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THE MECHANISM FOR CELLULAR UPTAKE, STORAGE AND RELEASE OF DAUNORUBICIN

STUDIES ON FIBROBLASTS IN CULTURE

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Abstract—The mechanism for uptake, storage and release of daunorubicin have been studied in cultured fibroblasts. Analysis by high performance liquid chromatography of cells incubated with daunorubicin revealed that the major part of the accumulated drug did not undergo metabolic transformation. Small amounts of daunorubicinol and aglycone were formed. [^{3}H]-daunorubicin was used to study membrane fluxes of the drug under different conditions. Metabolic inhibitors enhanced the influx of [^{3}H]-daunorubicin and, under certain conditions, also reduced its efflux, indicating that the cells have an active mechanism for the outward transport of the drug. The very high intracellular drug accumulation is due to trapping in nuclei and lysosomes. Cell fractionation techniques have been used to study drug trapping under various conditions. Nuclear storage of daunorubicin is probably due to binding to DNA. Metabolic inhibitors, as well as lowering the incubation temperature, reduced the lysosomal trapping, supporting the hypothesis that the low pH in these organelles is maintained by a proton pump and that the drug is trapped in the protonated form. A hypothesis is presented, which by combining the mechanisms for membrane transport and intracellular storage of daunorubicin, gives also an explanation for the observed differences in the cellular accumulation and subcellular distribution of daunorubicin and its 14-hydroxy derivative, doxorubicin. Daunorubicin is more lipophilic than doxorubicin and will therefore diffuse faster through the cell membrane. Assuming that both substances have the same affinity for the proposed active outward transport mechanism, this will lead to a higher steady-state level of daunorubicin in the cytosol and as a consequence to a higher lysosomal storage level if the drug in the lysosomes is in equilibrium with that in the cytosol. The similarity in nuclear storage capacity for the two substances can be explained by saturation of the available storage sites.

The cellular accumulation of DNR† markedly exceeds that of its 14-hydroxy-derivative, DOX [1–4]. In cultured rat embryo fibroblasts, we have found that both drugs are almost exclusively localized in nuclei and lysosomes [4]. Under steady-state conditions, the nuclear concentrations of DNR and DOX are very similar, whereas the lysosomal concentration of DNR is much higher than of DOX [4, 5]. The similarity in the nuclear trapping of the two drugs can be explained by saturation of available binding sites, since the drug molecules intercalate in the DNA double helix [6, 7]. Since DNR and DOX are weak bases, their accumulation in the lysosomes can be explained by trapping of protonated drug molecules [8]. However, it is not possible to explain the difference in lysosomal accumulation on this basis, since the p*Ka*'s of DNR and DOX are very similar [3, 9].

A hypothesis has been presented to explain the differences in cellular accumulation and subcellular distribution of the drugs, based on our studies on cultured fibroblasts [5]. Since DNR is more lipophilic than DOX [10, 11], it can be expected to enter the cells faster, either by simple diffusion or by a carrier-mediated process involving binding to a membrane constituent. Evidence has been presented for an active outward transport mechanism of anthracyclines in cultured fibroblasts [5] as well as in Ehrlich ascites cells [11, 12] and P388 leukaemia cells [13]. The outflow of DNR and DOX is very similar when fibroblasts have been preloaded with the drugs to the same intracellular concentration [14]. Therefore, assuming that the drugs have the same affinity for the proposed transport mechanism, the difference in influx rate leads to a higher steady-state concentration of DNR in the cell sap, as compared to that of DOX. If, as suggested by de Duve *et al.* [8], the lysosomal drug is in equilibrium with that in the cell sap, this would explain the higher lysosomal accumulation of DNR.

In the present investigation, the proposed model for membrane transport and intracellular storage of anthracyclines has been further tested experimentally in cultured fibroblasts. [^{3}H]-DNR has been used to study drug fluxes across the plasma membrane under different conditions. Furthermore, the metabolism of DNR in cultured fibroblasts has been studied using h.p.l.c.

