Biochemical Pharmacology, Vol. 29, pp. 1035–1040.

0006-2952/80/0401-1035 $02.00/0

# COMPARATIVE STUDIES OF THE ACCUMULATION OF DOXORUBICIN AND DOXORUBICIN–DNA IN VARIOUS CELL LINES

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(*Received* 31 *May* 1979; *accepted* 8 *November* 1979)

**Abstract**—The interaction of doxorubicin and doxorubicin-$^{125}$I-labelled DNA with rat fibroblasts, rat hepatoma, mouse leukaemia and Schmidt–Ruppin sarcoma cells has been studied. When doxorubicin was added to the culture medium, the drug was accumulated as a function of its external concentration and of the time of incubation. The accumulation of doxorubicin was considerably lower when cells were incubated in presence of doxorubucin–DNA. $^{125}$I-labelled material is bound to the cells at 0° and 37°. At this latter temperature, in the absence of cells, DNA degradation products are recovered in the culture medium, resulting from the addition of serum. The presence of fibroblast and leukaemia cells, but not of hepatoma and sarcoma cells, increases considerably the amount of degradation products recovered in the medium. These results are discussed in view of different mechanisms of drug uptake. For the free drug a simple permeation through the cell membrane is envisaged, as previously reported. For doxorubicin–DNA, two possible mechanisms can be considered: endocytosis and release of the drug inside the lysosomes or uptake of the free drug resulting from a partial dissociation of the complex in the culture medium.

Anthracyclines, and doxorubicin (DOX)* in particular, are very potent antitumoral drugs, the use of which is severely restricted by their cardiotoxicity. The binding of daunorubucin and doxorubicin to high molecular weight DNA has been shown experimentally to decrease the toxicity and to increase the activity of the drugs [1, 2]. The use of anthracycline–DNA complex was proposed on the basis of the lysosomotropic drug carrier hypothesis, which assumes that tumor cells and to a lesser extent, normal cells might endocytose DNA complexes and release the drug intralysosomally.

Although the cellular pharmacokinetic and the subcellular localization of DOX have been studied in various cell types [3–5], very little is known with regard to the interaction of anthracyclines–DNA complexes with cultures cells [6].

In this paper, we report data on the accumulation of DOX and DOX–DNA by a normal cell line (rat embryo fibroblasts: RF) and by three cultured tumoral cell lines (mouse leukemic cells: L1210 cells; rat hepatoma cells: HTC cells; and Schmidt–Ruppin sarcoma cells: SR cells).

In a forthcoming paper, we will present data on the subcellular localization of DOX and DOX–DNA in RF and L1210 cells as determined by cell fractionation techniques.

## MATERIALS AND METHODS

*Cell cultures.* Fibroblasts (RF) were isolated from rat embryos (Wistar strain) and cultivated according to Tulkens *et al.* [7]. Cells were cultured in 20 cm$^2$ Petri dishes in a modified Eagle Dulbecco medium containing 10 per cent of newborn calf serum; only first or second confluent subcultures were used.

Since 1972, L1210 ascitic cells obtained from leukemic $DBA_2$ mice (kindly provided by Drs. C. Gosse and J. Morizet: Institut Gustave Roussy, Villejuif, Paris, France), have been cultured continuously in RPMI 1640 medium, supplemented with 10 per cent foetal calf serum according to Moore *et al.* [8].

Hepatoma tissue culture cells (HTC cells), supplied by Dr. B. Hogan (Brighton, U.K.), are derived from the Morris transplantable hepatoma No. 7288 C [9]. The cells were cultivated in suspension according to Samuels and Tomkins [10] in a 213 medium supplemented with 10 per cent foetal calf serum.

Schmidt–Ruppin sarcoma cells [11] (ATCC-CCL47) were purchased from Flow Laboratories (Irvine, Scotland). They were grown in Eagle Dulbecco medium containing 15 per cent newborn calf serum and were subcultivated weekly.

