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TRANSPORT AND BINDING OF DAUNORUBICIN, ADRIAMYCIN, AND RUBIDAZONE IN EHRLICH ASCITES TUMOUR CELLS

TORBEN SKOVSGAARD

The Finsen Institute, Department of Internal Medicine,
Strandboulevarden 49, 2100 Copenhagen Ø, Denmark

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Abstract—The determinants of cellular net uptake of three anthracyclines—daunorubicin, adriamycin, and rubidazone—were studied in Ehrlich ascites tumour cells *in vitro*. A striking finding was the marked effect of the pH on net uptake in the way that lowering of the pH decreased the rate of uptake and steady-state level. As pK_A of the drugs proved to be 8·15–8·45, this finding suggests a penetration of the drugs by passive transport of the unionized form of the molecules. Several findings indicated an uphill efflux of the drugs from the cells. (1) The estimate of the cytoplasm/medium concentration ratio at steady-state for daunorubicin and rubidazone was considerably below that predicted when assuming passive distribution. (2) The cellular accumulation of the drugs was significantly enhanced by sodium azide. (3) The cellular release of the drugs was inhibited by sodium azide. Together these findings suggest a membrane transport of the drugs as a "leak-and-pump" system. Studies of the intracellular binding showed that the affinity of adriamycin for isolated nuclei and for total cellular homogenate was about 50 per cent higher than that of daunorubicin; rubidazone possessed the lowest affinity for cellular binding sites. The affinity of the drugs for intracellular binding sites was not influenced by the pH.

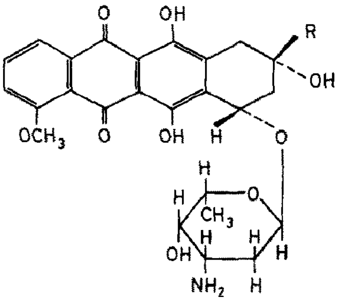
The results indicate that the net uptake of anthracycline derivatives in these tumour cells is determined by: (1) the permeability of the membrane to the unionized form of the molecule; (2) the specificity of the drug for the mechanism of active efflux; (3) the relationship between intracellular and extracellular pH; (4) the pK_A value of the drug; (5) the affinity of the drug for the intracellular binding sites.

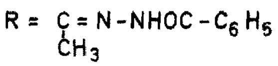
The anthracycline antibiotics play a prominent role in the treatment of leukemias and solid tumours in man [1–4]. The mechanism of their cytotoxic action is attributed mainly to an interaction with DNA [5, 6]. As the therapeutic target is localized intracellularly, cellular uptake is a decisive factor in the biological and therapeutic effect of drugs belonging to this group. Thus, *in vitro* the inhibitory effect of daunorubicin on nucleic acid synthesis has proved higher than that of adriamycin in equimolar concentrations; but if the inhibition is adjusted for cellular uptake, the effect of adriamycin was at least equal to that of daunorubicin [7]. Acquired resistance to anthracyclines *in vitro* [8] as well as *in vivo* [9–12] has been shown to be accompanied by decreased cellular uptake. Although the anthracyclines have been extensively investigated, little is known about the exact mode of cellular uptake [7, 13–15]. In a subline of the Ehrlich ascites tumour resistant to daunorubicin there are indications of an active efflux of this drug [16].

The present study is concerned with details of membrane transport and intracellular binding of the anthracyclines in Ehrlich ascites tumour cells and with evaluating the importance of chemical modification of the molecule in this respect. As representative compounds of the anthracycline group, three derivatives now on clinical trial were chosen, viz. daunorubicin, adriamycin, and rubidazone (Fig. 1).

MATERIALS AND METHODS

Tumour cells. A hypotetraploid Ehrlich ascites tumour was maintained by weekly transplantation of 0.2 ml undiluted ascitic fluid into first-generation hybrids of female random-bred Swiss mice and male inbred DBA mice. The mice were killed by cervical dislocation 6–8 days after tumour inoculation, ascitic fluid was removed with a Pasteur pipette and placed on ice. To eliminate contamination with plasma and red cells, pooled ascitic fluid was washed four times in ice-cold Ringer solution. Cell agglutination was prevented by addition of heparin (100 IE/ml) to the initial washing fluid. Washing was followed by centrifugation 900 *g* for 4 min. Packed cell volume was determined by centrifugation of samples at 5500 *g* for



Rubidazone R =

$\underset{CH_3}{C}=N-NHOC-C_6H_5$

Daunomycin R = $COCH_3$

Adriamycin R = $COCH_2OH$

Fig. 1. Structural formulae of daunorubicin, adriamycin, and rubidazone.

8 min in a haematocrit centrifuge. Suspensions of cells with a density of 1% by volume were prepared and preincubated 5–10 min before exposure to drug. Cell viability was estimated by the ability to exclude 0·1% nigrosin. After incubation for 1 hr in the phosphate medium, the uptake of dye was 10, 6, and 9 per cent at pH 6.4, 7.4, and 7.8 respectively.

