A Fickian Diffusion Transport Process
with Features of Transport Catalysis

Doxorubicin Transport in
Human Red Blood Cells

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ABSTRACT   The transport of the antineoplastic drug doxorubicin (Adriamycin) in human red blood cells was investigated by measuring the net efflux from loaded cells. Previous data indicated that doxorubicin transport was a Fickian diffusion transport process of the electrically neutral molecule through the lipid domain of the cell membrane (Dalmark, 1981 [In press]). However, doxorubicin transport showed saturation kinetics and a concentration-dependent temperature dependence with nonlinear Arrhenius plots. The two phenomena were related to the doxorubicin partition coefficient between 1-octanol and a water phase. This relationship indicated that the two phenomena were caused by changes in the physicochemical properties of doxorubicin in the aqueous phase and were not caused by interaction of doxorubicin with cell membrane components. The physicochemical properties of doxorubicin varied with concentration and temperature because of the ability of doxorubicin to form polymers by self-association in aqueous solution like other planar aromatic molecules through pi-electron orbital interaction. The hypothesis is proposed that doxorubicin transport across cell membranes takes place by simple Fickian diffusion.

INTRODUCTION

Saturation kinetics and nonlinear Arrhenius plots are usually regarded as indicative of transport facilitation and catalysis by membrane proteins or lipid-protein complexes. This conclusion presupposes that the physicochemical properties of the permeant in the water phases on both sides of the cell membrane are independent of concentration and temperature. However, many organic molecules are able to form molecular associations like dimers and polymers through self-association by hydrophobic forces in aqueous solutions without forming any covalent bonds. Such molecular associations are dependent on both concentration and temperature. In such cases, features of a carrier-mediated transport process may occur, although the transport of the permeant takes place by simple Fickian diffusion because the diffusion

coefficients of the various molecular aggregates in the cell membrane may be different. In the extreme case, only the monomer permeates the cell membrane with the polymers being impermeants. The transmembraneous transport is linearly related to concentration, but with increasing concentration more molecules are in the impermeant polymer conformation—which means that the apparent membrane permeability coefficient decreases with concentration. The ability to form molecular associations is affected by temperature, which affects the Brownian movements.

Here we describe the concentration and temperature dependence of the transport of an organic molecule, doxorubicin, in human red blood cells. Doxorubicin (Adriamycin) is one of the most widely used and potent drugs against human cancer (Blum, 1975). Doxorubicin belongs to the anthracycline group of chemotherapeutics (Ollis, 1961). Its therapeutical action is probably linked to its ability to intercalate within the DNA helix in the cell nucleus and thereby destroy the function of the DNA (Pigram et al., 1972). Doxorubicin (Fig. 1) is a glycosidic antibiotic constituted by a pigmented aromatic aglycone
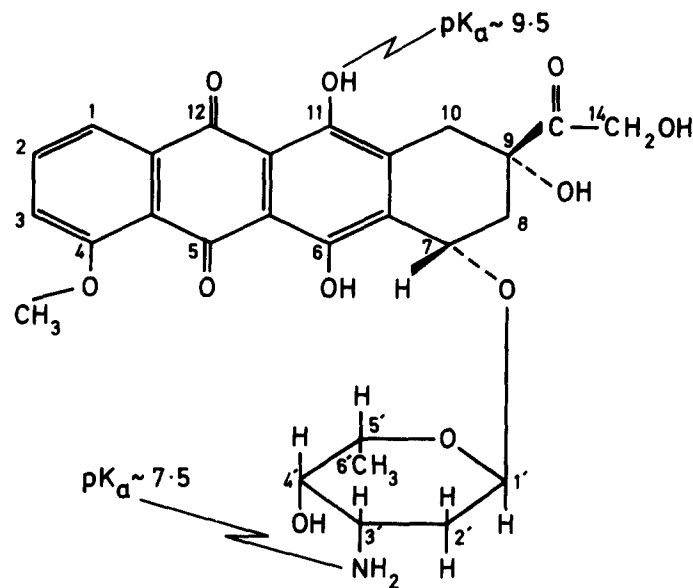

FIGURE 1.   The chemical structure of the doxorubicin (Adriamycin) monomer. Mol wt, 544.

(doxomycinone) linked to an amino-sugar (doxosamine). It has previously been shown that doxorubicin transport in human red blood cells apparently takes place by Fickian diffusion of the electrically neutral molecule through the lipid domain of the cell membrane because (*a*) the transport is accelerated by 1-alcohols, phloretin, and local anesthetics, (*b*) the transport increases with pH, and (*c*) the transport is not inhibited by several inhibitors of facilitated transport systems in red cells (Dalmark, 1981 [In press]). However, the present

paper demonstrates that doxorubicin transport in red cells shows saturation kinetics and a concentration-dependent temperature dependence. Both phenomena are explained by the ability of doxorubicin to form aggregates in aqueous solutions with concentration and decreasing temperature through self-association. In a previous report the existence of a carrier-mediated doxorubicin transport based on indications of saturation kinetics of doxorubicin influx in human red blood cells was postulated (Mikkelsen et al., 1977).

The ability of doxorubicin to self-associate in water and to intercalate with DNA appears to be a function of the electronic properties of the aromatic doxomycinone portion of the doxorubicin. In the 1930's Hückel described the classical double bonds of aromatic hydrocarbons and conjugated systems as corresponding to mobile pi-electrons that moved around the entire conjugated system in molecular orbitals. The idea of molecular orbitals has been of particular importance in discussing molecular associations and electronic spectra. It was observed in the German dye industry more than 50 years ago that dyes containing conjugated ring systems decreased in relative light absorbance and often changed color with concentration, indicating molecular associations that had pi-electron orbital interactions. It was an early observation that anthracycline antibiotics were subjected to light-absorbance quench with increasing concentration (Bachur et al., 1970). Later the self-association of daunomycin (Barthelemy-Clavey et al., 1974) and ultimately, the self-association of doxorubicin (Eksborg, 1978; Righetti et al., 1979) was demonstrated in nonbiological systems.

