.1 mM ethylenediaminetetraacetate (EDTA) HCl buffer (pH 7.4). Membranes were sedimented by centrifugation at $20000 \times g$ for 20 min, then washed four times by centrifugation at $20000 \times g$ for 20 min in a 9-fold volume of Tris-EDTA buffer. White membranes were thus obtained as described in Ref. 18. Protein concentration was determined according to the Lowry procedure [19]; 10^{10} cells yielded a mean of 4.6 ± 0.6 mg protein (ten experiments). The colorimetric ammonium ferrothiocyanate method [20] was used to determine membrane phospholipids. A mean value of $2.5 \cdot 10^8$ phospholipids per cell was thus obtained.

In some experiments, prior to the ghost preparation, the washed RBC were desialylated by the action of neuraminidase: 1 volume of packed RBC were incubated for 90 min at 37°C with gentle shaking with 60 mIU (international units) *V. cholerae* neuraminidase per $8 \cdot 10^9$ RBC in 2 volumes of PBS as previously described [21]. In each experiment, an aliquot of 60 mU of *V. cholerae* neuraminidase, dissolved in CH_3COONa, 0.05 M; NaCl, 0.15 M; $CaCl_2$, 9 mM (pH 5.5) was diluted in 2 ml PBS prior to the ghost preparation, as described above. Free sialic acid was determined in the supernatant of the centrifugation by the thiobarbituric reaction as described by Warren [22].

It has already been shown [21] that this experimental procedure allows the release of a mean of $94 \pm 3.5\%$ of the total sialic acid from human RBC in accordance with Nordt et al. [23,24]. In the present study, membranes from intact RBC contain 42 μg of sialic acid per mg of protein, whereas membrane from desialylated RBC contain 3.1 μg of sialic acid per mg of protein.

In other experiments prior to the ghost preparation, the washed RBC were incubated for 1 h at 37°C with either pronase or trypsin at an enzyme concentration of 0.5 mg per $8 \cdot 10^9$ RBC in 2 volumes of PBS. It has been shown previously that under these experimental conditions, surface glycopeptides are released from the RBC [25]. Pronasic glycopeptides contains about 2/3 of the neutral hexose, fucose, hexosamine and sialic acid content of the erythrocyte membranes, whereas tryptic glycopeptides contains less than 1/2 of the hexose and sialic acid content from the RBC [25]. In the present study, membranes from pronase-treated RBC contain 14 μg of sialic acid per mg protein, whereas membrane from trypsin-treated RBC contain 22 μg of sialic acid per mg protein.

In some experiments, erythrocytes ghosts were subsequently fractionated into glycoproteins on one hand, and lipids and glycolipids on the other, by extraction of the membranes with chloroform/methanol (2:1, v/v) as described by Hamaguchi and Cleve [18]. The fractions were then concentrated under a stream of nitrogen. The presence of glycoproteins in the upper fraction was assessed by polyacrylamide gel electrophoresis. In other experiments, spectrin was extracted from the membranes by a 30-min incubation at 37°C of 1

vol. membrane with a 20 vol. soiution of 0.1 mM EDTA, 0.5 mM mercaptoethanol and 0.03 mM phenylmethylsulfonylfluoride (pH 9). The spectrin-depleted membranes were spun at $17000 \times g$ for 20 min and the supernatant was collected; the membranes were washed once with the Tris-EDTA buffer (pH 7). Depletion of spectrin was assessed by polyacrylamide gel electrophoresis.

Results

Interaction of adriamycin with human erythrocyte membranes: circular dichroism study

The interaction of adriamycin with erythrocyte ghosts was followed using circular dichroism spectroscopy. In order to avoid artefacts due to the interaction of adriamycin with any heparin which may have remained in the ghost preparation, the ghosts used in that study were prepared form blood collected on adenosine/citrate/dextrose (ACD).

The interaction of adriamycin with ghosts was immediate and the same CD spectrum was observed whatever the length of the incubation period, ranging from 1 min to 1 h.

