Biochemical Pharmacology, Vol. 43, No. 12, pp. 2575–2580, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

THERMODYNAMICS OF THE ANTHRACYCLINE–NUCLEI INTERACTIONS IN DRUG-RESISTANT AND DRUG-SENSITIVE K562 CELLS

JOLANTA TARASIUK* and ARLETTE GARNIER-SUILLEROT†
Laboratoire de Chimie Bioinorganique (LPCB URA CNRS 198), UFR de Médecine et Biologie Humaine, 74, rue Marcel Cachin, 93012 Bobigny Cedex, France

(*Received* 29 *October* 1991; *accepted* 9 *March* 1992)

Abstract—Fluorescence emission spectra from anthracycline-treated cells suspended in buffer have been used to measure the uptake of three anthracycline derivatives: Adriamycin® (ADR), 4′-*o*-tetrahydropyranyl-Adriamycin (THP-ADR) and aclacinomycin (ACM) in drug-sensitive and drug-resistant K562 cells. The concentration of drug bound to the nucleus and free in the cytoplasm, at steady state, as well as the concentration of drug bound to the nucleus at equilibrium state have been determined at temperatures ranging from 6° to 40°. The enthalpies for the binding of ADR, THP-ADR and ACM to nuclei equal -35 ± 3, -35 ± 3 and -30 ± 3 kJ/mol, respectively. These values compare with the enthalpies of binding of these drugs to naked DNA.

Multidrug resistance (MDR‡) of human cancer cells is a major problem in chemotherapy. In the development of MDR by malignant cells, a MDR gene (*mdr* 1) is frequently overexpressed in animal tumor models and in some human neoplasms [1, 2]. The overexpression of a heterogeneous group of high molecular mass membrane glycoproteins, termed P-glycoprotein, has been the most consistent phenotypic marker of MDR in cultured cells. Several lines of evidence suggest that P-glycoprotein is an energy-dependent efflux pump that maintains low intracellular drug levels [3, 4].

An important aim now is to be able to develop means of counteracting insensitivity to drugs. Cells exhibiting the MDR phenotypes can be made more sensitive to the cytotoxic effects of drugs by treatment with compounds such as calcium channel blockers [5–7] and the search for agents which can potentiate drug sensitivity at a clinically achievable concentration is a very important point. This requires the precise knowledge of the role of P-glycoprotein.

A study of the mechanism of outward transport involves a determination of the osmotic active drug concentration in the cytoplasm during the efflux process. We have shown recently that such determination was possible by taking into account the fact that the amount of drug intercalated in the nucleus is in equilibrium with the free drug in the cytoplasm, this equilibrium being pH dependent [8–11].

This equilibrium is also temperature dependent and in the present study we have undertaken a systematic study of the temperature dependence of the uptake of anthracycline derivatives by drug-resistant and drug-sensitive K562 cells. We have determined the enthalpies of formation of the drug–nucleus complex inside the cell at steady state as well as those of the drug–DNA complex. In both cases, the values compare and are characteristic of an exothermic process.

MATERIALS AND METHODS

Drugs and chemicals

Purified Adriamycin® (ADR), 4′-*o*-tetrahydropyranyl-Adriamycin (THP-ADR) and aclacinomycin A (ACM) were kindly provided by Laboratoire Roger Bellon (Neuilly, France). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11{,}500\ M^{-1}\,cm^{-1}$ for ADR and THP-ADR, and $\varepsilon_{436} = 11{,}150\ M^{-1}\,cm^{-1}$ for ACM. As anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just before use. Verapamil was from the Sigma Chemical Co. (St Louis, MO, U.S.A.)

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 9.5 mM HEPES buffer (plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose) at pH 7.2.

Absorption spectra were recorded on a Cary 219 spectrophotometer. Experiments were conducted in 1-cm quartz cuvette containing 2 mL of buffer under continuous stirring. The desired temperature was maintained using a circulating thermostated water bath.

