These results indicate that cinchocaine is more effective than procaine in inhibiting [^{14}C]ACh and choline release from synaptosomes, and this correlates with their relative local anaesthetic potency. There is a substantial evidence [7–9] which suggests that local anaesthetics bind specifically to Na^+ channels of the nerve membrane, preventing the normal Na^+ flux and as a result causing a nerve conduction block. The effect of procaine and cinchocaine in reducing [^{14}C]ACh release in high K^+ medium (in the absence of ouabain) suggests that they cause a reduction in synaptosomal depolarization by the same mechanism. This is also supported by the fact that the concentrations of the two anaesthetics used in these experiments, although slightly lower, are still within the range that has been used previously. For example, concentrations of about 1 mM procaine and 0.5 mM cinchocaine have been employed by Narahashi *et al.* to inhibit Na^+ conductance in nerve membrane [10, 11].

In these experiments, cinchocaine reversed the effect of ouabain on [^{14}C]ACh release even in depolarizing concentrations of high K^+. Its effect was even more marked on ouabain-induced [^{14}C]choline efflux, suggesting an additional effect of ouabain. In our previous work we showed that the effect of ouabain on [^{14}C]ACh and choline release is independent of Ca^{2+} [1]. In addition to its specific effect on Na^+–K^+ ATPase activity and the related ACh release, it seems likely that ouabain at a concentration of 200 μM has a detergent action which would increase the leakiness of the synaptosomal membrane. Such leakiness would be reversed or counteracted by the membrane stabilization that is a feature of local anaesthetic action [12].

Summary. Procaine and cinchocaine do not affect Na^+–K^+ ATPase but reverse the [^{14}C]ACh and [^{14}C]choline release evoked by ouabain with a relative effectiveness correlating with their local anaesthetic potency. They reduce the [^{14}C]ACh release evoked by high K^+. In addition, cinchocaine reversed the [^{14}C]ACh and [^{14}C]choline efflux induced by ouabain in synaptosomes already depolarized by high K^+. This suggests that ouabain (200 μM) has an additional detergent-type action that can be counteracted by local anaesthetic membrane stabilization.

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Uptake and metabolism of doxorubicin in isolated perfused rat lung*

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Primary bone and soft-tissue sarcomas frequently and almost exclusively metastasize to the lungs [1, 2]. Without treatment, over 75% of patients developing pulmonary metastatic osteosarcoma die within 12 months [3]. Surgical procedures have proven successful with a 26% five-year survival for soft-tissue sarcoma and 28% for osteogenic sarcoma [4–6]. At present, doxorubicin (DOX) is considered the most useful anticancer agent for the treatment of these forms of cancer with a 33–40% remission rate in patients with soft tissue sarcomas [7, 8]. However, a significant proportion of patients developing pulmonary metastatic disease are unresectable and unresponsive to chemotherapy.

Recently, studies were undertaken to develop an isolated perfused lung procedure for the treatment of patients with metastatic soft-tissue sarcomas to the lungs where other surgical and chemotherapeutic approaches have been exhausted [9]. The procedure entails short-term isolated pulmonary perfusion with blood concentrations of doxorubicin normally not tolerated *in vivo*. It is hoped that such a procedure may offer a means to deliver tumoricidal drug concentrations but avoid systemic toxicity.

Little is known regarding the disposition of anthracyclines in lung although this organ is often the site of drug

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action. Marafino *et al.* [10] have reported that the half-life of DOX-derived radioactivity in lung is 4–5 hr and that, following an intravenous injection of [^{14}C]DOX, significant covalent binding of ^{14}C was evident in the lungs. Whole animal studies may not reflect the kinetics of DOX in pulmonary tissue because of the influence of extrapulmonary tissue on the distribution and metabolism of the drug. In the present study, an isolated rat lung preparation was used as a means to investigate the uptake, metabolism and efflux of DOX in lung tissue.