The binding force of the intercalation of the planar aromatic doxomycinone within the DNA helix is probably also hydrophobic through pi-electron interaction and identical to the stabilizing force of the DNA helix itself. The main stabilizing factor of single- and double-stranded DNA molecules is the hydrophobic force through vertical base stacking with pi-electron interaction (Crothers and Zimm, 1964). This hypothesis is strongly supported by the observation that doxorubicin forms complexes with nucleotides, nucleosides, and DNA-derived bases (Dalmark and Johansen, 1981).

It is possible to test the influence of concentration and temperature on doxorubicin self-association because the doxomycinone is linked to doxosamine, which has a pK of the amino group between 7 and 8 at body temperature (Arcamone et al., 1972; Dalmark, 1981 [In press]). This means that doxorubicin exists partly as a monovalent cation and partly as an electrically neutral molecule at physiological pH and temperature. Mainly neutral molecules self-associate for electrostatic reasons, but random association of the complexes with the doxorubicin cations turns the complexes into more hydrophilic, electrically charged aggregates. The latter reaction is demonstrable by a decrease in the 1-octanol/water partition of doxorubicin. The aim of this paper was to test the hypothesis that the concentration and temperature dependence of doxorubicin transport in human red cells correlated with the influence of concentration and temperature on the doxorubicin 1-octanol/water partition. If so, then the doxorubicin saturation kinetics and temperature dependence are caused by changes in the physicochemical prop-

erties of doxorubicin in the water phase and are not caused by interactions of doxorubicin with cell membrane components. In this case, the previous and present data are consistent with the perception of doxorubicin transport in red cells as a Fickian diffusion transport process. A preliminary report of this work has been presented elsewhere (Dalmark and Storm, 1980).

METHODS AND CALCULATIONS

Freshly drawn, heparinized human red blood cells were washed three times with a plasma-like salt solution with the following composition: 144 mM Na, 3.2 mM K, 1.5 mM Ca, 1 mM Mg, 150 mM Cl, 1.1 mM phosphate, 27 mM sucrose, 5 mM glucose (37°C, pH 7.3). The cells were resuspended (hematocrit, 0.4) and equilibrated with doxorubicin at pH 7.3 (37°C). The doxorubicin was added to the cell suspensions from a doxorubicin stock solution. The stock solution was prepared by dissolving a weighed amount of doxorubicin powder in the salt solution. The cell suspensions were centrifuged at 10°C (80,000 g, 20 min). The supernate and the packed, loaded cells were separated after the centrifugation and kept at 10°C until the efflux experiments were carried out. The amount of extracellularly trapped medium was ~2% (wt/wt) of packed cells.

Efflux Experiments

The efflux experiments were performed by injecting ~500 μl of packed, loaded cells into 30 ml of initially doxorubicin-free efflux medium vigorously stirred by a Teflon-coated magnetic bar at the appropriate pH and temperature. The composition of the efflux medium was identical with the incubation medium except for the absence of doxorubicin. The rate of doxorubicin efflux was measured by serially isolating 2 ml of cell suspension, which was instantaneously cooled to 0°C. The cooling procedure completely stopped the doxorubicin efflux (Dalmark, 1981 [In press]). The supernate was collected after centrifugation of the cell suspension at 3°C (5 × 10³ rev/min, 5 min). The doxorubicin concentration in the supernate was measured at room temperature. The rate of doxorubicin efflux as a function of doxorubicin concentration in cell water, i.e., the doxorubicin concentration in the incubation medium, was measured at pH 7.3 (37°C). The effect of temperature on doxorubicin efflux was determined with cells from one single cell batch equilibrated with doxorubicin at pH 7.3 (37°C). The efflux of doxorubicin was measured at the various temperatures without further acid-base titrations of the cells. The effect of temperature on the cellular pH of the red cells at physiological pH (Fig. 2A) and on the pK of the amino group of doxorubicin (Fig. 2B) gave linear plots with slightly different slopes. This difference was taken into account in the following calculations (Discussion). The pH of the efflux medium was adjusted to the appropriate pH at the various temperatures before the injection of the loaded cells as calculated from the data in Fig. 2A. The cell suspension was during the efflux experiments mainly buffered by the cellular content of hemoglobin (Dalmark, 1976).

Measurement of pH

The pH measurements were carried out electrometrically with a voltmeter (pH-meter 64; Radiometer, Copenhagen, Denmark) and a combined small glass electrode with a calomel reference electrode (GK2401C; Radiometer). The function of the pH electrode was checked repeatedly by a two-buffer test. The pH of the cell suspensions were recorded continuously during the experiments. The cellular pH was measured

with the pH-electrode submerged in the lysed, packed cells at the appropriate temperature. The packed cells were lysed by freezing and thawing three times by submerging a glass tube with the cells in a dry ice-ethanol mixture. The change of pH in the packed loaded cells with temperature is given in Fig. 2 *A*. The heat of ionization was 33 kJ/mol (7.95 kcal/mol) in this pH range. This observation was consistent with previously published data (Dalmark, 1980).