MATERIALS AND METHODS

Drugs. DNR hydrochloride and [^{3}H]-DNR hydrochloride (0.5 mCi/mg) were kindly supplied by

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† Abbreviations used: DNR (daunorubicin), DOL (daunorubicinol), DOX (doxorubicin or adriamycin), h.p.l.c. (high performance liquid chromatography).

Rhône-Poulenc S.A. (Paris, France). The radiochemical purity of the [^{3}H]-DNR was checked by thin layer chromatography on silica gel plates (CH_2Cl_2-CH_3OH-HCOOH-H_2O, 85 : 15 : 2 : 1). More than 90 per cent of the radioactivity was found to be associated with the drug.

To study the effects of metabolic inhibitors on the transport and storage of DNR, a combination of iodoacetate (Merck A.G., Darmstadt, Germany) and antimycin A (Sigma Chemical Co., St Louis, MO) was used as described previously [5].

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.* [15]. Cells from the first or second subculture were incubated with drugs as soon as they reached confluency. Unless otherwise stated, all incubations were performed at 37°.

Experiments on influx of [^{3}H]-*DNR.* Cells grown in Falcon T-flasks (25 cm^2 growth surface, which at confluency corresponds to about 1 mg cell protein) were incubated for 10 hr with 5 ml of culture medium containing DNR (17.5 μM). Thereafter, a trace amount of [^{3}H]-DNR (1.7 nmole, which corresponds to 2 per cent of the total DNR present) was added with or without metabolic inhibitors. The cells were incubated further, washed and harvested as described previously [4]. Subsequently, the radioactivity associated with the cells was determined. The results have been calculated as the percentages of added radioactivity found associated with the cells.

Experiments on efflux of [^{3}H]-*DNR.* Cells grown in Roux flasks (200 cm^2 growth surface, which at confluency corresponds to about 10 mg cell protein) were incubated with 50 ml of culture medium containing [^{3}H]-DNR (17.5 μM) for 30 min or 10 hr. Thereafter, the cells were washed once and subsequently reincubated with 50 ml of fresh medium in the presence or absence of metabolic inhibitors. After different periods of time, 1-ml aliquots of the incubation medium were withdrawn and the amount of radioactivity which had leaked out from the cells was determined. The results have been calculated as the percentages of cell-associated radioactivity released into the medium during the reincubation period. The radioactivity which had accumulated in the cells at the end of the preloading period was determined from the amount of radioactivity left in the incubation medium after that period.

Cell fractionation procedures. Confluent cells were incubated in Roux flasks containing 50 ml of culture medium and appropriate amounts of the drugs. For each experiment, five Roux flasks, corresponding to about 50 mg of cell protein, were used. After incubation, the cells were washed, harvested and homogenized as described previously [4]. A nuclei-free cytoplasmic extract was prepared by differential centrifugation of the homogenate and then submitted to isopyknic centrifugation as described by Tulkens *et al.* [15]. The subcellular distribution of DNR, its metabolites and certain marker enzymes was plotted in the form of standardized histograms as described by Leighton *et al.* [16].

Assays. Marker enzymes, protein and DNR-related fluorescence (parent drug + metabolites) were assayed as described previously [4, 15]. In cer-

