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Proton Exchange in DNA–Luzopeptin and DNA–Echinomycin Bisintercalation Complexes: Rates and Processes of Base-Pair Opening†

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ABSTRACT: Imino proton exchange studies are reported on the complexes formed by bisintercalation of luzopeptin around the two central A·T pairs of the d(CCCATGGG) and d(AGCATGCT) duplexes and of echinomycin around the two central C·G pairs of the d(AAACGTTT) and d(CCAAACGTTTGG) duplexes. The depsipeptide backbone of the drugs occupies the minor groove of the complexes at the bisintercalation site. The exchange time of the amide protons of the depsipeptide rings provides a lower estimate of the complex lifetime: 20 min at 15 °C for the echinomycin complexes and 4 days at 45 °C for the luzopeptin complexes. The exchange time of imino protons is always shorter than the complex lifetime. Hence, base pairs open even within the complexed oligomers. For the two base pairs sandwiched between the aromatic rings of the drug, the base-pair lifetime is strongly increased, and the dissociation constant is correspondingly reduced. Hence, the lifetime of the open state is unchanged. This suggests similar open states in the free duplex and in the complex. In contrast to the sandwiched base pairs, the base pairs flanking the intercalation site are not stabilized in the complex. Thus, the action of the bisintercalating drug may be compared to a vise clamping the inner base pairs. Analysis suggests that base-pair opening may require prior unwinding or bending of the DNA duplex.

Luzopeptin and echinomycin are bifunctional intercalating agents of related structure which possess antimicrobial and antitumor activities (Rose et al., 1983; Ward et al., 1965). They consist of two aromatic rings attached by a cyclic (luzopeptin) or a bicyclic (echinomycin) peptidic linker (Chart I). Both drugs bisintercalate in DNA, with the cyclic peptidic linker in the minor groove.

The quinoline rings of luzopeptin bisintercalate at the d(C–A) d(G–T) step of the d(CATG) (Zhang & Patel, 1991) and d(GCATGC) (Searle et al., 1989) sequences, spanning the two central A·T pairs. All the base pairs of the d(-CATG-) sequence retain the Watson–Crick alignment.

The quinoxaline rings of echinomycin span the two C·G pairs of the d(-ACGT-) and d(-TCGA-) sequences. The A·T pairs flanking the intercalation sites may adopt the Hoogsteen

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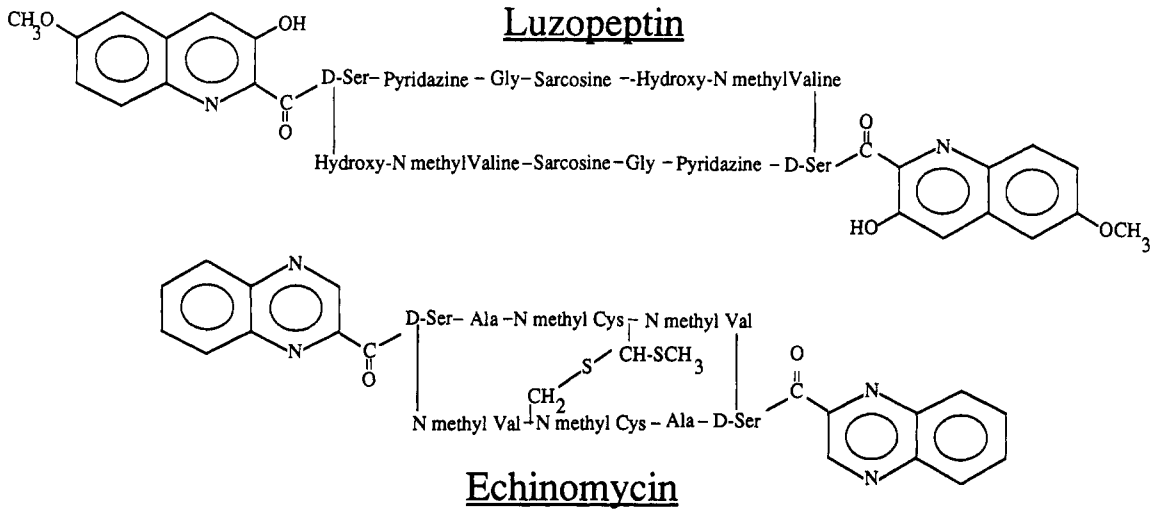
Chart I



pairing as in the complexes of d(ACGT) (Gao & Patel, 1987), d(ACGTACGT) (Gilbert et al., 1989), and d(CGTACG) (Ughetto et al., 1985), or they may retain the Watson–Crick pairing as in the complexes of d(TCGA) (Gao & Patel, 1987), d(AAACGTTT) (Gao & Patel, 1988), d(TCGATCGA) (Gilbert & Feigon, 1991).

In the present work, we study the kinetics of base-pair opening in luzopeptin and echinomycin complexes, using NMR of exchangeable protons. Four complexes are examined: those formed by luzopeptin with d(CCCATGGG) and with d(AGCATGCT) and those formed by echinomycin with d(AAACGTTT) and with d(CCAAACGTTTGG).

One goal of this study is to characterize the kinetic parameters of base-pair motion in complexes of DNA with bis-intercalating drugs, in the perspective of the biochemical activity of these antitumor drugs. Another goal is to contribute to the experimental characterization of base-pair opening pathways and of the open state of the base pairs.

On the basis of imino proton exchange catalysis by ammonia (Leroy et al., 1988), we find that the lifetime of the two base pairs sandwiched by the aromatic rings of luzopeptin and echinomycin is enormously longer than in the free duplexes, whereas base pairs outside are much less affected. The bis-intercalating drug thus seems to act as a vise, clamping the inner base pairs. This point will be examined further by comparison with complexes formed by intercalation of nogalamycin (a monointercalator which has the same site specificity as luzopeptin) on the d(CCCATGGG) and on the d(AGCATGCT) duplexes, with a stoichiometry of two nogalamycin molecules per duplex (manuscript in preparation).

Base-pair stability is altered similarly to base-pair lifetime, thus reinforcing the contrast between inner and outer base pairs. The lifetime of the open state, on the other hand, is not much altered, be it for inner or outer base pairs.

In order to ascertain that the base-pair opening process described above occurs in the complex, we determined the lifetime of the complex itself by proton exchange of the amide protons of the drug.

