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Transport and Storage of Daunorubicin and Doxorubicin in Cultured Fibroblasts[1]

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ABSTRACT

For elucidation of the mechanism behind the differences in cellular accumulation of daunorubicin and doxorubicin, the uptake and subcellular localization of these drugs were studied in cultured fibroblasts by cell fractionation techniques. Daunorubicin and doxorubicin accumulated to the same saturation level in nuclei, whereas the lysosomal concentration of daunorubicin always exceeded that of doxorubicin. No saturation of lysosomes could be achieved under the experimental conditions used. In both cellular storage pools, more of the drug was found when the cells had been incubated in a medium at pH 7.8 than after incubation at pH 6.9. Metabolic inhibitors enhanced the accumulation of both drugs under conditions under which most of the drug was stored in nuclei. On the other hand, low incubation temperature inhibited drug accumulation under conditions under which most of the drug was stored in lysosomes. The accumulation of *N*-acetyldaunorubicin markedly exceeded that of *N*-acetyldoxorubicin, although these derivatives were not stored in significant amounts in either nuclei or lysosomes. Our results support the hypothesis that transport of daunorubicin and doxorubicin across the plasma membrane occurs by a "leak and pump" system, the leak being inward diffusion of non-ionized drug molecules and the pump being an active efflux. Since daunorubicin is less polar than is doxorubicin, it can be assumed to diffuse faster across biological membranes, which should lead to a higher cytoplasmic steady-state level. On this basis a hypothesis is presented to explain the observed differences in cellular accumulation and subcellular distribution between daunorubicin and doxorubicin.

INTRODUCTION

DNR[3] and DOX show similar DNA-binding properties *in vitro* (20). However, the cellular uptake of DNR markedly exceeds that of DOX (1, 3, 10, 13). Using cultured rat embryo fibroblasts, we have confirmed that the accumulation of DNR is greater than that of DOX (11). We also have found that both drugs are exclusively localized to nuclei and lysosomes but to quite different proportions, so that the nuclear concentrations of DNR and DOX are very similar (11).

Since the mechanism behind the differences in cellular pharmacokinetics of DNR and DOX is not clear, we have performed further studies on the cellular uptake and subcellular localization of these drugs and their *N*-acetyl derivatives in cultured fibroblasts under different conditions.

MATERIALS AND METHODS

Drugs. DNR hydrochloride (Cerubidine) was kindly supplied by Rhône-Poulenc S. A. (Paris, France), and DOX hydrochloride (Adriblastina) was supplied by Farmitalia (Milan, Italy). The *N*-acetyl derivatives of the drugs were kindly prepared by Dr. R. Baurain, International Institute of Cellular and Molecular Pathology, Brussels, Belgium, from the parent compounds as described by Yamamoto *et al.* (19). Iodoacetic acid (Merck AG, Darmstadt, Germany) was neutralized with NaOH. Antimycin A (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol so that the ethanol concentration in the culture medium was 0.2%. In controls without antimycin A, ethanol was added to the same concentration. *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 4-(2-hydroxyethyl)-1-piperazine-*N'*-2-ethanesulfonic acid, and 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid were purchased fom Sigma.

Cell Culture. Rat embryo fibroblasts were obtained and cultured in a modified Eagle-Dulbecco's medium (pH 7.4) as described by Tulkens *et al.* (14). For incubations with drugs, cells from the first or second subculture were used after reaching confluency.

Uptake Experiments. Cells were grown in Falcon T-flasks (25-sq cm growth surface, which at confluency corresponds to about 1 mg cell protein). The incubation was started by changing the culture medium to a fresh medium (5 ml) containing appropriate amounts of drugs. After incubation the cells were washed and harvested as described previously (11). Unless otherwise stated, all incubations were performed at 37°.

Cell Fractionation Procedures. Confluent cells were incubated in Roux flasks (200-sq cm growth surface, corresponding to about 10 mg cell protein) containing 50 ml of culture medium and appropriate amounts of the drugs. In experiments at different pH's, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (10 mM), 4-(2-hydroxyethyl)-1-piperazine-N'-2-ethanesulfonic acid, (15 mM), and 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (10 mM) were added to the culture medium, and the pH was adjusted by the addition of NaOH or HCl as described by Eagle (7).