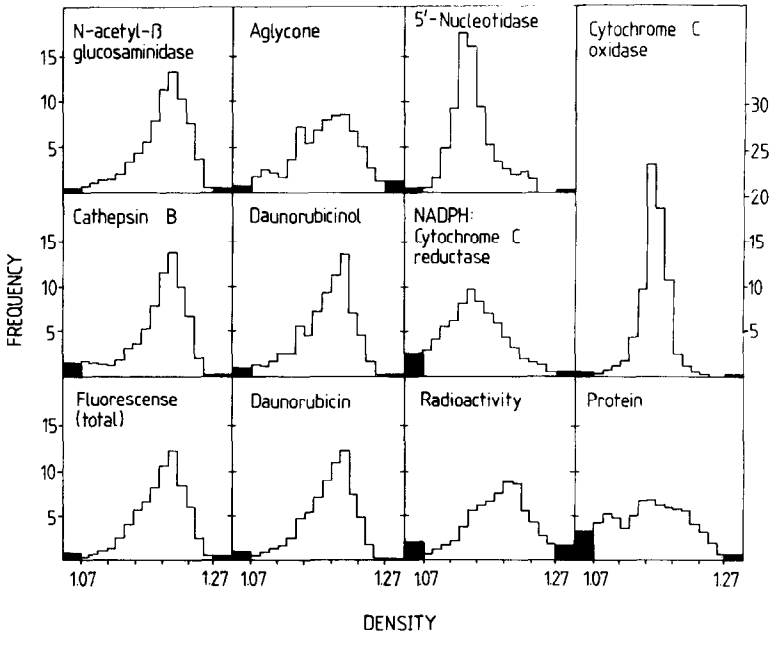

Fig. 1. Distribution of total fluorescence, radioactivity, DNR, DOL, aglycone, marker enzymes and protein in the cytoplasmic extract of fibroblasts incubated for 10 hr with DNR at an extracellular concentration of 17.5 μM. [^{3}H]-DNR (final concentration : 0.35 μM) was added 30 min before harvesting the cells. The frequency (ordinate) is $\Delta Q/(Q \times \Delta\delta)$, where ΔQ is the amount of constituent in the section, Q, is the total amount of constituent in all sections and $\Delta\delta$ is the density increment for each section (0.0133). Filled areas represent the amount of constituent equilibrating at densities below 1.07 and above 1.27, respectively.

tain experiments, DNR and its metabolites were measured separately using h.p.l.c. [17, 18]. Radioactivity was determined in a liquid scintillation spectrometer (Packard Instruments Inc., Downers Grove, IL). For determination of radioactivity, 1-ml aliquots of the sonicated cell suspensions (15 sec at 75 W, 20 kHz: Sonifer B-12, Branson Sonic Power Co., Dannbury, CT) as well as 0.1-ml samples of the incubation media diluted to 1 ml with H_2O, were acidified with 10 μl of acetic acid before adding 10 ml of Aqualuma (Lumac, Meise, The Netherlands). Under these conditions, the influence of quenching was negligible. Between 89.8 and 102.2 per cent of the radioactivity added to the incubations could be recovered at the end of the incubation period in the cells, incubation media and wash fluids.

Statistical methods. The effect of metabolic inhibitors on the fluxes of [^{3}H]-DNR across the plasma membrane was evaluated by analysis of variance.

RESULTS

After incubation of cultured fibroblasts with DNR (17.5 μM) for 10 hr, 15 per cent of the accumulated drug was identified by h.p.l.c. as DOL and a few per cent as aglycone. The subcellular distribution of DNR and its metabolites is shown in Fig. 1. The distribution of total fluorescence, DNR and DOL was very similar to that of *N*-acetyl-β-glucosaminidase and cathepsin B, marker enzymes for lysosomes. On the other hand, the distribution of cytochrome *c* oxidase (marker enzyme for mitochondria), NADPH:cytochrome *c* reductase (endoplasmic reticulum) and 5′-nucleotidase (plasma membrane) was quite different. The distribution of the aglycone was similar to that of the lysosomal marker enzymes, although some material equilibrated at lower densities. However, most daunorubicin was not metabolized. Therefore, in experiments on drug accumulation only total fluorescence was measured.