Methods

NMR Samples. The oligonucleotides were synthesized on a 10-μmol scale by the β-cyanoethyl phosphoramidite method and purified by high-pressure chromatography as previously described (Kochoyan et al., 1990). The complexes were prepared in an NMR tube containing a solution of free duplex ($\approx$200 A_{260} units in 0.3 mL, 0.1 M NaCl) by addition of an excess of drug ($\approx$25%). The mixture was shaken at 50 °C until complete disappearance in the NMR spectrum of the free duplex imino proton lines. Luzopeptin and echinomycin being nearly insoluble in water, the solutions were then centrifuged to remove the excess of drug. Unless otherwise stated, the excess of free duplex was always less than 5% of the complex concentration.

For real time exchange experiments, the complex concentration was adjusted to about 0.5 mM. Exchange time measurements vs ammonia were performed with complex concentrations ranging from 0.5 to 1 mM. Ammonia was added to the samples from a 6.5 M stock solution, pH 8.8. The sample pH was checked with a microelectrode and adjusted with HCl or NaOH, 0.1–0.5 M.

When necessary, ammonia was removed from the sample by dialysis in Visking dialysis tubes (6–8-kDa cutoff). The ammonia concentration in the dialysis tube decreased with a time constant of 5 min. No loss of luzopeptin was detected after 5 h of dialysis against a 20 mM NaCl solution. When the echinomycin complexes were treated according to the same protocol, about 5% of the bound echinomycin was lost. The stoichiometry of the samples was then restored by addition of drug.

Proton Exchange Formalism. Exchange of the imino proton of a base pair is a two-step process requiring disruption of the base pair followed by chemical exchange. In the open state, the chemical step is catalyzed by a proton acceptor such as ammonia or the hydroxyl ion, or by a proton acceptor intrinsic to the nucleic acid. The exchange rate k_i of the imino proton of an isolated nucleoside can be expressed as a function of the collision rate k_{coll}, the catalyst concentration [C], and the difference ΔpK, between the nucleoside and catalyst pKs:

$$k_i = k_{coll}[C]/(1 + 10^{\Delta pK}) \quad (1)$$

If the added catalyst has full access to the imino proton in the open pair, the exchange rate k_{tr}^{add} is the same as in the monomer except for the difference between the monomer and the duplex coefficients of translation diffusion (Guéron & al., 1989). Therefore

$$k_{tr}^{add} \approx k_i/[1 + (M_c/M_n)^{1/3}] \equiv k_i' \quad (2)$$

where M_c and M_n are the molecular masses of catalyst and monomer.

In case of restricted access, we write $k_{tr}^{add} = \alpha k_i'$, where α is an accessibility factor which may depend on the exchange catalyst. The time for imino proton exchange may be written

in terms of the base-pair lifetime τ_0 and of the apparent base-pair dissociation constant αK_d:

$$\tau_{ex} = \tau_0 + 1/(k_i'\alpha K_d) \quad (3)$$

When the catalyst concentration is sufficiently high, i.e., $k_i'\alpha K_d \gg 1$, exchange occurs every time the base pair opens. At infinite catalyst concentration, τ_{ex} extrapolates to τ_0, the base-pair lifetime. At lower catalyst concentrations, the exchange rate is proportional to the fraction of the time during which the imino proton is exposed. By comparison with the exchange time $1/k_i$ of the imino proton in the monomer, τ_{ex} yields the apparent base-pair dissociation constant αK_d. The lifetime of the open state τ_{open} is equal to $K_d\tau_0$, and the available parameter $\alpha K_d\tau_0 \equiv \alpha\tau_{open}$ is denoted the "apparent lifetime" of the open state.

In the absence of added catalyst, the imino proton exchange time τ_{AAC} remains finite. Exchange still takes place via transient base-pair opening, but it is catalyzed, very probably, by a proton acceptor of the duplex itself, the cyclic nitrogen of the complementary base. Exchange would occur via proton transfer to a water molecule linking the imino proton to this group (Guéron et al., 1987).

The equation corresponding to eq 2 is

$$\tau_{AAC} = \tau_0 + 1/(K_d k_{tr}^{int}) \quad (4)$$

where k_{tr}^{int} is the transfer rate due to internal catalysis in the open state. Since $\tau_{AAC} \gg \tau_0$, the exchange time in the absence of added catalyst is related to the base-pair dissociation constant rather than to the base-pair lifetime.

NMR Methods. The NMR measurements of the complex d(AAACGTTT)–echinomycin were performed mostly on a Bruker AM300 spectrometer, and those of the other complexes were performed on the 360-MHz spectrometer built at Ecole Polytechnique. The H_2O signal was suppressed with the JR sequence (Guéron et al., 1991).

The methods for measuring imino proton exchange times and base-pair lifetimes have been described (Kochoyan et al., 1987; Leroy et al., 1988a). The exchange times longer than 1 min were determined in real-time exchange experiments by dilution of a concentrated protonated sample into D_2O. Exchange times shorter that 2 s were obtained from the rate of magnetization transfer from water (Forsen & Hoffmann, 1963). The exchange contribution of added ammonia was determined from the variation of the longitudinal relaxation, using the relation $1/\tau_{ex} = 1/T_{1cat} - 1/T_{10}$, where T_{1cat} and T_{10} are the relaxation times measured with and without catalyst. Exchange times in the millisecond range were obtained from the line broadening Δ observed upon catalyst addition, by the relation $\tau_{ex} = 1/(\pi\Delta)$.

The relative uncertainty on the base-pair lifetimes is estimated to be ±15% for the longer values and ±50% for the smaller values; that on the apparent dissociation constants is ±20%.

Results

1. Proton Exchange in the Absence of Ammonia

1.1. Luzopeptin Complexes. Figure 1 presents a real-time exchange experiment performed by dilution into D_2O of the H_2O solution of the d(CCCATGGG)–luzopeptin complex at 15 °C, pH 8.3. The imino proton and the resolved amino protons (8.31, 6.92, and 6.81 ppm) of the C·G pairs, as well as the quinoline OH3 proton, exchange so quickly that they are absent even in the first spectrum (B), whereas the imino proton of A4·T5 exchanges in 3.6 h.

The exchange time of the quinoline OH3 proton, 0.4 s under these conditions of temperature and pH (Figure 2), was obtained by the magnetization transfer method. The exchange time of the imino proton of the C3·G6 pair is estimated to be 10 s by extrapolation to 15 °C of the exchange times measured by magnetization transfer between 25 and 65 °C (Figure 2).

