Biochemical Pharmacology, Vol. 25, pp. 2085–2089. Pergamon Press, 1976. Printed in Great Britain.

INHIBITION OF OXIDATIVE PHOSPHORYLATION IN TUMOR CELLS AND MITOCHONDRIA BY DAUNOMYCIN AND ADRIAMYCIN

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(*Received* 25 *September* 1975; *accepted* 20 *February* 1976)

Abstract The binding of copper to daunomycin has been investigated. It is concluded that the strength of binding is not large enough for the 1:2 copper–daunomycin complex to exist *in vivo*. Daunomycin and adriamycin inhibit glutamate- and pyruvate-malate-dependent oxidative phosphorylation in bovine heart mitochondria and adriamycin uncouples this process as well. No inhibition of Ehrlich ascites tumor cell or mitochondrial respiration by daunomycin is observed at concentrations much larger than those used for heart. In conjunction with the work of others, these results suggest a role for the inhibition of oxidative phosphorylation in the cardiac toxicity of these anthracycline drugs.

The anthracycline drugs, adriamycin and daunomycin, are clinically useful antitumor agents thought to elicit their effects through direct binding to DNA [1]. However, employment of these drugs is limited by cardiac toxicity, seen in animals and man [2]. Baja *et al.* [3] recently described the cardiac ultrastructural changes which follow exposure to daunomycin. Among them were morphological alterations similar to those seen in ischemia and chronic hypoxia. Another brief report has outlined the uncoupling of oxidative phosphorylation in rat liver mitochondria by copper ion plus daunomycin and indicated that this metal–drug combination reduced delayed toxicity in animals without decreasing therapeutic effectiveness [4]. After completion of this study, the investigation of Gosalvez *et al.* [5], which showed that adriamycin and daunomycin alone inhibit oxidative phosphorylation in rat liver mitochondria and decrease respiration in Ehrlich ascites tumor cells, became available. Within this context the current study was undertaken to examine the relevance of copper complexes of these anthracycline drugs to the lessening of cardiac toxicity and to explore aspects of their effects on oxidative phosphorylation in mitochondria from beef heart and tumor cells. In fact, the results focus primarily on the inhibition of these systems by adriamycin and daunomycin.

MATERIALS AND METHODS

Materials. Daunomycin was purchased from P & L Biochemicals, lot number 410381 or from Sigma, lot number 113C-0420. Adriamycin (10 mg A:50 mg lactose)* was a gift from Dr. Michael Stein (manufactured by Farmitalia, Milan, Italy, batch No. 43 and 63). Human outdated plasma was obtained from the Milwaukee County Blood Center and was centrifuged before use.

Stability of Cu daunomycin complex in plasma. The details of the method are described elsewhere [6].

Mitochondrial preparations. Bovine heart mitochondria were prepared by minor modification of the method of Smith [7]. The isolated mitochondria had the following average respiratory control ratios (state 3/state 4) using various substrates: glutamate, 3.0; pyruvate-malate, 3.0; and succinate, 2.1. The typical rate of oxygen consumption in state 3 was 40 nmoles O_2/min/mg for glutamate, and 55 nmoles O_2/min/mg for succinate. P/O ratios for these preparations averaged 2.3 for glutamate, 2.5 for pyruvate-malate, and 1.8 for succinate as substances.

Ehrlich ascites tumor mitochondria were isolated by the method of Thorne and Bygrave [8]. The average respiratory control ratios were 2.2 for pyruvate-malate or succinate. State 3 rates of oxygen consumption were 4 nmoles O_2/min/mg of protein for glutamate and pyruvate-malate and 7 nmoles O_2/min/mg for succinate. P/O ratios for pyruvate-malate or succinate ranged between 1.8 and 2.0.