Nuclei. Isolated nuclei were prepared by a method described by Danø [9] by homogenization after swelling of the cells in a hypotonic buffer. The preparation contained 1–3 per cent apparently intact cells. The loss of nuclei during the preparation (20–30 per cent) is compensated for by the use of a higher cell concentration initially to make the DNA content in the suspension of nuclei comparable to the DNA content in a 1% cell suspension [16].

Cell homogenate. The homogenization was performed as described for isolated nuclei except that no compensation for loss of nuclei was included, and no washing procedure was performed after homogenization. The cell content in the final suspension of homogenate corresponds to a 1% cell suspension.

Buffers, incubation and sampling procedure. For all washing procedures a Ringer solution was used containing: 148 mM Na^+, 5.2 mM K^+, 151 mM Cl^-, 1.7 mM Ca^{2+}, 1.2 mM Mg^{2+}, 1.2 mM SO_4^{2-} and 3.0 mM orthophosphate (pH adjusted to 7.45). The standard medium was a phosphate buffered medium (modified after Bloch–Frankenthal *et al.* [17]), which at pH 7.45 contained 57.0 mM NaCl, 5.0 mM KCl, 1.3 mM $MgSO_4$ and 60 mM sodium phosphate; at other pH levels NaCl was adjusted so that isotonia was maintained. To reduce the tendency of the cells to form clumps during the incubation, calcium was omitted from the suspending medium. In specific experiments with long-term incubation, glucose was added to a concentration of 10 mM. Isolated nuclei and cell homogenate were incubated in a buffer of the following composition: Tris–HCl 50 mM, NaCl 92 mM, KCl 5.0 mM, $MgSO_4$ 1.3 mM and $CaCl_2$ 4.0 mM.

Incubation was carried out in a water-thermostated shaking incubator in air atmosphere. The suspensions were incubated at 37° for varying periods in Ehrlenmeyer flasks. In each experiment the pH in the suspensions was followed by a pH meter (PHM 27 Radiometer, Copenhagen). During the incubation of cells the pH in the suspension decreased by 0.04–0.20 unit per hour if glucose was present. The most pronounced drop was seen when the initial pH in the solution was high (7.8), as a consequence of the small buffer capacity at this pH-level. In suspensions without glucose and in suspensions of nuclei or cell homogenate pH remained essentially unchanged during the incubation. After incubation the suspensions were cooled to 0–4° and pelleted by centrifugation at 2000 *g* for 4 min. Unless otherwise stated cells and nuclei were washed twice with ice-cold Ringer solution, whereas no washing procedure was performed for cell homogenate. Before determination of drug in the medium from the cell homogenate the supernatant was further centrifuged 15000 *g* for 20 min.

Determination of drug uptake. Two methods were used. (1) In most experiments drug uptake was calculated by subtraction of drug in medium and wash fluid from the amount added to the suspension. The concentration of drug in medium and wash fluid was determined spectrophotometrically at 480 nm (Unicam SP 1800 spectrophotometer); the drug content of the combined wash fluids ("weakly adsorbed fraction") was for cells less than 10 per cent and for isolated nuclei less than 20 per cent of that of the medium. (2) In some experiments the drug uptake was determined by measuring the total drug fluorescence extracted from the drained cell pellet after two cold washes. Drug was extracted with 0.3 N HCl–50% ethanol solution according to Bachur *et al.* [18]. The fluorescence of the supernatant solution was determined in Aminco Bowmann spectrofluorometer (excitation, 470 nm; fluorescence 585 nm). No significant difference could be demonstrated between the results of the two methods for determination of drug content. For spectrophotometric and fluorometric measurements standards were used. The standards were adjusted by means of spectrophotometric measurements of dilutions in methanol using the maximums: daunorubicin at 490 nm $E^{1\%}_{1\,cm} = 218$, adriamycin at 479 nm $E^{1\%}_{1\,cm} = 219$, and rubidazone at 480 nm $E^{1\%}_{1\,cm} = 190$. In all cases the weight statements for the drugs refer to the hydrochloride.

Efflux experiments. Suspensions of cells were preloaded with drug at varying concentrations for 30 min at 37° in the phosphate medium without glucose. After incubation the cells were washed twice at 0–4° to remove extracellular drug. The cells were suspended in the same medium at 0° and the volume per cent of cells determined. From this suspension aliquots containing 5 μl packed cells were transferred to 10 ml drug-free medium at 37°. After varying incubation times the cells were pelleted by cold centrifugation (2000 *g* for 4 min).