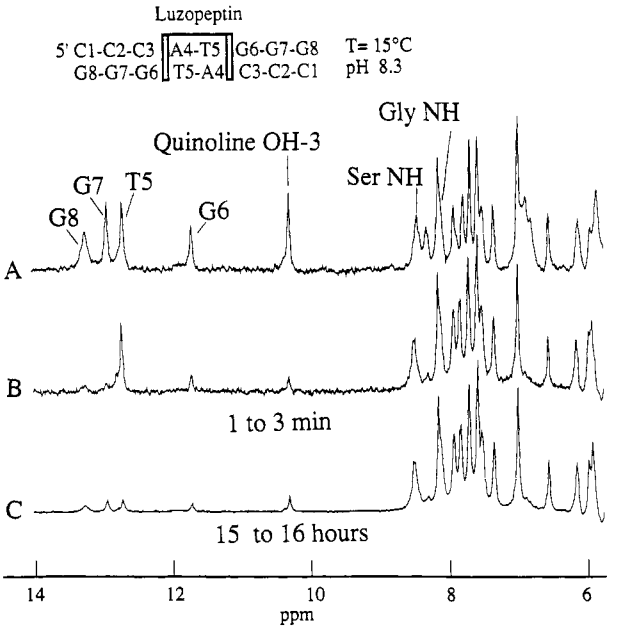


FIGURE 1: Real-time solvent exchange experiment in the d-(CCCATGGG)–luzopeptin complex: (A) reference spectrum in 90% H_2O; (B) 3 min after solvent change (120 scans); (C) 16 h after solvent change (3600 scans). Note the residual intensity, e.g., between 14 and 10 ppm, corresponding to 13% residual H_2O in the solvent. Spectra were normalized for the number of scans. Experimental conditions: 0.1 M NaCl; pH 8.3; recurrence time 0.5 s.

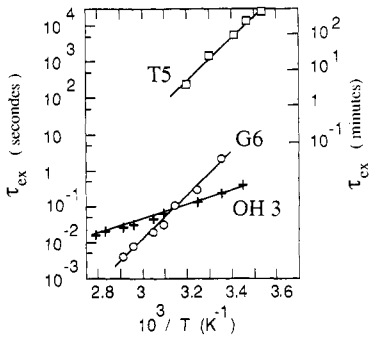


FIGURE 2: Proton exchange times vs temperature in the d-(CCCATGGG)–luzopeptin complex. Exchange times of the imino proton of T5 were obtained by the real-time method. Those of the hydroxyl proton of the quinoxaline ring of luzopeptin and of the imino proton of G6 were deduced from the rate of magnetization transfer from water (Leroy et al., 1988a). Experimental conditions: 0.1 M NaCl; T = 15 °C; pH 8.3.

At 15 °C, the deuteration of the Ser and Gly amide protons of the drug is negligible up to 16 h after the solvent change (Figure 1). At 45 °C, their exchange times are identical: $\tau_{ex} \approx 4$ days. For reference, we note that the exchange time of the amide proton in random polyD,L-alanine is 0.7 s at 15 °C, pH 8 (Englander & Poulsen, 1969).

The activation energies, determined by the Arrhenius relation from the temperature dependence of exchange times (Figure 2), are respectively 120 kJ/mol for T5 and G6 imino protons and 30 kJ/mol for the quinoline OH3 proton.

The pH dependence of the exchange time of the imino proton of T5 reveals three different mechanisms (Figure 3). Below pH 4, exchange is acid-catalyzed, maybe in relation to adenine protonation (pK_a = 3.7). Between pH 5 and pH 7.5, the exchange time is maximum at τ_{AAC} = 9.7 h, a value 5

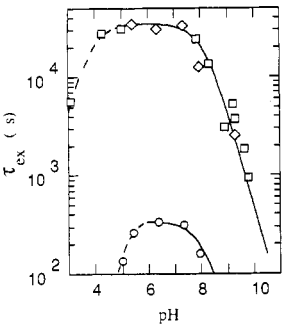


FIGURE 3: Imino proton exchange times vs pH in the luzopeptin complexes at 15 °C: A4·T5 imino proton (□) in the d(CCCATGGG)–luzopeptin complex; A4·T5 imino proton (◇) and C3·G6 (○) in the d(AGCATGCT)–luzopeptin complex. At high pH, exchange is catalyzed by hydroxyl ions. Exchange in the pH-independent region is controlled by internal catalysis. The reduction of the exchange times at low pH indicate an exchange process catalyzed by H^+. The solid curves are computed according to $\tau_{ex} = (1/\tau_{AAC} + k_i'[OH^-]\alpha K_d)^{-1}$ with $\alpha K_d = 3 \times 10^{-9}$ and $\tau_{AAC} = 9.7$ h for the imino proton of T5 in both complexes, and with $\alpha K_d = 4 \times 10^{-7}$ and $\tau_{AAC} = 5$ min for the imino proton of G6 in the d(AGCATGCT)–luzopeptin complex. The values of k_i' were obtained from eq 2 with the k_i values given in the text.

orders of magnitude larger than that commonly measured for the A·T imino protons in a free duplex (Guéron et al., 1987). At high pH, exchange is catalyzed by OH^-. We measured the exchange time for free thymidine at 15 °C, τ_{ex} (s) = $1/((6 \times 10^{10})[OH])$. From the data of Figure 3, eq 3 then gives an apparent dissociation constant $\alpha K_d = 4.5 \times 10^{-9}$ for the A4·T5 pair.

Real-time exchange experiments performed on the d(AGCATGCT)–luzopeptin complex show a similar exchange behavior, except that exchange of the imino proton of the C3·G6 pair is slow enough in this complex to be measured in real-time experiments (Figure 3). With the exchange time of the free guanosine at 15 °C, τ_{ex} (s) = $1/((7 \times 10^{10})[OH])$, we compute an apparent dissociation constant $\alpha K_d = 4 \times 10^{-7}$ for the C3·G6 pair. The pH dependence of the exchange time in the A4·T5 imino proton in the d(AGCATGCT)–luzopeptin complex is identical to that of its counterpart in the d(CCCATGGG)–luzopeptin complex (Figure 3).

