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ADRIAMYCIN INACTIVATES CYTOCHROME C OXIDASE BY EXCLUSION OF THE ENZYME FROM ITS CARDIOLIPIN ESSENTIAL ENVIRONMENT

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Adriamycin and several derivatives were found to inhibit the last oxidation site of the respiratory chain (cytochrome c oxidase EC.1.9.3.1.) both on mitochondria and on purified reconstituted systems. A new mechanism of membrane enzyme inactivation is proposed to explain the experimental results : adriamycin does not interact directly with cytochrome c oxidase but inactivates it by changing the cardiolipin environment essential for its activity. In presence of adriamycin, cardiolipin is extracted from the lipid surrounding environment of cytochrome c oxidase and segregates in a separate phase inaccessible for the enzyme. We suggest that other cardiolipin dependent enzymes could be inactivated by adriamycin.

INTRODUCTION

Adriamycin and derivatives play a prominent role in the treatment of leukemias and solid tumors in man (1,2,3). Among this class of compounds, adriamycin is one of the most promising. However, adriamycin cardiotoxicity places a limit on the total dose that may be given (4). The development of the cardiac failure is characterized by a good correlation with the impairment of mitochondrial functions (5,6,7) without perturbation of the sliding of actin and myosin filaments across each other (7). Necco showed that the toxic manifestations of adriamycin on single cardiac cells can be suppressed by addition of ATP until complete consumption of the furnished ATP (8).

We bring here evidence that the inhibition of the last oxidation site of the respiratory chain (cytochrome c oxidase (EC 1.9.3.1.)) could participate to the adriamycin cardiotoxicity process. The mechanism of the cytochrome c oxidase inhibition by adriamycin and derivatives is shown to be due to the complexation of the enzyme lipid environment rather than to a drug-enzyme direct interaction. Most precisely, the drug-lipid complexation seems to induce the exclusion of cytochrome c oxidase from its essential cardiolipin environment.

MATERIALS AND METHODS

L-α-dimyristoylphosphatidylcholine (DMPC), DL-α-dipalmitoylphosphatidic acid, cardiolipin (bovine heart), were purchased from Sigma Chemical Co..

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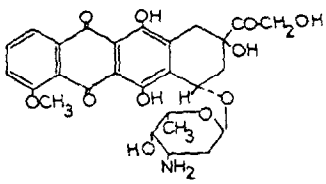
N-acetyl-adriamycin was a gift of Prof. A. TROUET and Dr R. BAURIN (Laboratoire de Chimie Physiologique, Université de Louvain). Adriamycin and other derivatives (Fig.1) were generously supplied by Dr J. HILDEBRAND (Institut J. Bordet) and by Dr C. DESLOVER (Farmitalia). After beef heart mitochondria preparation (9), extraction and purification of cytochrome c oxidase were realized as described elsewhere (10,11,12). Further depletion of phospholipids has been carried out using the procedure of M. FRY (13). Purified and phospholipid depleted cytochrome c oxidase was stored at -20°C for several weeks without loss of activity. All chemicals were of analytical grade and water was triple-distilled. Buffered solutions (Tris-HCl 10^{-2}M, pH 7.4) were used. Cytochrome c oxidase activity was measured by the rate of oxidation of reduced cytochrome c. Cytochrome c was reduced by dithionite. Excess of dithionite was eliminated by gel filtration (Sephadex G25). Multilamellar liposomes were prepared as described (14). Care was taken to work in N_2 atmosphere. Differential scanning calorimetry spectra were recorded on a Setaram 111 using 100 µl inox cells.