A nuclei-free (verified by light microscopy) cytoplasmic extract was prepared and analyzed by isopyknic centrifugation as described previously (11, 14). The distributions of

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[3] The abbreviations used are: DNR, daunorubicin; DOX, doxorubicin (Adriamycin); N-acetyl-DNR, *N*-acetyldaunorubicin; N-acetyl-DOX, N-acetyldoxorubicin.

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DNR and DOX between the nuclei and the lysosomes were determined after correcting the drug content of the nuclear fraction for contamination by lysosomal drug as described previously (11).

Assays. DNR, DOX, and protein and marker enzymes were assayed as previously described (11). The *N*-acetyl derivatives of DNR and DOX were assayed as the parent compounds.

RESULTS

The accumulation kinetics of DNR and DOX in the nuclear and lysosomal compartments were determined by incubation of fibroblasts for different periods of time (up to 10 hr) and subsequent fractionation (Chart 1). The distribution of drug between the 2 storage pools varied with the incubation time. In nuclei the accumulation of DNR reached a plateau within 30 min, and a similar level of DOX was reached after 3 hr. The lysosomal levels of both drugs continued to increase after steady-state conditions had been reached in the nuclei, suggesting saturation of nuclear storage sites. At all times the lysosomal content of DNR greatly exceeded that of DOX. It should be noted that total accumulation of both drugs in fibroblasts reaches steady state in 6 to 10 hr (11).

Accumulation of DNR and DOX in nuclei and lysosomes as a function of extracellular drug concentration is shown in Chart 2. The incubation time was limited to 2 hr because of drug toxicity at the highest concentrations. Lysosomal accumulation of DNR increased linearly with drug concentration, whereas nuclear accumulation leveled off. In the concentration range studied, the nuclear accumulation of DOX did not reach the same level as that of DNR, which can be explained by the short incubation time. Lysosomal levels of DOX remained low at all drug concentrations.

The effect of pH on total cell accumulation of DNR and DOX (17.5 μM) is shown in Table 1. The incubation time was limited to 2 hr to keep the pH in the medium constant

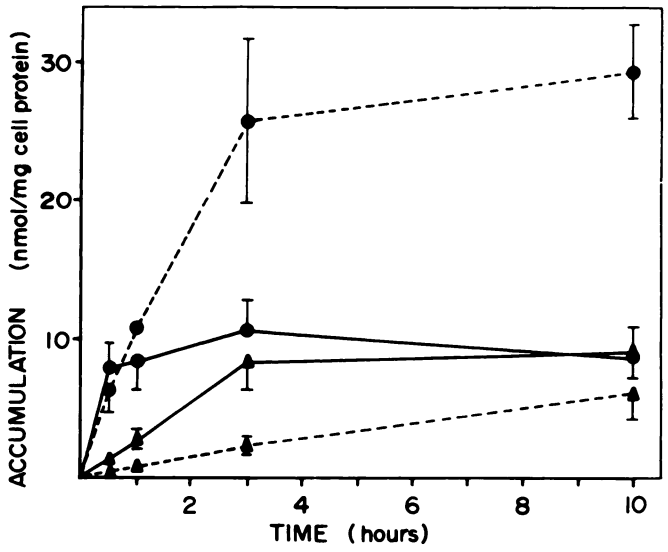

Chart 1. Time courses for the accumulation of DNR (●) and DOX (▲) in nuclei (——) and lysosomes (----) of fibroblasts incubated in the presence of the drug at an extracellular concentration of 17.5 μM. *Bars*, S.D. for 2 to 6 experiments.