Tumorigenicity of the three tumor cell lines was routinely checked on rats (Newborn Amsterdam for SR cells and Buffalo for HTC cells) and on $DBA_2$ mice for L1210 cells.

In our experimental conditions, L1210 and HTC cells were maintained in Falcon tubes containing, respectively, 1.5 to 2 × 10$^6$ and 0.5 to 1 × 10$^6$ cells (*ca.* 0.2 mg of cell protein) in 4 ml culture medium; RF and SR cells were cultivated in 20 cm$^2$ Petri dishes to confluency (0.5 to 1 mg of cell protein per dish).

*Drug.* Lyophilized doxorubicin hydrochloride was dissolved in distilled water to 10 mg/ml (17.5 mM), further diluted in 0.15 M NaCl and used extemporaneously.

A DOX–DNA complex was prepared with a ratio of 20 nucleotides per molecule of drug [1]. In brief, 9.5 ml of DNA at a concentration of 2.34 mg/ml were mixed with 0.5 ml of DOX at a concentration of 4 mg/ml (7 mM).

*Abbreviations used: DOX, doxorubicin; DOX–DNA, DOX complexed to $^{125}$I-labelled DNA; HTC, hepatoma tissue culture; L1210, L1210 leukaemia; RF, rat embryo fibroblasts; SR, Schmidt–Ruppin sarcoma; TCA, trichloracetic acid.

For all the experiments DNA was labelled with $Na^{125}I$ [12] before binding to DOX.

*Accumulation experiments.* DOX or DOX–DNA were added to the complete culture medium. The pH of the culture medium was 7.8 for RF and SR cells and 7.7 for L1210 and HTC cells and values found unchanged at the end of the experiment. After incubation, RF and SR cells which adhere to dishes were washed three times with phosphate buffered saline at 4° and then scraped with a rubber policeman in 1.3 ml of distilled water at 4°. HTC and L1210 cells, maintained in suspension, were centrifuged at 4° for 10 min at 800 (HTC cells) or 1200 (L1210 cells) r.p.m. (IEC Centrifuge, rotor No. 253), washed two times with phosphate buffered saline and finally resuspended in 1.3 ml of distilled water.

*Assays.* After incubation of the cells with DOX-$^{125}I$ labelled DNA, radioactivity was assayed in 1 ml of the culture medium (Autogamma Scintillation Spectrometer, Packard 5130 model, Packard Instruments) and on the supernatant fraction obtained after precipitation with 2 ml of 20% TCA and centrifugation. Cell-associated radioactivity was measured on 0.1 ml of cell lysate and on the supernatant fraction obtained after precipitation of 1 ml of the cell sample with 2 ml of 20% TCA. This latter supernatant fraction was also used to determine by fluorimetry (Perkin–Elmer fluorimeter, with an excitation wavelength of 481 nm and an emission of 570 nm) the amount of drug associated to the cells. Standards of free and complexed DOX were systematically included. Protein was determined by the method of Lowry *et al.* [13].