Metabolism. To evaluate the extent of cell metabolism during the incubation, drugs were extracted from cells, which had been incubated for 1 hr with 10 μg/ml and then were washed twice. To prevent hydrolysis of the drug molecules after incubation, no acidification was used in the subsequent procedure. Drugs were extracted from cells by chloroform–methanol (2:1 by vol). After centrifugation the organic phase was evaporated to dryness. The residue was dissolved in *n*-butanol and chromatographed on Silica gel plates in chloroform–methanol–water (80:20:3 by vol). The quantification of the conversion of drugs was determined according to Cradock *et al.* [19]. The spots were localized under 366 nm light by comparison with spots obtained with controls of the authentic compound. The spots identified as the authentic compound as well as the residue of the chromatogram were carefully scraped from the plate and extracted separately with 0.3 N HCl–50% ethanol. After centrifugation, fluorescence of the supernatant solutions was determined, and the percentage of metabolites was related to total drug fluorescence. In extracts from cells 89–91 per cent was recovered; no major difference was found between the three analogues. The hydrolysis of rubidazone to daunorubicin was investigated by incubation in water at 37° for 15 min–4 hr. By the procedure described above, but with acetone–formamide (95:5 by vol) as mobile phase, no significant hydrolysis occurred within the incubation period.

Chloride distribution. Ten-ml cell suspensions of 1%

by vol were incubated with $^{36}Cl^-$ for 30 min at 37°. [^{3}H]methoxyinulin was added to the suspensions as a marker of extracellular space in the cell pellet. After centrifugation the medium was preserved for analysis, and the wet weight of the samples was determined. Dry weight of the cell pellet was determined after drying overnight at 65°. Intracellular water was calculated from the difference between the wet weight and dry weight less the [^{3}H]inulin space in the cell pellet. Appropriate correction for $^{36}Cl^-$ in trapped medium was made in calculations of intracellular $^{36}Cl^-$. Dried samples were digested by addition of 2000 µl 0.5 N KOH and incubation at 80° for 1 hr. Two-hundred-µl samples from medium and cell extract were transferred to scintillation vials containing 10 ml hyamine–toluene–ethanol scintillation solution (composition: POP: 3.5 g: POPOP: 70 mg, toluene: 700 ml, ethanol (96% by vol) 300 ml; hyamine 75 ml). ^{3}H and $^{36}Cl^-$ were counted simultaneously in a Beckman LS-250 liquid scintillation spectrometer.

Determination of pK_A *values.* To determine the pK_A value for the three anthracyclines titration was performed at 25° by a research pH-meter (PHM 64 Radiometer, Copenhagen) with metrohm combined pH-electrode. The pK_A values were determined from the titration curves after titration with 0.1 M KOH. pK_{A1} was for daunorubicin 8.25, for adriamycin 8.15, and for rubidazone 8.45. pK_{A2} was for all drugs > 9.5 and pK_{A3} > 10.1. For comparison, pK_A of adriamycin was found to be 8.22 in another study [20].

Chemicals. Daunorubicin and adriamycin, both as hydrochlorides, were obtained from Farmitalia Co., Milan. Rubidazone (benzoyl-hydrazone daunorubicin) was kindly supplied by Rhone-Poulenc Research Laboratory, Paris, France. According to the analysis performed by the Rhone-Poulenc laboratory, the rubidazone product contained 3–9 per cent daunorubicin. Sodium azide was obtained from Merck, Darmstadt, Germany. [^{3}H]methoxyinulin (55.5 µCi/mg) was obtained from New England Nuclear, Germany, $^{36}Cl^-$ was added as 0.5 N HCl (7.4 µCi/mg Cl^-) from AEK Risø, Roskilde, Denmark.

RESULTS

Time-course of uptake. Figure 2 shows the time-course of net uptake of daunorubicin, adriamycin, and rubidazone. The drugs were added to the cell suspension to a concentration of 10 µg/ml at zero time. A considerable difference in the time-course of net uptake for the three drugs was demonstrated. Thus, the uptake of daunorubicin was much more rapid than that of rubidazone and adriamycin. For daunorubicin and rubidazone a steady state was reached within 30 and 90 min respectively, while with adriamycin the cell content was increasing during the whole period of incubation. For all drugs a pronounced intracellular accumulation took place; at steady state the cell/medium concentration was 380 for daunorubicin compared to about 75 for rubidazone. As illustrated for daunorubicin (the dashed line) a minor part of the drug was removed by the two cold washes included in the procedure; this component is referred to as the "weakly adsorbed fraction".

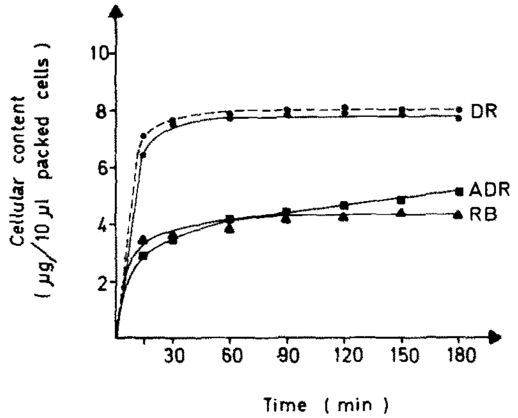


Fig. 2. Time-course of net uptake of daunorubicin (DR), adriamycin (ADR), and rubidazone (RB) in Ehrlich ascites tumour cells. Suspension of cells (10 µl/ml) were incubated at 37° in standard medium (pH 7.45) containing 10 mM glucose. Drug, 10 µg/ml, was added at time 0. Drug remaining in medium and in two batches of wash fluid obtained at 0–4° was determined spectrophotometrically, and the cellular drug content was calculated by subtraction from the total amount added. The dotted line indicates the uptake of daunorubicin uncorrected for drug in wash fluid. Average of results from two experiments done on different days.