Interaction of adriamycin with intact erythrocyte ghosts. The CD spectrum adriamycin is largely dependent on the association state of the drug [26,27]. At pH 7.2 and 10^{-5} M, adriamycin is fully in the monomeric form and the visible CD spectrum consists of one positive band at 465 nm. When the concentration is increased, this band splits into a doublet, characteristic of the associated dimeric form, with a positive band at 460 nm and a negative one at 530 nm. The amplitude of the negative band is indicative of the number of molecules in the dimeric form.

In the following experiments, concentrations of adriamycin ranging from $2 \cdot 10^{-4}$ to $4 \cdot 10^{-4}$ M were used: at these concentrations the percentage of dimer varied from 60 to 80% Fig. 1 shows the CD spectrum of $2.9 \cdot 10^{-4}$ M adriamycin in the presence of increasing amounts of erythrocyte ghosts. When the concentration of phospholipids increased up to about $0.9 \cdot 10^{-3}$ M (i.e., about $2 \cdot 10^9$ cells/ml), one different spectral pattern was obtained suggesting the occurrence, in this range of concentrations, of one type of binding site of adriamycin to ghosts. Due to light scattering by

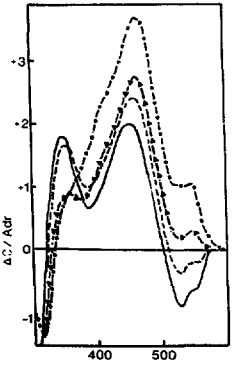

Fig. 1. Circular dichroism spectra of adriamycin in the presence of erythrocyte ghosts. Experimental conditions: 290 μM adriamycin (Adr), Hepes buffer, (pH 7.2) and 0.1 M KCl. The molar ratio of phospholipids to adriamycin was 0 (———), 0.85 (— — —), 1.9 (▲———▲), 3.9 (●———●).

the ghosts, it was impossible to obtain reliable data at molar ratios of phospholipids to adriamycin of greater than 6. It was, thus, impossible to show the existence of a second type of binding site of adriamycin to ghosts as has previously been achieved with negatively charged phospholipid-containing small or large unilamellar vesicles [10]. We checked that the addition of phosphate ions did not modify significantly the interaction of adriamycin with ghosts. The slight modification observed was assigned to the variation of ionic strength.

The variation of $\Delta\epsilon$ at 530 nm as a function of the molar ratio of phospholipids to adriamycin was used to monitor the fixation of adriamycin to ghosts (Fig. 2). At 530 nm, the values of $\Delta\epsilon$ are $\Delta\epsilon_m = +0.2$ and $\Delta\epsilon_d = -1.3$ for free adriamycin in the monomeric and the dimeric form, respectively, and $\Delta\epsilon_b = +0.8$ for bound adriamycin. For each value of the phospholipid-to-adriamycin molar ratio, $\Delta\epsilon$ at 530 nm is the sum of three terms $\Delta\epsilon = 1/C_t(\Delta\epsilon_d C_d + \Delta\epsilon_m C_m + \Delta\epsilon_b C_b)$ where C_d, C_m, C_b and C_d are the concentrations of adriamycin in the dimeric and monomeric state,

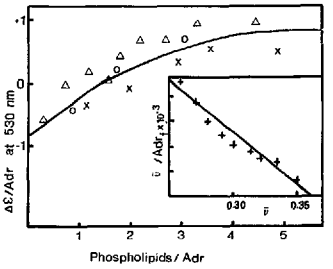

Fig. 2. Circular dichroism spectra of adriamycin in the presence of erythrocyte ghosts. $\Delta\epsilon$ values at 530 nm are plotted as a function of the molar ratio of phospholipids to adriamycin. Experimental conditions: 300 μM adriamycin (Adr), Hepes buffer, (pH 7.2) and 0.1 M KCl. Three independent experiments were performed. Inset: Scatchard plot analysis of the binding data of adriamycin to erythrocytes ghosts. $\bar{\nu}$, adriamycin bound per phospholipid; Adr_f, free adriamycin concentration.