Cell lines and cultures

Anthracycline-sensitive and -resistant K562

* Present address: Department of Pharmaceutical Technology and Biochemistry, Technical University, 80952 Gdansk, Poland.

† Corresponding author: A. Garnier-Suillerot, Laboratoire de Chimie Bioinorganique, Université Paris-Nord, 74, rue Marcel Cachin, 93012 Bobigny, France. Tel. (33) 1 48 38 77 48; FAX (33) 1 48 38 77 77.

‡ Abbreviations: ADR, Adriamycin; THP-ADR, 4′-*o*-tetrahydropyranyl-Adriamycin; ACM, Aclacinomycin A; MDR, multidrug resistance.

erythroleukemia cells were a gift of Dr Tapiero (Departement de Pharmacologie Cellulaire, ICIG, 94800 Villejuif, France). They were grown in RPMI medium supplemented with L-glutamine and 5% fetal calf serum at 37° in a humidified atmosphere of 95% air and 5% CO_2. Cultures initiated at a density of 10^5 cells/mL grew exponentially to about 10^6 cells/mL in 4 days. For the assays, in order to have cells in the exponential growth phase, culture was initiated at 5×10^5 cells/mL and cells were used 24 hr later; they were then at about 8×10^5 cells/mL. The cell population was analysed using a cytofluorometer. The proportion of cells in the G_1, S and G_2 phases was 26%, 63% and 12% for drug-sensitive cells and 34%, 53% and 13% for drug-resistant cells, respectively. These values are the means of four determinations. Cell viability was assessed by Trypan blue exclusion. The cell number was determined by microscopic analysis. By evaluation of growth inhibition a "resistance factor" was obtained by dividing the IC_{50} of resistant cells by the IC_{50} of the corresponding sensitive cells. The resistant factors obtained were 28, 8 and 3 for ADR, THP-ADR and ACM, respectively.

Drug accumulation

Determination of the amount of drug intercalated between the base pairs in the nucleus at steady state and at equilibrium state. Anthracyline uptake by the cells was followed using a new fluorometric method previously described [8, 10, 11]. Using this method it is possible: (i) to accurately quantify the amount of anthracycline intercalated inside the nucleus at steady state and at equilibrium state and (ii) to follow the kinetics of drug uptake as incubation of cells with the drugs proceeds without compromising cell viability. This method, which is 5–10 hr less time consuming than procedures currently in use [12, 13], is based on the observation that fluorescence of anthracycline is only quenched when intercalated between the base pairs of DNA and that transport across the cell membrane is the rate-limiting step [8]. The measurements were done using an excitation wavelength of 480 nm for ADR and THP-ADR, and 436 nm for ACM, and slit widths of 10, 10, 2, 2 nm; the fluorescence emission was measured at 590 nm.

All experiments were conducted in a 1-cm quartz cuvette containing 2 mL of buffer. The temperature was maintained at the desired value using a circulating thermostatted water bath. In a typical experiment, 2×10^6 cells were suspended in 2 mL of glucose-containing HEPES buffer under continuous stirring. Twenty microlitres of the stock anthracycline solution were quickly added to this suspension yielding an anthracycline concentration, C_T. The decrease in fluorescence intensity F at 590 nm, in the case of ADR and THP-ADR, and at 585 nm, in the case of ACM, was followed as a function of time.

After some time, depending on the derivative of anthracycline used, the curve $F = f(t)$ reached a plateau and the fluorescence intensity was equal to F_n (Fig. 1). The drug–cell system was thus at steady state and the drug concentration C_n intercalated between the based pairs in the nucleus was $C_n = C_T(F_0 - F_n)/F_0$ where F_0 is the fluorescence intensity at $t = 0$ of an anthracyline solution, C_T. Once steady state was reached, cell membranes were solubilized with 0.05% Triton X-100 yielding the equilibrium state which was characterized by a new value of the fluorescence intensity F_N which depends on the value of pH_e, the extracellular pH [9–11]. The concentration C_N of drug intercalated between the base pairs in the nucleus at equilibrium was $C_N = C_T(F_0 - F_N)/F_0$. Under our conditions Triton X-100 was not able to release anthracycline from its intercalative binding site to DNA and the quenching of anthracycline fluorescence by the interaction of the drug with cells was not due to the formation of non-fluorescent drug metabolites [8].