For transport across the cell membrane an important point is the extent of ionization of the penetrating molecules, as it is generally agreed that ionized molecules penetrate the membrane poorly by simple diffusion [21]. Concerning the anthracyclines, a general chemical characteristic is the amino group in the sugar moiety which implies that these drugs behave as weak bases. As the pK_A of the analogues was determined as 8.2–8.5 (see Materials and Methods), the fraction of positively charged molecules will increase when the pH in the solution is decreased.* As the phenolic hydroxy groups at positions 5 and 12 have

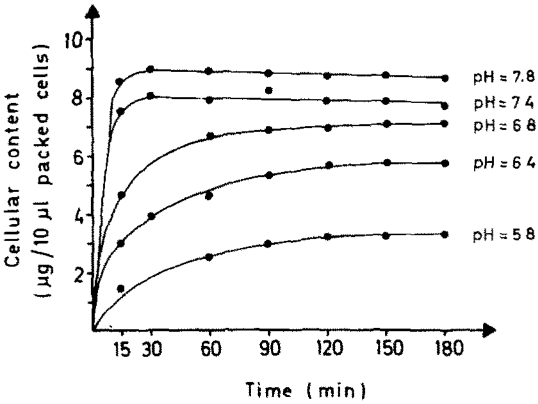


Fig. 3. Effect of the pH of the medium on time course of uptake of daunorubicin. Cells were incubated in standard medium containing 10 mM glucose and with pH adjusted to specified values, and 10 µg/ml was added at time 0. Experimental conditions as described in Fig. 2.

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*The relationship between ionized molecules (I) and unionized molecules (U) is expressed in the Henderson–Hasselbalch equation (1): $pH = pK_A + \log([U]/[I])$.

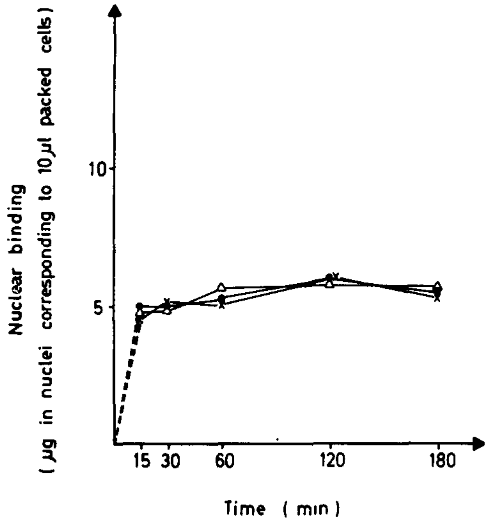

Fig. 4. Binding of daunorubicin to isolated nuclei from Ehrlich ascites tumour cells at varying pH of the media. Suspension of isolated nuclei was prepared from a cell suspension of 13 μl whole cells/ml, resulting in a final suspension of nuclei with a DNA content corresponding to a cell suspension of 10 μl/ml. The suspension of nuclei was incubated at 37° in Tris–HCl buffer at pH 7.8 (×), 7.4 (●) and 6.4 (△). Daunorubicin at 10 μg/ml was added at time 0, and the uptake was determined by fluorometric measurements of the extracts of the nuclei.

a pK_A about 10, the dissociation of these groups plays no role at physiological pH. To investigate the importance of the dissociation in cellular uptake, the time-course of the uptake of daunorubicin was studied at varying pH levels. Figure 3 illustrates the time-course of uptake of daunorubicin at pH 7.8, 7.4, 6.8, 6.4, and 5.8. At these pH levels the percentage of total drug in the unionized form was calculated as 26.2, 12.4, 3.4, 1.4, and 0.4 respectively. The uptake is seen to be considerably influenced by pH in the medium, in the way that lowering of the pH decreased the rate of uptake and the steady-state level. Thus, the cell/medium concentration ratio at pH 7.8 was about 800, whereas the corresponding ratio at pH 5.8 was about 50. In repeated experiments the cell content of daunorubicin at pH 7.8 showed a maximum, followed by a slightly descending slope, probably a consequence of the inevitable drop in pH in medium with time (see Materials and Methods). A corresponding influence of pH on cellular uptake was found for adriamycin and rubidazone (results not shown).

Binding of anthracyclines. Several investigations have shown that the anthracyclines after penetration of the cell membrane bind mainly to DNA in the nucleus [5, 22–24]. To test whether pH influences the nuclear binding of the anthracyclines, the time-course of binding to isolated nuclei was studied at varying pH levels. As illustrated for daunorubicin (Fig. 4), the binding was not found to be influenced by a change in pH in the medium from 7.8 to 6.4. The ionized and the unionized molecules, therefore, seem to have the same affinity for DNA.

