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INHIBITION OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION BY ADRIAMYCIN

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The antitumour antibiotic, adriamycin, inhibited oxidative phosphorylation in freshly prepared mitochondria from the heart, liver and kidney of the rat. It abolished respiratory control and stimulated ATPase activity. Sccinate oxidation by heart mitochondria was extremely sensitive to the drug when hexokinase was present in the reaction medium. The sensitive site has been identified to lie in the region between the succinate dehydrogenase flavoprotein and ubiquinone of the respiratory chain.

Introduction

The anthracycline antibiotic, adriamycin, ranks among the most potent anticancer drugs known at present. However, the use of this drug in the chemotherapy of cancer is severely restricted by its extreme toxicity. Among the more pronounced toxic side effects, mention could be made of severe, cumulative, dose-dependent cardiomyopathy leading to congestive heart failure, nephrotoxicity and liver necrosis [1,2]. Functional impairment of the organs has been found to coincide generally with ultrastructural and biochemical lesions at the cellular and subcellular level. One of the striking effects seen in cardiac muscles of patients who succumbed to myocardial infarction following adriamycin therapy was the swelling of mitochondria and the lysis of mitochondrial membranes and cristae [3,4]. Experimental animals also showed mitochondrial damage on treatment with adriamycin or structurally related compounds [5,6]. Structural and functional damage to mitochondria is therefore believed to be a direct effect of the drug and considered responsible for myocyte death [1,7–9].

The relation between mitochondrial injury and cardiomyopathy was further emphasized by the observations that adriamycin inhibited oxidative activity of mitochondria [10–12] and complexed with cardiolipin [13], and that the toxic effects of the drug on isolated cardiac cells could be suppressed by the addition of ATP [14]. It has also been claimed that ubiquinone displayed antidote effects against adriamycin-induced cardiotoxicity [1,15,16].

In the studies quoted above, attention was confined to the heart tissue. Since the kidney and liver are also susceptible to adriamycin toxicity, a comparative study of the toxic effects of the drug on heart, liver and kidney mitochondria was made. The results on the inhibition of oxidative phosphorylation in vitro presented in this paper reveal a hexokinase-dependent sensitization of heart mitochondria to adriamycin toxicity.

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Abbreviations: $\phi_{1/2}$, concentration of adriamycin required for 50% inhibition of activity; TMPD *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

Materials and Methods

Preparation of mitochondria. Male albino Wistar rats (140–150 g) maintained on commerical Hind Lever diet were used for the preparation of mitochondria. Liver and kidney mitochondria were prepared according to the procedure of Johnson and Lardy [17] with minor modifications [18]. Heart mitochondria were also prepared by the same precedure without the use of bacterial protease. The mitochondrial pellet, in all cases, was washed once, suspended in 0.25 M sucrose and used with minimum delay.

Assay of oxidative phosphorylation. Manometric determinations of oxidative phosphorylation were made in a Gilson differential respirometer as described earlier [19]. Phosphorylation coupled specifically to the first and second sites was measured using ferricyanide as electron acceptor [20]. Polarographic measurements of oxygen uptake were made using a K-ICT-C oxygraph fitted with a Clark oxygen electrode. The reaction system and procedure were essentially the same as described earlier [21].

Assay of enzymes. The activity of ATPase (ATP phosphohydrolase, EC 3.6.1.3) was determined as described earlier [19]. The content of mitochondrial protein (250 μg) and duration of incubation (maximum 2 min) were adjusted to ensure linearity. Succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) activity was determined manometrically using phenazine methosulphate as electron acceptor [22]. Succinate-ubiquinone reductase activity was estimated by measuring the reduction of externally added ubiquinone using 2,2-dipyridyl and ferric chloride [23]. The rate of oxidation of chemically reduced ubiquinone [24] was also measured by the same technique. In these experiments, ubiquinone-9 was used, being the major lipid quinone in rat mitochondria [25]. The reduction of cytochrome *b* was followed in an Aminco DW 2a dual-wavelength recording spectrophotometer [26].

Protein was measured by the biuret method [27], deoxycholate being used for solubilization. All solutions were prepared in water, double-distilled in an all quartz apparatus. Solutions were adjusted to pH 7.4 before use. Sucrose solutions were prepared fresh daily.