Mitochondrial studies. Drugs were prepared as aqueous solutions (pH 7.0) and preincubated with mitochondria at 0°C for 30 min in a medium containing 0.25 M sucrose and 1 mM EDTA in the case of tumor mitochondria. Mitochondrial function was measured at ambient temperatures in a suspension containing 0.015 M KCl, 0.030 M KH_2PO_4, 0.045 M sucrose, 0.005 M $MgCl_2$, and 0.025 M Tris at pH 7.20. The rate of oxygen uptake upon addition of 0.09 M substrate is defined as state 4. A limiting amount of ADP was then added to examine the state 3 rate and the extent of phosphorylation.

The titration experiments reported here are the compilations of results from several mitochondrial preparations and illustrate the reproducibility of inhibition between batches of mitochondria in these studies. To normalize differences in the rate of oxygen consumption in various preparations, each point on the titration curves is the ratio of respiration rates in experimental and control samples times 100, which have been determined in tandem. Hence, ordinates in Figs. 4–6 represent per cent of control rates of

*Abbreviations: adriamycin, A; and daunomycin, D.

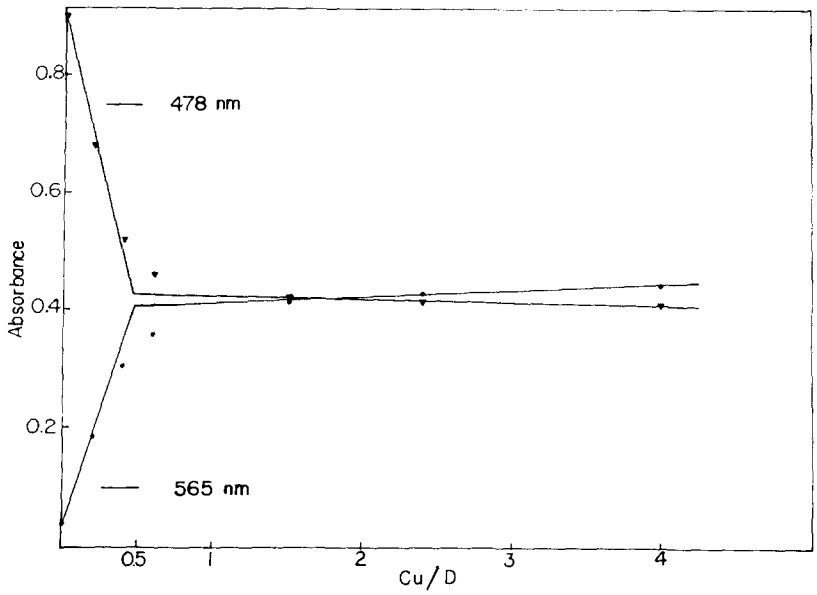

Fig. 1. Titration of 8.35×10^{-5} M daunomycin by Cu^{2+} at pH 7.0.

oxygen uptake for drug-treated mitochondria. Likewise, P/O ratios were compared in this fashion.

RESULTS

Stability of the Cu(II)-daunomycin complex. The spectrophotometric titration of daunomycin with $CuSO_4$ is shown in Fig. 1. Using wavelengths characteristic of ligand and metal complex, 478 and 568 nm, respectively, a 1:2 copper to daunomycin complex is observed to form. However, as illustrated in Fig. 2, further additions of copper ion cause further spectral perturbations which are not complete at Cu/D of 30:1.

Because the 1:2 complex is partially dissociated at the stoichiometric endpoint of the titration, indicating a thermodynamically weak metal ligand system, the stability of this chelate in human plasma was exam-