RESULTS AND DISCUSSION

Cytochrome c oxidase activity was measured on beef heart mitochondria by the rate of oxidation of reduced cytochrome c. Activity change was evaluated in mitochondria for several adriamycin derivatives (Fig.2). We recently demonstrated that subtile structural modifications of the adriamycin molecule were sufficient to modulate its affinity for cardiolipin (15). Figure 3 indicates a striking linear relationship between the inhibiting capacity of these derivatives on the cytochrome c oxidase activity and their affinity for cardiolipin. This result suggests strongly that adriamycin inactivates cytochrome c oxidase by complexation of its cardiolipin specific environment. To confirm this hypothesis, cardiolipin depleted cytochrome c oxidase was prepared (13) and further reactivated by addition of cardiolipin or phosphatidic acid. Addition of other purified lipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol) did not activate significantly cytochrome c oxidase (data not shown). Affinity of adriamycin is about 80 times higher for cardiolipin than for phosphatidic acid (16). In complete agreement with our model, the adriamycin concentration required to inhibit 50% of the cytochrome c oxidase activity was 80 times higher in the phosphatidic acid reconstituted system than in the cardiolipin reconstituted system (Fig.3). This last experiment demonstrates definitively that only the drug-lipid interaction is responsible of the enzyme inactivation. It is of interest to remember that adriamycin, cinerubin and rubidazone, the most effective inhibitors, interact specifically with the polar head of cardiolipin (15) while nogalamycin and rhodomycin which penetrate deeper and without specifi-



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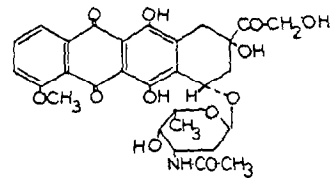

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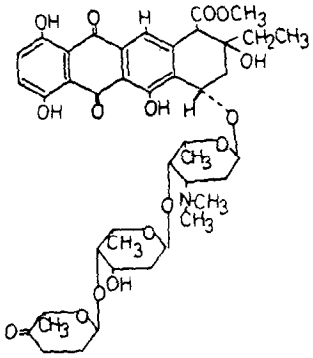

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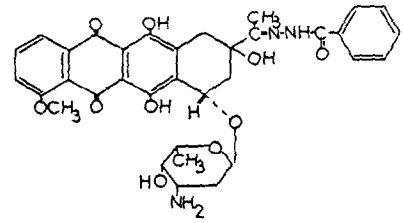

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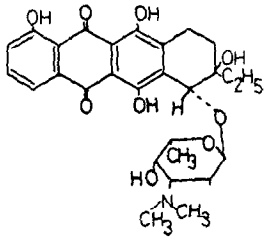
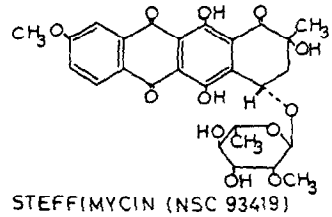
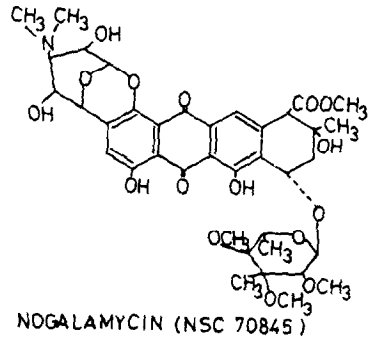


RHODOMYCIN (NSC 102813)



STEFFIMYCIN (NSC 93419)



NOGALAMYCIN (NSC 70845)

Figure 1 : Structure of adriamycin and derivatives.

city in model membranes are less effective. Moreover, steffimycin which is uncharged and penetrates without specificity in model membranes does not perturbate the cytochrome c oxidase activity. If we compare the inhibition drugs sequence with their octanol-water partition coefficient (17), no correlation would appear. This kind of behaviour is thus quite different from the

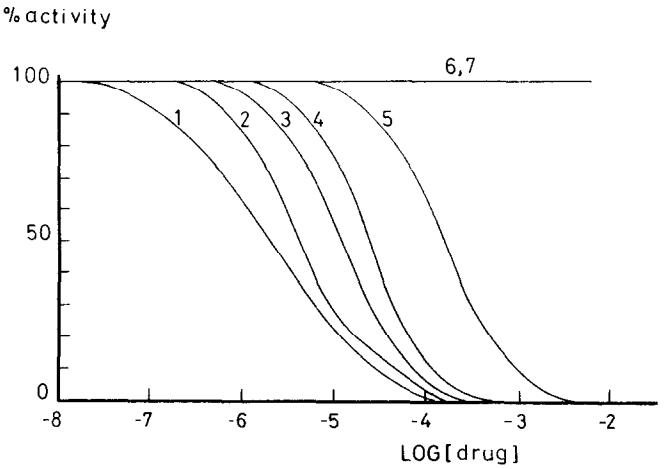


Figure 2 :