1.2. AAACGTTT–Echinomycin Complex. Although the 2-fold symmetry of the d(AAACGTTT) duplex is partly lost upon binding of echinomycin, the two G imino protons which are far from the asymmetric -$CH_2SCH(SCH_3)$- group of the drug are not resolved and their deuteration follows a single exponential in the real-time experiment shown in Figure 4. The τ_{AAC} value measured for the imino proton of G5 in the complex (Figure 5) is about 100 times longer than in the free duplex. Exchange of the imino proton of the A3·T6 pair is much faster. It was measured by magnetization transfer from water and from the imino line broadening above pH 7. From the OH^- catalysis of G5 and T6 imino protons, by reference to the exchange time of the free monomers, we calculate the apparent dissociation constants, $\alpha K_d \leq 2 \times 10^{-9}$ for the C4·G5 pair and $\alpha K_d = 5 \times 10^{-3}$ for the A3·T6 pair.

The resolved amide protons of the alanine residues (9.8 and 9.6 ppm) and the nonresolved amide protons of the serine residues (8.1 ppm) of echinomycin in the complex exchange at the same rate, with the same pH dependence. The activation energy (170 kJ/mol) for amide proton exchange was determined from exchange times measured at 15 and 25 °C in real time and at 50 and 60 °C by magnetization transfer.

1.3. Effect of the Complex Stoichiometry on Proton Exchange Rates. In the case of the AAACGTTT–echinomycin complex, real-time exchange experiments were carried out in

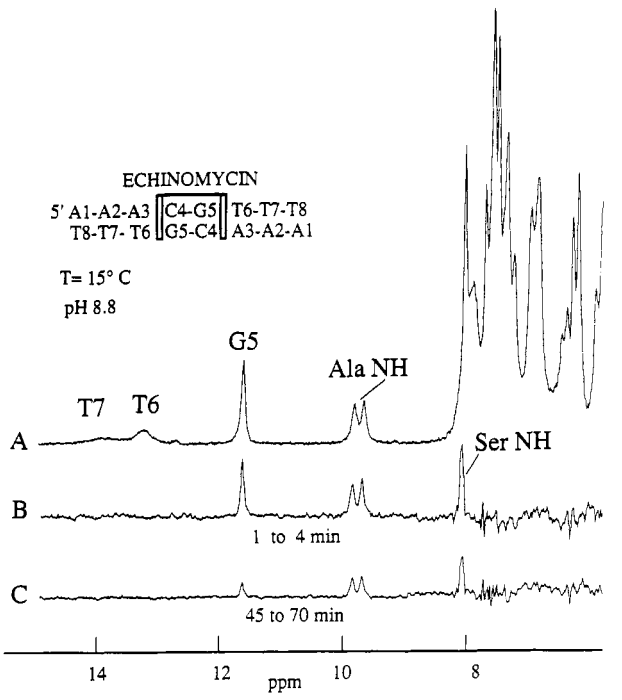


FIGURE 4: Real-time solvent experiment in the d(AAACGTTT)–echinomycin complex. Spectrum A: reference spectrum in 90% H_2O. The protonated complex was diluted in D_2O at $t = 0$. The difference spectra, B and C, were obtained by subtracting the spectrum recorded 24 h after transfer of a concentrated protonated sample in D_2O from those recorded in the time intervals indicated on the figure. The spectra are multiplied by a function which corrects for the amplitude response of the JR sequence (Guéron et al., 1991). Experimental conditions: 0.1 M, NaCl; T = 15 °C; pH 8.8.

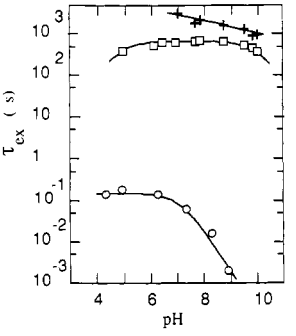


FIGURE 5: The effect of pH on proton exchange in the d(AAACGTTT)–echinomycin complex at 15 °C: (+) echinomycin alanine and D-serine amide protons; (□) G5 imino proton; (○) T6 imino proton. The pH-independent exchange time of G5 is 650 s and that of T6 is 0.15 s. The apparent dissociation constant computed according to the procedure described in Figure 3 is 5×10^{-3} for the A4·T6 pair. The small sensitivity of the G5 imino proton to hydroxyl catalysis indicates an apparent dissociation constant smaller than 2×10^{-9}.

the presence of an excess of free duplex (0.33 free duplex per complex), at least 6 times larger than the largest excess (0.05 free duplex per complex) in the nominal stoichiometric conditions (see Methods). Measurements at 15 °C, pH 7 and pH 9, revealed no change in any of the exchange times.

2. Exchange Catalysis by Ammonia

2.1. Luzopeptin Complexes. The effect of ammonia on the imino proton spectrum of the d(CCCATGGG)–luzopeptin complex at 35 °C, pH 8.8, is shown in Figure S1 (supplementary material). The terminal G8 imino proton exchanges too rapidly to be observed, even in the absence of added catalyst. The G7 imino proton broadens and becomes unobservable as ammonia is added. The sensitivity of the hydroxyl

Table I: Base-Pair Lifetime (τ_0), Apparent Dissociation Constant (αK_d), and Apparent Open-State Lifetime ($\alpha\tau_{open}$) in the Free d(CCCATGGG) and d(AGCATGCT) Duplexes and in Their Complex with Luzopeptin

	free d(CCCATGGG)			d(CCCATGGG)–luzopeptin complex		
T (°C)	τ_0 (ms)	$\alpha K_d \times 10^{-6}$	$\alpha\tau_{open}$ (ns)	τ_0 (ms)	$\alpha K_d \times 10^{-6}$	$\alpha\tau_{open}$ (ns)
			Base Pair A4·T5			
5	3	8.5	25	–	–	–
15	0.7	19	13	–	0.004[a]	–
25	0.3	63	19	–	–	–
35	–	–	–	517	0.09	46
45	–	–	–	223	0.18	40
55	–	–	–	87	0.33	29
65	–	–	–	50	1.1	55
			Base Pair C3·G6			
5	6	2	13	–	–	–
15	4	8.3	33	23	1.7	39
25	2	46	92	7.5	6	45
35	–	–	–	1	17	17
	free d(AGCATGCT)			d(AGCATGCT)–luzopeptin complex		
T (°C)	τ_0 (ms)	$\alpha K_d \times 10^{-6}$	$\alpha\tau_{open}$ (ns)	τ_0 (ms)	$\alpha K_d \times 10^{-6}$	$\alpha\tau_{open}$ (ns)
			Base Pair A4·T5			
15	1	18	18	–	0.003[a]	–
35	–	–	–	610	0.039	24
45	–	–	–	300	0.16	47
55	–	–	–	120	0.3	39
			Base Pair C3·G6			
15	5	4.7	23	15	0.17	2.5
25	–	–	–	9	0.6	5.4
35	–	–	–	2	3	6
45	–	–	–	≈0.5	10	≈5
55	–	–	–	–	50	–

[a] This dissociation constant is obtained from exchange catalysis by OH^-. Other dissociation constants are obtained from exchange catalysis by NH_3. Dashes indicate that this value was not measured.

proton of the quinoline ring (10.3 ppm) to NH_3 catalysis indicates good accessibility of this group. The exchange time of the amide protons of luzopeptin is too long ($\tau_{ex} > 3$ s) to be measured by saturation transfer, even at 65 °C and in the presence of ammonia (data not shown).