bound adriamycin and total adriamycin, respectively. The equilibrium of dimerization of adriamycin is $K_d = C_d/2C_m^2$, $K_d = 1.2 \cdot 10^4$ [10]. Consedering that $C_t = Cm + C_d + C_b$, the following equation is obtained $2K_d(\Delta\epsilon_d - \Delta\epsilon_b)C_m^2 + (\Delta\epsilon_m - \Delta\epsilon_b)C_m + (\Delta\epsilon_b - \Delta\epsilon)C_t = 0$. Its resolution yielded for each value of $\Delta\epsilon$ (i.e., for each value of the phospholipid-to-adriamycin molar ratio) the corresponding value of C_m. The concentration of free adriamycin, $C_f = C_m + C_d$, and bound Adr. C_b, as a function of the phospholipid-to-adriamycin molar ratio were thus calculated. The binding data were thus analyzed by Scatchard plots (Fig. 2, inset).

In order to gain better insight into the possible binding sites of adriamycin at erythrocyte ghosts, these have been modified in different ways.

Interaction of adriamycin with neuraminidase-, trypsin- or pronase-treated ghosts. The modifications of the CD spectrum of adriamycin in the presence of ghosts treated with either neuraminidase, in order to remove free sialic acid, or trypsin or pronase, in order to remove sialoglycopeptides, were qualitatively as well as quantitatively identical to those observed with intact cells.

Interaction of adriamycin with spectrin-depleted ghosts. Here again the same CD spectral modifications as those found with intact ghosts were observed, strongly suggesting that the interaction of adriamycin with spectrin, if present, is very weak.

Direct binding measurements of adriamycin to intact erythrocyte ghosts, pronase-treated ghosts and spectrin-depleted ghosts were performed by determining the concentration of free drug that remained in the supernatant after centrifugation. Under conditions similar to those described above, we determined that in the three cases, the same amount of adriamycin was bound per erythrocyte ghost.

Circular dichroism studies of the interaction of adriamycin with various components of erythrocyte ghosts

Interaction of adriamycin with phosphatidylserine-containing SUV. Phosphatidylserine accounts for about 16% of the total phospholipids present in the erythrocyte ghosts [28]. It bears one negatively charged phosphate group and, from this point of view, it constitutes the essential part of the negatively charged groups containing phospholipids. The other negatively charged phospholipids of erythrocyte ghosts are phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidic acid (PA) which account for 1.2, 0.8, 1.4 and 2.2% of the total phospholipids present, respectively [28]. It was, thus, relevant to determine the binding of adriamycin to PS-containing SUV.

In the following experiments, concentrations of adriamycin ranging from $2 \cdot 10^{-4}$ to $4 \cdot 10^{-4}$ M were used: at these concentrations, the percentage of dimer varied from 60% to 80%. Fig. 3 shows the CD spectrum of $3 \cdot 10^{-4}$ M adriamycin in the presence of increasing amounts of PS-containing SUV. The SUV were prepared with PC and PS in a molar ratio of 10 : 1. When the molar ratio of PS to adriamycin was varied from 0 to 2, two different spectral patterns were obtained. As can be seen in Fig. 3, when the molar ratio of PS to adriamycin was increased from 0 to about 1, the CD spectrum changed from that typical of adriamycin in the dimeric form to another form (hereafter labeled I) which exhibited a positive band at 470 nm and a shoulder at 530 nm. A further increase of the molar ratio of PS to

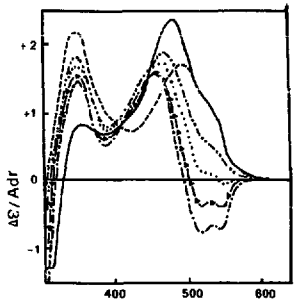

Fig. 3. Circular dichroism spectra of adriamycin in the presence of PS-containing SUV. Experimental conditions; 300 μM adriamycin (Adr), Hepes buffer (pH 7.2) and 0.1 M KCl. The SUV were prepared with. PC and PS in a molar ratio 10:1. The molar ratio of PS to adriamycin was 0.06 (·–·–·), 0.17 (▲———▲), 0.39 (·····), 0.62 (··–···–), 1.07 (———) 3.30 (— — —).