The time course for the accumulation of radioactivity in fibroblasts after the addition of a trace amount of [^{3}H]-DNR to the incubation medium is shown in Fig. 2. The cells had been preloaded with unlabelled DNR to steady-state conditions, which

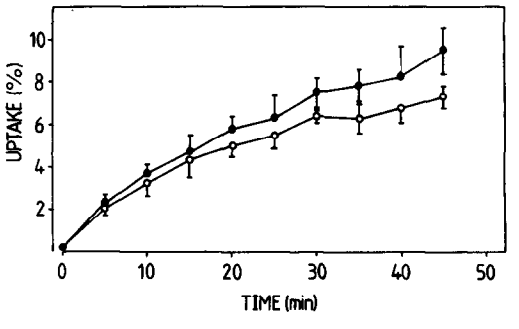

Fig. 2. Uptake of radioactivity into fibroblasts preloaded with unlabelled DNR (17.5 μM for 10 hr). Thereafter, [^{3}H]-DNR (final concentration 0.35 μM) was added with (●——●) or without (○——○) metabolic inhibitors (antimycin A, 2 μM + iodoacetate, 1 mM). Mean values ± S.D. of 4 experiments are shown.

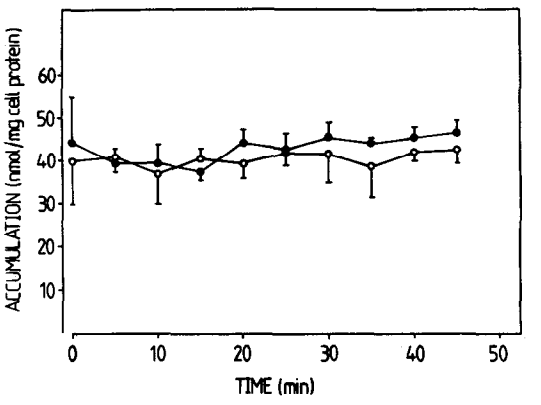

Fig. 3. Total cellular drug content before and various times after the addition of [^{3}H]-DNR in the experiments shown in Fig. 2.

is reached in 6–10 hr [4]. The radioactivity associated with the cells increased with time and after 45 min it constituted about 7 per cent of the radioactivity added. Metabolic inhibitors, added simultaneously with the [^{3}H]-DNR, enhanced the accumulation of radioactivity ($P < 0.01$). However, the inhibitors did not increase the total cellular drug content (Fig. 3).

The subcellular distribution of the radioactivity after the addition of a trace amount of [^{3}H]-DNR to fibroblasts is shown in Fig. 1. The cells had been preloaded with unlabelled DNR to steady-state conditions. [^{3}H]-DNR was then added and the cells fractionated 30 min thereafter. The distribution of radioactivity in the cytoplasmic extract corresponded very well to the distribution of DNR as well as to that of *N*-acetyl-β-glucosaminidase and cathepsin B, marker enzymes for the lysosomes. Calculation of the distribution of DNR between nuclei and lysosomes, as previously described [4], showed that 34 per cent of the accumulated radioactivity was localized in the nuclei, as compared to only 13 per cent of the total DNR.

Figure 4 shows the effect of metabolic inhibitors

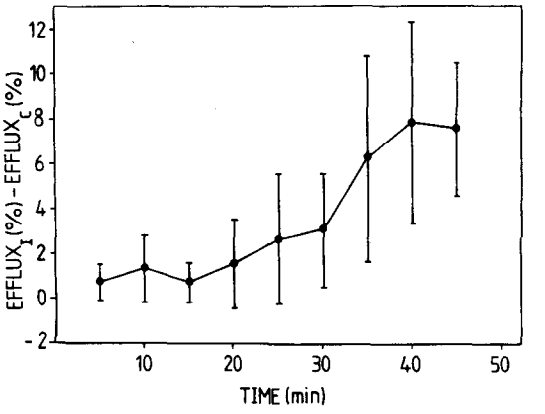

Fig. 4. Effect of metabolic inhibitors on the efflux of radioactivity from fibroblasts preloaded for 10 hr with [^{3}H]-DNR (17.5 μM), and then washed and reincubated in fresh medium with ($Efflux_I$) or without ($Efflux_C$) metabolic inhibitors (antimycin A, 2 μM + iodoacetate, 1 mM). Mean values ± S.D. of 3 experiments are shown.