The pK of the amino group of doxorubicin was determined electrometrically by titration with NaOH of a 1-mM doxorubicin-distilled water solution under a carbon dioxide-free, nitrogen atmosphere at various temperatures. Fig. 2 *B* shows the shift of
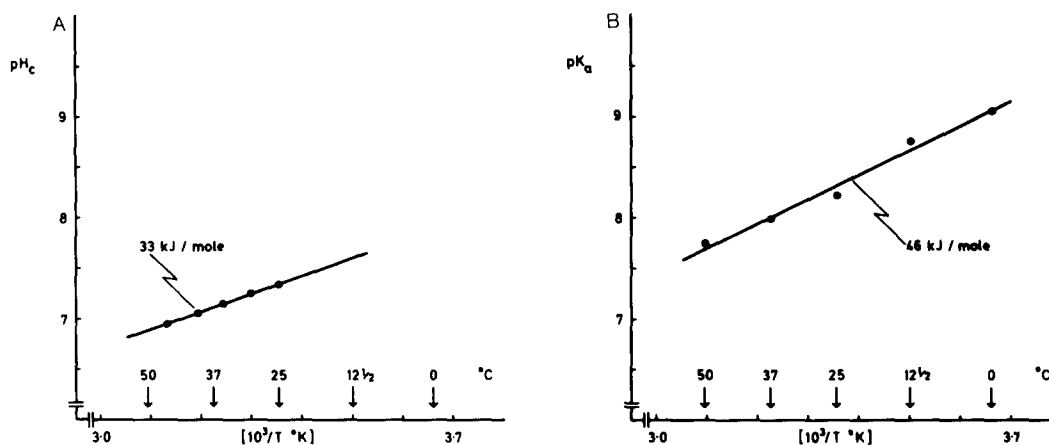

FIGURE 2. (*A*) The influence of temperature on the pH of red blood hemolysate. The relationship between pH cell (y) vs. the reciprocal absolute temperature $(10^3/T)$ (x) between 25 and 46°C was described by the equation: y = 1.757 x + 1.488 (r = 0.99). The Arrhenius activation energy was 33 kJ/mol (7.95 kcal/mol). (*B*) The influence of temperature on the pK of the amino group of doxorubicin. The relationship between pK (y) vs. the reciprocal absolute temperature $(10^3/T)$ (x) between 0 and 50°C was described by the equation: y = 2.387 x + 0.306, r = 0.98. The Arrhenius activation energy was 46 kJ/mol (10.9 kcal/mol).

the pK with temperature. The heat of ionization was 46 kJ/mol (10.9 kcal/mol). This value was consistent with the ionization enthalpy of other amino group-containing compounds like tris(hydroxymethyl)aminomethane (Tris) (Bates, 1973). The pK of the doxorubicin amino group varied with the ionic strength like the pK of Tris. The pK increased ~0.18 pH units when the ionic strength was increased from 0.001 to 0.15 by addition of KCl. These observations were used in the following experimental designs.

Partition Coefficient

The partition coefficient of doxorubicin between 1-octanol and a 0.05M Tris-distilled water solution was determined at the appropriate temperature and pH. The octanol/buffer partition coefficient was used as an indicator of the red cell membrane/buffer partition coefficient because the octanol/buffer partition coefficient has been shown

to be five times the cell membrane/buffer partition coefficient for many compounds (Seeman, 1972). The doxorubicin was dissolved in 18 ml Tris-buffer solution and equilibrated at the appropriate temperature and pH. Three initial 2-ml samples were collected and 12 ml 1-octanol was added under vigorous stirring by a magnetic bar to the remaining 12 ml of Tris-buffer. After 10, 20, and 40 min, respectively, a 2-ml sample was drawn from the octanol and buffer phases after spontaneous separation of the two phases. The doxorubicin equilibration was obtained within the first 10 min. The doxorubicin was extracted from the octanol phase by shaking 1 ml octanol with 1 ml of 1 N HCl solution at room temperature. The doxorubicin concentration was determined in the buffer phase by spectrophotofluorometry. The partition coefficient [(micrograms per milliliter octanol)/(micrograms per milliliter Tris-buffer)] was calculated from (a) the amount of doxorubicin that disappeared from the Tris-buffer phase as well as from (b) the amount of doxorubicin in the acid-water extraction solution of the 1-octanol phase—both are expressed as fractions of the average amount of doxorubicin in the Tris-buffer solution at equilibrium. The recovery of doxorubicin from the octanol phase was ~90%.

The partition coefficient was measured as a function of doxorubicin concentration at equilibrium at pH 7.3 (37°C). No shift of the pH in the Tris-buffer solution was observed after mixing of the Tris-buffer with octanol. The effect of temperature on the partition coefficient was determined using one single batch of Tris-buffer solution equilibrated with doxorubicin at pH 7.3 (37°C). The doxorubicin partition coefficient was measured at the various temperatures without further acid or base titration of the doxorubicin-Tris-buffer solution to keep the ionization of doxorubicin constant at the various temperatures. The effect of temperature on the pK of a Tris-buffer (Bates, 1973) and doxorubicin is identical (Fig. 2 B) because the determining group in both cases is a primary amino group.

Doxorubicin Concentration

The determination of doxorubicin concentration in the stock solution was carried out by spectrophotometry (SP 1800; Pye Unicam Ltd., Cambridge, England) of an acidic methanolic solution (>97% [vol/vol] methanol) of doxorubicin at 478 nm. The doxorubicin was obtained commercially (Adriamycin; Farmitalia Co., Milan, Italy). The doxorubicin concentration before reading was adjusted to give an absorbance of 0.1–0.3 units to obtain a linear relationship between absorbance and drug concentration. An extinction coefficient of $E_{1\,cm}^{1\%}$ = 225 was used (Arcamone et al., 1972). The doxorubicin concentration in the efflux medium was measured by spectrophotofluorometry (Aminco Bowman; American Instrument Co., Silver Spring, Md.) with excitation and emission wavelengths of 470 nm and 590 nm, respectively, at room temperature and pH 3. The linear relation of fluorescence to drug concentration was tested. The doxorubicin concentration was measured in red cells and in the incubation medium by extracting ~100 μl weighed, packed cells and 200 μl of incubation medium with 10 or 5 ml of extraction solution (75% [vol/vol] acetone, 25% [vol/vol] N,N-dimethylacetamide), respectively, after hypotonic hemolysis of the cells with 0.5 ml of water. The extraction solution with cells or medium was shaked for 10 min at room temperature and a water-clear supernate was obtained after centrifugation at room temperature (4 × 10^3 rev/min, 5 min). The doxorubicin concentration was measured by spectrophotofluorometry after acidification. Appropriate standards were used and linearity of fluorescence vs. drug concentration was tested. The doxorubicin recovery from cells was 90–95%.