Determination of C_i the osmotic active drug concentration inside the cells at steady state. C_i, the osmotic active drug concentration inside the cell (alternatively, the free drug concentration in the cytoplasm) was determined using a method that we have described previously [10, 11]. Briefly, the method is based on the following observations: (i) the drug bound to the nucleus is in equilibrium with the free drug in the medium around the nucleus; and (ii) the nuclei from resistant and sensitive cells have the same ability to bind anthracycline [9–11]. The affinity of the drugs to the nucleus also depends on the pH value of the medium around the nucleus [9–11]. Thus, when sensitive cells are placed in a medium with approximately the same pH as in the cytoplasm (pH_i), and when Triton X-100 is added in order to permeabilize the membrane, no modification of the amount of drug bound to the nucleus is observed since $pH_e = pH_i$ and, therefore, the free drug concentrations outside the cell and inside the cell (C_i) are the same. From these observations, we inferred that at pH_i the drug concentration in the nuclei (C_n) and cytoplasm (C_i) of cells at steady state can be mimicked by placing cells, whose membranes have been permeabilized by the addition of Triton X-100, in a medium where $pH_e = pH_i$. For drug concentrations lower than 1 μM, we observed that the plot of C_n versus C_E was linear, i.e. $C_E = \alpha C_N$, α depending on the nature of the drug, on the pH and on the temperature. Thus for each drug and at every temperature value, when $pH_e = pH_i = 7.2$, the cytoplasm free drug concentration C_i at steady state is given by the simple relation $C_i = (C_E/C_N)C_n$.

DNA preparation

High molecular mass calf thymus DNA was purchased from Sigma and dissolved in HEPES buffer for 3 hr under vigorous stirring. An absorption coefficient of 13,200 $M^{-1}\,cm^{-1}$ (bp) was used to calculate DNA concentrations from absorbance measurements at 260 nm.

Temperature dependence of the binding of ADR, THP-ADR and ACM to naked DNA. A fluorescence method was used to determine the concentration of bound and free drug as a function of temperature. The experiments wre performed in HEPES buffer at pH 7.2. A stock solution of DNA was added to 2 mL anthracycline solution (1 μM) in 5-μL increments. The DNA concentration was varied from 0 to 100 μM (bp). Following each addition, the fluorescence intensity at 590 nm was recorded. The fluorescence intensity in the presence of DNA (F)