The times for catalyst-induced exchange of the imino protons of G7 and T5 vary linearly with the inverse of the ammonia concentration, in the complex and in the free duplex (Figure 6). In Figure 7 (left), the base-pair lifetimes obtained by extrapolation to infinite ammonia concentration are plotted vs the reciprocal temperature, together with the apparent dissociation constant (center) and open-state lifetime (bottom). Although it was impossible to measure the lifetimes in both duplex and complex at the same temperature, extrapolation shows that at 30 °C (and probably over a large temperature range) the lifetime of A4·T5 is 4 orders of magnitude longer in the complex. The activation energy for opening (70 kJ/mol) is similar in the free duplex and in the complex.

The effects of ammonia on the exchange rates of the hydroxyl proton of luzopeptin and of the imino protons of the d(AGCATGCT)–luzopeptin complex are similar to those observed on the d(CCCATGGG)–luzopeptin complex. The base-pair lifetimes, dissociation constants, and open-state lifetimes determined from the exchange catalysis by ammonia of the imino proton of G6 and T5 are listed in Table I.

In both complexes, the lifetime and the stability of the A4·T5 pairs sandwiched by the aromatic rings of the drug are considerably enhanced with respect to the free duplex. In contrast to the A4·T5 pair, the kinetic parameters for the C3·G6 pair adjacent to the luzopeptin binding site are changed by factors smaller than 10.

2.2. Echinomycin Complexes. The effects of pH and ammonia on the imino proton spectrum of the d(AAACGTTT)–echinomycin complex at 38 °C are shown in Figure S2. The time for catalyst-induced exchange of the G5 imino proton is plotted vs the inverse of the ammonia concentration in the left panel of Figure S3. For comparison, the free duplex values are plotted in the right panel.

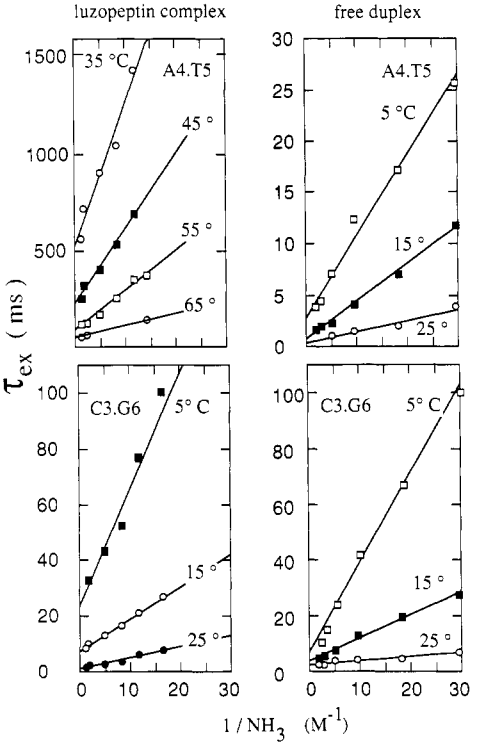


FIGURE 6: Temperature dependence of the exchange catalysis by ammonia of T5 (top panels) and G6 imino protons (bottom panel) in the d(CCCATGGG)–luzopeptin complex (left side) and in the free d(CCCATGGG) duplex (right side). The base-pair lifetimes obtained by extrapolation to infinite ammonia concentration and the apparent dissociation constants obtained from the slope of τ_{ex} vs $1/NH_3$ are plotted vs temperature in Figure 7 (left panel).

Table II: Base-Pair Lifetime, Apparent Dissociation Constant, and Apparent Open-State Lifetime of the C4·G5 Pair in the d(AAACGTTT) Free Duplex and in the Echinomycin Complex

	free d(AAACGTTT) duplex			d(AAACGTTT)–echinomycin complex		
T (°C)	τ_0 (ms)	$\alpha K_d \times 10^{-6}$	$\alpha\tau_{open}$ (ns)	τ_0 (ms)	$\alpha K_d \times 10^{-6}$	$\alpha\tau_{open}$ (ns)
5	16	1	16	–	0.005	–
10	13	1.2	16	–	–	–
15	8	2.9	23	–	≤0.002[a]	–
25	3	27	81	220	0.12	26
38	–	–	–	66	0.19	12
50	–	–	–	22	0.51	11
61	–	–	–	9	3.2	29

[a] This dissociation constant is obtained from exchange catalysis by OH^-. Other dissociation constants are obtained from exchange catalysis by NH_3.