Cellular Water Content

The cellular water content was measured by drying packed red cells to a constant weight (24 h, 105°C). The water content was expressed as kilograms water per kilogram cell solids. 1 kg cell solids is equivalent to 3×10^{13} red cells (Funder and Wieth, 1966).

Calculations

The rate of doxorubicin efflux was determined from the relationship between fractional cellular doxorubicin content and the time of sampling after injection of the loaded packed cells into the efflux medium. The fractional cellular content was calculated from the doxorubicin concentration in the efflux medium at the time of sampling, C_t, and at equilibrium, C_∞. The equation describing the time dependence of doxorubicin efflux was

$$\ln(1 - C_t/C_\infty) = -bt. \tag{1}$$

The slope, b, was put equal to the rate coefficient of doxorubicin efflux (k^0) as the hematocrit was low (0.01–0.02). The apparent doxorubicin permeability coefficient, P_T (cm/s), was defined as

$$P_T = k^0 \, (V/A), \tag{2}$$

where V was the cellular water volume (grams of water per 3×10^{13} cells) and A the surface area of 3×10^{13} cells with the assumption of a cell surface of 142 μm^2 per single cell.

RESULTS

The results will be described in two sections. The first section deals with the concentration dependence of doxorubicin transport at 37°C (pH 7.3). The second one describes the influence of temperature on doxorubicin transport at high and low doxorubicin concentrations. The biological data are compared in both sections with the partition coefficient of doxorubicin between a 1-octanol and a water phase under conditions comparable to those of the biological experiments. Doxorubicin was concentrated in the red cells relatively to the cell suspending salt solution. The distribution ratio was approximately seven (kilograms red cells per kilogram salt solution) at pH 7.3 (37°C). The distribution ratio was independent of the doxorubicin concentration between 1 and 500 μM. In the following sections, the doxorubicin transport data are correlated to the doxorubicin concentration in the extracellular fluid after the initial loading and equilibration of the cells with doxorubicin. The remainder of the cellular doxorubicin was assumed to be adsorbed to various cellular compounds (Discussion).

Fig. 3 shows the rate of doxorubicin efflux from cells of various doxorubicin concentrations into initially doxorubicin-free media (37°C, pH 7.3). The one-half-time of the initial doxorubicin efflux was ~2 min at concentrations <10 μM. The one-half-time of efflux increased with increasing doxorubicin concentration. The one-half-time was increased to 14 min at a cellular doxorubicin concentration of 516 μM.

Fig. 4B shows the apparent doxorubicin permeability coefficient as a function of the cellular doxorubicin concentration (37°C, pH 7.3). The permeability coefficient was ~2.4 × 10^{-7} cm/s at concentrations of <20 μM doxorubicin. The permeability decreased with increasing doxorubicin concentration to ~20% of the permeability observed at low concentrations. Fig. 4A shows the partition of doxorubicin between 1-octanol and a Tris-buffer solution as a function of the doxorubicin concentration in the Tris-buffer at
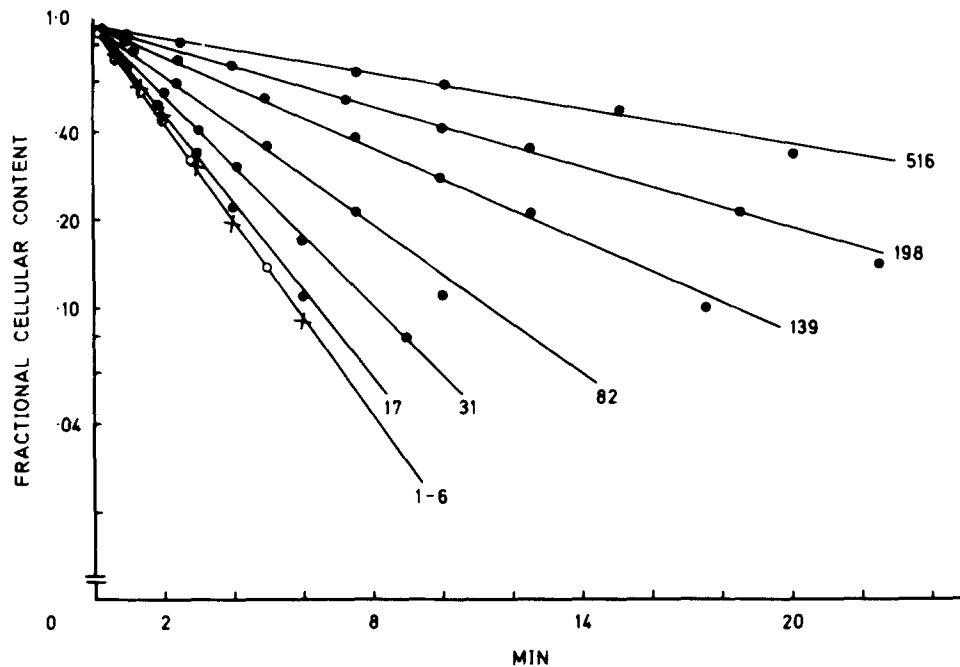

FIGURE 3. Rate of doxorubicin efflux from red cells with various doxorubicin concentrations in the cell water (μM) into initially doxorubicin-free media at pH 7.3 (37°C). The cells were at equilibrium with the efflux medium except with regard to doxorubicin. The fractional cellular doxorubicin content was determined from the formula $1 - C_t/C_\infty$, where C_t and C_∞ were the doxorubicin concentration in the medium at the time of sampling and at equilibrium, respectively. The abscissa indicates the time after injecting the doxorubicin loaded cells into the efflux medium.