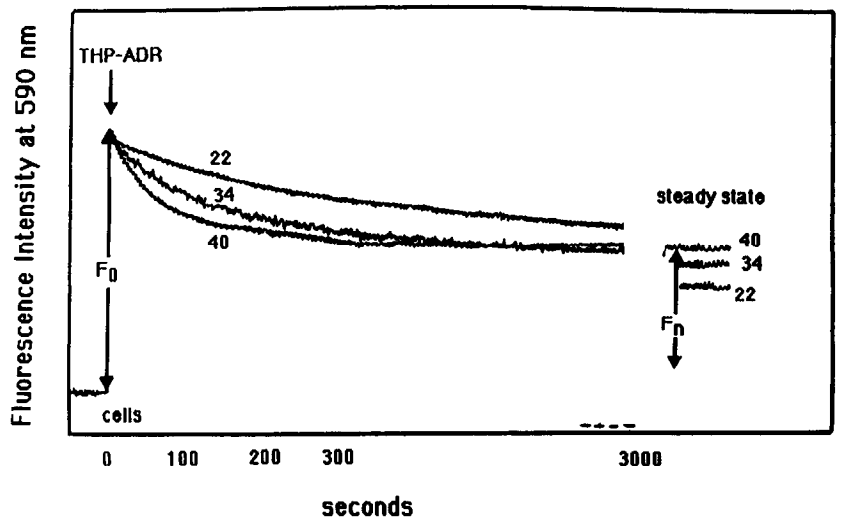

Fig. 1. Uptake of THP-ADR by drug-sensitive K562 cells at different temperatures. F, the fluorescence intensity at 590 nm (λ_{ex} = 480 nm) is recorded as a function of time. Cells (2×10^6) are suspended in a cuvette filled with 2 mL of buffer at $pH_e = 7.2$ under vigorous stirring. At $t = 0$, 20 μL of a 100 μM stock THP-ADR solution are added to the cells yielding a $C_T = 1\ \mu M$ THP-ADR solution; the fluorescence intensity is then F_0. The slope of the tangent to the curve $F = f(t)$ at $t = 0$ is $(dF/dt)_{t=0}$ and the initial rate of uptake $V_+ = (dF/dt)_{t=0}(C_T/F_0)$ (M/sec). Once steady state is reached, the fluorescence intensity is F_n and the concentration of drug intercalated between the base pairs in the nucleus is $C_n = C_T(F_0 - F_n)/F_0$. The uptake was followed at temperatures of 40°, 34° and 22°.

and in the absence of DNA (F_0) was used to calculate the concentration of free (D_f) and bound (D_b) drug, according to the relation

$$[D_f] = C_T(F/F_0 - \rho)/(1 - \rho).$$

C_T is the concentration of drug and ρ is the ratio of the observed quantum yield of fluorescence of totally bound drug to that of free drug. The quantity ρ was determined to be 0.04, 0.05 and 0.01 for ADR, THP-ADR and ACM, respectively.

RESULTS

Temperature dependence of the uptake of THP-ADR by drug-resistant and drug-sensitive cells

The uptake of THP-ADR by drug-resistant and drug-sensitive cells was recorded under the following conditions: a constant initial concentration C_T of drug equal to 1 μM was added to 10^6 cells/mL suspended in a glucose-containing HEPES buffer at pH 7.2 at various temperatures ranging from 8° to 40°. Figure 1 shows the records of typical experiments performed at 40°, 34° and 22° in the case of drug-sensitive cells. As can be seen, as the temperature decreased the initial rate of uptake decreased but the concentration of drug intercalated at the steady state (C_n) increased. Similar experiments were performed at temperatures ranging between 40° and 6°.

Temperature dependence of the concentration C_N of THP-ADR intercalated between the base pairs in the nucleus at the equilibrium state

In the case of experiments performed at temperature higher than 35° steady state was reached within about 15 min. However, at lower temperature the time required to reach steady state increased strongly as the temperature decreased. Nevertheless, after about 15 min, Triton X-100 was added to the suspension and equilibrium state was obtained. Figure 2 shows the plot of C_N as a function of temperature. As can be seen C_N is the same for drug-sensitive and drug-resistant cells. This corroborates the previous observation that the binding properties of the nucleus do not depend on the degree of resistance of the cells [9, 10].

Temperature dependence of the concentration C_n of THP-ADR intercalated between the base pairs in the nucleus at the steady state

The determination of C_n at different temperatures was performed under the following conditions: 10^6 cells/mL were incubated with 1 μM THP-ADR at 37°. Steady state was reached within about 10 min and the corresponding value of C_n was measured. Then the temperature was cooled to 35° and C_n was measured after 30 min incubation at this temperature.

The temperature was thus progressively cooled by 5° step down to 10°. At each step C_n was measured after 30 min incubation at the chosen temperature. Figure 3a shows the plot of C_n as a function of temperature for both drug-sensitive and drug-resistant cells.