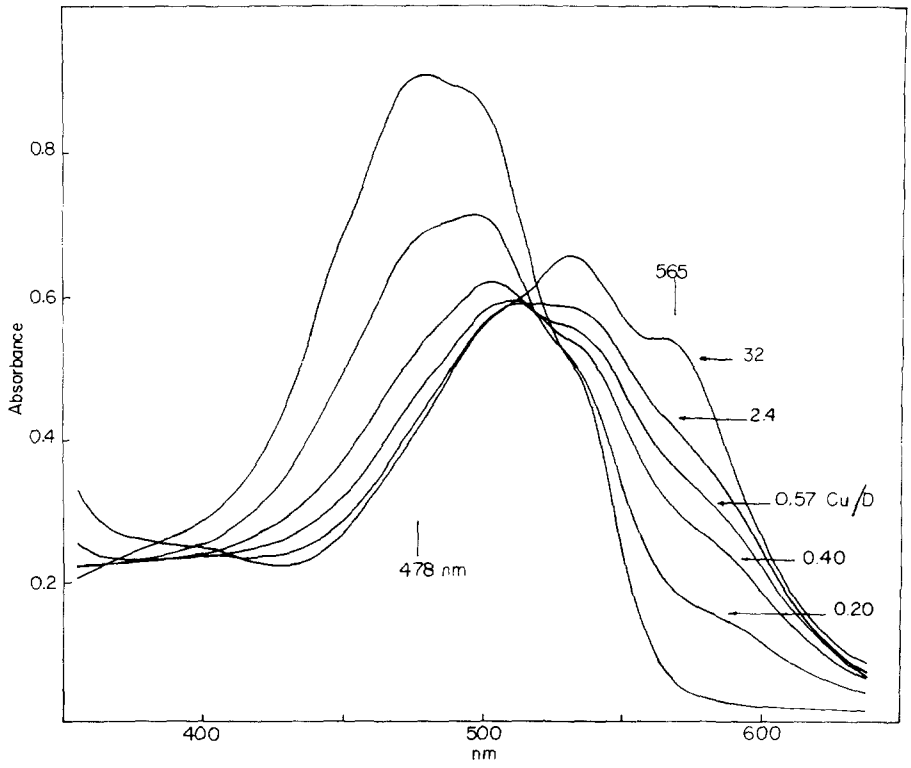

Fig. 2. Visible spectra of Cu^{2+}–D titration shown in Fig. 1.

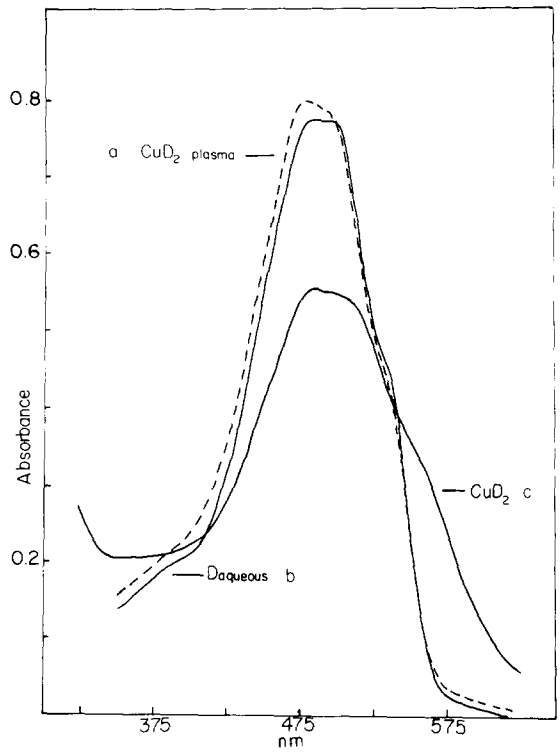

Fig. 3. Reaction of CuD_2 with plasma. (a) Spectrum of 3.7×10^{-5} M CuD_2 in human plasma ( ); (b) spectrum of 7.4×10^{-5} M D in saline at pH 7 ( ); and (c) spectrum of 3.7×10^{-5} M CuD_2 in saline at pH 7.