equilibrium (37°C, pH 7.3). The partition coefficient was ~1.9 at concentrations of <20 μM doxorubicin. The partition coefficient decreased with concentrations >20 μM to ~20% of the coefficient observed at low concentrations. The solid line of Fig. 4B indicates the apparent doxorubicin permeability calculated from the partition coefficient data of Fig. 4A. A permeability coefficient of 2.4 × 10^{-7} cm/s was anticipated at low doxorubicin concentra-
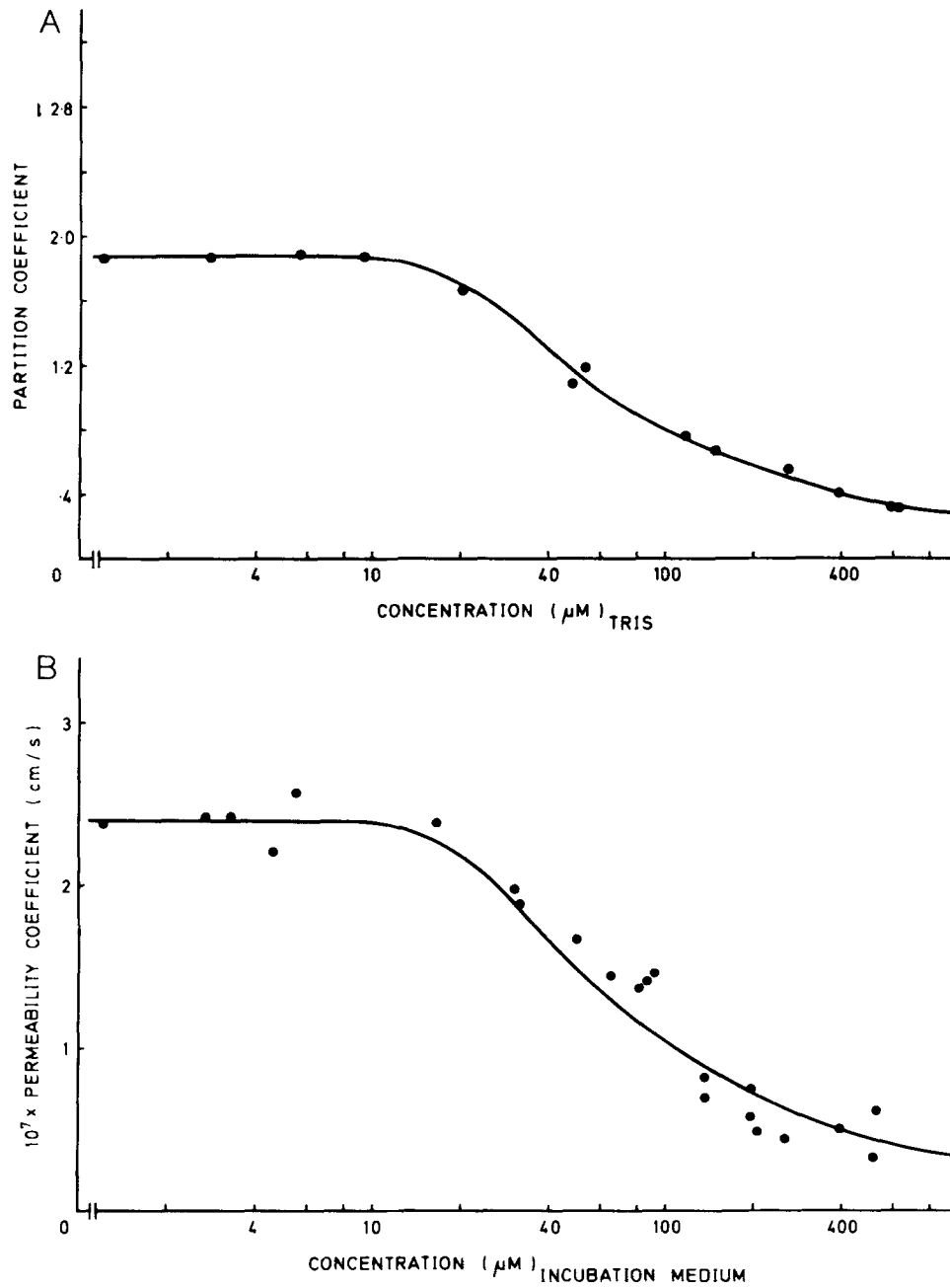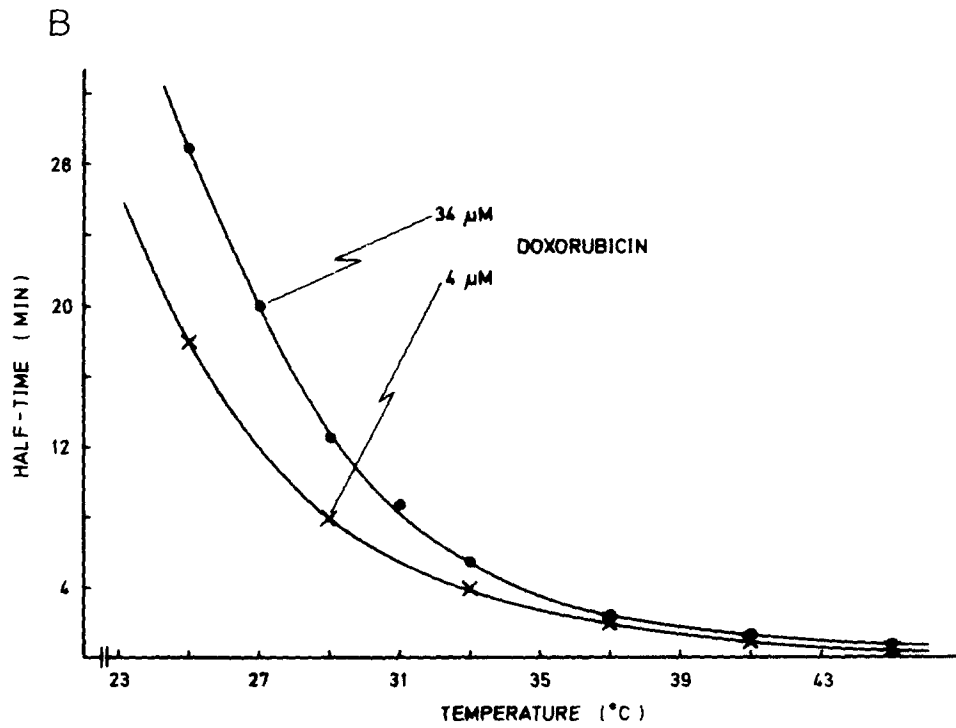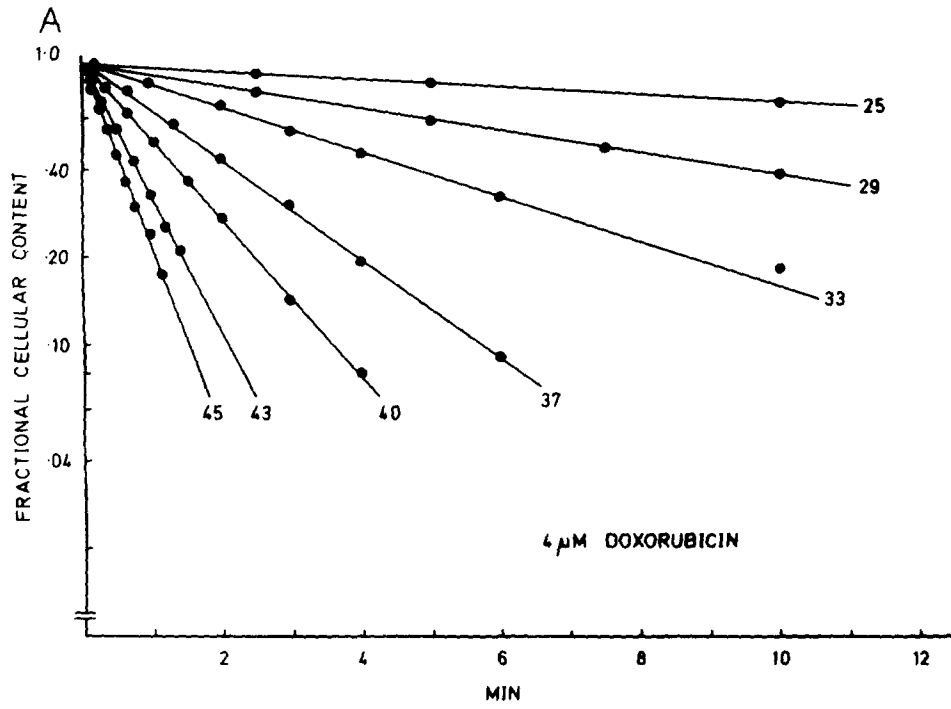
FIGURE 4.   (*A*) The partition coefficient of doxorubicin between 1-octanol and a Tris-buffer solution [(mol^{-1} 1-octanol)/(mol^{-1} Tris-buffer)] as a function of the doxorubicin concentration in the Tris-buffer at equilibrium (37°C, pH 7.3). (*B*) The apparent doxorubicin permeability coefficient as a function of the doxorubicin concentration in the cell water (37°C, pH 7.3).