At the end of the experiment the temperature of the suspension was warmed up from 10° to 37° and left for 1 hr. The new value of C_n thus obtained was the same as that obtained at the beginning of the experiment. We inferred that the process was completely reversible.

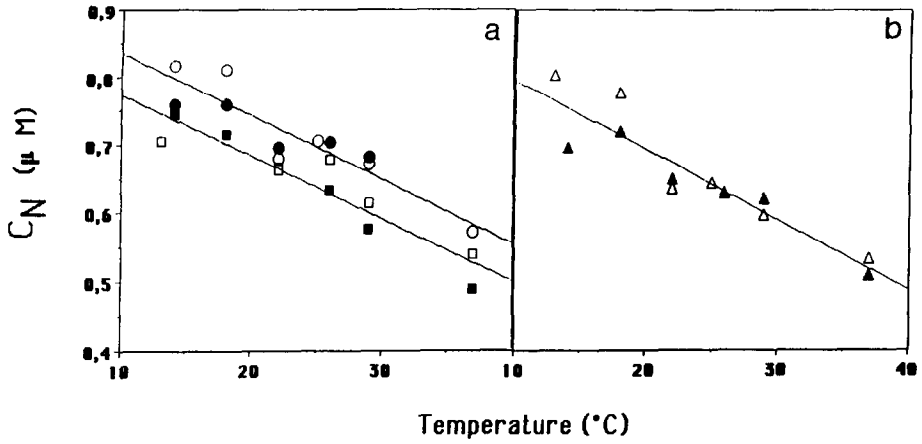

Fig. 2. Uptake of THP-ADR (△▲), ACM (□■) and ADR (○●) by drug-sensitive (▲■●) and drug-resistant (△□○) cells. C_N the molar concentration of drug bound to the nuclei at equilibrium state has been plotted as a function of temperature. The experimental conditions are as described in the legend to Fig. 1. After about 15 min incubation 5 μL 20% Triton X-100 were added. The lines drawn have been least-squares fitted to the data. The correlation coefficients are higher than 0.92.

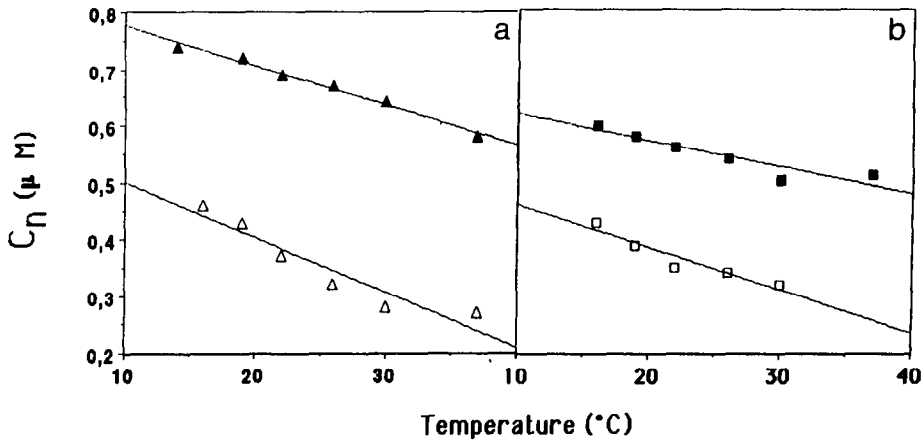

Fig. 3. Uptake of THP-ADR (△▲) and ACM (□■) by drug-sensitive (▲■) and -resistant (△□) cells. C_n the molar concentration of drug bound to the nuclei at steady state, has been plotted as a function of temperature. Cells (10^9/L) were incubated with 1 μM drug in glucose-containing buffer at pH 7.2 and at 37°. The temperature was slowly cooled to 5°. The cells were kept at each temperature for 30 min to ensure that steady state was reached. C_n was calculated as described in Materials and Methods. The lines drawn have been least-squares fitted to the data. The correlation coefficients are higher than 0.92.