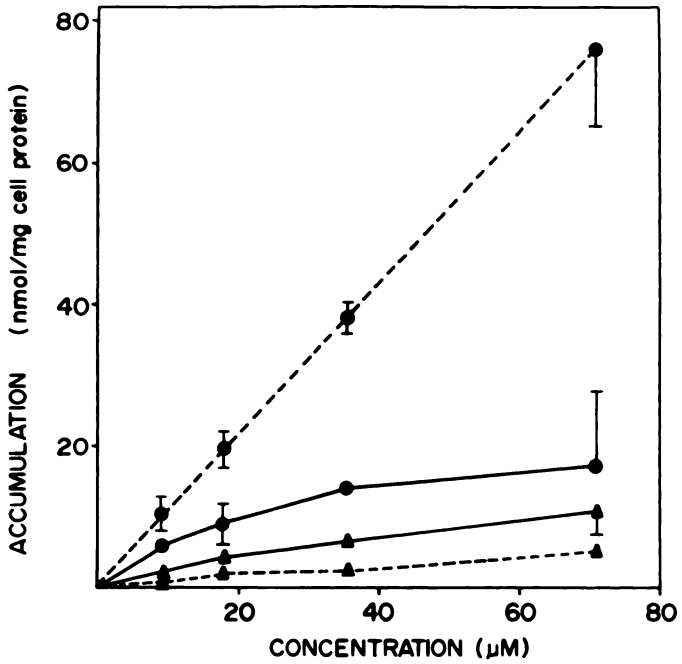

Chart 2. Effect of drug concentration on the accumulation of DNR (●) and DOX (▲) in nuclei (——) and lysosomes (----) of fibroblasts after 2 hr. *Bars*, S.D. for 2 experiments.

Table 1

Effect of pH on the accumulation of DNR and DOX in fibroblasts

Cells were incubated for 2 hr at an extracellular drug concentration of 17.5 μM.

	Drug accumulation (nmol/mg cell protein)	
pH	DNR	DOX
6.9	15.19 ± 6.81[a]	2.58 ± 0.46
7.4	28.34 ± 5.57[b]	6.33 ± 1.48[b]
7.8	38.85 ± 2.83	10.70 ± 2.62

[a] Mean ± S.D. for 2 experiments.
[b] Values refer to experiments shown in Chart 2.

throughout the incubation. The accumulation of both drugs was about 3 times greater at pH 7.8 than it was at pH 6.9. From the nuclear and lysosomal contributions to the total cell accumulation (shown in Chart 3), it is evident that the enhancing effect of increasing pH on drug accumulation applies to both compartments.

Cellular accumulation of DNR and DOX (17.5 μM for 10 hr) as a function of incubation temperature is shown in Chart 4. The accumulation of both drugs was very temperature sensitive and was significantly ($p < 0.05$) inhibited by even a modest decrease in incubation temperature from 37 to 28°.

For study of the effect of metabolic inhibitors on drug accumulation, a combination of iodoacetate (1 mM) and antimycin A (2 μM) was used. Iodoacetate inhibits the glycolytic enzyme 3-phosphoglyceraldehyde dehydrogenase (17), whereas antimycin A blocks electron transport in the respiratory chain (16). A combination of these inhibitors depleted the cellular ATP level within 1 hr, as measured by the technique of Lowry *et al.* (9), and the cells thereafter detached from the growth surface. The inhibitors increased the accumulation of both DNR ($p < 0.01$) and DOX ($p < 0.05$) in the fibroblasts during incubations for 30 min (Chart

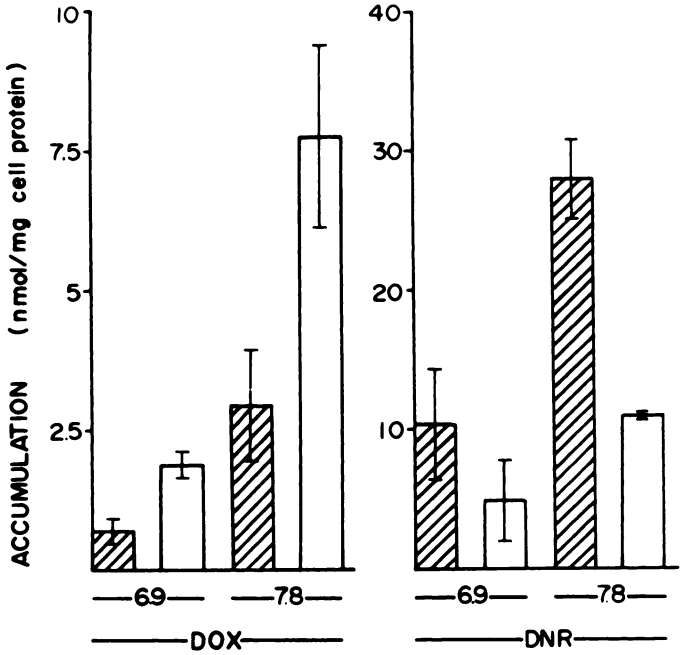

Chart 3. Effect of pH on the accumulation of DNR and DOX in nuclei (*open boxes*) and lysosomes (*hatched boxes*) of fibroblasts incubated for 2 hr in the presence of the drug at a concentration of 17.5 μM. *Bars*, S.D. for 2 experiments.