A

FRACTIONAL CELLULAR CONTENT

1·0

·40

·20

·10

·04

25
29
33
37
40
43
45

4 µM DOXORUBICIN

MIN

B

HALF-TIME (MIN)

28

20

12

4

34 µM   DOXORUBICIN
4 µM

TEMPERATURE (°C)

tions. Fig. 4 demonstrates that the doxorubicin permeability coefficient varied with concentration, as did the doxorubicin partition coefficient.

Fig. 5 A shows the rate of doxorubicin efflux from cells with 4 μM doxorubicin into initially doxorubicin-free media at various temperatures. The experiments were carried out with cells from one single batch equilibrated with doxorubicin at pH 7.3 (37°C). The efflux of doxorubicin was measured at the various temperatures without further acid-base titration of the cells. The pH of the efflux media was titrated in accordance with the results of Fig. 2 A before the injection of the cells. The doxorubicin efflux was highly temperature dependent (Fig. 5 A). Fig. 5 B shows that the one-half-time of doxorubicin efflux decreased from 18 min (25°C) to 27 s (45°C) at low cellular doxorubicin concentrations. A larger one-half-time of doxorubicin efflux was observed at higher doxorubicin concentration at all temperatures investigated (Fig. 4 B).

Fig. 6 B shows an Arrhenius plot of the doxorubicin permeability between 25 and 45°C at two doxorubicin concentrations (4 and 34 μM). Linear and parallel graphs were observed at both concentrations at high temperatures with an Arrhenius activation energy of 138 kJ/mol (33 kcal/mol) equivalent to a Q_{10} of 5.7. However, the permeability decreased more than expected at low temperatures. The deviation from linearity was a function of the doxorubicin concentration. The nonlinearity was observed at 35°C at 34 μM doxorubicin in contrast to a value of 25°C at 4 μM. The permeability coefficient at 34 μM doxorubicin decreased to ~65% of the value at 25°C extrapolated from the linear portion of the Arrhenius graph observed at high temperatures. Fig. 6 A shows the influence of temperature (12.5–65°C) on doxorubicin partition between 1-octanol and a Tris-buffer solution at two doxorubicin concentrations (4 and 34 μM). The experiments were carried out with one single batch of Tris-buffer solution equilibrated with doxorubicin at pH 7.3 (37°C). The doxorubicin partition coefficient was measured at the various temperatures without further acid-base titration to keep the doxorubicin ionization constant. The heat of ionization of the amino groups on doxorubicin and Tris-buffer is identical (Methods). An altered doxorubicin partition under these conditions indicated an influence of other factors than the influence of temperature on the intrinsic hydrogen ion dissociation of the doxorubicin amino group. Fig. 6 A shows a smaller partition coefficient at higher doxorubicin concentration at all temperatures investigated (Fig. 4 A). The two graphs converge with increasing temperature. The partition coefficient at low doxorubicin concentration was constant from 65 to 25°C, after which it decreased. The partition coefficient at high doxorubicin concentration decreased at temperatures <40°C. The partition coefficient at 34 μM decreased at 25°C to ~55% of the value observed at high temperatures. Fig. 6 demonstrates that the nonlinearity of the Arrhenius plots of the doxorubicin