Methods and Materials

Chemicals. [14-^{14}C]Doxorubicin HCl (sp. act. = 17.2 mCi/mmole), doxorubicin HCl, daunorubicin HCl, doxorubicinol HCl and doxorubicinone HCl were provided by Dr. Robert Engle, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD, U.S.A. All other reagents were of analytical grade. Radiopurity of [^{14}C]DOX was greater than 98% as determined by high pressure liquid chromatography using conditions outlined below.

Isolated perfused rat lung. The lungs of male Sprague–Dawley rats (180–220 g) were perfused *in situ* as previously described [11]. Lungs were perfused for up to 20 min with drug ranging from 1 to 100 μM following which the lungs were frozen for later analysis. Samples of the perfused effluent were also analyzed for DOX. Drug efflux was measured by perfusion with 1 μM DOX for 10 min followed by a 10-min perfusion with drug-free buffer. Lung wet to dry weight ratios did not differ from control lungs (5.80 ± 0.15; mean ± S.D.) at any concentration of drug.

DOX assay. Perfusion medium (1 ml) or lung homogenate (0.4 ml or an appropriate dilution at the higher perfusate DOX concentrations; homogenized in 4 vol. water) was vortexed with 400 μl of 0.05 M borate buffer (pH 9.8), 50 μl daunorubicin (internal standard; 10 μg/ml) and 6 ml chloroform-methanol (4:1). After centrifugation, the organic phase was collected, evaporated to dryness, taken up in 200 μl methanol by sonication in an ultrasonic water bath, and 20–100 μl injected into a Waters high pressure liquid chromatograph. A C_{18}-phenyl column (Waters Associates, U.S.A.; I.D. 3.9 mm, length 30 cm) was used with a mobile phase of 35% acetonitrile in formate buffer (pH 4.0). DOX was detected using a Schoeffel FS 980 fluorescence detector fitted with a deuterium lamp and using an excitation wavelength of 228 nm and an emission filter of 550 nm. At a flow rate of 1.5 ml/min, the retention times for doxorubicinol, DOX, doxorubicinone and daunorubicin were 3.15, 4.14, 7.46 and 7.61 min respectively. The coefficient of variation for the assay was less than 8% over the concentration range of 25–1000 pmoles/ml of perfusate or lung homogenate.

DOX metabolism and covalent binding. Metabolism and covalent binding of DOX were investigated after perfusing rat lungs with 1 μM [^{14}C]DOX for 10 min. Following perfusion, the lungs were homogenized at 4° in 10 vol. water. An aliquot (1 ml) of homogenate was extracted five times with 6 ml $CHCl_3$–CH_3OH (4:1, v/v) after the addition of 1 ml of 0.05 M borate buffer (pH 9.8). The organic extracts were pooled and evaporated to dryness under N_2. The residue was reconstituted in 200 μl CH_3OH, and 10–50 μl was analyzed by high pressure liquid chromatography using a flow rate of 1.5 ml/min. The HPLC mobile phase was collected in 15-sec fractions, and radioactivity in each was quantified by liquid scintillation counting. Extraction of ^{14}C was 98 ± 0.2%, and recovery of radioactivity from the high pressure liquid chromatographic column was 96.2 ± 2.8%.

A second aliquot (1 ml) of lung homogenate was used to quantify covalent binding. After the addition of 200 μl of 50% trichloroacetic acid (TCA), the mixture was centrifuged at 1000 *g* for 10 min. The precipitated pellet was then washed extensively with warm methanol until radioactivity in the methanol phase was not different from background (five to eight washings). The precipitated pellet was dissolved in 2 M NaOH at 80° following which ^{14}C content was quantified. Protein concentration in the solubilized fraction was measured by the Bradford method [12].