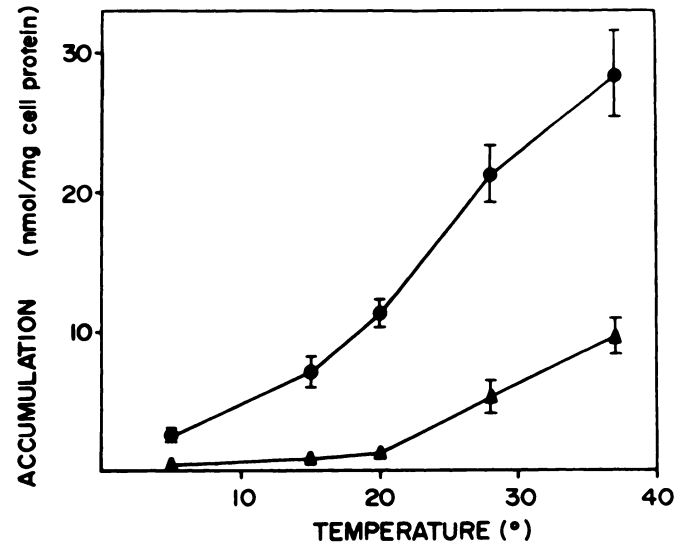

Chart 4. Effect of temperature on the accumulation of DNR (●) and DOX (▲) in fibroblasts incubated for 10 hr in the presence of an extracellular concentration of 17.5 μM. *Bars*, S.D. for 4 experiments.

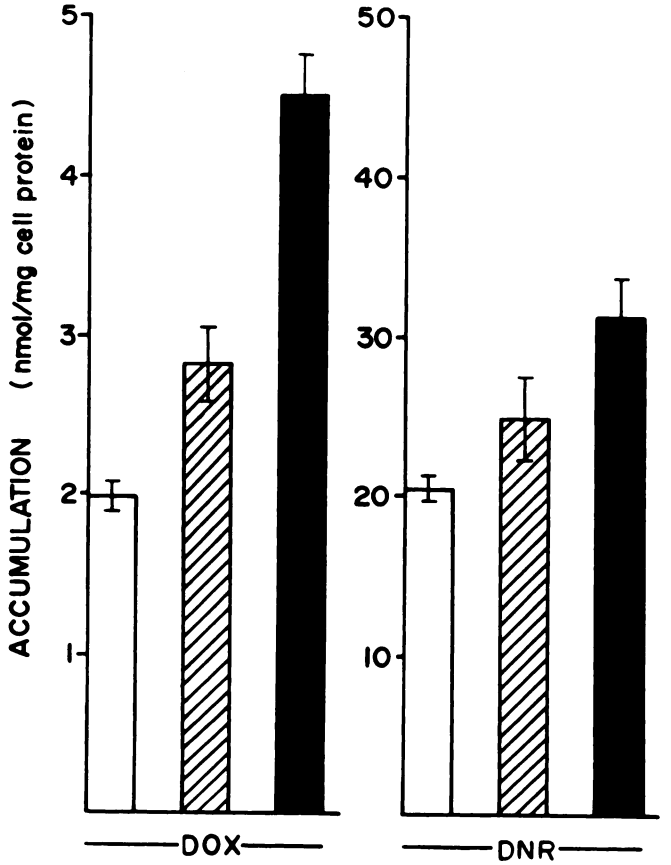

Chart 5. Effect of metabolic inhibitors [iodoacetate (1 mM) + antimycin A (2 μM)] on the accumulation of DNR and DOX in fibroblasts incubated in the presence of the drug for 30 min at an extracellular drug concentration of 35 μM. *Open boxes*, drug accumulation in the absence of inhibitors; *hatched boxes*, drug accumulation in the presence of inhibitors; *solid boxes*, drug accumulation in the presence of inhibitors. The inhibitors were added 15 min before DNR or DOX. *Bars*, S.D. for 3 experiments.