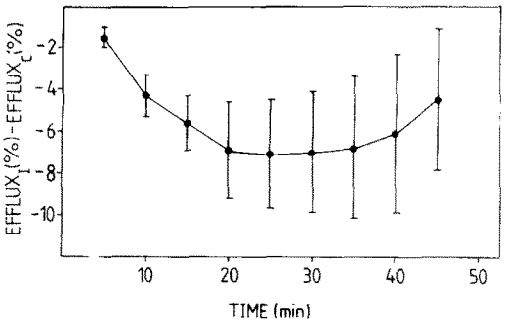

Fig. 5. Same type of experiments as in Fig. 4, except that the fibroblasts were preloaded with [^{3}H]-DNR for only 30 min instead of 10 hr. Mean values ± S.D. of 3 experiments are shown.

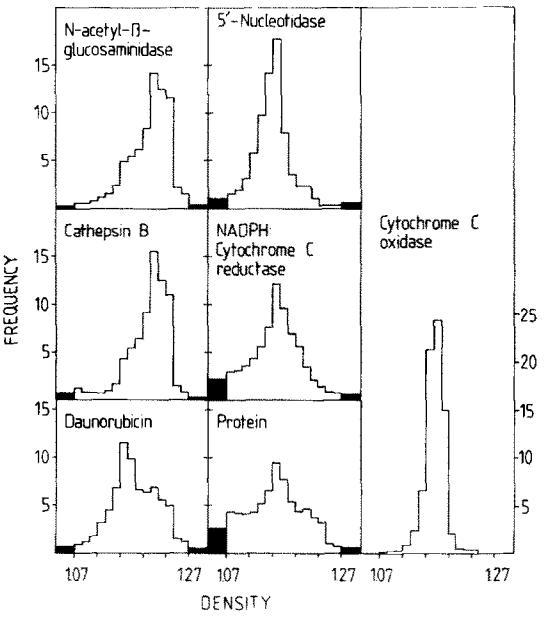

Fig. 7. Subcellular distribution of DNR, marker enzymes and protein in the cytoplasmic extract of fibroblasts previously incubated with the drug at 20° (17.5 μM for 10 hr).

on the efflux of radioactivity from preloaded fibroblasts. After incubation with [^{3}H]-DNR (17.5 μM) for 10 hr, the cells contained 30.8 ± 3.6 nmoles DNR per mg cell protein (mean ± S.D., N = 6). They were then rapidly washed and subsequently reincubated in fresh medium. In the absence of inhibitors, 11.8 ± 2.9 per cent (mean ± S.D., N = 3) of the cellular radioactivity was found in the extracellular medium after a washout period of 45 min. The presence of metabolic inhibitors during the washout period increased the efflux of radioactivity ($P < 0.01$).

Since the subcellular distribution of DNR changes with the incubation time [5], the efflux of radioactivity has also been studied after a preloading period of only 30 min, when the cells contained 4.8 ± 0.9 nmoles DNR per mg cell protein (mean ± S.D., N = 6). In this case, the relative efflux was considerably higher than that after a preloading period of 10 hr. Thus 32.7 ± 4.9 per cent (mean ± S.D., N = 3) of the accumulated radioactivity was recovered in the washout medium within 45 min in the absence of metabolic inhibitors. Figure 5 shows the effect of metabolic inhibitors on the efflux. In contrast to what was observed when the cells had been preloaded for 10 hr, the inhibitors reduced the efflux ($P < 0.01$). The effect of the inhibitors increased with time to reach a maximum after 20–30 min and seemed to decrease thereafter.