Temperature dependence of the uptake of ACM by drug-resistant and drug-sensitive K562 cells

The experiments were performed under exactly the same conditions as those described for THP-ADR. The variation in C_N and C_n as a function of temperature is shown in Figs 2a and 3b, respectively.

Temperature dependence of the uptake of ADR by cells

The kinetics of uptake of ADR by cells is very slow; at 37° it is about 60-times slower than that of THP-ADR. Under these conditions it was very difficult to reach steady state at low temperature. We have thus determined the concentration of ADR bound to the nucleus at the equilibrium state only (Fig. 2a).

Enthalpy estimates of the binding of ADR, THP-ADR and ACM to naked DNA

Binding data from fluorescence titration experiments were cast into the form of a Scatchard plot of $\nu/[D_f]$ versus ν, where ν is the number of moles of anthracycline bound per mole DNA base pairs. The data were fit to the neighbor-exclusion model [14]:

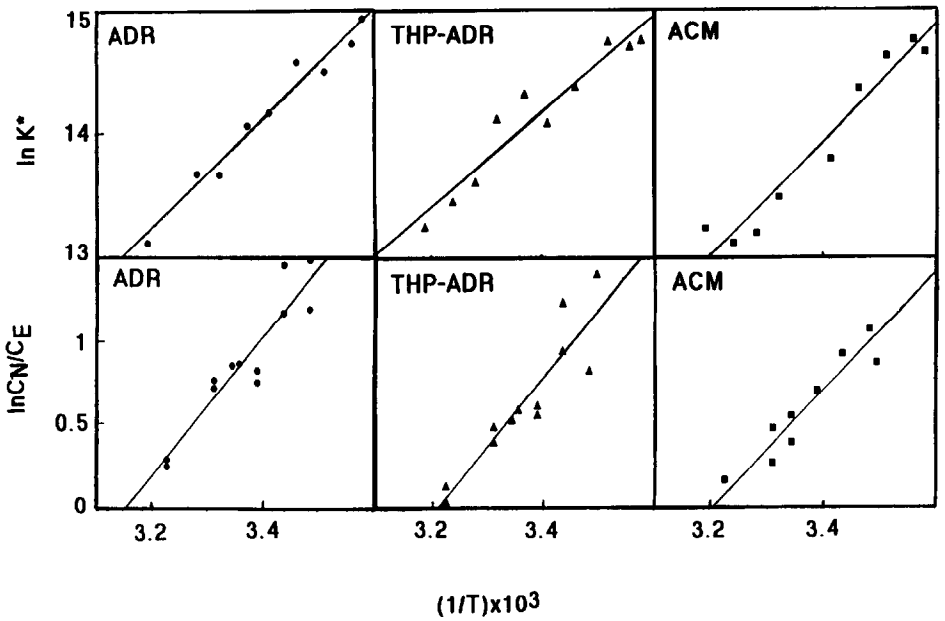

Fig. 4. Binding of ADR, THP-ADR and ACM to naked DNA and to cell nuclei. lnK^* has been plotted as a function of $1/T$ (upper curves). K^* is the overall binding constant of the drug to naked DNA. lnC_N/C_E has been plotted as a function of $1/T$ (lower curves). C_N and C_E are the concentrations of drug bound to the nuclei and free in the cytoplasm at equilibrium state, respectively.

Table 1. Thermodynamic parameters for the interaction of anthracycline with DNA and cell nuclei

	ΔH^0 (kJ/mol)	
	Drug–DNA	Drug–nuclei
ADR	−36 ± 3	−35 ± 3
THP-ADR	−32 ± 3	−35 ± 3
ACM	−39 ± 3	−30 ± 3

The enthalpies ΔH^0 were obtained from the slopes of van't Hoff plots.

$$\nu/[D_f] = K^*(1 - n\nu)[(1 - n\nu)/1 - (n-1)\nu]^{n-1}$$

where K^* is the binding constant to an isolated DNA binding site and n is the exclusion parameter.