Inhibition of cytochrome oxidase activity. Freshly extracted beef heart mitochondria were incubated for 20 minutes in presence of : 1)adriamycin, 2)cinerubin, 3)rubidazone, 4)nogalamycin, 5)rhodomycin, 6)N-acetyl-adriamycin, 7)steffimycin.
The reaction medium contained 0.028 mg of mitochondrial proteins dissolved in 1.9 ml of buffer Tris-HCl 10 mM, pH 7.4, Triton X100 1% (v/v). The reaction was initiated by addition of 100 µl of reduced cytochrome c (final concentration 31 µM). Ferricyanide was used to stop the reaction and to oxidize completely cytochrome c. Initial rates of oxidation of cytochrome c were measured spectrophotometrically at 550 nm.

mechanism of action of adriamycin and related compounds on NADH dehydrogenase and succinoxidase developed by FOLKERS (18,19,20).

In order to elucidate how the complexation of cytochrome c oxidase boundary lipids could induce the enzymic inhibition, two mechanisms can be envisaged.

1. The tightly bound cardiolipin molecules are complexed and their new properties make them ineffective in activating cytochrome c oxidase.
2. The drug-cardiolipin complex segregates in a separate lipid phase. The lipid environment of cytochrome c oxidase is then constituted by other phospholipids such as phosphatidylcholines which don't allow the enzyme activation.

Differential scanning calorimetry (DSC) measurements are strongly favourable to the second hypothesis. Indeed, multilamellar liposomes made of cardiolipin and dimyristoylphosphatidylcholine (DMPC) were formed and analyzed by DSC. The transition usually observed at 23.4°C with pure DMPC liposomes is abolished as a consequence of a modification of the DMPC-DMPC interactions induced by cardiolipin (Fig.4). However, subsequent addition of adriamycin restores the transition peak characterizing a pure DMPC phase (Fig.4). This result demonstrates that the adriamycin-cardiolipin complex segregates in the lipid matrix (Fig.5). This is thermodynamically likely since complex formation decreases electrostatic repulsion between cardiolipin molecules. Moreover, interactions

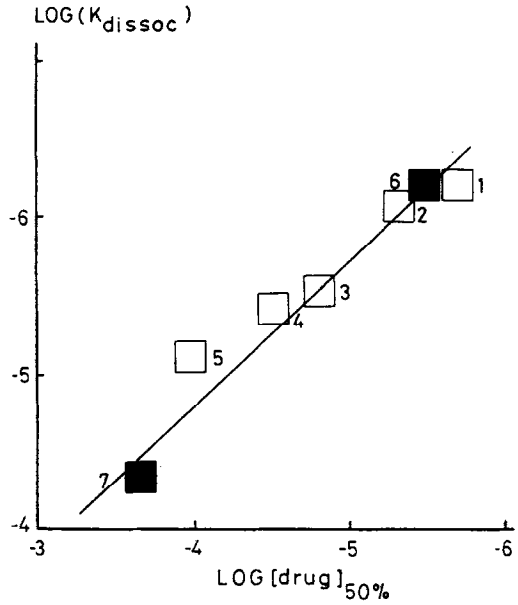


Figure 3 :

□ Relation between the drug concentration inhibiting 50% of cytochrome c oxidase activity on mitochondria and the dissociation constant of the cardiolipin-drug complex. 1- adriamycin, 2- cinerubin, 3- rubidazone, 4- nogalamycin, 5-rhodomycin.