Table 1 shows the nuclear binding at pH 7.45 for the three drugs at equilibrium. The results demonstrate a significantly ($P < 0.01$) higher binding of adriamycin than of daunorubicin and rubidazone. Expressing the affinity by the ratio (amount of drug bound)/(concn of free drug), the affinity of adriamycin for isolated nuclei was about 50 per cent higher than that of daunorubicin. At low drug concentration the nuclear affinity of rubidazone was close to that of daunorubicin, but if 10 μg/ml were added the affinity of rubidazone was found to be 36 per cent below that of daunorubicin.

Figure 5 shows the amount of drug bound to cell homogenate (including the "weakly adsorbed fraction") as a function of the concentration of the drug in the medium at equilibrium (free drug). It appears that when the binding to cell homogenate was below 7–9 μg/10 μl packed cells, there was for each of the drugs proportionality between the amount of drug bound and the corresponding concentration of free drug. Consequently, differences in the binding in this interval reflect differences in the affinity between drug and cell components. As demonstrated with nuclei, the affinity of adriamycin was significantly stronger than that of daunorubicin, and rubidazone possessed the weakest affinity of the three drugs investigated. At a binding to cell homogenate above 9 μg/10 μl packed cells, a saturation gradually occurred. As complete saturation was not obtained, this experiment does not permit any conclusion concerning differences in total binding capacity between the three drugs. To estimate the fraction of drug bound to nuclei, the binding (including the "weakly adsorbed fraction") of the three drugs to isolated nuclei is plotted on the same figure. It appears that the nuclear binding may account for about 70 per cent of the total cellular

Table 1. Nuclear binding of daunorubicin, adriamycin, and rubidazone

	Initial concn in suspending medium (μg/ml)	Nuclear binding at equilibrium, μg drug in nuclei corresponding to 10 μl packed cells (mean ± S.E.M.)
Daunorubicin	0.25	0.138 ± 0.003
	10.0	5.33 ± 0.07
Adriamycin	0.25	0.160 ± 0.002
	10.0	6.43 ± 0.22
Rubidazone	0.25	0.132 ± 0.003
	10.0	4.32 ± 0.09

Suspension of isolated nuclei prepared from 13 μl whole cells/ml (see Material and Methods) incubated at 37° with drugs for 1 hr in Tris buffer adjusted to pH 7.45. After incubation the nuclei were washed twice, and the amount of drug taken up was determined by fluorometric measurements of the extraction medium. The mean value is based on five determinations.

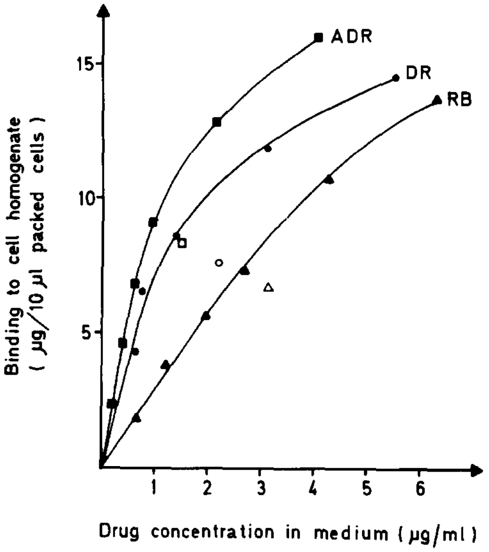

Fig. 5. Binding of daunorubicin (DR), adriamycin (ADR), and rubidazone (RB) to cell homogenate corresponding to a 1% cell suspension plotted (closed symbols) as a function of drug concentration in the medium at equilibrium. Open symbols represent nuclear binding when 10 µg/ml drug was added to the suspension at time 0, including "weakly adsorbed fraction". The concentration of drug in medium was determined spectrophotometrically, and the amount of drug bound was calculated by subtraction from the total amount added. The suspending medium was buffered with Tris–HCl and adjusted to 7.45. The incubation time was 60 min (a separate experiment had shown equilibrium at that time). Each point represents the mean of duplicate determinations from two separate experiments done on different days.

binding at a certain equilibrium concentration in the medium. No significant effect on binding of the drugs to cell homogenate could be demonstrated by sodium azide (10 mM) or by a change in the pH in the medium from 6.4 to 7.8 (results not shown).

When comparing Figs. 2 and 5, the distribution of drug across the membrane at steady state may be estimated. At 10 µg/ml added to the suspension of cells the cell/medium concentration ratio at steady state (including the "weakly adsorbed fraction") was 400 (corresponding to an uptake of 8.0 µg/10 µl "packed cells" at 2.0 µg/ml in the medium (Fig. 2)). Fig. 5 shows that an uptake of 8.0 µg/10 µl "packed cells" in cell homogenate equilibrates with 1.20 µg/ml. Assuming that the drug determined in the supernatant of the homogenate represent free drug this is the best obtainable estimate of the drug concentration on the cytoplasmic side of the membrane. The finding in one study [15] of a high intralysosomal accumulation of the anthracyclines was considered. In our cells, however, this could be ruled out as differential centrifugation according to de Duve *et al.* [25] showed less than 10 per cent of the drug present in the lysosomal fractions. That is, the cytoplasm/medium distribution ratio C_i/C_e (using the subscripts *i* and *e* for intracellular and extracellular respectively) for daunorubicin at steady state is estimated to be 0.60; for rubidazone the corresponding ratio is estimated to be 0.29.