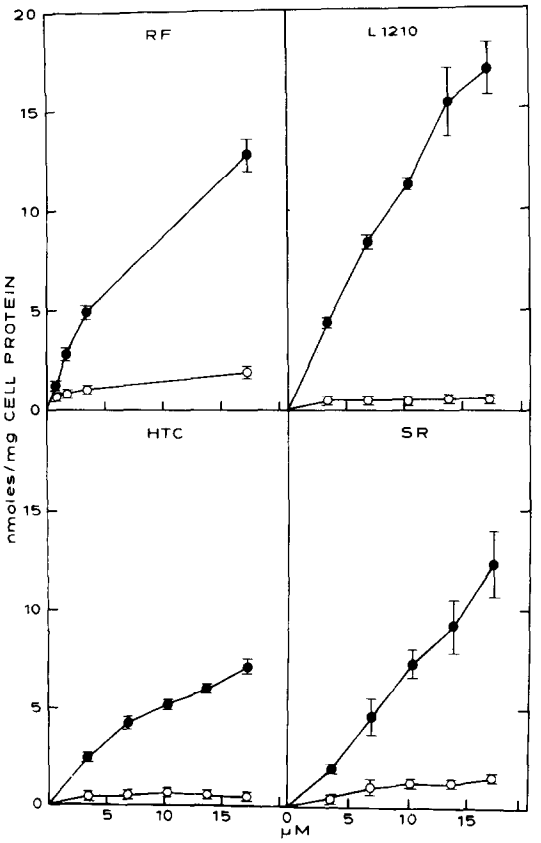


Fig. 1. Accumulation of DOX by RF, L1210, HTC and SR cells. Cells (0.2–0.5 mg of protein) incubated 3 hr at 37° in 4 ml of culture medium supplemented with various concentration of DOX (●) or DOX–DNA (○). Drug was assayed and accumulation determined as described in Materials and Methods. Results are expressed in nmoles of DOX/mg of cell protein. Mean of 3 experiments ± S.D.

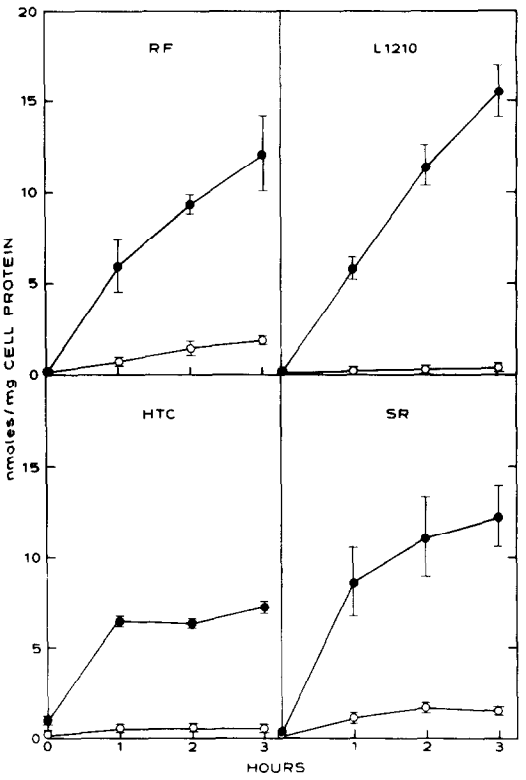


Fig. 2. Time course of accumulation of DOX by RF, L1210, HTC and SR cells. Cells (0.2–0.5 mg protein) were incubated for various times at 37° in the presence of 4 ml of culture medium supplemented with DOX (●; external concentration 17.5 μM) or DOX–DNA (○; external concentration in DOX 17.5 μM, which corresponds to 117 μg of DNA/ml). Drug was assayed and accumulation determined as described in Materials and Methods. Results are expressed in nmoles of DOX/mg of cell protein. Mean of 9 experiments for RF and L1210 cells and 3 experiments for HTC and SR cells ± S.D.

*Materials.* All chemicals were of analytical grade. They were purchased from E. Merck A.G. (Darmstadt, Germany). DNA type VII from herring sperm was obtained from Sigma Chemical Co. (St. Louis, MO); doxorubicin (Adriblastine) from Farmitalia-Benelux (Bruxelles, Belgium); $Na^{125}I$ from IRE (Fleurus, Belgium); culture medium (RPMI 1640, Swims $H_{20}$), foetal calf serum and newborn calf serum from Gibco Biocult (Paisley, U.K.).

Wistar and Buffalo rats were bred in a local animal house. $DBA_2$ mice were obtained from Charles River (Aubin-lez-Elboeuf, France).