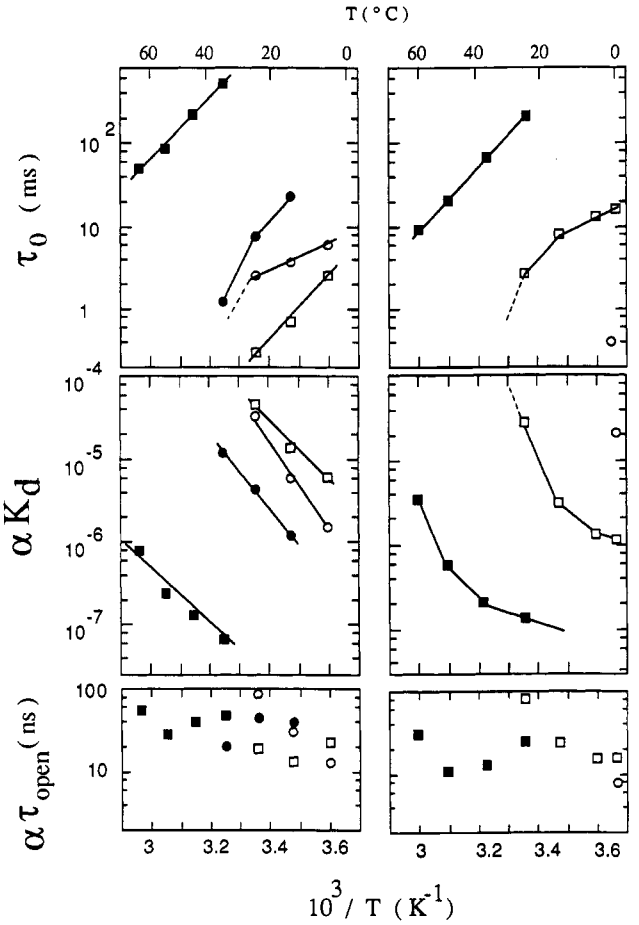


FIGURE 7: Base-pair lifetime, τ_0, apparent dissociation constant of base pairs, αK_d, and apparent open-state lifetime, τ_{open}, in complexes (solid symbols) and in free duplexes (open symbols). Left panels (with/without luzopeptin): A4·T5 (■,□) and C3·G6 (●,○) in the d(CCCATGGG)–luzopeptin complex and free duplex. Right panels (with/without echinomycin): C4·G5 (■,□) and A3·T6 (□) in d(AAACGTTT)–echinomycin complex and free duplex.

The base-pair lifetimes, the dissociation constants, and the open-state lifetimes of A3·T6 and C4·G5 in the free duplex and for C4·G5 in the complex are listed in Table II and are plotted vs temperature in the right panels of Figure 7. At 15 °C, complexation increases the lifetime and the stability of the C4·G5 pair about 40-fold. The activation enthalpy for C4·G5 opening in the complex and in the free duplex are in the same range (56 and 45 kJ/mol, respectively).

The line width of the T6 imino proton at 13.07 ppm and that of the imino proton at 13.8 ppm (presumably T7) indicates that these protons exchange quickly, even at pH 7. The imino proton of T6 exchanges more rapidly in the complex than in the free duplex. Its NMR line becomes too broad to be observed as ammonia is added (Figure S2).

Table III: Base-Pair Lifetime, Apparent Dissociation Constant, and Apparent Open-State Lifetime of the C6·G7 Pair in the CCAAACGTTTGG–Echinomycin Complex

T (°C)	τ_0 (ms)	$\alpha K_d \times 10^{-6}$	$\alpha\tau_{open}$ (ns)
38	78	0.15	12
50	28	0.42	12
61	14	1	14

The base-pair lifetime and the apparent dissociation constant of the C6·G7 pair in the d(CCAAACGTTTGG)–echinomycin complex were determined from the exchange catalysis by ammonia of the imino proton of G7 (Figure S4) and are listed in Table III. Spectral overlap of the signals of the T imino protons, exchange-broadened in the presence of ammonia, prevents a quantitative determination of the A·T-pair lifetimes. The upper limit for the lifetime of A5·T8 is estimated to 3 ms.

Up to 50 °C, the base-pair kinetics for the C6·G7 pair in the d(CCAAACGTTTGG)–echinomycin complex and for C4·G5 in the d(AAACGTTT)–echinomycin complex are similar (Tables II and III). This shows that C4·G5 opening is not related to fraying. At 60 °C, the effect of fraying appears in the lesser stability of C4·G5 in the AAACGTTT–echinomycin complex.

Discussion

The results presented above establish that bisintercalation of luzopeptin and echinomycin slows down tremendously the imino proton exchange in base pairs sandwiched by the aromatic rings of the drugs. In contrast, exchange catalysis in pairs flanking the intercalation site is much less reduced. It is even enhanced in the AAACGTTT–echinomycin complex (Figure S2).

In order to interpret the exchange catalysis, one needs to know whether exchange occurs from the complex or via a pathway involving complete or partial complex disruption.

1. Kinetics of Complex Dissociation

Not much is known about the complex dissociation pathway, about the importance of partial dissociation, or about the exchange of the amide protons in the complex of echinomycin with the specific AAACGTTT sequence.

The significant observation for the present work is that the exchange time of the serine and alanine amide protons of echinomycin is order of magnitudes slower than that of the imino proton which is used to derive the base-pair lifetime (20 min vs 0.4 s at 15 °C).

As usual, it must be considered whether exchange (of the amide protons) is limited by catalysis or opening. The lack of a strong pH dependence indicates the latter. Hence, the amide proton exchange time is probably equal to the time for partial or complete disruption. We note that these times are unaffected by an excess of free duplex.

Another argument for opening-limited amide proton exchange is provided by the identical values of the amide proton exchange rates of alanine (hydrogen-bonded to the N3 nitrogen of G5) and of D-serine (not hydrogen-bonded) (Gao & Patel, 1987).

The similarity between the amide exchange times reported here and the complex disruption times measured by the technique of detergent sequestration (Fox et al., 1981) and by the analysis of the transcription rate of a DNA fragment complexed to the drug (Phillips et al., 1990) provides further support for our interpretation.

In the case of luzopeptin, the same arguments apply. The observation that the lifetime of the CCCATGGG–luzopeptin complex is much longer than that of the AAACGTTT–echinomycin complex is consistent with the prediction made on the basis of electrophoretic measurements (Huang et al. 1983; Fox et al., 1988) that luzopeptin has a higher affinity for DNA than echinomycin.

2. *Base-Pair Kinetics in the Free Duplexes*

In B DNA, typical lifetimes at 15 °C for internal pairs range from 0.5 to 7 ms for A·T pairs and from 7 to 40 ms for G·C pairs. The apparent dissociation constant is around 10^{-5} for A·T pairs and 10^{-6} for G·C pairs, leading to open-state lifetimes in the range of 10–100 ns. In duplexes terminated with G·C pairs, fraying affects only the two external pairs when the temperature is less than 25 °C (Guéron et al., 1989).

The lifetimes and apparent dissociation constants determined in the free duplexes studied in this work are typical of the values in duplexes of different length and composition (Leroy, 1990). In the d(AAACGTTT) duplex, the short lifetime of the A3·T6 pair (≈0.4 ms at 0 °C) and its poor stability (Figure 7) are probably due to fraying effects, as already observed in the case of duplexes terminating with A·T pairs (Kochoyan et al., 1988; Leroy et al., 1991). The break at 15 °C in the plot of the lifetime of C4·G5 in d(AAACGTTT) vs the reciprocal temperature and the increased open-state lifetime indicate propagation of the fraying into the center of the duplex.