adriamycin gave rise to a shift of this band from 470 nm to 500 nm and to a decrease of its amplitude. This CD pattern will thereafter be labelled II. These two CD patterns strongly suggest the existence of two different types of binding site of adriamycin to PS-containing SUV. Analogous data have previously been obtained with negatively charged phospholipid-containing SUV, i.e., cardiolipin-, phosphatidic acid- and phosphatidylglycerol-containing SUV and it has been shown that the two CD patterns were characteristic of two types of adriamycin binding to the negatively charged SUV: type I in which electrostatic interactions occur between the negatively charged phosphate and the positively charged amino group of the sugar moiety of adriamycin, and type II in which the dihydroxyanthraquinone moiety is, in addition, embedded in the phospholipid bilayer. The CD spectral modification of adriamycin being the same with the four negatively charged phospholipid-containing SUV, we can infer that the same two types of binding of adriamycin to these SUV occur. The value of $\Delta\epsilon$ at 540 nm yielded quantitative information for adriamycin binding site I, and its variation as a function of the molar ratio PS/adriamycin has been plotted in Fig. 4. Taking into account the dimerization equilibrium of free adriamycin ($K_d = 1.2 \cdot 10^4$) and the data of Fig. 4, the concentrations of bound adriamycin (I plus II) and free adriamycin as a function of the PS-to-adriamycin molar ratio were calculated. The binding data were analyzed using Scatchard plots. Since two different binding sites are involved, the plot should exhibit two slopes. In fact, only one slope is observed (Fig. 4, inset). This can easily be explained by the fact that the plot was drawn at a molar ratio of PS to adriamycin in the range 0.1–1, where binding site II, which is clearly the stronger, is saturated. The slope gives the stability constant for binding site I, $K_1 = 1.7 \cdot 10^5$ and the intercept $n_1 + n_{II} = 1.05$, where n_1 and n_{II} are the number of adriamycin molecular bound per PS in site I and site II, respectively.

Interaction of adriamycin with pure PC SUV. Experiments similar to those described above were performed in the absence of PS. The CD spectral pattern corresponding to site I was not observed. Nevertheless, some modifications of the CD spectrum were observed: a simultaneous decrease of the amplitude of the bands at 528 and 460 nm. This decrease was rather smooth, and the plots of

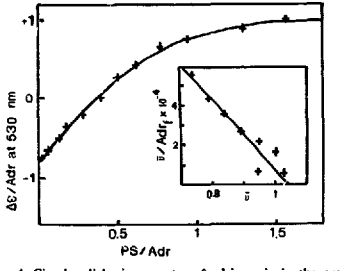

Fig. 4. Circular dichroism spectra of adriamycin in the presence of PS-containing SUV. $\Delta\epsilon$ values at 520 nm have been plotted as a function of the molar ratio of PS to adriamycin (Adr). Experimental conditions: 300 μM Adr, Hepes buffer (pH 7.2) and 0.1 M KCl. The SUV were prepared with PC and PS in 9 molar ratio of 10:1, $t = 22°C$. Inset: Scatchard plot analysis of the binding data of adriamycin to PS-containing SUV. $\bar{v}$, adriamycin bound per PS, Adr_f, the free adriamycin concentration.

$\Delta\epsilon$ at 528 and 460 nm as a function of the molar ratio of PC to adriamycin did not attain a plateau even at molar ratios higher than 30. These modifications were taken as an indication that adriamycin interacts with PC; this binding site was labelled II′; its spectral pattern is reminiscent of that of adriamycin bound to site II. This site is weak as, at a concentration of 300 μM adriamycin, a PC-to-adriamycin molar ratio equal to about 30 is required to ensure 50% binding of Adr to site II′.

Interaction of adriamycin with erythrocyte lipid-containing SUV. SUV were prepared from total lipid extract of erythrocytes. The absence of proteins from the preparation was checked using the Lowry procedure [19].