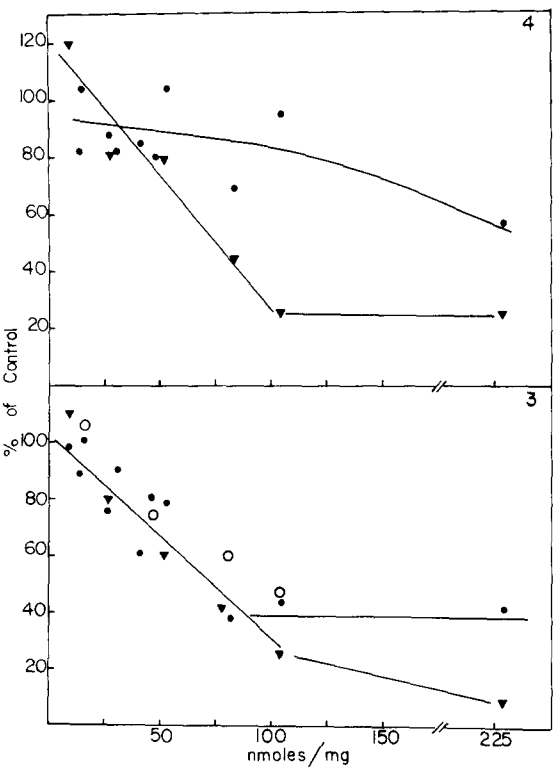

Fig. 4. Reaction of adriamycin with bovine heart mitochondria: state 4 and state 3 effects. Key: (▼) glutamate as substrate; (●) pyruvate-malate as substrate; and (○) P/O ratio for pyruvate-malate.

ined to see if it could exist as the metal complex in the presence of ubiquitous biological ligands such as amino acids and proteins. Figure 3 shows the results of placing a 1:2 mixture of Cu^{2+} and daunomycin in plasma. The spectrum of the ligand is observed immediately (a) in place of the distinctly different spectrum of the copper complex (c) found in the aqueous medium. For comparison, an equal concentration of daunomycin in saline provides a very similar visible spectrum (b). It is concluded, therefore, that the copper complex is not sufficiently stable to exist at all *in vivo*. Hence, the complicated nature of the titration results was not further explored.

Effects of adriamycin and daunomycin on oxidative phosphorylation in bovine heart mitochondria. Figures 4–6 summarize the results of studies of the influence of adriamycin and daunomycin on oxidative phosphorylation using several different preparations of bovine heart. The results are displayed as per cent of control vs nmoles drug/mg of protein so that data from different preparations can be normalized to the same scale. In this work, drugs were incubated with mitochondria for at least 30 min to assure completion of reaction.

Adriamycin inhibits state 3 respiration using glutamate, pyruvate-malate, and succinate as electron donors with 50 per cent inhibition occurring between 75 and 100 nmoles/mg of drug. The P/O ratios for pyruvate-malate and succinate decrease similarly over the same range of concentrations, indicating an uncoupling reaction is also involved. An interesting dif-

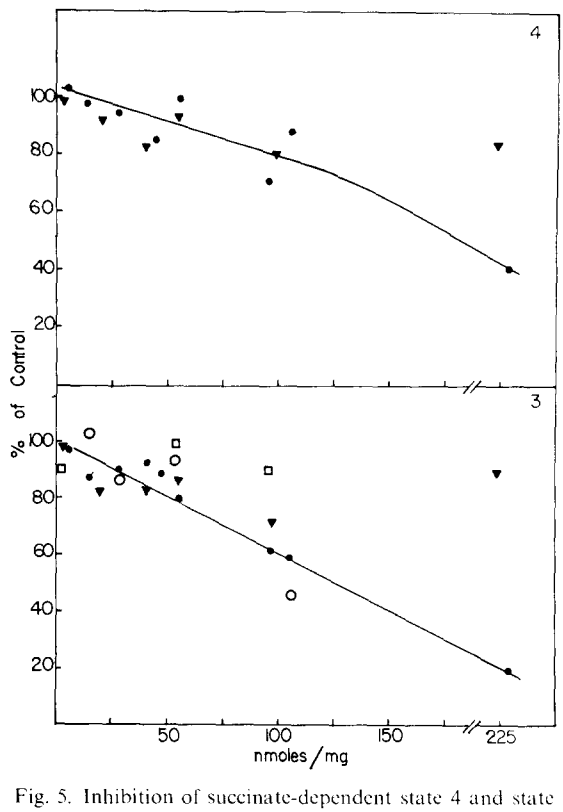

Fig. 5. Inhibition of succinate-dependent state 4 and state 3 respiration in bovine heart by adriamycin and daunomycin. Key: (●) adriamycin effects on respiration; (○) adriamycin effects on P/O ratio; (▼) daunomycin effects on respiration; and ( ) daunomycin effects on P/O ratio.