Results

Oxygen uptake and the P / O ratio

Substrate oxidation by heart, kidney and liver mitochondria of the rat was inhibited when adriamycin was added to the reaction system. Both oxidation and phosphorylation were progressively inhibited with increasing concentrations of the antibiotic until about 75% inhibition was achieved. From regression equations correlating activity (rate of oxygen uptake or of phosphorylation) with the concentration of the drug in the reaction system, the concentrations required for 50% inhibition ($\phi_{1/2}$) was calculated. The values of $\phi_{1/2}$ for oxidation and phosphorylation are presented in Table I. Since the experimental conditions for the determination of oxidative phosphorylation (manometric, using about 2 mg of mitochondrial protein) were the same, the values of $\phi_{1/2}$ reveal the relative potency of adriamycin as an inhibitor of the function of three types of mitochondria.

The data presented in Table I highlight the striking sensitivity of succinate oxidation in heart mitochondria to inhibition by the antibiotic. In kidney mitochondria also succinate oxidation was more sensitive to the drug than was the oxidation of NAD^+-linked substrates. Electron transport and coupled phosphorylation in the cytochrome oxidase region of the respiratory chain was least sensitive to the drug, the $\phi_{1/2}$ for the oxidation of ascorbate + TMPD being almost double that for the oxidation of NAD^+-linked substrates.

The results in Table I hinted at the possibility of coupled phosphorylation being more sensitive to the drug than was electron-transfer activity. This was particularly true of kidney mitochondria oxidizing pyruvate + malate, the $\phi_{1/2}$ for inhibition of phosphorylation being significantly lower ($P < 0.01$) than that for the inhibition of oxidation. In the case of both kidney and liver mitochondria, concentrations of adriamycin near the $\phi_{1/2}$ caused a significant lowering of the P/O ratio (30 to 60%).

It may be pertinent to mention here that at low concentrations of the antibiotic (below 0.2 mM), malate and succinate oxidation by rat liver mitochondria showed a small (15–20%) but consistent stimulation in the rate of oxidation and phosphorylation. The reason for this was not explored.

TABLE I

POTENCY OF ADRIAMYCIN IN THE INHIBITION OF OXIDATIVE PHOSPHORYLATION BY RAT HEART, LIVER AND KIDNEY MITOCHONDRIA

Oxidative phosphorylation was measured manometrically [19]. Regression lines for the inhibitor effect were fitted by the least-squares method. The correlation coefficients (r) ranged from 0.81 to 0.99, the lower values of r being for the inhibition of ascorbate + TMPD oxidation. The values of $\phi_{1/2}$ (adriamycin concentration for 50% inhibition) were calculated from regression equations. The mean ± S.D. of three to six independent determinations (mitochondrial preparations) are given.

Substrate	Reaction	$\phi_{1/2}$ (μM)		
		Heart	Liver	Kidney
Glutamate (pyruvate) + malate	Oxidation	593 ± 40	778 ± 42	895 ± 161
	Phosphorylation	530 ± 26	741 ± 108	490 ± 89 [b]
Succinate	Oxidation	5.5 ± 0.2	805 ± 124	590 ± 117 [a]
	Phosphorylation	5.2 ± 0.1	715 ± 93	490 ± 50
Ascorbate + TMPD	Oxidation	1633 ± 252 [b]	1170 ± 60 [b]	1193 ± 233 [b]
	Phosphorylation	1350 ± 200 [b]	800 ± 207	1160 ± 274 [b]

[a] $P < 0.05$.
[b] $P < 0.01$.

Respiratory control

It was felt that the values of $\phi_{1/2}$ obtained using the manometric method for the determination of oxidative phosphorylation may not give a true index of the inhibitor potency of adriamycin because of the possibility of non-specific binding of the antibiotic to yeast hexokinase which was present in fair amounts in the reaction system. Moreover, the large excess of hexokinase would tend to minimize the uncoupling action of the drug by making ATP unavailable for hydrolysis [28]. To eliminate these possibilities, oxidative phosphorylation was measured by the polarographic method.

Addition of adriamycin to freshly prepared liver or kidney mitochondria resulted in a partial stimulation (50%) of 'State 4' respiration (ADP exhausted; see Ref. 29), decrease in respiratory control and inhibition of State 3 oxidation. Some typical results are shown in Fig. 1. The increase in State 4 respiration is consistent with the decrease in the P/O ratio observed in the manometric assay. It may, however, be noted that the decrease in respiratory control index was caused not by a rapid increase in State 4 respiration but primarily by the inhibition of State 3 respiration. Consequently, the disappearance of the State 3-State 4 transition did not occur even at concentrations of the drug as high as 400 μM.