In a typical experiment increasing concentrations of SUV were added to a $3 \cdot 10^{-4}$ M adriamycin solution. The molar ratio of the total phospholipid concentration to adriamycin was varied from 0.5 to 12. For the lower values of the molar ratio, i.e., up to about 8, we observed the formation of a precipitate and it was impossible to record CD spectra. Such a precipitate was not observed with ghosts. The amount of precipitate formed was checked using absorption at 700 nm. The amount of precipitate reached a maximum value at a molar ratio of phospholipid to adriamycin equal to about 5. However, at molar ratios higher than 8, no precipitate formation occurred and the CD spectrum is shown in Fig. 5. As can be seen, this CD spectrum is closely related to that obtained with erythrocyte ghosts: both spectra exhibit a positive band at 465 nm with shoulders at 415 and 530 nm and a negative band at 315 nm.

Interaction of adriamycin with mucopolysaccharides: circular dichroism study

Fig. 6 shows the CD spectrum of $4.5 \cdot 10^{-5}$ M adriamycin in the presence of increasing amounts of chondrointin sulfate. Due to the formation of precipitate, it was impossible to use concentrations of Adr higher than $8 \cdot 10^{-5}$ M. The molar ratio of 1 disaccharide unit of chondroitin sulfate per adriamycin was increased from 0 to 2. At the concentration of adriamycin used, the percentage of dimer was 25%. When the molar ratio of disaccharide unit to adriamycin was varied from 0 to 2, one spectral pattern was obtained. As can be seen

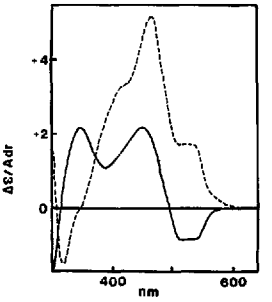

Fig. 5. Circular dichroism spectra of adriamycin (Adr) in the presence of SUV prepared from total lipid extract of erythrocyte. Experimental conditions: 300 μM adriamycin, Hepes buffer (pH 7.2) and 0.1 M KCl. The molar ratio of phospholipids to adriamycin was 0 (———), 10 (— — —).

in Fig. 6, when the molar ratio of disaccharide unit to adriamycin was varied from 0 to about 1, the CD spectrum changed from that typical of

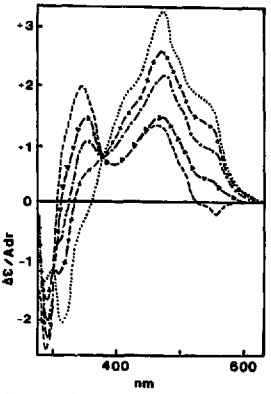

Fig. 6. Circular dichroism spectra of adriamycin (Adr) in the presence of chondroitin sulfate. Experimental conditions: 46 μM adriamycin, Hepes buffer, (pH 7.2) and 0.1 M KCl. The molar ratio of chondroitin sulfate unit to adriamycin was 0 (— — —), 0.24 (▲———▲), 0.47 (·—·—·), 0.71 (×———×), 1.9 (·····).

adriamycin to one which exhibited a positive band at 465 nm and a shoulder at 540 nm; the positive band at 350 nm was replaced by a negative one at 320 nm. Very well defined isodichroic points are present at 380 and 305 nm. A further increase of the molar ratio of disaccharide units to adriamycin up to 2 did not give rise to further modification of the CD spectral pattern. This suggest the existence of only one type of binding site of adriamycin to chondroitin sulfate. The variation of $\Delta\epsilon$ at 320 nm as a function of the disaccharide unit-to-adriamycin molar ratio was used to monitor the fixation of adriamycin to this site (Fig. 7). At this wavelength, the values of $\Delta\epsilon$ are $+1$ and -0.3 for free adriamycin in the monomeric and dimeric forms, respectively. Thus, taking into account the dimerization equilibrium of free adriamycin ($K_d = 1.2 \cdot 10^4$) and the data of Fig. 6, the concentrations of bound and free adriamycin as a function of the disaccharide unit-to-adriamycin molar ratio were calculated. The binding data were analyzed by Scatchard plots. The slope gives the stability constant for the binding site, $K = 6.7 \cdot 10^5 \pm 1.5 \cdot 10^5$ and the intercept $n = 1,.1$ (Fig. 7).