■ Relation between the drug concentration inhibiting 50% of cytochrome c oxidase activity in the cardiolipin reconstituted system 6- and in the phosphatidic acid reconstituted system 7- . For the reconstitution, cardiolipin or phosphatidic acid was dissolved in a buffer Tris-HCl 10 mM, pH 7.4, Triton X100 5% (v/v) containing the purified (12) and phospholipid depleted (13) beef heart cytochrome c oxidase. Triton X100 was dialyzed for 48 h against a buffer Tris-HCl 10 mM, pH 7.4. Phosphatidic acid was found to be as effective as cardiolipin in reconstitution, both restoring about 95% of the specific activity of cytochrome c oxidase.

between neighbouring adriamycin residues conduct to the formation of a cardpack-stacked complex (16). These interactions will stabilize the complex and favour the surface aggregation. The good correlation observed between the impairment of mitochondrial functions induced by adriamycin and derivatives and their affinity for cardiolipin (15) suggests the hypothesis that this segregation of a mitochondrial lipid must play a prominent role in the adriamycin cardiotoxicity.

Finally, a classical way to explain membrane enzyme inactivation is to suppose a direct drug-enzyme interaction. The data presented here demonstrate another mechanism of inactivation which requires a highly specific drug-lipid interaction. Adriamycin was shown to satisfy this condition. From a general point of view, drugs with a high affinity for well defined lipids would offer an elegant way to inactivate selectively membrane enzymes. Moreover, it can be suggested that membrane enzymes with different conformational and biological properties but which need the same lipid environment could be

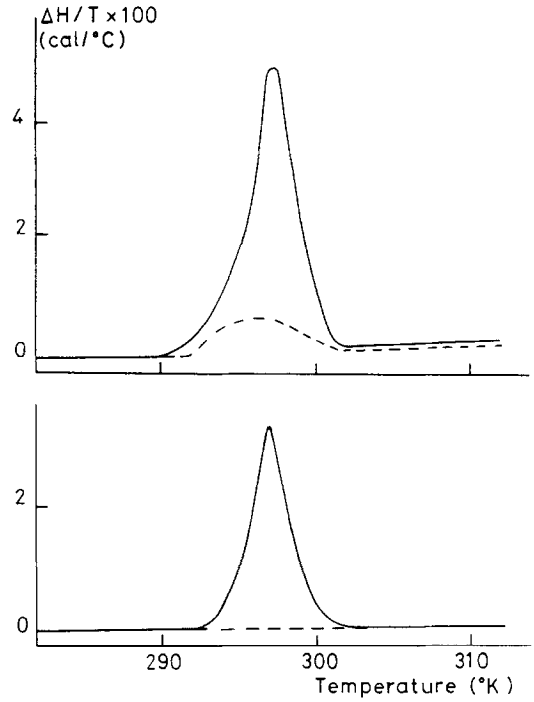


Figure 4. :

Differential scanning calorimetry heating curves of DMPC-cardiolipin multilamellar liposomes in absence (dotted line) and in presence of adriamycin (full line). The mixture contained 10% (w/w) of cardiolipin (upper part of the figure) and 30% (w/w) of cardiolipin (lower part of the figure). For each spectra, 100 µl (20 mM final lipid or adriamycin concentration) solution were used. The reference is constituted by 100 µl of the buffer solution (Tris-HCl 10 mM, pH 7.4). The heating rate is 2°C/min. Before the measurements, the samples were left to stand at room temperature (20°C) for 6 hours. Slightly different results are obtained if this delay is not respected.

inactivated by the same probe. This possibility is under investigation in our laboratory for NADH dehydrogenase and cytochrome c reductase for which a cardiolipin requirement has been demonstrated (21).

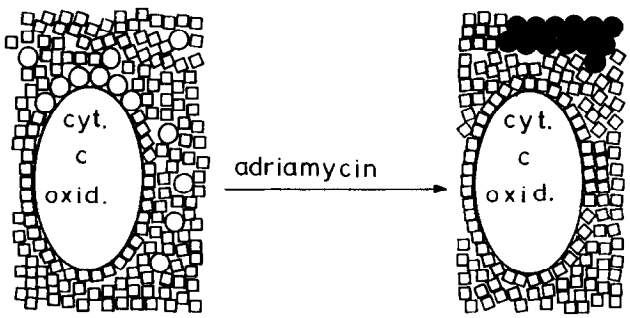


Figure 5 :

Schematic representation of the enzyme inactivation mechanism. Cytochrome c oxidase in its lipid environment. □ Neutral lipid, ○ cardiolipin, ● cardiolipin-adriamycin complex.

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