In the initial stages, respiratory control decreased linearly with increase in the concentration of antibiotic. From regression equations (r = 0.97–0.99), $\phi_{1/2}$ for decrease in the respiratory control index was calculated as 151 ± 45 μM for glutamate + malate and 76 ± 10 μM for succinate oxidation by liver mitochondria. For kidney mitochondria oxidising pyruvate + malate the value was 127 ± 48 μM.

Under the experimental conditions employed, the antibiotic inhibited active oxidation (State 3; see Ref. 29) of substrates in a sigmoidal fashion. The $\phi_{1/2}$ values calculated from Hill plots are presented in Table II. In the case of kidney and liver mitochondria, the $\phi_{1/2}$ values generally agree with those obtained for the manometric assay (Table I). Even though the greater sensitivity of succinate oxidation in heart mitochondria to inhibition by the antibiotic is reflected in the results presented in Table II, $\phi_{1/2}$ was 20-times higher than that obtained in the manometric assay. Preincubation (up to 10 min) of mitochondria and adriamycin exerted no influence on the $\phi_{1/2}$ values. A study of the effects of different components of the reaction system of the manometric assay on the pattern of inhibition of State 3 oxidation by adriamycin in the polarographic assay revealed that the presence of hexokinase in the reaction

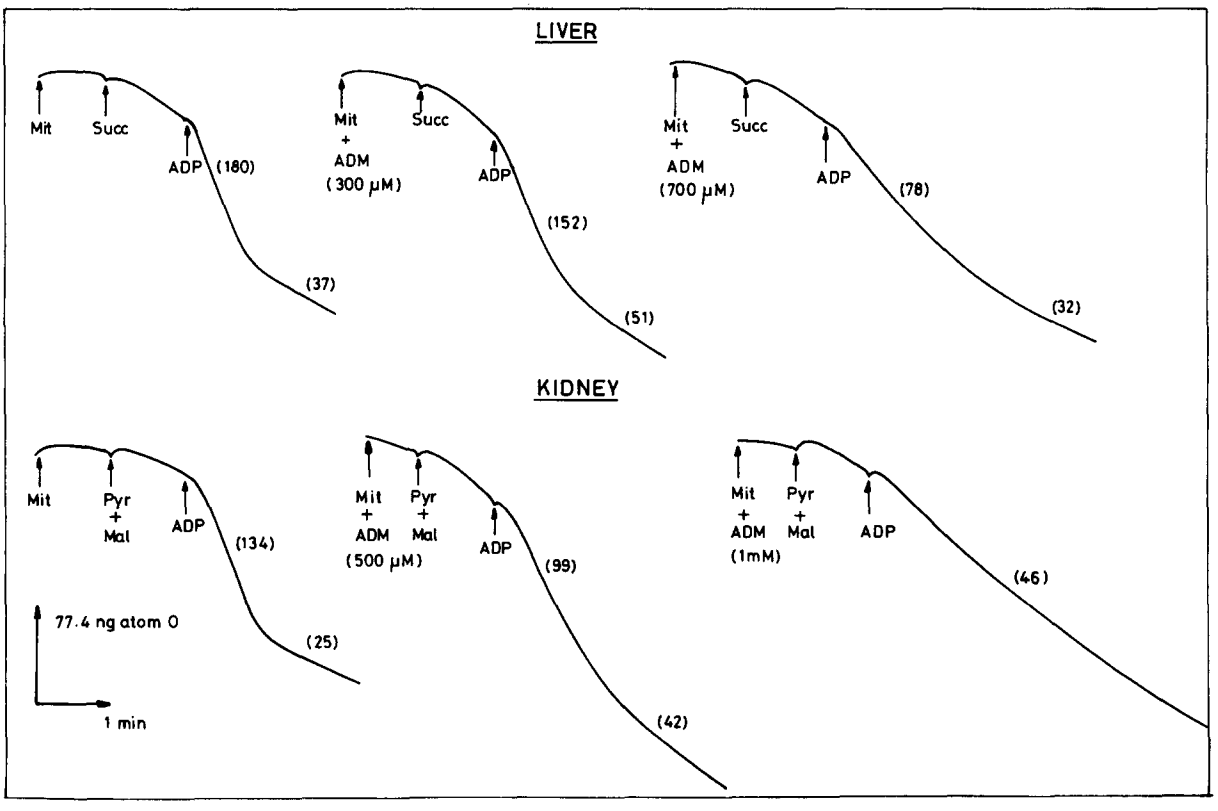

Fig. 1. Effect of adriamycin on respiratory control in liver and kidney mitochondria. Freshly isolated rat liver mitochondria (0.84 mg) and kidney mitochondria (1.1 mg protein) were used in a reaction volume of 1.4 ml for the determination of respiratory control by the polarographic method as given in Materials and Methods. The values in parentheses refer to the rates of oxygen uptake (ngatom O/min per mg protein) on stirring in 176 nmol ADP (State 3) and on exhaustion of the added ADP (State 4; see Ref. 29). The concentration of adriamycin (ADM) is indicated. Mal, malate; Mit, mitochondria; Pyr, pyruvate; Succ, succinate.

TABLE II

POTENCY OF ADRIAMYCIN IN THE INHIBITION OF STATE 3 OXIDATION OF SUBSTRATES BY RAT HEART, LIVER AND KIDNEY MITOCHONDRIA

State 3 oxidation [29] was measured polarographically with freshly prepared mitochondria. In all cases about 1 mg of mitochondrial protein was used in the reaction system. The values of $\phi_{1/2}$ calculated from Hill plots are the mean ± S.D. of four independent determinations (samples of mitochondria).