The subcellular distribution of DNR in a cyto-

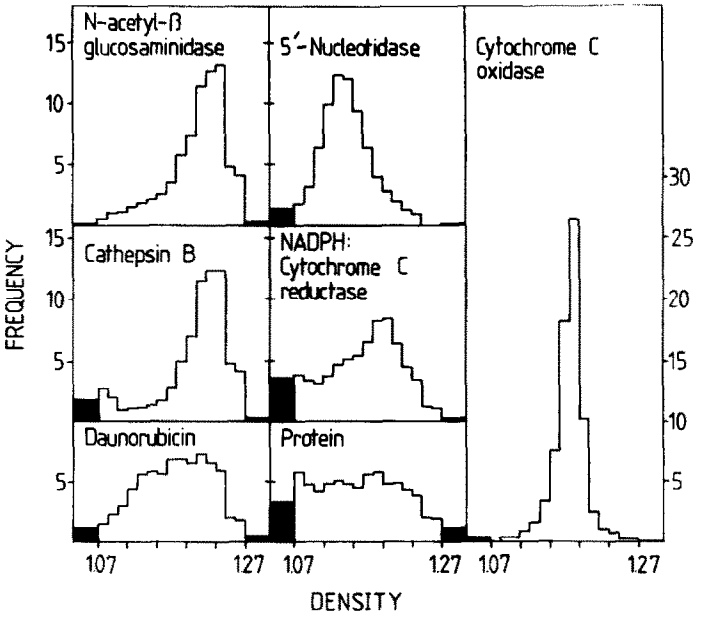

Fig. 6. Subcellular disbribution of DNR, marker enzymes and protein in the cytoplasmic extract of fibroblasts previously incubated for 50 min with the drug (17.5 μM) in the presence of metabolic inhibitors (antimycin A, 2 μM + iodoacetate, 1 mM).

plasmic extract prepared from fibroblasts incubated with the drug (17.5 μM for 50 min) in the presence of metabolic inhibitors is shown in Fig. 6. Under these conditions, most drug equilibrated at lower densities than the lysosomal enzymes, *N*-acetyl-β-glucosaminidase and cathepsin B. The drug concentration in the nuclei cannot accurately be determined since the nuclear fraction is heavily contaminated with cytoplasmic organelles in various proportions [4]. To make an appropriate correction for the amount of drug in the organelles contaminating the nuclear fraction, the concentration of drug in the various organelles must be known.

Figure 7 shows the intracellular localization of DNR in a cytoplasmic extract prepared from fibroblasts incubated at 20° with the drug (17.5 μM) for 10 hr. As after incubation with metabolic inhibitors, only a minor part of the drug equilibrated at the same densities as the lysosomal enzymes. The major part equilibrated at the same densities as NADPH:cytochrome *c* reductase, marker enzyme for endoplasmic reticulum and 5'-nucleotidase, marker enzyme for the plasma membrane. The drug distribution profile most resembled that of NADPH:cytochrome *c* reductase.

DISCUSSION

During the incubation of cultured fibroblasts with DNR, only a minor part of the accumulated drug was metabolized. The 13-hydroxy derivate, DOL, was the major metabolite formed. This corresponds to the *in vivo* situation in both animals [19, 20] and man [21, 22].

Our results on the intracellular distribution of DNR and its metabolites confirm that the amino group of DNR plays a crucial role in the lysosomal trapping of the drug. Thus, the aglycone was not localized in the lysosomes to the same extent as DNR and DOL. Previous observations show that the *N*-acetyl-derivatives of DNR and DOX are not trapped in the lysosomes [5].

In the present study, [^{3}H]-DNR has been used to measure fluxes of DNR across the cell membrane. There is good evidence that the radioactivity measured represents DNR. More than 90 per cent of the radioactivity in the starting material was associated with DNR, as judged from the results of thin layer chromatography. Furthermore, after incubation of fibroblasts with [^{3}H]-DNR, the subcellular distribution of the radioactivity was very similar to that of DNR as assayed by fluorometry.

Metabolic inhibitors enhanced the influx of [^{3}H]-DNR and, under certain conditions, reduced its efflux from the preloaded fibroblasts. These observations support the hypothesis that the fibroblasts have an active efflux mechanism for DNR [5]. However, the metabolic inhibitors only reduced the efflux of [^{3}H]-DNR from cells preloaded for a short period of time (30 min); they enhanced the efflux from cells preloaded to steady-state conditions (10 hr).