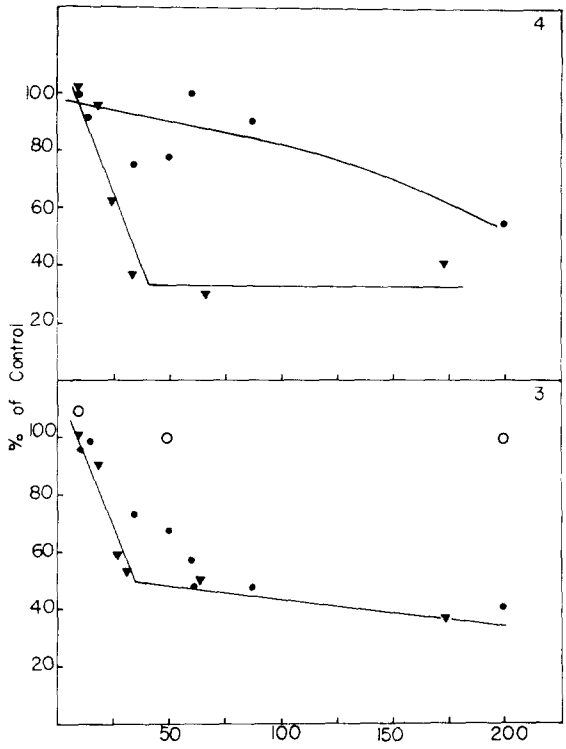

Fig. 6. Reaction of daunomycin with bovine heart mitochondria: state 4 and state 3 effects. Key: (▼) glutamate as substrate; (●) pyruvate-malate as substrate; and (○) P/O ratio for pyruvate-malate.

ference in state 4 is observed. Whereas pyruvate-malate and succinate show a gradual decrease in respiration over the range of drug to mitochondria used, glutamate-dependent oxygen consumption is much more sensitive to adriamycin, paralleling the inhibition seen in state 3.

Daunomycin behaves somewhat differently with bovine heart mitochondria. There seems to be little effect on state 4, state 3, or P/O ratio using succinate as substrate. However, a decrease in respiration occurs with either pyruvate-malate or glutamate as substrate. The 50 per cent inhibiting level of daunomycin is about 30 nmoles/mg and 60 nmoles/mg, respectively, for these electron donors. However, the P/O ratio for pyruvate-malate seems normal over the range of drug concentrations employed, in contrast to the results with adriamycin. Finally, with glutamate and pyruvate-malate, the features of state 4 inhibition of respiration are qualitatively similar to those for the case of adriamycin.

Effects of daunomycin on oxidative phosphorylation in Ehrlich ascites mitochondria and tumor cells. Over the range of 0–300 nmoles/mg of mitochondria, daunomycin has no consistent effect upon state 4 or state 3 rates of oxygen consumption. Per cent of control values for state 4 and state 3 averages 90 ± 30 and 90 ± 15 respectively. Likewise, at 76, 147 and 220 nmoles/mg, this compound shows no inhibition of respiration of isolated Ehrlich ascites tumor cells.

DISCUSSION

The fact that adriamycin and daunomycin can bind metals has been previously demonstrated, using a variety of metals such as $Al_2(SO_4)_3$, $MgCl_2$, and $CuSO_4$ [9]. In the present work, it is shown that a 1:2 copper daunomycin complex forms. However, from the form of the titration curve, it is evident that the formation constant for the complex is small. That the metal–ligand interaction is not strong enough for the complex to exist in a typical biological environment containing a variety of competing ligands for copper is shown by the results involving the reaction of the complex with human plasma. Complete dissociation of the complex occurs immediately.