## RESULTS

### *Incubation with DOX*

In a first set of experiments (Fig. 1), accumulation of DOX by RF, L1210, HTC and SR cells were

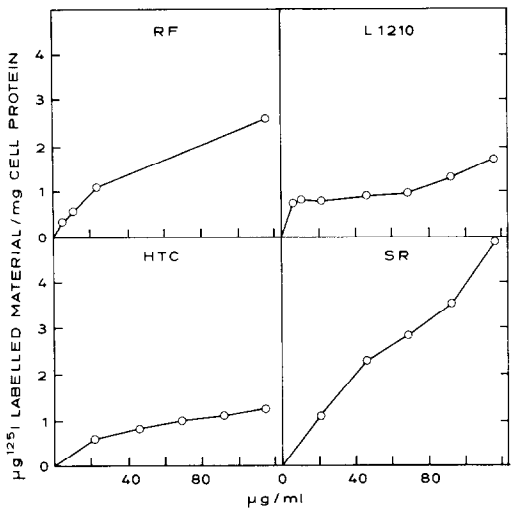


Fig. 3. Accumulation of $^{125}$I-labelled material by RF, L1210, HTC and SR cells. Cells (0.2–0.5 mg protein) were incubated for 3 hr at 37° in 4 ml of culture medium supplemented with various concentrations of DOX–DNA (0–117 μg/ml, which corresponds to 0–17.5 μM in DOX). Radioactivity was assayed and accumulation determined as described in Materials and Methods. Results were expressed in μg of DNA equivalents/mg of cell protein. Mean of 3 experiments.

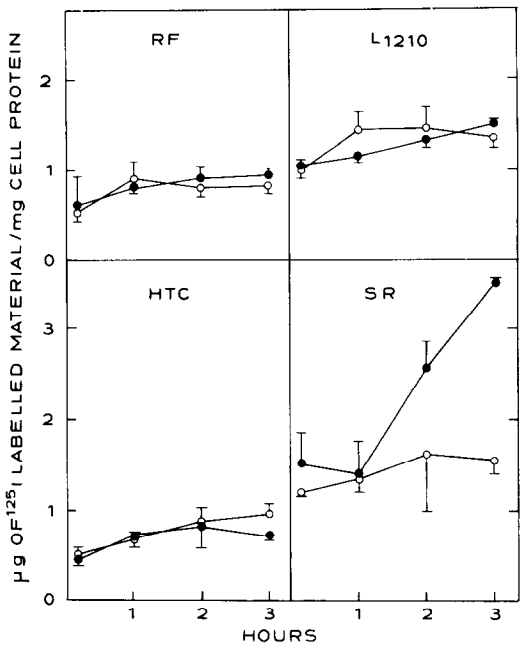


Fig. 4. Accumulation of $^{125}$I-labelled material by RF, L1210, HTC and SR cells. Cells (0.2–0.5 mg protein) were incubated for various times at 37° (●) and 0° (○) in the presence of 4 ml of culture medium supplemented with 117 μg/ml of DOX–DNA (17.5 μM in DOX). Radioactivity was assayed and intracellular accumulation determined as described in Materials and Methods. Results are expressed in μg of DNA equivalents/mg of cell protein. Mean of 3 experiments ± S.D.

determined after 3 hr of incubation at 37° in presence of various concentrations of the drug. In the four cell types, the accumulation increased as a function of the external concentration, but different levels were reached.

The accumulation of DOX was also recorded as a function of the incubation time at 37° (Fig. 2). At an external concentration of 17.5 μM it increased with time in RF, L1210 and SR cells, whereas a plateau was reached after 1 hr incubation in the presence of HTC cells.

*Incubation with DOX–DNA*

*Accumulation of DOX.* In a second set of experiments, cells were incubated for 3 hr at 37° in the presence of increasing concentrations of DOX–DNA. The accumulation of the drug is much lower (Fig. 1), and a saturating level is reached nearly in all cell types.

The accumulation of DOX as a function of the incubation period with DOX–DNA (17.5 μM, i.e. 117 μg of DNA/ml) is illustrated in Fig. 2.