3. *Base-Pair Kinetics in the Complexes*

Since the complex lifetimes are much longer than the imino proton exchange times, imino proton exchange is controlled by processes occurring in the complex and may be used to derive base-pair kinetics in the complex.

3.1. Base Pairs between the Two Intercalated Moieties. In the four complexes, the base pairs sandwiched by the drug seldom open and are very stable.

Thus, in the d(CCCATGGG)–luzopeptin complex (left panel, Figure 7), the lifetime of the A4·T5 pair is larger by about 4 orders of magnitude than that in the free duplex (top) and the apparent dissociation constant is less by the same factor (middle plot). The product $\alpha K_d \tau_0$ and hence the apparent open-state lifetime of A4·T5 is not, or hardly, affected (bottom plot).

In the d(AAACGTTT)–echinomycin complex, the lifetime of the C4·G5 pair at the intercalation site is about 70 times longer than in the free duplex, and the apparent open-state lifetime is unchanged (right panel, Figure 7).

3.2. Base Pairs Outwardly Adjacent to an Intercalating Moiety. The enhanced lifetime and stability observed for the sandwiched base pair are conspicuously absent in the case of the outwardly adjacent base pairs.

Luzopeptin Complexes. The activation energy for C3·G6 opening in the d(CCCATGGG)–luzopeptin complex (100 kJ/mol) is larger than that measured in the free duplex (45 kJ/mol). But the lifetime and stability are enhanced by factors less than 10 in the temperature range studied (left panel, Figure 7).

The larger stabilization observed for the C3·G6 pair upon luzopeptin binding to d(AGCATGCT) is mainly due to the short open lifetime of this pair (Table I) and it accounts for the slower exchange rate of this proton in the absence of added catalyst (Figure 3).

Echinomycin Complexes. In the d(AAACGTTT)–echinomycin complex, the apparent dissociation constant of A3·T6 obtained from the exchange broadening vs pH ($\alpha K_d = 5 \times 10^{-3}$ at 15 °C; Figure 5) indicates that this pair is even less stable than in the free duplex, where we measure $\alpha K_d = 2 \times 10^{-5}$.

A destabilization of A·T pairs adjacent to the intercalation site of echinomycin is also observed in the d(ACGTACGT)–echinomycin complex (Gilbert et al., 1989). It is probably a consequence of the structural constraints which force this pair to adopt a Hoogsteen conformation in some sequences (Ughetto et al., 1985; Gao & Patel, 1987; Gilbert et al., 1989).

In the d(CCAAACGTTTGG)–echinomycin complex, the lifetime of A5·T8, $\tau_0 \leq 3$ ms at 15 °C, is shorter or comparable to values previously measured in free duplexes for A·T pairs in a comparable environment (Kochoyan et al., 1987).

4. *Base-Pair Opening Process*

The most striking property of the luzopeptin and echinomycin complexes is the considerable enhancement of base-pair lifetime and stability observed for the sandwiched (inner) base pairs, and for them only. It is as though the bisintercalating drug acts as a vise, clamping down the inner base pairs. Meanwhile, the lifetime of the open state of these base pairs is unaffected.

What do these observations suggest about the geometry of the opening process and about the ways in which the drug interferes with the relevant motions of the nucleic acid? Note that the opening geometry is presently unknown even for noncomplexed double-stranded nucleic acids. Opening by base rotation toward the major groove has been proposed (Ramstein & Lavery, 1990).

The bisintercalator drug consists of three parts, two intercalating aromatic moieties and one cyclic peptidic linker which is located in the minor groove. Let us first consider these parts separately.

The peptidic linker occupies the minor groove at the position of the inner base pairs, and by steric hindrance alone it probably inhibits any opening toward the minor groove.

It could also inhibit opening toward the major groove, due to hydrogen bonding between the linker and a base. Such bonding occurs between the glycine amide proton of luzopeptin and O2 of thymine T3 in the complex with d(CATG) (Zhang & Patel, 1991), as well as between the alanine amide proton of echinomycin and N3 of guanosine G3 in the complex with d(ACGT) (Gao & Patel, 1988). However, this cannot, per se, explain the slow kinetics of inner base pairs since the effect does not occur for outer base pairs, despite hydrogen bonding to the linker, as in the case of G4 in the luzopeptin complex with d(CATG) and, therefore, presumably also for G6 of d(CCCATGGG).

A third possibility would be that opening in the free duplex is nearly always toward the minor groove. This opening direction is ruled out in the complex, and only opening toward the major groove would remain, thus explaining the increased base-pair lifetime. A difficulty in this interpretation is that it supposes different open states for the free duplex and the

complex, whereas the observation that the open-state lifetimes are in the same range (Figure 7) suggests that the open state is the same.

Turning now to the aromatic moieties, their intercalation would be expected to affect similarly the base pairs on either side; one inner, one outer. Hence, intercalation, per se, cannot explain the large effect on the inner base pairs.

Although data for detailed comparisons are lacking, we note that the above considerations are consistent with properties of other complexes of DNA. Thus, slow base-pair opening is observed in complexes with nonintercalating minor-groove binders such as chromomycin (Leroy et al., 1991) and netropsin (Charretier, 1990). As for the monointercalator actinomycin D (manuscript in preparation), its effects are much less pronounced than those observed here.

The above considerations suggest that the kinetic effect of the bisintercalator may be due to the cooperative action of its different parts. One possibility is that in the free duplex base-pair opening involves a distortion of the double helix, such as unwinding or bending, both of which would lead to unstacking of a base pair from its neighbors. This unstacking might by itself provide a pathway for exchange, or more probably, it would be a prerequisite for lateral opening of the base pair toward one groove, leading to exchange. This would be consistent with molecular modeling investigations which predict a coupling between base-pair opening and bending (Ramstein & Lavery, 1988).

The effect of the double intercalator would be to clamp together the inner base pairs, thus strongly inhibiting the distortion of the double helix. An increase of the required distortion energy by 9 kT (22 kJ/mol) would reduce its probability by the required factor of 10^4. The opening process would be the same as in the free duplex, and steric hindrance by the linker suggests that this is toward the major groove. The lack of effect on outer base-pair opening is expected since they are not "clamped".

We are thus lead to consider base-pair opening not as a fluctuation of the equilibrium structure of DNA but as a motion which is secondary to a fluctuation, such as unwinding or bending, which by itself does not necessarily lead to imino proton exchange. This model is certainly not proven by the evidence presently available, but it is susceptible to further experimental investigation.