5). The effects were even more pronounced when the cells had been pretreated with the inhibitors for 15 min before the addition of the drug.

Fibroblasts incubated for 10 hr with DNR or DOX (17.5 μM) lost 26% of their DNR and 12% of their DOX after being washed and reincubated for 6 hr in a fresh medium without drug. Nuclear and lysosomal drug contents after the washout period are shown separately in Chart 6. Only the nuclear levels were significantly ($p < 0.05$) reduced during the washout period.

Cellular uptake of the N-acetyl derivatives of DNR and DOX (160 μM) is shown in Chart 7. The accumulation of N-acetyl-DNR was about 3 times greater than that of N-acetyl-DOX. Assuming that there was a uniform intracellular distribution of the drugs and that 1 mg of fibroblast protein corresponds to a volume of 5 μl (15), the cell:medium concentration ratio at steady state was about 15 for *N*-acetyl-DNR and 5 for N-acetyl-DOX. No contamination of the *N*-acetyl derivatives by the parent compounds could be detected by thin-layer chromatography on silica gel plates (CH_2Cl_2:CH_3OH:HCOOH:H_2O, 85:15:2:1).

The distribution of N-acetyl-DNR and protein and marker enzymes after isopyknic centrifugation of a cytoplasmic extract of fibroblasts incubated previously with the drug (160 μM for 10 hr) is illustrated in Chart 8. The results are presented as standardized histograms as described by Leighton *et al.* (8). The distribution of N-acetyl-DNR was diffuse and did not correspond to the distribution of any marker enzyme. The highest concentrations were found in the lightest fractions containing soluble constituents. The drug concentration in the nuclear fraction was very low (1.40 nmol/mg cell protein).

DISCUSSION

Cultured fibroblasts store DNR and DOX in nuclei and lysosomes. The nuclear trapping can easily be explained by

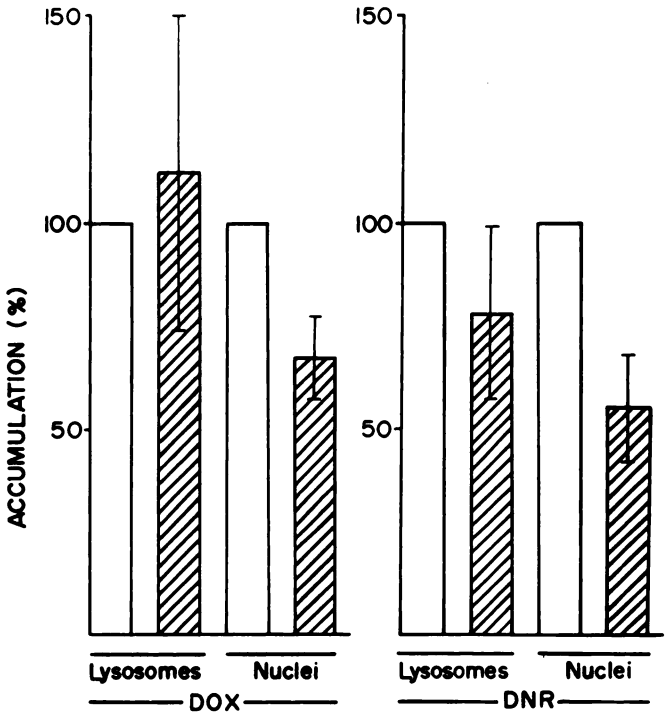

Chart 6. Nuclear and lysosomal concentrations of DNR and DOX in fibroblasts after a washout period of 6 hr (*hatched boxes*) after previous incubation of the cells for 10 hr at an extracellular drug concentration of 17.5 μM. The results are expressed as percentages of the drug contents in cells incubated only for the initial 10 hr (*open boxes*). *Bars*, S.D. for 3 experiments.