Substrate	$\phi_{1/2}$ (μM)		
	Heart	Liver	Kidney
Glutamate (pyruvate)+malate	766 ± 55	549 ± 21	724 ± 37
Succinate	121 ± 26 [a]	532 ± 66	541 ± 111

[a] $P < 0.01$.

system enhanced the potency of the drug for the inhibition of succinate oxidation in heart mitochondria, the $\phi_{1/2}$ decreasing from 121 ± 26 to 27 ± 8 μM.

It may be mentioned that the degree of inhibition achieved at any given concentration of adriamycin depended on the amount of mitochondrial protein in the reaction system. Thus, when the protein concentration was doubled, the inhibition was decreased by half (data not shown).

Ferricyanide reduction

The results in Table I indicated that the terminal part of the electron-transport chain was relatively less sensitive to the inhibitory action of the drug. In order to narrow down the locus of interaction of adriamycin with the respiratory chain, the effect of the antibiotic on substrate-dependent

ferricyanide reduction was tested. Under phosphorylating conditions, ferricyanide accepts electrons from the respiratory assembly by interacting with a component on the oxygen side of the second coupling site. Contrary to the earlier belief, cytochrome *c* does not appear to be the interacting component [21].

The values of $\phi_{1/2}$ given in Table III compare favourably with those given in Tables II and III for liver mitochondria. In the case of heart mitochondria, NADH-linked reduction of ferricyanide appeared more sensitive to hinhibtion by the antibiotic than was oxygen uptake. When succinate was used as the electron donor, phosphorylation showed the characteristic high sensitivity to adriamycin ($\phi_{1/2} = 7$ μM). However, the $\phi_{1/2}$ for ferricyanide reduction was about 10-times as high. This discrepancy was traced to the large amount of antimycin-insensitive ferricyanide reduction appearing in the presence of the inhibitor. Thus, in the presence of 50 μM adriamycin, more than 50% of the electron-transport activity was antimycin insensitive and therefore non-phosphorylating.

Reduction of cytochrome b

The 'hexokinase effect' is seen clearly in the inhibition of the rate of reduction of cytochrome *b*. Inhibition of the transfer of electrons to cytochrome *b* prolonged by more than 50% the onset of anerobiosis and the rate of reduction of cytochrome *b* (Fig. 2). At the concentrations employed, the antibiotic alone did not cause any perceptible decrease in the rate of reduction of cytochrome *b*.