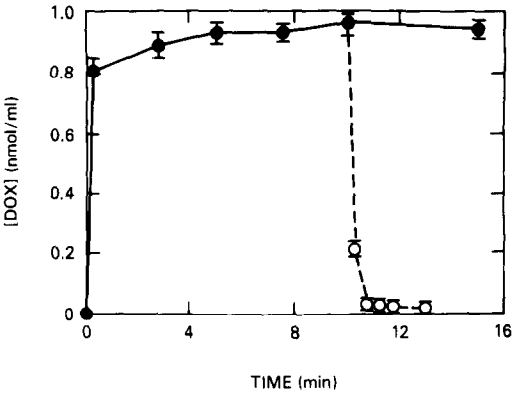

Fig. 1. DOX concentration in the effluent of rat lungs perfused with 1 μM drug (●—●). Each point represents the mean ± S.E. of three observations. Efflux of DOX was measured by changing the perfusion medium to drug-free buffer after 10 min (○--○).

Data analysis. Linearity of the uptake curves was assessed by step-wise polynomial regression using a Marquardt–Levenberg nonlinear least-squares regression algorithm. All data have been expressed as mean ± S.E.

Results and Discussion

DOX uptake and efflux. The time–concentration profile of DOX in the effluent of rat lungs perfused with 1 μM drug is shown in Fig. 1. Steady-state was reached after approximately a 2 min perfusion at which time the effluent concentration represented between 90 and 95% of the inflowing concentration. If the perfusion medium was changed to drug-free buffer following a 10 min perfusion, efflux of DOX from the tissue could be observed (Fig. 1, hatched curve). The drug concentration in the effluent rapidly fell to approximately 30 pmoles/ml by 2 min post washout and could not be detected by 4 min.

The lung concentration of DOX increased linearly with time, reaching 12.3 ± 0.7 nmoles/g lung by 20 min (Fig. 2). The rate of DOX accumulation in the lungs was 0.52 ± 0.03 nmole per g per min. Efflux of DOX from the lungs

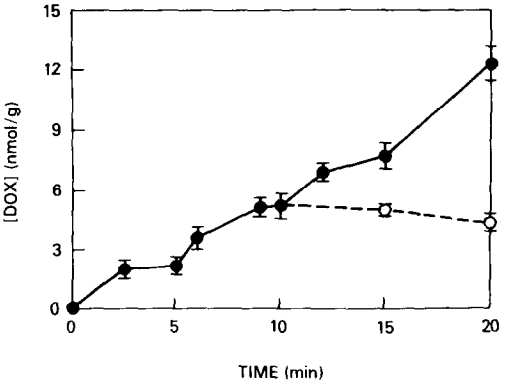

Fig. 2. DOX concentration in tissue of rat lungs perfused with 1 μM drug (●—●). Each point represents the mean ± S.E. of four to six lungs. Efflux of DOX was measured by changing the perfusion medium to drug-free buffer after 10 min (○--○).

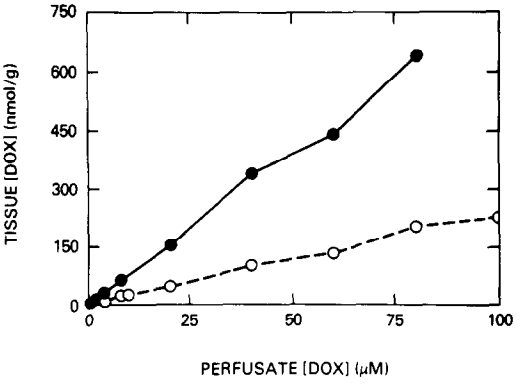

Fig. 3. Lung DOX concentrations after 5 (○--○) and 15 (●—●) min of perfusion with various drug concentrations. Each point is the mean of two observations. The range at each point was less than 10% of the mean.