Yesair *et al.* [4] have reported that a 1:1 copper daunomycin mixture suppresses reductive cleavage of the drug by liver homogenates and stimulates respiration in isolated liver mitochondria. According to titration data in Fig. 1, a 1:2 complex forms, implying that 50 per cent of the copper in Yesair's system may not have been complexed with drug. Hence, these effects may be due to Cu^{2+} alone or to CuD_2 in the absence of strong competing ligands as are found in plasma. However, the observations of effects of 1:1 Cu D of Cu A *in vivo* cannot be due to the presence of the chelate form of these drugs.

Turning to the influence of daunomycin and adriamycin on oxidative phosphorylation, titration-like behavior is observed for the inhibition of state 4 and state 3 respiration of bovine heart mitochondria. While there are general similarities between the two drugs, there is a definite difference in the sensitivity of the mitochondria to the two closely related compounds and a difference in the ability of the drugs to uncouple oxidative phosphorylation. However, of most interest was the total lack of effect of daunomycin against Ehrlich ascites cell mitochondria over concentrations ranging several times in excess of those used with the heart system. This was unexpected for, in other work with copper, zinc and cadmium bis(thiosemicarbazones), inhibition patterns have been similar in these two types of mitochondria.*,†,‡ To extend the dichotomy further, no inhibition of respiration of Ehrlich tumor cells *in vitro* could be obtained at levels of daunomycin as high as 200 nmoles/mg of cells. Hence, while antitumor effects against the Ehrlich cell seem unrelated to inhibition of oxidative phosphorylation, it may be that the sensitivity of heart tissue to daunomycin and adriamycin is connected with the inhibition of mitochondrial respiration observed in these studies.

In support of this possibility are studies of Baja *et al.* [3], who find that, in part, the subcellular derangement of cardiac cells intoxicated with anthracyclines is similar to damage produced by ischemia and chronic hypoxia. The lack of oxygen in a tissue results in an inability to generate ATP. No matter how much oxygen is supplied, inhibition or uncoupling of the energy transfer system in mitochondria also results in an inability to generate ATP.

* C. Chan-Stier, D. Minkel and D. H. Petering. Accepted in *Bioinorg. Chem.*

† D. Minkel, C. Chan-Stier and D. H. Petering, manuscript submitted for publication.

‡ D. Solaiman and D. H. Petering, unpublished observations.

Viewing the results of Gosalvez *et al.* [5] within this context, a generally consistent picture develops of a possible basis for the tissue selectivity of anthracycline drug-induced toxicity. They find that adriamycin and daunomycin inhibit glutamate-malate-dependent oxidative phosphorylation in rat liver at very large concentrations of drug to protein, on the order of 300–500 nmoles/mg for 50 per cent inhibition of state 3 and succinate-dependent phosphorylation at considerably larger concentration ratios. Likewise, tumor cells are inhibited only at very high levels of these compounds. However, in one experiment which compared rat heart and liver mitochondrial responses to adriamycin under conditions of equal cytochrome *a* concentration, the heart particles were distinctly more sensitive than the liver system. For instance at 100 μM drug, respiration in heart mitochondria was depressed 40 per cent, with only a corresponding 10 per cent suppression in liver.

Since adriamycin and daunomycin distribute themselves similarly among a variety of organs of the rat at early times after administration, the specific cardiac toxicity does not appear to be due to a preferential accumulation of the drug in the heart [10]. However, given the necessity of heart muscle to carry out oxidative phosphorylation on a continual basis more efficiently than any other tissue, it may be proposed that inhibition of this process contributes to the specific toxic effect of these drugs on heart muscle.

Acknowledgements—This research was supported by a grant, CA16156, from the National Institutes of Health. K. M. wishes to acknowledge several helpful discussions with Dr. Michael Stein, Medical College of Wisconsin.

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