*Accumulation of $^{125}$I-labelled material by the cells.* The accumulation of $^{125}$I-labelled material by cells incubated for 3 hr at 37° with various concentrations of DOX–DNA increases with the external concentration up to different levels (Fig. 3). In all cases, more than 90 per cent of the cell-associated labelled material was precipitated by TCA. If RF, L1210 or HTC cells are incubated for various times at 0° or 37° with DOX–DNA, similar amounts of $^{125}$I-labelled material are bound by the cells and this binding is nearly immediate. With SR cells, the binding of $^{125}$I-labelled material at 4° is also immediate, but more material is accumulated at 37° after 1 hr.

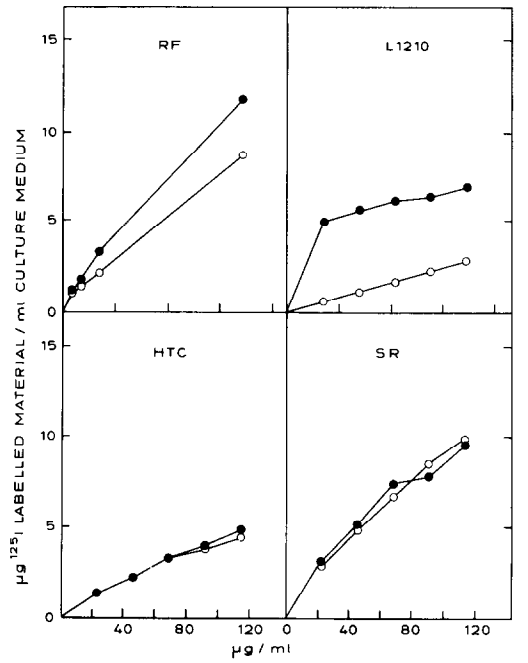


Fig. 5. Solubilization of $^{125}$I-labelled DNA into fragments soluble in TCA. Complete culture medium (4 ml) was incubated for 3 hr at 37° with increasing concentrations of DOX–DNA, in the presence (●) or absence (○) of cells. Radioactivity after precipitation was assayed as described in Materials and Methods. Results are expressed in μg of DNA equivalents/ml of culture medium. Mean of 3 experiments.

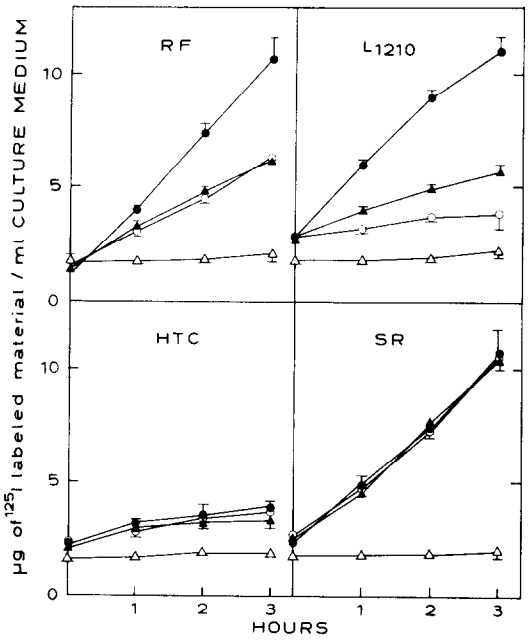


Fig. 6. Solubilization of $^{125}$I-labelled DNA into fragments soluble in TCA. Complete culture medium (4 ml) was incubated for various times at 37° with 117 μg DNA/ml (17.5 μM in DOX). Radioactivity was assayed as described in Materials and Methods. Results are expressed in μg of DNA equivalents/ml of culture medium. Mean of 3 experiments ± S.D.

Culture medium with serum incubated in presence of cells (●).

Culture medium with serum incubated in absence of cells (○).

Culture medium without serum (△).

Culture medium with serum previously incubated for 24 hr at 37° in the presence of cells and then incubated in the absence of cells (▲).

*Digestion of $^{125}$I-labelled material in the medium.* The amounts of $^{125}$I-labelled material soluble in TCA were systematically determined in the culture medium. Immediately after dilution in culture medium, about 97 per cent of the $^{125}$I-labelled material can be precipitated by TCA.