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FIGURE 5 (*opposite*). (*A*) The rate of doxorubicin efflux from cells with a doxorubicin concentration in the cell water of 4 μM at various temperatures (°C). (*B*) The one-half-time (min) of doxorubicin efflux from cells with a doxorubicin concentration in the cell water of 4 μM or 34 μM at various temperatures.

permeability coefficient correlated with the temperature dependence of the doxorubicin partition coefficient at the various concentrations.

DISCUSSION

The aim of this work was to test the hypothesis that the concentration and temperature dependence of doxorubicin transport in human red blood cells correlated with the influence of concentration and temperature on the doxo-
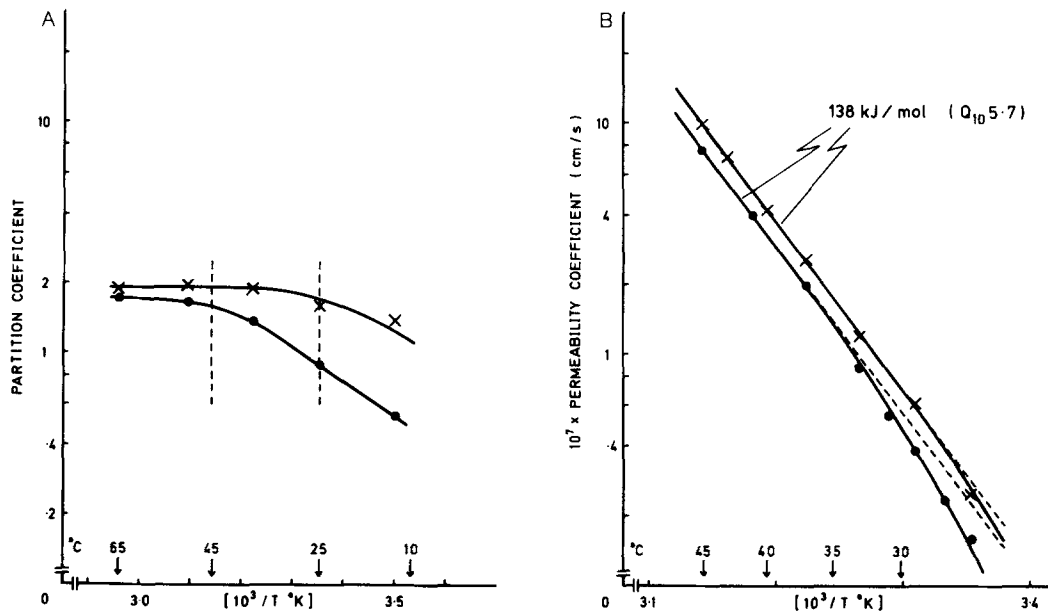

FIGURE 6. (A) The partition coefficient of doxorubicin between 1-octanol and a Tris-buffer [(mol^{-1} 1-octanol)/(mol^{-1} Tris-buffer)] at equilibrium as a function of temperature (12.5–65°C). The doxorubicin concentration in the Tris-buffer at equilibrium at 37°C (pH 7.3) was either 4 (X) or 34 μM (●). (B) The apparent doxorubicin permeability coefficient as a function of temperature (25–45°C). The experiments were carried out with two separate cell batches equilibrated with doxorubicin at 37°C (pH 7.3) to a final cellular doxorubicin concentration in the cell water of either 4 (X) or 34 μM (●).

rubicin 1-octanol/water partition. If so, the previous and present data are consistent with the perception of doxorubicin transport in red cells as a Fickian diffusion transport process. It appears from Figs. 4 and 6 that the criteria of the hypothesis qualitatively were fulfilled.

The doxorubicin transport was determined by measuring the doxorubicin efflux as a function of the doxorubicin concentration in the medium at equilibrium after the initial loading of the cells with doxorubicin. This concentration was assumed to be equal to the doxorubicin concentration in the cell water. However, the concentration of doxorubicin in the red cells depended on the composition of the cell-suspending medium. The distribution ratio was ~7 at pH 7.3 (37°C), but decreased to ~1.5 when the cells were

suspended in their own plasma at the same pH and temperature (M. Dalmark, unpublished observation). The doxorubicin-binding capacity of the cells was large because the distribution ratio was unaltered in the doxorubicin concentration range of the suspending medium between 1 and 500 μM. This indicates that proteins and lipoproteins have a small doxorubicin affinity but a large binding capacity. The protein binding is expected because proteins contain many aromatic amino acids capable of forming molecular associations through pi-electron interaction. The effect of plasma on the distribution ratio seems to justify the interpretation of doxorubicin distribution as being passive, like the distribution of other aromatic compounds like salicylate (Dalmark and Wieth, 1972). The concentration of an amino group-containing compound in the cell water is close to the concentration of the cell-suspending medium when the pH values of cell water and medium are close to each other (cf. Fig. 2A).

Fig. 4 shows the apparent permeability coefficient for doxorubicin as a function of the doxorubicin concentration in the incubation medium correlated quantatively with the doxorubicin 1-octanol/water partition coefficient as a function of the doxorubicin concentration in the water phase.