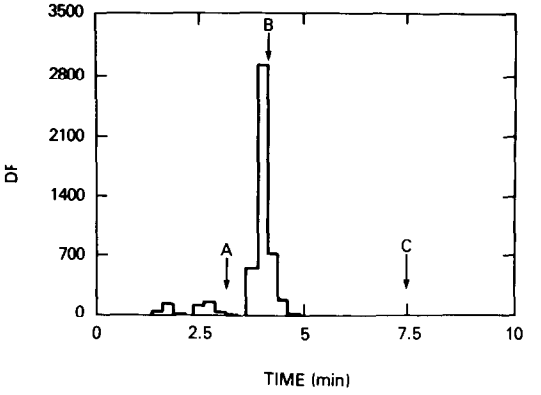

Fig. 4. High pressure liquid chromatogram of radioactivity extracted from rat lungs perfused with 1 μM [^{14}C]DOX for 10 min. The retention times for doxorubicinol (A), DOX (B), and doxorubicinone (C) are shown.

was considerably slower than uptake and was linear over the first 10 min (Fig. 2). By the end of the washout period, 82% of the accumulated unchanged DOX still remained in the lung. These results indicate that rat lung contains an accumulation process, such as tissue binding or facilitated transport, for DOX. Further, since the rate of uptake was some 40-fold greater than the rate of efflux, a tissue to medium ratio of approximately this value could be expected at equilibrium. Figure 3 illustrates the linear relationship between inflowing DOX concentration and lung DOX concentration attained after 5 and 15 min of perfusion. Over a drug concentration range of 1 to 100 μM, accumulated DOX was directly related to the perfusate concentration ($r = 0.99$). The binding of DOX to the perfusate constituents as determined by equilibrium dialysis was constant (51 ± 2%) over the concentration range used in the present study (unpublished data).

A high pressure liquid chromatographic tracing of DOX-derived radioactivity indicated that at least two metabolites were present in lung tissue after a 10 min perfusion (Fig. 4). The first peak eluted with the void volume of the column and accounted for 4.1% of the total radioactivity. The second peak accounted for 4.7% of the total radioactivity and eluted with a retention time of 2.6 min. Neither peak corresponded to the retention time of authentic doxorubicinol or the aglycone doxorubicinone. However, the shorter retention times of the metabolites, compared to DOX itself, indicate that both are more polar compounds than the parent drug.

DOX-derived radioactivity associated with TCA-precipitated cell components accounted for 4.8% of the total lung radioactivity and was equivalent to 4.7 ± 0.5 pmoles/mg protein or 429 ± 46 pmoles/g lung. In mice, Marafino *et al.* [10] found that lung irreversibly bound 87 pmoles ^{14}C/mg protein following an intravenous injection of 5 mg [^{14}C]DOX/kg. Ghezzi *et al.* [13] reported that DOX-derived radioactivity was bound covalently to rat liver microsomal macromolecules *in vitro*: binding was dependent on the presence of viable microsomes and NADPH, suggesting that it was metabolically mediated. Whether the covalent binding of DOX is the result of oxidative or reductive metabolism is unknown. However, DOX has been shown to undergo cytochrome P-450 reductase-dependent reduction to a semiquinone [14]. More recently, Scheulen and Kappus [15] demonstrated that purified cytochrome P-450 reductase metabolizes DOX to an intermediate that covalently binds to bovine serum albumin under aerobic conditions. Ultraviolet spectra of the drug–protein complex indicated the presence of the anthracycline moiety in the product.

The present study has shown that rat lung is able to extract DOX from the vasculature but at a relatively low rate. Within 20 min of perfusion, lung concentrations of DOX were considerably higher than inflowing concentrations. Furthermore, significant biotransformation of the drug occurred within 10 min of the perfusion. The high drug levels achieved in the tissue suggest that an *in situ* isolated lung perfusion technique may be advantageous over systemic administration, provided adverse toxicity is not experienced at these concentrations. The toxicity of DOX in lung is presently under investigation, using an *in situ* isolated dog lung preparation.

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Inhibition of soluble 5′-nucleotidase from rat brain by different xanthine derivatives

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There is increasing evidence that adenosine plays a role in the regulation of brain function [1–3]. Similarly there is good evidence that xanthine derivatives, such as theophylline and caffeine, may exert many of their actions due to adenosine receptor antagonism [2–4]. However, xanthine derivatives may affect not only the actions of adenosine, but also its formation. Thus it has been shown that xanthine derivatives are able to inhibit 5′-nucleotidase [5–7].