Conclusion

This study extends to bisintercalating drug–oligomer complexes the characterization of the DNA base-pair kinetic parameters. We have determined a lower limit for the lifetimes of luzopeptin and echinomycin complexes and established that the exchange catalysis of imino protons may be interpreted in terms of base-pair motion occurring within the complex.

The lifetime of base pairs sandwiched between the aromatic rings of the drug is strongly increased. The similarity of the base-pair open-state lifetimes in the complexes with those in the free duplexes points to a common base-pair opening pathway toward the major groove.

These properties suggest that base-pair opening is slowed down by clamping action of the drug and that it may require prior unwinding or bending of the double helix, in the complex as well as in the free duplex.

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Supplementary Material Available

Four figures showing the proton exchange broadening for increasing concentrations of ammonia in the d-(CCCATGGG)–luzopeptin complex (Figure S1); the effect of pH and ammonia in the AAACGTTT–echinomycin complex (Figure S2); the exchange time vs ammonia of the G5 imino proton in the AAACGTTT duplex, free or complexed with echinomycin (Figure S3); and the temperature dependence of the exchange catalysis by ammonia of the G5 imino proton in the CCAAACGTTTGG–echinomycin complex (Figure S4) (4 pages). Ordering information is given on any current masthead page.

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Lateral Interactions of Pig Apolipoprotein A-1 with Egg Yolk Phosphatidylcholine and with Cholesterol in Mixed Monolayers at the Triolein–Saline Interface†

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ABSTRACT: Interfacial tensions of egg yolk phosphatidylcholine (PC) and cholesterol monolayers adsorbed at the triolein–saline interface were measured in the presence and absence of pig apolipoprotein A-1 (apoA-1) in the saline phase. In the absence of apoA-1, the adsorptions of PC and cholesterol at the interface from the triolein phase are cooperative, showing large lateral attractive interactions between the PC molecules and the cholesterol molecules in the monolayer. In the presence of apoA-1, the PC adsorption is anti-cooperative, indicating strong lateral attractive interactions between the PC and the apoA-1 molecules, i.e., apparently, repulsive lateral interactions between the PC molecules. On the other hand, lateral interactions of very low magnitude are observed between the cholesterol and apoA-1 molecules in the monolayer. Values of the lateral interaction energy are evaluated from the adsorption data by the Defay–Prigogine–Flory theory of monolayers. The large difference in lateral interaction energy with apoA-1 between PC and cholesterol in a mixed monolayer is discussed in connection with current problems in lipoprotein catabolism: reverse cholesterol transport, alterations in affinity of lipid particles to apoA-1, and formation of high-density lipoproteins and abnormal lipoproteins.

Interactions among phosphatidylcholine (PC),[1] free cholesterol (Chol), and apolipoproteins at a lipoprotein surface are considered to be important in determining the metabolic fate of the lipoprotein in plasma. Plasma HDL's or reconstituted HDL particles act as efficient acceptors for Chol in reverse cholesterol transport (Brown et al., 1980; Pittman et al., 1987). Even bilayer vesicles of PC or sphingomyelin are capable of extracting Chol from cells in the culture without apolipoprotein of HDL's (Williams et al., 1984; Williams & Scanu, 1986). Apolipoprotein (apoA-1, apoA-2, or apoE)-containing vesicles are more effective in extracting Chol from cultured cells than either the proteins or PC alone (Oram et al., 1983) and mobilize intracellular deposits of esterified Chol from cells (Williams et al., 1984; Ho et al., 1980). Free apolipoproteins (apoA-1, apoA-2, or apoE) also interact with Chol-loaded macrophages to remove Chol. The products of the interaction are rich not only in Chol but also in PC (Hara & Yokoyama, 1991), and PC is assumed to play important roles in the extraction of Chol by apoA-1, apoA-2, or apoE.

Nascent chylomicrons and VLDL's usually contain a low content of Chol (Miller & Small, 1983). During the lipolysis of TG-rich lipoprotein particles in plasma, Chol is transferred from other lipoproteins and cellular elements of the blood (Atokinson & Small, 1986). The chol accumulation at the particle surface is assumed to affect the binding of apolipoproteins. The composition of the exchangeable apolipoprotein (apoA-1, apoA-2, apoC, and apoE, etc.) at the surface probably controls the metabolic fate of the particles (Gotto et al., 1986). Derksen and Small (1989) have shown that the binding capacities of apoA-1 and apoE to TG–PC emulsion particles decrease sharply when the Chol content is increased.

In vitro, single-bilayer vesicles acquire apoA-1 from HDL and are converted into small protein–phospholipid discs (Jonas, 1986) and serve as substrates for lecithin–cholesterol acyltransferase (LCAT) (Albers et al., 1986). These processes can occur, however, only if the mole ratio of Chol/PC is not larger than 0.5 (Fielding et al., 1972; Tall & Lange, 1978). An abnormal lipoproteins, known as lipoprotein X's (LP-X's), are Chol-rich single-bilayer vesicles containig albumin (30% of the protein), apoC (50% of the protein), and a trace of apoA-1 (LCAT activator) (Hauser et al., 1977; Kostner & Laggner, 1989). LP-X's are poor substrates for LCAT (Patsch, 1977) and cannot undergo the above transformations, thus accumulating in plasma (Untracht, 1982).

The mechanism of redistribution of soluble apolipoproteins among lipoprotein particles during the catabolism in plasma has not been completley defined. A more rigorous understanding of the lipoprotein metabolism requires a quantitative evaluation on interactions among PC, Chol, and soluble apolipoproteins. In this work, we measured the interfacial tensions of mixed monolayers of apolipoproteins from pig HDL with PC and with Chol at the TG [triolein (TO) in this work]–saline interface. The PC monolayer at the TO–saline interface studied is a proper model for the surface of TG-rich lipoproteins: chylomicrons, VLDL's, and TG-rich HDL_2. The

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[1] Abbreviations: PC, egg yolk phosphatidylcholine or phosphatidylcholine; Chol, (free) cholesterol; apoA-1, pig or human apolipoprotein A-1; TG, triglyceride; TO, triolein; VLDL's, very low density lipoproteins; HDL's, high-density lipoproteins; LP-X's, abnormal lipoprotein-X's; LCAT, lecithin–cholesterol acyl transferase.

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