In order to understand these results, we have to bear in mind that the intracellular localization of the drug under these two conditions is very different. After incubation for 30 min, 60 per cent of the accumulated drug is stored in the nuclei and 40 per cent in the lysosomes, as compared to 20 per cent in the nuclei and 80 per cent in the lysosomes after incubation for 10 hr [5]. The lysosomal trapping of a weak base like DNR is probably due to the low intralysosomal pH [8, 23]. If this is maintained by a proton pump, as suggested by de Duve *et al.* [8], it should be dependent on the energy supply. The present results showing that both metabolic inhibitors and low incubation temperature reduced the lysosomal storage of DNR support this idea. If the metabolic inhibitors reduce the lysosomal storage capacity leading to leakage of DNR from the lysosomes, this may explain why they increased the efflux of DNR from cells in which 80 per cent of the accumulated drug was localized in the lysosomes.

Thus, we have evidence that an intact energy metabolism is of importance for the efflux of DNR across the plasma membrane, as well as for the lysosomal storage of the drug. A balance between these two effects can explain why the addition of metabolic inhibitors to cells preloaded with DNR did not increase the total cellular drug content. It has previously been found that when drug and inhibitors are added simultaneously to cells, the inhibitors increase the drug accumulation [5]. It is now possible to explain why this effect was less pronounced for DNR than for DOX, since lysosomal trapping is much more important for DNR [4].

When a trace amount of [^{3}H]-DNR was added for a short time to fibroblasts preincubated with unlabelled DNR to steady-state conditions, the nucleo-lysosomal distribution ratio was higher for the radioactivity than for the total drug. This indicates that drug initially bound to the nuclei is redistributed to the lysosomes. Previously, it has been found that the efflux of DNR from loaded fibroblasts during a washout period mainly occurs from the nuclei [5].

The efflux of [^{3}H]-DNR from preloaded fibroblasts calculated as a percentage of accumulated drug was much higher when the cells had been preloaded for 30 min than after preloading for 10 hr. This can also be explained by the difference in intracellular localization under these different conditions, since nuclear storage is much more important after loading for 30 min and the efflux mainly occurs from the nuclei [5].

The present results support the hypothesis that membrane transport of DNR in cultured fibroblasts occurs as a 'leak and pump' system, the leak being inward diffusion of nonionized drug molecules and the pump an active efflux. The very high intracellular accumulation is due to trapping in nuclei and lysosomes. Nuclear storage can be explained by binding to DNA. In the cytoplasm the drug is concentrated in lysosomes in the protonated form, as a result of the low pH. Both storage processes are dependent on the presence of an intact amino group. Assuming that other anthracyclines have the same affinity for the proposed active outward transport mechanism, the steady-state concentrations in the cytoplasm as well as in the lysosomes will depend on the inward transport rate. DOX, being less lipophilic than DNR, can be expected to diffuse across the plasma membrane at a slower rate. This provides a plausible explanation for all the differences we have observed between DNR and DOX concerning cellular accu-

mulation and subcellular distribution [4, 5]. It can also explain the observation of Bachur *et al.* [1] that the accumulation of various anthracyclines in L 1210 leukaemia cells was linearly related to the lipophilicity of the drugs.

An alternative explanation for the increased cellular uptake of daunorubicin as well as for the reduced drug efflux caused by a combination of antimycin A and iodoacetate could be that the treatment with the metabolic inhibitors decreased the intracellular pH. Antimycin A inhibits electron transport in the mitochondrial respiratory chain [24] and has been found to increase the cellular production of lactic acid [25]. The principal effect of iodoacetate is to inhibit the glycolytic enzyme 3-phosphoglyceraldehyde dehydrogenase [26] and it has been found to reduce the production of lactic acid from glucose and other glycolytic substrates [25]. Therefore treatment of the fibroblasts with antimycin A alone should lead to a higher accumulation of acid metabolites as compared to treatment with the combination of antimycin A and iodoacetate. However, we have found that antimycin A alone does not influence the cellular accumulation of daunorubicin. Furthermore, a pronounced intracellular accumulation of acid metabolites should also increase the steady-state level of daunorubicin after the addition of the inhibitors to preloaded fibroblasts. However, this treatment only increased the turnover of accumulated drug, not the steady-state level. Therefore, it is unlikely that changes of the intracellular pH during the incubations play a significant role for the observed effects caused by the metabolic inhibitors.