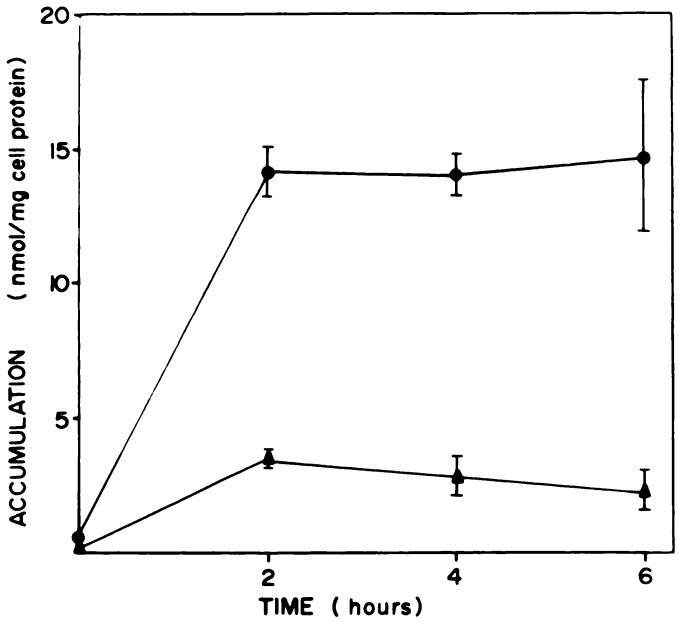

Chart 7. Time courses for the accumulation of N-acetyl-DNR (●) and N-acetyl-DOX (▲) in fibroblasts incubated at an extracellular drug concentration of 160 μM. *Bars*, S.D. for 4 experiments.

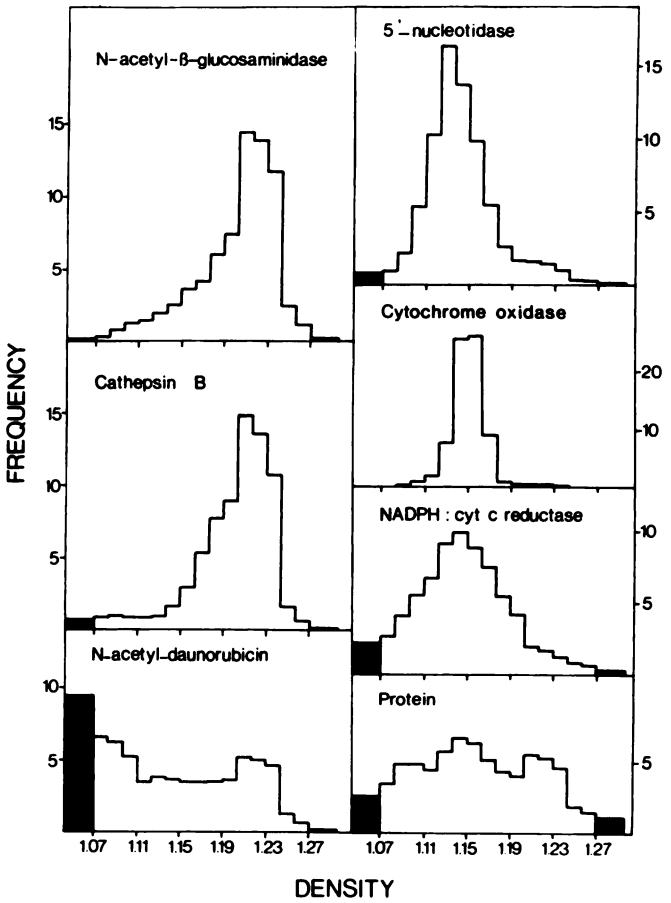

Chart 8. Distribution of N-acetyl-DNR and protein and marker enzymes in the cytoplasmic extract of fibroblasts incubated for 10 hr in the presence of an extracellular drug concentration of 160 μM. The frequency (*ordinate*) is $\Delta Q/(Q \times \Delta\zeta)$, where ΔQ is the amount of constituent in the section, Q is the total amount of constituent in all sections, and $\Delta\zeta$ is the density increment for each section (0.0133). *Filled areas*, amount of constituent equilibrating at a density below 1.07 and above 1.27, respectively; *NADPH: cyt c reductase*, NADPH:cytochrome *c* reductase.