According to Milne *et al.* [26] the theoretical distribution ratio of a weak base at equilibrium (assuming free permeability of the unionized molecules (U) and complete impermeability to the ionized molecules (I)) is:

$$R = \frac{C_i}{C_e} = \frac{[\mathrm{U}_i] + [\mathrm{I}_i]}{[\mathrm{U}_e] + [\mathrm{I}_e]} = \frac{1 + 10^{(\mathrm{p}K_A - \mathrm{pH}_i)}}{1 + 10^{(\mathrm{p}K_A - \mathrm{pH}_e)}}. \qquad (2)$$

Assuming that both chloride and hydrogen ions are distributed passively, pH_i may be determined by using the Gibbs–Donnan relationship:

$$[\mathrm{Cl}_i^-]/[\mathrm{Cl}_e^-] = [\mathrm{H}_e^+]/[\mathrm{H}_i^+]. \qquad (3)$$

According to Lassen *et al.* [27] chloride is distributed passively in Ehrlich ascites tumour cells. The distribution of H^+ is still subject to discussion, but there is indication of a passive distribution across the erythrocyte membrane [28].

The distribution ratio $[\mathrm{Cl}_i^-]/[\mathrm{Cl}_e^-]$ was measured to 0.52 ± 0.02 (S.E.M.) at $\mathrm{pH}_e = 7.25$ (pH in medium containing glucose after one hour's incubation). The corresponding pH_i is calculated to 6.97 which is in accordance with the value measured by the DMO method in Ehrlich ascites tumour cells [29, 30]. Using $\mathrm{p}K_A$ determined by titration (see Material and Methods) the ratio C_i/C_e, assuming passive distribution (equation (2)), is 1.8 for daunorubicin as well as rubidazone.

The distribution ratio C_i/C_e at equilibrium of daunorubicin and rubidazone, assuming a passive process, thus exceeds that obtained by estimating the intracellular free drug by factor 3.0 and 6.2 respectively. This finding suggests an uphill outward transport against the concentration gradient.

Energetics of cellular uptake. Figure 6 shows the importance of energy requirement for the time-course of uptake. As illustrated for rubidazone, omission of glucose from the medium did not significantly influence the uptake during 1 hr of incubation. However, if sodium azide, an uncoupler of the oxidative phosphorylation, was added to the suspension a significant enhancement of net uptake was seen (for

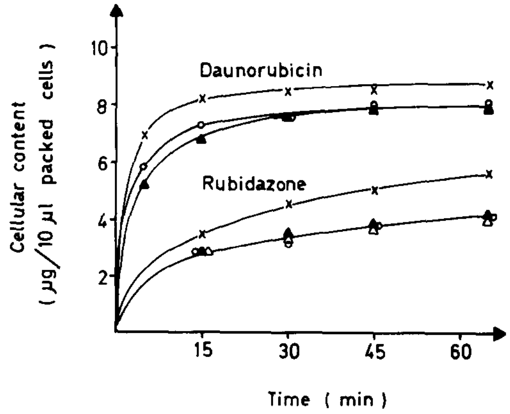

Fig. 6. The effect of inhibition of cell metabolism on time-course of uptake of rubidazone and daunorubicin. Experimental conditions as described in Fig. 2. Symbols: (▲) standard medium containing 10 mM glucose, (△) standard medium without glucose, (×) standard medium containing 10 mM sodium azide, and (○) standard medium containing 10 mM glucose and 10 mM azide.

daunorubicin the cell/medium concentration ratio increased from 380 to 900 at equilibrium). For adriamycin a corresponding effect of sodium azide was demonstrated (data not shown).

The effect of sodium azide could be a consequence of an acidification of the intracellular compartment which, according to equation (2) would result in an increased intracellular drug accumulation. To evaluate this point the distribution ratio of chloride across the membrane was measured in standard medium and in the same medium including 10 mM sodium azide (30 min incubation). In standard medium $[Cl_i^-]/[Cl_e^-]$ was 0.62 ± 0.02 (S.E.M.) compared to 0.68 ± 0.02 (S.E.M.) if sodium azide was added. This difference is significant ($P < 0.05$) and corresponds (equation (3)) to an increase in pH_i of 0.03 unit caused by sodium azide. A corresponding effect of azide on the chloride distribution ratio has been shown for L1210 leukemia cells [31]. The enhancement of net uptake by sodium azide is compatible with an energy-dependent active efflux. As addition of glucose reversed the effect of sodium azide, glycolysis and oxidative phosphorylation may probably substitute one another in providing energy for this mechanism.