Reduction of exogenous ubiquinone

Our attempts to measure the extent of reduction of endogenous ubiquinone in heart mitochondria in the presence of the antibiotic, both by the extraction procedure and by the spectrophotometric method [30], were not successful. Therefore, we measured the influence of the drug

TABLE III

POTENCY OF ADRIAMYCIN IN THE INHIBITION OF FERRICYANIDE REDUCTION BY RAT HEART AND LIVER MITOCHONDRIA

The reaction system was essentially the same as that used for the manometric assay except for the presence of cyanide and ferricyanide. Regression lines for the inhibitor effect were fitted by the least-squares method ($r = 0.95$–1.00). The values of $\phi_{1/2}$ are the mean ± S.D. of three to five independent determinations (mitochondrial samples). The initial rates for ferricyanide reduction for pyruvate + malate by heart and liver mitochondria were 712 ± 54 and 250 ± 13, respectively, and for phosphorylatoin 545 ± 63 and 183 ± 11. When succinate was the electron donor the rates of reduction of ferricyanide by heart and liver mitochondria were 919 ± 110 and 583 ± 75 and for phosphorylation the respective rates were 254 ± 19 and 1949 ± 23 nmol/min per mg protein.

Substrate	Reaction	$\phi_{1/2}$ (μM)	
		Heart	Liver
Glutamate (pyruvate) +	Ferricyanide reduction	131 ±21	743 ± 70
	Phosphorylation	83 ± 9 [b]	627 ± 42
Succinate	Ferricyanide reduction	57 ± 7	705 ± 90
	Phosphorylation	6.5 ± 1.6 [b]	550 ± 20 [a]

[a] $P < 0.05$.
[b] $P < 0.01$.

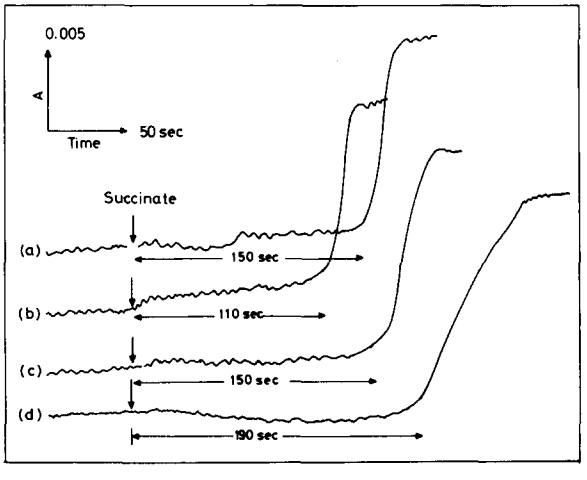

Fig. 2. Effect of hexokinase on the inhibition of cytochrome *b* reduction in rat heart mitochondria by adriamycin. The reaction system (1 ml) was the same as that used for the manometric determination of oxidative phosphorylation except that yeast hexokinase was added as indicated. Mitochondria (2 mg) were incubated in the reaction medium for 1 min either alone (a) or with the addition of 1 mg hexokinase (b), 100 μM adriamycin (c), or hexokinase + adriamycin (d), before addition of succinate. The rate of reduction of cytochrome *b* was followed by the increase in absorbance at 562–577 nm. The addition of succinate and the time taken for oxygen depletion are indicated.

TABLE IV

EFFECT OF HEXOKINASE ON ADRIAMYCIN-MEDIATED INHIBITION OF UBIQUINONE REDUCTION AND REOXIDATION IN RAT HEART MITOCHONDRIA

The reaction system (1 ml) was essentially the same as used for the manometric assay except that hexokinase (1 mg) was added as indicated. Succinate-ubiquinone reductase was assayed in the presence of cyanide (3 mM) and ubiquinone-9 (1 mM). The oxidation of chemically prepared ubiquinol (1 mM) was assayed in the presence of rotenone. Mitochondria (1–1.5 mg) were incubated with hexokinase and adriamycin as indicated for 10 min before the start of the reaction and continued after addition of substrate for 5 min (reduction) or 10 min (oxidation). After stopping the reaction with perchloric acid the amount of reduced quinone was estimated colourimetrically after extraction into cyclohexane [23,24], hydroquinone being used as standard. The values (expressed as nmol/min per mg protein) are the mean ±S.D. of four independent determinations.

Addition	Ubiquinone reduction	Ubiquinol oxidation
None	16.4±2.3	22.7±2.3
Adriamycin (50 μM)	14.4±2.9	25.9±2.6
Hexokinase (1 mg)	12.9±2.8	20.5±2.1
Hexokinase + adriamycin	4.0±0.9	23.2±2.3

on the rate of reduction of exogenously added ubiquinone. It is known that in heart mitochondria exogenous ubiquinone accepts electrons from the endogenous quinone [24]. The results in Table IV clearly show that the succinate cytochrome *c* reductase segment of the respiratory chain is the sensitive region for inhibition by adriamycin. It may also be noted that the electron-transport chain-mediated oxidation of exogenous ubiquinol was not inhibited by adriamycin.