binding to DNA (6, 12, 20). Both drugs accumulated in the nuclei to a similar saturation level of about 10 nmol/mg cell protein. Assuming that all of the drug in the nuclei is bound to DNA, we can estimate that fibroblast DNA has a binding capacity of 0.1 drug molecule per mononucleotide molecule since the DNA concentration is 35 μg/mg cell protein (C. Godfredsen, personal communication). *In vitro*, calf thymus DNA binds 0.16 to 0.2 drug molecule per mononucleotide molecule (20). The lower binding capacity in intact cells could be explained by the association of nuclear DNA to proteins such as histones. The saturation of nuclear binding sites would also explain why the specific inhibitory activity of DNR and DOX decreases with increasing cellular drug concentrations (1).

Since DNR and DOX are weak bases, their accumulation in the acid lysosomal compartment can be understood if the lysosomal membrane is much less permeable for the protonated drug than for the unprotonated form (5). It is not possible, however, to explain the difference in lysosomal accumulation on this basis since the pK_a's of DNR and DOX are very similar (13).

The low concentration of N-acetyl-DNR in nuclei and lysosomes indicates that the amino group of the anthracyclines, necessary for their strong interaction with DNA (2) and for their basic properties, plays a crucial role for the cellular storage of the drugs.

The penetration of DNR and DOX into the cells does not seem to be the result of an active inward transport since metabolic inhibitors increase the rate of drug uptake. Our

results are compatible with a mechanism of penetration by passive diffusion of non-ionized molecules since the accumulation of both DNR and DOX decreases at low-medium pH (6.9), when the degree of protonation increases, and increases when the pH is raised to 7.8. A penetration by diffusion is also compatible with the correlation that can be established between the higher uptake of DNR and its higher lipophilicity (1, 13) as compared to those of DOX.

The accumulation of DOX (and probably also that of DNR) occurs at a higher rate in the nuclei than in the lysosomes. Conversely, during the washout period, nuclei release the drugs faster than do the lysosomes. These differences could be explained if we consider that the nuclear membrane is incomplete and porous (18). Consequently, the diffusion across the nuclear membrane should be faster than that across the lysosomal membrane.

The increased accumulation of DNR and DOX in the presence of metabolic inhibitors suggests that the fibroblasts have an active efflux mechanism. Evidence for such a mechanism for DNR has been presented for Ehrlich ascites cells (4, 13).

The decrease in drug accumulation, observed on lowering the incubation temperature, is more difficult to interpret, but it does not necessarily contradict the hypothesis of an active efflux since we do not know how temperature affects diffusion and storage of anthracyclines. It should also be borne in mind that the experiments with metabolic inhibitors have been performed for a short time (30 min), when most of the drug is stored in nuclei, whereas the experiments on the effect of temperature have been performed for 10 hr, when lysosomal storage is much more important. According to the observations of de Duve *et al.* (5), the acid pH in lysosomes could be maintained by an energy-dependent proton pump. If this hypothesis is correct, a lowering of temperature could decrease the lysosomal drug trapping by increasing the lysosomal pH.

The accumulation of N-acetyl-DNR exceeds that of N-acetyl-DOX. Since neither of these derivatives is stored in nuclei or lysosomes, the explanation for this difference has to be sought at the plasma membrane level. N-Acetyl-DNR, being less polar, can be assumed to diffuse faster across biological membranes. The difference in accumulation could therefore be explained by a slower diffusion of N-acetyl-DOX in combination with an extrusion mechanism.

We can thus formulate a hypothesis that could explain the difference in the cellular accumulation of DNR and DOX. Both drugs enter by diffusion and are actively transported outward. Consequently, the concentration of both drugs in the cell sap will be very low, but it will be higher for DNR than for DOX. Equilibration with the nuclei surrounded by a porous membrane will occur relatively quickly and result in similar nuclear concentrations after saturation of all of the DNA-binding sites. The higher lysosomal accumulation of DNR will on the other hand be the result of the slower equilibration between the cell sap and the lysosomes and the much higher storage capacity of the latter organelles. Further studies are needed to confirm this hypothesis in other cell types and to evaluate its significance for drug-resistant cells.

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