Linear least-squares fit of lnK^* vs $1/T$, T = temperature, were used to estimate the enthalpy from the slope according to the van't Hoff relationship $[\mathrm{d}\ln K^*/\mathrm{d}(1/T)] = -\Delta H^\circ/R$ (Fig. 4). The values obtained are reported in Table 1.

Enthalpy estimates of the binding of anthracycline derivatives to cell nuclei

In the case of the interaction of anthracycline with cell nuclei we formed the simple hypothesis that the drug–nucleus interaction can be represented by the equilibrium:

$$\text{Nuclei} + \text{Drug} \rightleftarrows \text{Drug bound}.$$

Taking into account that the DNA concentration [N] present in the nuclei is large as compared with the drug concentration, it can be stated that the concentration of DNA not bound to the drug is equal to [N] and remains nearly constant throughout the experiments performed with the same number of cells in the same proliferative state. Under these conditions and in the case of experiments performed at equilibrium state, i.e. in the presence of Triton X-100, the overall binding constant of the drug to the nuclei may be defined as $K_{\text{nucleus}} = C_N/C_E[N]$ where C_E is the free drug concentration. The exact value of [N] is unknown. However the determination of the enthalpy from the slope of lnK_{nucleus} vs $1/T$ does not require this knowledge, the slope of lnC_N/C_E vs $1/T$ being the same since [N] is constant.

In the case of cells at steady state, the overall binding constant of the drug to the nuclei may be defined as $K_{\text{nucleus}} = C_n/C_i[N]$ where C_i is the osmotic active drug concentration in the cytoplasm. As we have shown that when $pH_e = pH_i$, $C_n/C_i = C_N/C_E$, it is obvious that the enthalpies are the same.

DISCUSSION

The data reported in this paper concerns the binding of three anthracycline derivatives with DNA and with cell nuclei.

Recently, Lane *et al.* [15] have studied the temperature dependence of ADR uptake and cytotoxicity. They have reported: (i) a loss of cytotoxicity below 20° which appears to be limited to a structural change in the cell membrane; and (ii) that the uptake of ADR measured at steady state decreases systematically as the temperature

decreases. The latter data mean that the binding of ADR to the nucleus inside the cell should be an endothermic process. However, it is well-documented that the binding of anthracycline derivatives either to free DNA or to nucleosomes is an exothermic process [16–19].

These discrepancies between these two types of data prompted us to determine the enthalpy for the binding of anthracycline derivatives to (i) naked DNA and (ii) cell nuclei.

The enthalpy values that we have determined for the binding of THP-ADR, ACM and ADR compare with the literature data. However, our data concerning the uptake of THP-ADR and ACM are at variance with those of Lane *et al.* [15] concerning ADR. We observed that the uptake of THP-ADR and ACM at steady state increases as the temperature decreases. Our interpretation of the discrepancy between both sets of data is that in the case of ACM and THP-ADR, which are drugs which enter the cells very rapidly [8, 10, 11], it is possible to reach steady state even at low temperatures. However, in the case of ADR, which enters the cells very slowly, the time required to reach steady state is very long: about 2 hr at 37° compared with 10 min for THP-ADR. Under these conditions, when the temperature decreases the time required to obtain steady state for ADR becomes considerable and steady state is certainly not reached within 2 hr as is proposed by Tritton *et al.* [15].

The enthalpy values obtained for the binding of the three drugs to nuclei at equilibrium state or at steady state in the case of THP-ADR and ACM compare with those obtained with naked DNA.

In conclusion, we have shown that the binding of anthracycline derivatives to cell nuclei is, as is the binding of anthracycline to DNA or nucleosomes, an exothermic process. This conclusion is not surprising. However, as it has been reported previously that the uptake of ADR, measured at steady state, decreased systematically as the temperature decreased [15], it was necessary to clear up this point.