Efflux experiments. Figure 7 demonstrates the unidirectional efflux of daunorubicin, adriamycin and rubidazone from Ehrlich ascites tumour cells preloaded to nearly equal drug content and resuspended in a large volume of drug-free medium at 37°. For each of the drugs biphasic curves were obtained with an initial rapid loss after which the rate of release became essentially constant for at least 10 min. In comparison of the linear phase of the efflux of the three drugs in standard medium the release of daunorubicin and rubidazone was nearly equal (1.5% per min) whereas the release of adriamycin was considerably slower (0.7% per min). In a similar experiment with release of daunorubicin from isolated nuclei (data not shown) the rate of release was of the same order of magnitude as for whole cells, indicating that the release from the nuclei in this medium is the rate-limiting process.

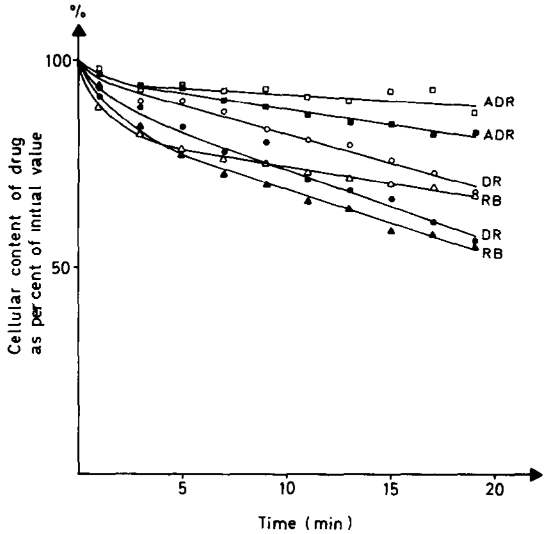

Fig. 7. Efflux of daunorubicin (DR), adriamycin (ADR), and rubidazone (RB) from Ehrlich ascites tumour cells. Cells were incubated for 30 min at 37° in standard medium without glucose containing drugs at 5–20 μg/ml, so that comparable levels of uptake were obtained with the three drugs (3.1–3.6 μg/10 μl packed cells). Cells were collected, then resuspended in a large volume of the same medium without additions (closed symbols) or in a medium containing 10 mM sodium azide (open symbols). Cellular content of drug was measured fluorometrically at intervals and data are plotted as per cent of cellular content present at end of preloading.

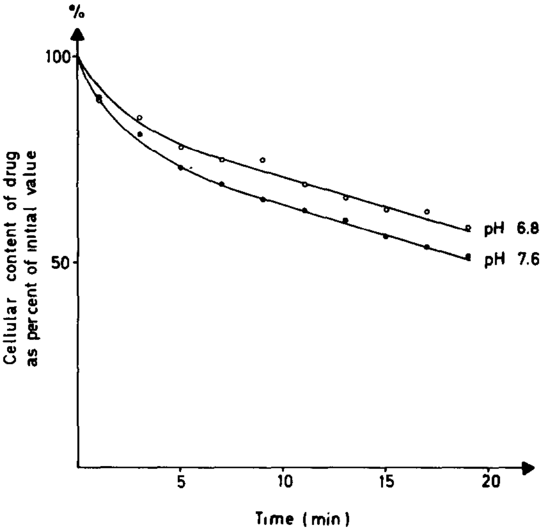

Fig. 8. Efflux of daunorubicin at pH 7.6 and 6.8 in standard medium. Experimental conditions as described in Fig. 7.

For all the drugs the release was significantly inhibited by 10 mM sodium azide. This finding may be expressive of inhibition of an energy-dependent efflux resulting in a higher cytoplasmic concentration and consequently a slower release from the nuclei. Figure 8 shows that apart from the initial rapid phase, the efflux was nearly equal at pH_e 7.6 and 6.8.

If the unidirectional efflux was continued by repeated resuspensions in fresh standard medium, the intracellular drug content after 2 hr was reduced to 8 per cent for daunorubicin and rubidazone and to 27 per cent for adriamycin (duplicate experiments). These data indicate that although the anthracycline binds with strong affinity to intracellular binding sites, most of the drug is freely exchangeable.

DISCUSSION

The data in the present study suggest that the membrane transport of the anthracyclines in Ehrlich ascites tumour cells consitutes a "leak-and-pump" system, the leak being a passive transport of the unionized form of the molecules and the pump an uphill efflux.

The lack of effect of pH on cellular binding and release of the drugs indicates that the marked effect of pH on the net rate of uptake is mainly due to an effect on influx. This finding is compatible with a diffusion mechanism of the penetration, mainly in the unionized form, in accordance with the pH partition theory [26, 32]. The hypothesis of penetration by a diffusion process is supported by the finding of a linear rate-concentration relationship for daunorubicin in a previous study in our Laboratory [16]. As pointed out by Wilbrandt *et al.* [33], however, a transport which follows diffusion kinetics may reflect a carrier transport with a high value of Km. If this is the case, the effect of pH on the rate of uptake must indicate that the specificity of the carrier for the drug is considerably influenced by the ionization of the amino group in the sugar moiety of the molecule.