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REFERENCES

1. N. R. Bachur, M. Steel, W. D. Meriwether and R. C. Hildebrand, *J. med. Chem.* **19**, 651 (1976).
2. D. S. Chervinsky and J. J. Wang, *J. Med.* **7**, 63 (1976).
3. T. Skovsgaard, *Biochem. Pharmac.* **26**, 215 (1977).
4. G. Noël, C. Peterson, A. Trouet and P. Tulkens, *Eur. J. Cancer* **14**, 363 (1978).
5. C. Peterson and A. Trouet, *Cancer Res.* **38**, 4645 (1978).
6. E. Calendi, A. Di Marco, M. Reggiani, B. Scarpinato and L. Valenti, *Biochim. biophys. Acta* **103**, 25 (1965).
7. W. J. Pigram, W. Fuller and L. D. Hamilton, *Nature New Biol.* **235**, 17 (1972).
8. C. De Duve, T. De Barsy, B. Poole, A. Trouet, P. Tulkens and F. Van Hoof, *Biochem. Pharmac.* **23**, 2495 (1974).
9. T. Skovsgaard and N. I. Nissen, *Dan. med. Bull.* **22**, 62 (1975).
10. D. W. Wilson, D. Grier, R. Reimer, J. D. Bauman, J. F. Preston and E. J. Gabbay, *J. med. Chem.* **19**, 381 (1976).
11. T. Skovsgaard, *Biochem. Pharmac.* **27**, 1221 (1978).
12. K. Danö, *Biochim. biophys. Acta* **323**, 466 (1973).
13. M. Inaba, H. Kobayaski, Y. Sakurai and R. K. Johnson, *Cancer Res.* **39**, 2200 (1979).
14. G. Noël, A. Trouet, A. Zenebergh and P. Tulkens, in *EORTC International Symposium Adriamycin Review* (Eds. M. Staquet, H. Tagnon, Y. Kenis, G. Bonadonna, S. K. Carter, G. Sokal, A. Trouet, M. Ghione, C. Praga, L. Lenaz and O. S. Karim), p. 99. European Press Medikon, Ghent (1975).
15. P. Tulkens, H. Beaufay and A. Trouet, *J. Cell Biol.* **63**, 383 (1974).
16. F. Leighton, B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler and C. De Duve, *J. Cell Biol.* **37**, 482 (1968).
17. R. Baurain, M. Masquelier, C. Peterson, D. Deprez-De Campeneere and A. Trouet, *Current Chemother. Proc. 10th Congr. Chemother.* Vol. 2, 1130 (1978).
18. R. Baurain, A. Zenebergh and A. Trouet, *J. Chromat.* **157**, 331 (1978).
19. N. R. Bachur, *Biochem. Pharmac.* Suppl. 2, 207 (1974).
20. D. W. Yesair, E. Schwartzbach, D. Shuck, E. P. Denine and M. A. Asbell, *Cancer Res.* **32**, 1177 (1972).
21. N. R. Bachur, *J. Pharmac. exp. Ther.* **177**, 573 (1971).
22. S. Eksborg and H. Ehrsson, *J. Chromat.* **152**, 211 (1978).
23. S. Ohkuma and B. Poole, *Proc. natn. Acad. Sci. U.S.A.* **75**, 3327 (1978).
24. W. W. Wainio, in *The Mammalian Mitochondrial Respiratory Chain* (Ed. W. W. Wainio), p. 112. Academic Press, New York (1970).
25. B. Diamant and C. Peterson, *Acta physiol. scand.* **83**, 324 (1971).
26. J. L. Webb, in *Enzyme and Metabolic Inhibitors* (Ed. J. L. Webb), Vol. 3, p. 1. Academic Press, New York (1966).