Acknowledgements—This investigation was supported by Université Paris Nord, le centre National de la Recherche Scientifique, l'institut Curie, l'Association pour la Recherche sur le Cancer and the Polish Minister of National Education.

REFERENCES

1. Riordan JR and Ling V, Genetic and biochemical characterization of multidrug resistance. *Pharmacol Ther* **28**: 207–227, 1985
2. Pastan I and Gottesman MM, Multidrug-resistance in human cancer. *N Engl J Med* **316**: 1388–1393, 1987.
3. Skovsgaard T, Mechanism of resistance to daunorubicin in Ehrlich ascites tumor cells. *Cancer Res* **38**: 1785–1791, 1978.
4. Inaba M and Johnson RK, Uptake and retention of Adriamycin and daunorubicin by sensitive and anthracycline-resistant sublines of P388 leukemia. *Biochem Pharmacol* **27**: 2123–2130, 1978.
5. Tsuruo T, Lida H, Tsukagoshi Y and Sakuari Y, Potentiation of vincristine and adriamycin effects in human hemopoietic tumor cell lines by calcium antagonists and calmodulin inhibitors. *Cancer Res* **43**: 2267–2272, 1983.
6. Kessel D and Wilberding C, Mode of action of calcium antagonists which alter anthracycline resistance. *Biochem Pharmacol* **33**: 1157–1160, 1984.
7. Kessel D and Wilberding C, Anthracylcine resistance in P388 murine leukemia and its circumvention by calcium antagonists. *Cancer Res* **45**: 1687–1691, 1985.
8. Tarasiuk J, Frezard F, Garnier-Suillerot A and Gattegno L, Anthracycline incorporation in human lymphocytes. Kinetics of uptake and nuclear concentration. *Biochim Biophys Acta* **1013**: 109–117, 1989.
9. Frezard F and Garnier-Suillerot A, Comparison of the binding of anthracycline derivatives to purified DNA and to cell nuclei. *Biochim Biophys Acta* **1036**: 121–127, 1990.
10. Frezard F and Garnier-Suillerot A, Determination of the osmotic active drug concentration in the cytoplasm of anthracycline-resistant and -sensitive K562 cells. *Biochim Biophys Acta* **1091**: 29–35, 1991.
11. Frezard F and Garnier-Suillerot A, Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cells. *Eur J Biochem* **196**: 483–491, 1991.
12. Burke TG, Morin MJ, Sartorelli AC, Lane PE and Tritton TR, Function of the anthracycline amino group in cellular transport and cytotoxicity. *Mol Pharmacol* **31**: 552–556, 1987.
13. Skovsgaard T and Nissen NI, Membrane transport of anthracyclines. *Pharmacol Ther* **18**: 293–311, 1982.
14. McGhee J and von Hippel PH, Theoretical aspects of DNA–protein interactions: co-operative and non-co-operative binding of large ligands to a one-dimensional honmogenous lattice. *J Mol Biol* **86**: 469–489, 1974.
15. Lane P, Vichi P, Bain DL and Tritton TR, Temperature dependence studies of adriamycin uptake and cytotoxicity. *Cancer Res* **47**: 4038–4042, 1987.
16. Chaires JB, Dattagupta N and Crothers DM, Studies on interaction of anthracycline antibiotics and DNA: equilibrium binding studies on interaction of daunomycin with DNA. *Biochemistry* **21**: 3933–3940, 1982.
17. Chaires JB, Thermodynamics of the daunomycin–DNA interaction: ionic strength dependence of the enthalpy and entropy. *Biopolymers* **24**: 403–419, 1985.
18. Chaires JB, Dattagupta N and Green DM, Binding of daunomycin to calf thymus nucleosomes. *Biochemistry* **22**: 284–292, 1983.
19. Cera C, Palu G, Mgno SM and Palumbo M, *Nucleic Acids Res* **19**: 2309–2314, 1991.