The effect of pH on the steady-state level (Fig. 3) is probably in the main a consequence of displacement of the relation between pH_e and pH_i. As the anthracyclines are weak bases, they tend to accumulate in the more acidic compartment (equation 2). According to determinations performed by Poole *et al.* [30] on Ehrlich ascites tumour cells, pH_e is higher than pH_i when pH_e is higher than 6.9, whereas pH_i is higher than pH_e when pH_e is below 6.9. In other words the drug concentration in cytoplasm (which determines intracellular binding) is expected to decrease when pH_e decreases.

In a previous study from this laboratory [16] indications have been provided for an active efflux of daunorubicin from cells of a subline of Ehrlich ascites tumour cells resistant to this drug. The studies described in the present report provide indications of an uphill outward transport of the anthracycline antibiotics also in the wild-type tumour cells, *viz*: (1) The estimate of the cytoplasm/medium concentration ratio for daunorubicin and rubidazone at steady state was considerably below that predicted when assuming passive distribution. (2) The cellular accumulation of the drugs was significantly enhanced by the metabolic inhibitor sodium azide. (3) The cellular release of the drugs was inhibited by sodium azide.

However, the effect of azide on net uptake and efflux may also be explained by a fall of pH_i although measurements of chloride distribution ratio do not support this interpretation. On the other hand with another inhibitor of the oxidative phosphorylation as 2,4-dinitrophenol Pool [34] could demonstrate a fall of pH_i.

If the results obtained in the daunorubicin-resistant subline are compared with the present data, it appears that a much more pronounced efflux probably takes place in the resistant subline. This finding might suggest that the resistant subline has arisen by selection of a small population with a more pronounced activity of the efflux mechanism in the wild-type tumour. The results concerning energy dependence for the uptake (Fig. 6) indicate that the energy for the efflux is probably supplied by both glycolysis and oxidative phosphorylation. The present study does not permit any conclusion concerning differences in the specificity of the drugs for the mechanism of active efflux.

Like others [12, 14, 15], we found a considerably slower net uptake of adriamycin than of daunorubicin. This finding cannot be explained by a difference on the level of the binding, as the affinity of adriamycin exceeds that of daunorubicin. Neither can the efflux process, as the release of adriamycin was considerably slower than daunorubicin in the medium without inhibitor. Considering that adriamycin is a more polar drug than daunorubicin (as a consequence of the hydroxyl group at C-14), the difference in net uptake might reflect a difference in the diffusion resistance in the membrane. However, the results may also reflect a carrier transport with a higher value of K_m for adriamycin than for daunorubicin. The lower steady-state level of rubidazone compared to daunorubicin is probably partly expressive of the lower binding affinity of rubidazone compared to daunorubicin. However, the slower release of rubidazone compared to daunorubicin in the medium including inhibitor (Fig. 7) suggests that a slower membrane penetration of rubidazone also plays a role.

The degree of intracellular metabolic transformation of the anthracyclines has proved to vary widely in different tissue [35]. In our system the intracellular transformation of the three derivatives investigated was negligible within the incubation time. A correspondingly low metabolism has also been demonstrated in other studies [7, 14–16].

The high nuclear binding demonstrated for each of the drugs is compatible with a binding mainly to DNA in the nucleus, in agreement with other studies [5, 22–24]. Calorimetric data have revealed that at small amounts bound (the ratio antibiotic/DNA < 0.12) the drugs are strongly bound to DNA by intercalation; if more drug is bound, the binding is probably due to weaker electrostatic forces [36]. In the present study it was demonstrated that modification of the molecule, both at C-13 (rubidazone) and at C-14 (adriamycin) significantly influenced the binding properties. Comparing daunorubicin and adriamycin the difference in nuclear affinity was demonstrated both at low binding (the ratio antibiotic/DNA in the range of 0.07) and at high binding indicating that the modification of the molecule must influence both types of binding forces. In other studies, however the binding to calf thymus DNA has proved almost equal for daunorubicin and adriamycin [6, 14]. This discrepancy may be due to the difference in the preparations. Thus for ethidium bromide, which also bind to DNA by intercalation, a lower number of binding sites and a lower affinity was found to deoxyribonucleoproteins compared to naked DNA [37].

According to the results of the present study, the net uptake of the anthracycline derivatives in Ehrlich ascites tumour cells is determined by: (1) The permeability of the membrane to the unionized form of the molecule. (2) The specificity of the drug for the mechanism of active efflux. (3) The relationship between the intracellular and the extracellular pH. (4) The pK_A value of the drug and (5) the affinity of the drug for the intracellular binding sites.

As demonstrated in this study, small chemical modifications in the molecule may exert a considerable influence on several of the factors determining cellular uptake. By utilizing such information in creating new anthracycline, derivatives with a better therapeutic value of these drugs may be obtained.

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