In the presence of cells, the amount of material soluble in TCA increased as a function of the DOX–DNA concentration (Fig. 5) and of the time of incubation at 37° (Fig. 6).

This soluble material does not consist in free $^{125}$iodide, since after oxidation by $H_2O_2$ it is not extractable by chloroform [14] and since it does not behave as $^{125}$iodide during thin layer chromatography on silica gel (methanol, isopropanol, cyclohexane 40/40/20).

$^{125}$I-labelled material soluble in TCA is also recovered after incubation at 37° in the absence of cells, but only if serum is present in the culture medium (Figs. 5 and 6). With the single exception of L1210 cells, the incubation of DOX–DNA in the absence of cells in a medium which had been exposed to cells for 24 hr at 37° does not result in a significant increase of the amount of $^{125}$I-labelled material soluble in TCA. The results indicate that the presence of RF and L1210 cells, but not that of HTC and SR cells, increases the amount of $^{125}$I-labelled material soluble in TCA.

The amount of labelled material solubilized as a result of the presence of RF and L1210 cells increases as a function of incubation time (Fig. 6). With RF cells, it increases also as a function of the DOX–DNA concentration.

## DISCUSSIONS

As far as observation by phase contrast microscopy and determination of protein content of the cell culture are concerned, cell damage could not be detected in our experimental conditions. It must, however, be remembered that in similar conditions both DOX and DOX–DNA decrease rapidly thymidine uptake by L1210 cells [15].

In the four cell lines, accumulation of DOX present in the culture medium in its free form increases as a function of the incubation time and of the extracellular drug concentration (Figs. 1 and 2). Taking into account a cellular volume of, respectively, 5 and 6.8 μl/mg of cell protein for RF [16] and HTC cells [17] and assuming that 1 mg L1210 and SR cell protein correspond to 5 μl, one can estimate that cells incubated for 3 hr at 37° with 17.5 μM DOX concentrate the drug 140, 60, 180 and 140 times, respectively. After incubation of DOX–DNA we can calculate similarly that DOX is concentrated 22 times in RF cells, 8 times in SR cells and 5 times in HTC and L1210 cells.

At 0° the binding of $^{125}$I-labelled DNA is nearly immediate with the four cell types. All of the cell-associated $^{125}$I-labelled material is insoluble in TCA, with accumulation levels similar to those reached at 0°, SR cells excepted.

After incubation of DNA in culture medium at 37° in the absence of cells, increasing amounts of fragments soluble in TCA are produced as a function of time and concentration (Figs. 5 and 6). This digestion of DNA is related to the presence of serum, probably as a result of deoxyribonuclease activity as reported by Cox [18]. In the presence of RF and L1210 cells, higher amounts of $^{125}$I-labelled material soluble in TCA are recovered as a function of the duration of the incubation (Fig. 6). The amount of DNA solubilized by RF cells increases as a function of DOX–DNA concentration, whereas the digestion of DNA related to the presence of L1210 cells is not affected by DOX–DNA concentration (Fig. 5). In addition, the digestion of DNA and the appearance of TCA soluble material during an incubation in the presence of a medium which has been previously exposed to L1210 cells could suggest that these cells secrete deoxyribonucleases into the extracellular medium.

Rat fibroblasts accumulated to a similar extent DNA at 0° and 37° and release $^{125}$I-labelled fragments, soluble in TCA, into the culture medium as a function of the time of incubation and of the DOX–DNA concentration. These results are compatible with an endocytic uptake of DOX–DNA, a rapid intralysosomal digestion of DNA, followed by a release of the degradation products. L1210 cells as

RF accumulate equal amounts of $^{125}$I-labelled material at 0° and 37° and release degradation products. By contrast, however, a medium previously incubated with these cells is able to digest DNA into TCA soluble fragments. L1210 cells could therefore take up DOX–DNA by endocytosis, but also in addition secrete deoxyribonuclease which would then digest DNA extracellularly. HTC cells most probably do not endocytose DOX–DNA and no evidence of digestion had been found. SR cells bind DNA at 0° and accumulate more $^{125}$I-labelled material at 37°, but do not digest it, which could suggest either that the binding at the cell surface is higher at 37° than at 0° or that the cells accumulate DNA in an intracellular compartment inaccessible to hydrolases.