Similar experiments were performed with heparin. A spectral pattern similar to that described above was obtained with the same isodichroic points. The analysis of the binding data by Scatchard plots gives a stability constant $K = 4.7 \cdot 10^5 \pm 1.5$ and $n = 2.1$.

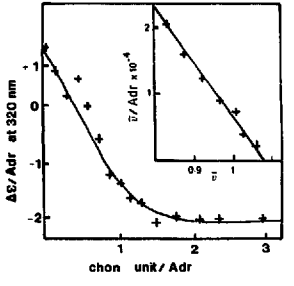

Fig. 7. Circular dichroism spectra of adriamycin (Adr) in the presence of chondroitin sulfate. $\Delta\epsilon$ at 320 nm has been plotted as a function of the molar ratio of chondroitin sulfate (chon) unit to adriamycin. Experimental conditions: 46 μM adriamycin, Hepes buffer (pH 7.2) and 0.1 M KCl. Inset: Scatchard plot analysis of the binding data of adriamycin to chondroitin sulfate. $\bar{\nu}$, adriamycin bound per chondroitin sulfate unit; Adr_f, free adriamycin concentration.

Discussion

Recently, several investigations have focussed on the interaction of adriamycin with synthetic plasma and mitochondrial membranes. The adriamycin binding to synthetic and mitochondrial membranes was investigated using resonance energy transfer between these drugs and two fluorescence probes, diphenylhexatriene and tryptophan [29]. The binding of adriamycin to synthetic membranes was also investigated using fluorescence anisotropy of the anthracyclines [30–32], fluorescence quenching of membrane-bound drug by membrane-impermeable iodide [32–34]. Both techniques have provided information on the relative location and dynamics of the drug within the membrane. A third approach, using circular dichroism has examined changes in anthracycline conformation and self-association as a function of binding of the drug to the membrane [10]. This study focussed on the point that the first interaction which takes place between adriamycin and DNA on the one hand and adriamycin and negatively charged phospholipids-containing vesicles on the other is an electrostatic one. The further interaction involving either the intercalation of the dihydroxyanthraquinone moiety of adriamycin between base pairs of DNA or the embedding of this moiety into the vesicle bilayer.

In the case of DNA, the only negatively charged groups which can interact with the positively charged amino acid of adriamycin are phosphate. However, in the case of membrane this is less obvious, as the negative charges are provided by phosphate and carboxylate groups. The data reported above show that the prime interaction between adriamycin and membrane, which is of an electrostatic nature, involved mostly the negatively charged phosphate and that the interaction with carboxylate is too weak to be detected using our techniques.

The weakness of the interaction between adriamycin and carboxylate is clearly detected in the mucopolysaccharide-adriamycin system which

is a relatively simple system. From the data reported above, we can infer that the number of adriamycin binding sites per disaccharide unit are 2.1 and 1.1 for heparin and chondroitin sulfate, respectively.

In each case, this represents, the average number of sulfate groups present in each subunit of the mucopolysaccharide. This strongly suggest that sulfate groups are the only binding site for adriamycin and that the participation of the carboxylate groups, if it occurs, is very small. The mean value for the stability constant is $K = (6 \pm 2) \cdot 10^5$. These data are slightly at variance with those previously obtained by Menozzi and Arcamone [35].

The weakness of the interaction between adriamycin and carboxylate is also detected in the interaction of adriamycin with pure PC SUV showing that at a concentration of 300 μM adriamycin, a molar ratio PC to adriamycin equal to about 30 is required to bind 50% of the drug.