The doxorubicin transport was strongly temperature-dependent. The temperature dependence owed both to the temperature dependence of the permeation process per se and to the variation of the fractional ionization of doxorubicin with temperature because the transport and the partition coefficient have been shown to be pH dependent (Dalmark, 1981 [In press]; Eksborg, 1978). The observed cellular pH as well as the pK of the amino group of doxorubicin was *linearly* related to the inverse absolute temperature (Fig. 2A and B). This linearity is to be expected (Bates, 1973) because histidine and amino groups are the major determinants of the cellular pH (e.g., Dalmark [1976 and 1980]) and the pH of a doxorubicin solution. The ionization of doxorubicin varied with temperature, although the variation was minimized by the applied technique. An increasing doxorubicin transport with temperature with a Q_{10} of 1.2 was expected from the shift in the ionization of doxorubicin because the heat of ionization of the amino group on doxorubicin exceeded that of the cellular pH. The activation energy of 138 kJ/mol (33 kcal/mol) observed at high temperature has to be corrected for 13 kJ/mol to obtain the Arrhenius activation energy of the doxorubicin permeation process per se of 126 kJ/mol (30 kcal/mol). A pronounced temperature dependence of the permeation process per se is expectable because doxorubicin has a high hydrogen-bonding capacity (Fig. 1). The retarding effect of hydrogen bonds between a permeant and the bulk water in the medium was pointed out by Stein (1967).

The nonlinearity of the Arrhenius plots of doxorubicin transport (Fig. 6B) was not explicable by the temperature-dependent shift of the fractional ionization of doxorubicin, but correlated semiquantitatively with the temperature dependence of the 1-octanol/water partition coefficient (Fig. 6A). It is a general observation that molecular associations through pi-electron interaction decrease with temperature, which affects the Brownian movements (e.g., Tsó et al. [1963]). The temperature dependence of self-association has

also been observed with another anthracycline (Barthelemy-Clavey et al., 1974).

It appears that both saturation kinetics and non-linear Arrhenius plots of doxorubicin transport qualitatively and semiquantitatively could be explained by aqueous aggregation. In this case, the previous (Dalmark, 1981[In press]) and present data are consistent with the perception of doxorubicin transport in human red blood cells as a Fickian diffusion transport process of the electrically neutral doxorubicin molecule through the lipid domain of the cell membrane. A carrier-mediated transport mechanism has been assumed to exist in the nucleated Ehrlich ascites tumor cells (Danoe, 1976; Skovsgaard, 1977 and 1978). The experimental data obtained with ascites cells and red cells are difficult to compare directly because the former have a nucleus, mitochondria, and an extensive cell surface coat (Smith and Levinson, 1979). The effect of pH on transport, however, was identical (Skovsgaard, 1977; Dalmark, 1981 [In press]). The cell membrane modifiers—1-alcohols and phloretin—accelerated the doxorubicin transport both in ascites cells and in human red blood cells in the same manner as these modifiers accelerate the transport of other lipophilic compounds (M. Dalmark and E. Hoffman, manuscript in preparation; Dalmark, 1981). Substrate inhibition and self-inhibition (Skovsgaard, 1978) can be expected from the present hypothesis because the addition of various anthracycline derivatives decreases the apparent permeability coefficient by aqueous aggregation. This assumption is in accordance with the inhibition of doxorubicin influx in human red cells by addition of various nucleotides which form molecular associations with doxorubicin (Dalmark and Johansen, 1981). The observed $K_{1/2}$ of doxorubicin influx in ascites tumor cells (Skovsgaard, 1978) is approximately one order of magnitude lower than the apparent $K_{1/2}$ of the doxorubicin 1-octanol/water partition at the same pH and temperature (Fig. 4A). However, one rarely, if ever, measures the concentration of the permeant at the place where the permeability is highest. Furthermore, the data indicated that doxorubicin concentration close to the rate-limiting barrier of the membrane was different from the concentration in the bulk solution because doxorubicin apparently was adsorbed to a large extent to the pronounced external surface coat of ascites cells (Danoe, 1976; Skovsgaard, 1978). On the other hand, the temperature dependence of doxorubicin transport in red cells and ascites cells appeared to be very different, with a Q_{10} of 1.2 of the latter cell type at body temperature. This difference cannot be explained by the present hypothesis because the amount of doxorubicin adsorbed to the cell surface coat appeared to be almost independent of the temperature (Skovsgaard, 1978).

Molecular association through pi-electron interaction in aqueous solutions has been observed for many organic molecules. Self-association of the various molecules has been observed in the micro- and millimolar range at room temperature. The self-association of anthracyclines at concentrations $>10^{-5}$ M has previously been observed by others (Barthelemy-Clavey et al., 1974; Eksborg, 1978; Righetti et al., 1979). The self-association of the heterocyclic acridine dyes is observed at 5×10^{-5} M (Albert, 1966) and that of the

antineoplastic heterocyclic drug actinomycin is observed at 2×10^{-4} M (Crothers et al., 1968). Heterocyclics derived from purine, pyrimidine, and indole (nucleotides, nucleosides, DNA-derived bases, and amino group-containing compounds like tryptophan, tryptamine, and serotinin) self-associate in the millimolar-concentration range (Tsó et al., 1963; Dimicoli and Hélène, 1973). It is a general observation that self-association decreases with temperature, which acts affects Browian movements (Tsó et al., 1963).

The influence of self-association on the transmembraneous transport is difficult to observe unless the polymers have a significantly lower permeability coefficient than the monomer. The latter situation is observed if the monomer exists both in a charged and an electrically neutral form, in which case the polymers are more hydrophilic than the permeating electroneutral monomer by random association of charged and uncharged molecules (e.g., doxorubicin). Self-association is much more difficult to observe by flux measurements when the permeant only exists as an electroneutral molecule because the diffusion coefficient of electroneutral molecules generally changes with the square root of the molecular weight (Stein, 1967). The energy necessary to carry a charged molecule through the low dielectric lipid membrane domain is probably very high in distinction to the requirement for translocation of electroneutral molecules (Parsegian, 1969).

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