If, as previously proposed [3], free DOX penetrates into the cell by permeation through the cell membrane and becomes trapped in the nuclei and lysosomes, the mechanism by which DOX is taken up by cells incubated with DOX–DNA still remains unelucidated. DOX–DNA could be taken up as such through endocytosis [2], DNA being digested in lysosomes and the released drug becoming segregated inside lysosomes [3] or gaining access to the nuclei. Alternatively, DOX–DNA could be dissociated in the extracellular medium [19, 20] and DOX taken up not as a DNA complex but as a free drug.

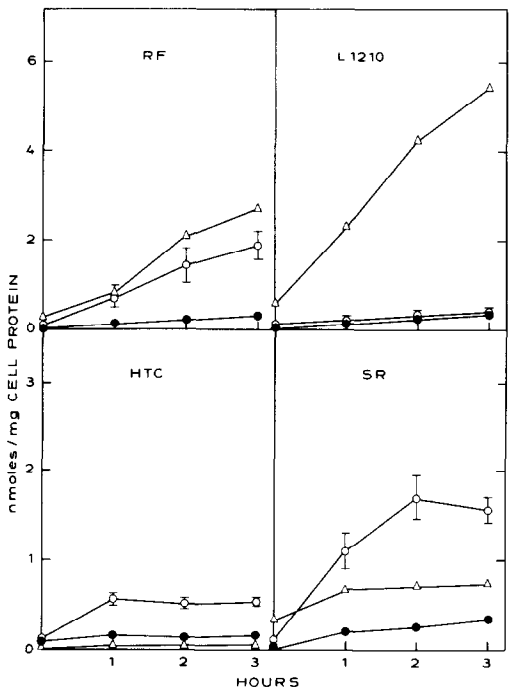


Fig. 7. Experimental and calculated amounts of DOX accumulated by RF, L1210, HTC and SR cells incubated for various times with 117 μg of DOX–DNA per ml (i.e. 17.5 μM of DOX).

Experimental data (○) come from Fig. 2. For the calculation, as previously described [19], we have assumed that DOX is accumulated as a result of the accumulation and the digestion of $^{125}$I-labelled material by the cells (△) or that DOX is taken up as the free drug after the partial dissociation of DOX–DNA (●).

The levels of DOX accumulated by RF cells incubated with DOX–DNA are higher than those we have previously calculated [19] from the partial dissociation of the complex in the medium and lower than those resulting from endocytosis and cell related digestion of DNA (Fig. 7). Our experimental data are, however, compatible with a mechanism of uptake implying the two hypotheses mentioned above, but considering in addition an active extrusion mechanism of DOX as previously reported for these cells [4]. The levels of DOX determined in L1210 cells correspond closely to the amount calculated on the basis of the partial dissociation of DOX–DNA in the medium. The high amount of DNA digested by the cells might be explained either by endocytosis of DOX–DNA, concomitant with an active extrusion of DOX, or by the digestion of DNA in the extracellular medium after secretion of hydrolytic enzymes. In the case of HTC cells and, to a lesser extent, SR cells, there is an agreement between the accumulation of DOX as determined experimentally and the sum of the values calculated on the basis of endocytosis and dissociation of DOX–DNA.

Our results indicate, therefore, that DOX is not accumulated to the same extent when cultured cells are incubated in the presence of the free drug or its DNA complex and that the uptake of DOX by cells incubated with DOX–DNA may be the result of different processes, such as endocytosis or diffusion following dissociation of the complex.

*Acknowledgements*—We are grateful to Dr. C. Moussebois and Mr. C. Gotfredsen for their helpful advice and we thank Mrs. F. Renoird-Andries for her excellent technical help. This work has been supported by the 'Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite' (Bruxelles, Belgium).

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