5′-Nucleotidase is present both as a soluble and as a membrane bound ecto-enzyme [8]. The latter form of the enzyme catalyzes the breakdown of AMP present on the outside of cells, and may be of importance in the salvage of purines from 'lost' purine nucleotides [9]. The intracellular, soluble form of the enzyme may be involved in the formation of intracellular adeonsine from AMP. Intracellular formation of adenosine may be of quantitatively greater importance than the formation from extracellular adenine nucleotides [10–12].

Whereas the earlier studies [5–7] demonstrated that the membrane bound form of the enzyme is inhibited by xanthine derivatives, the present study reports that the soluble enzyme also is inhibited by several xanthine derivatives. The structural requirements differ from those for adenosine receptor antagonism or phosphodiesterase inhibition.

Materials and methods

Male Sprague–Dawley rats (200 g) were killed by guillotine. The brains were rapidly dissected out, placed in ice-cooled saline and homogenized in 6 vol. of 0.25 M sucrose containing 50 mM Tris–Cl pH 7.4, 1 mM EDTA and 10 mM 2-mercaptoethanol. After an initial centrifugation at 800 *g* for 20 min to remove cell debris, the supernatant was centrifuged at 100,000 *g* for 45 min. The supernatant served as the source of soluble enzyme, the resuspended pellet (6 ml) as the source of membrane bound enzyme. The enzyme was stored frozen at −80°C in aliquots.

5′-Nucleotidase activity was assayed using ^{14}C-AMP as substrate by the method described earlier [5]. 50 μl suitably diluted enzyme was incubated for 5–60 min in a total volume of 1 ml of 50 mM Tris–Cl pH 7.8 containing 1 mM Mg acetate and labelled AMP as well as other additions as indicated. The assay was done at 30°C and was stopped by the addition of Zn-sulfate and Ba-hydroxide. Blanks were fortified with 30–50 μM α,β methylene ADP [13].

The particulate enzyme was diluted 25-fold and the assay was conducted for 30 min. The supernatant enzyme was used undiluted and the incubations lasted for 60 min. Under these conditions the assay was linear with time.

The conversions of ^{14}C-AMP and of ATP during incubation with the supernatant fraction was determined by HPLC as described earlier [20, 21].

The following drugs were used: 8-^{14}C-adenosine 5′-monophosphate (549 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. Theophylline and caffeine were obtained from ACO, Göteborg, Sweden; Xanthine, 1,7-dimethylxanthine, 7-methylxanthine, 3-methylxanthine, adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP) were from Sigma, St. Louis, MO; Verrophylline (3,7-dihydro-1,8-dimethyl-3-(2-methylbutyl)-1H-purine-2,6-dione) was a gift from Dr K. Murphy, Johns Hopkin, Baltimore, MD; Theobromine was from Astra, Södertälje, Sweden; 3-isobutyl-1-methylxanthine (IBMX) was from Aldrich Co., Rahaway, NJ; 7-benzyl-3-isobutyl-1-methylxanthine was synthesized by Dr G. Kjellin, AB Draco, Lund, Sweden, who also supplied enprofylline (3,7-dihydro-3-1H-purine-2,6-dione); 8-sulphophenyl-theophylline was a gift from Dr G.F. Bruns, Warner Lambert Co., Ann Arbor, MI.; 8-phenyl-theophylline from Calbiochem-Behring Co., CA. and 1,3-diethyl-8-phenylxanthine from Research Biochemicals Inc., Wayland MA were dissolved in a 1 mM solution of tetraphenylbromate; Pentoxiphylline and 1-(5′-oxohexyl)-3-methyl-7-propylxanthine (HWA 285) were gifts from Dr R. Sehleyerbach of Hoechst Ag., Wiesbaden, FRG; α,β-methylene adenosine 5′-diphosphate (AOPCP) was from P.L. Biochemicals, Milwaukee, WI.

Results and discussion

Approximately one fourth of the total 5′-nucleotidase