In the case of erythrocyte membranes, the lack of interaction of adriamycin with carboxylate is clearly shown by the observation that the spectral modifications are quantitatively and qualitatively the same, with either intact ghosts or neuraminidase- or trypsin-treated ghosts, i.e., deprived of 50–95% of their sialic acid content. On the other hand, the observation that the same spectral pattern was obtained with either intact ghosts or spectrin depleted-ghosts allows us to rule out the spectrin as the target of adriamycin interaction with erythrocyte ghosts.

Finally, the spectral modifications observed through adriamycin interaction with intact ghosts can be obtained through adriamycin interaction with SUV prepared from total lipid extracts of erythrocytes and with phosphatidylserine-containing SUV. These data strongly suggest that the electrostatic interaction occurs at the negatively charged-phospholipid level only. This is corroborated by quantitative analysis of the binding of adriamycin to ghosts and to SUV prepared from total lipid extracts of erythrocytes. Considering the data of Ferrell and Huestis [28], we can estimate the percentage of negatively charged phosphate groups per phospholipids. One can estimate that phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4,5-phosphate, phosphatidyl inositol and PS bear 3,2,1 and 1 negatively charged phosphate groups, respectively. This yields a total of 27% of once negatively charged phosphate group per total phospholipids.

Our data concerning the interaction of adriamycin with SUV, from total phospholipid extracts from ghosts, have shown that a maximum precipitate occurs at a phospholipid-to-adriamycin molar ratio equal to about 5, which yields a molar ratio of adriamycin negatively charged phosphate groups equal to about 0.8. We can tentatively suggest that the precipitation occurred when the entity SUV-adriamycin is neutral, i.e., when one positively charged adriamycin is bound to one negatively charged phosphate. We wish to emphasize that this is only a tentative explanation, as such a precipitation is not observed with other phospholipid-containing systems studied here.

In the case of intact erythrocyte ghosts, Scatchard plots gave an intercept $n = 0.35$ (Fig. 2 inset) indicating the fixation of 0.35 adriamycin per phospholipid. Considering that 27% of the phospholipids are negatively charged. One can estimate a mean value of 1.3 adriamycin per negatively charged phosphate with an apparent equilibrium constant $K = 3. \cdot 10^5$.

For the sake of convenience, all the data concerning the amount of adriamycin bound per negatively charged group (i.e., sulfate or phos-

TABLE I

INTERACTION OF ADRIAMYCIN WITH NEGATIVELY CHARGED MOLECULAR SYSTEMS

Systems	Adriamycin bound per negatively charged phosphate	K ($\times 10^5$)
SUV (PC-PS)	1.05	1.7
SUV (total phospholipids from ghosts)	0.75	–
Intact ghosts	1.3	3
Sialic acid-deprived ghosts	1.3	3
	Adriamycin bound per negatively charged sulfate	
Chondroitin sulfate	1.1	6.7
Heparin	2.1	4.7

K, apparent equilibrium constant.

phate) as well as the apparent equilibrium constant are tabulated in Table I.

All these studies could be relevant for several types of observation. First, concerning the interaction of adriamycin with membranes of normal cells, our data show that the primary interaction is specific and occurs at the level of negatively charged phosphate groups. Second, concerning the interaction of adriamycin with membranes of drug-resistant cells, it has been shown that drug accumulation in resistant cells is in most cases, modulated by phosphorylation of a plasma membrane glycoprotein [14], which means plasma membrane alterations with the appearance of negatively charged phosphate groups. On the other hand, very recently it was shown that the resistance of cells to adriamycin could be related to an inhibition of protein kinase C by this drug [15]. The dependence of protein kinase C activity on phospholipids and calcium has been well documented [36]. Phosphatidylserine appears most effective [37]. It has thus been suggested that the binding of adriamycin to PS could be responsible for the inhibition of protein kinase C activity by adriamycin [15]. On the other hand, although PS has been postulated most often as the phospholipid necessary to activate protein kinase C, it has been shown that other negatively charged phospholipids such as cardiolipin and phosphatidic acid can also activate it [38].

In this context, our data provide quantitative information that may guide further investigations designed to elucidate the mechanism by which cells become resistant to a wide array of toxic substances.

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