Succinate dehydrogenase activity of freshly isolated rat heart and liver mitochondria was not inhibited by adriamycin (1 mM) even when measured in the presence of hexokinase under phosphorylating conditions (data not shown).

Reverse electron transport

Adriamycin inhibited ATP-dependent, succinate-mediated reduction of NAD^+ by reverse electron transport in submitochondrial particles, the values of $\phi_{1/2}$ being 196 μM for the heart preparation and 210 μM for the liver preparation. These values compared favourably with the $\phi_{1/2}$ values for NADH oxidation in submitochondrial particles [31], but not for succinate oxidation (600–700 μM).

ATPase

The 50% stimulation of State 4 oxidation produced by adriamycin when added to tightly coupled mitochondria (Fig. 1) indicated that the drug could act as a partial uncoupler of oxidative phosphorylation. The effect of the compound on mitochondrial ATPase activity was therefore tested. The results presented in Fig. 4 show that adriamycin stimulated the ATPase activity of tightly coupled rat liver mitochondria. The concentration required (3 mM) is 3–4-times that required for the abolition of respiratory control.

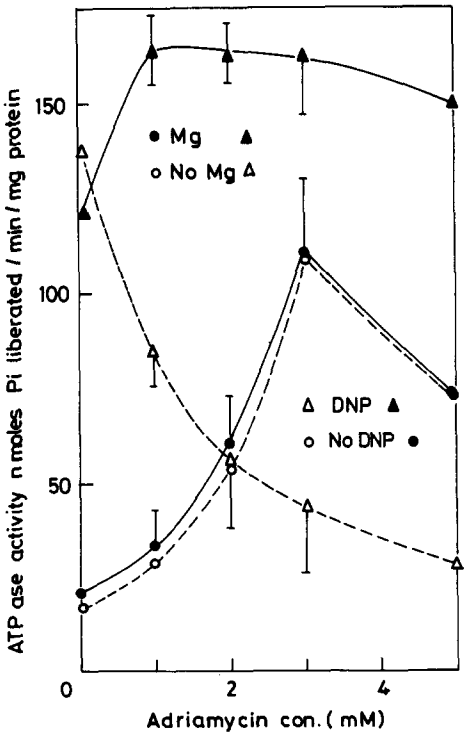

Fig. 3. Effect of adriamycin on the ATPase activity rat liver mitochondria. The reaction system contained 0.1–0.2 mg (protein) of freshly prepared rat liver mitochondria without (●, ○) or with (▲, △) 500 μM dinitrophenol and with (●, ▲) or without (○, △) 5 mM $Mgcl_2$. The values are the mean ± S.D. of four independent determinations. Some typical values of the S.D. are shown.

Classical uncoupling agents like dinitrophenol also show this differential potency towards stimulation of ATPase and respiratory uncoupling [32].

Adriamycin did not inhibit dinitrophenol-stimulated ATPase activity. In fact, it appeared to stimulate the enzyme activity even in the presence of dinitrophephenol. This suggested that the antibiotic might exert a disruptive action on the membrane. To test this, ATPase activity was assyed in the absence of Mg^{2+}. It may be noted that even in the absence of added magnesium, the drug stimulated ATPase activity like a true uncoupling agent. Detergents which disrupt membrane structure do not stimulate the ATPase activity of intact mitochondria in the absence of added Mg^{2+} [33,34]. Moreover, detergents drastically inhibit uncoupler-stimulated ATPase activity if Mg^{2+} is not added to the reaction system. The inhibition caused by adriamycin was not as severe as that observed with detergents like deoxycholate or fatty acids [34].

Discussion

Relevance for the study of the effects of anthracycline antibiotics on mitochondrial oxidative phosphorylation in vitro stems primarily from the observation [3] that on exposure to these drugs, cardiac ultrastructure shows morphological alterations similar to those seen in ischemia and chronic hypoxia. A complex of the antibiotic with copper was reported to show uncoupler action. The hypothesis [35] was advanced that the rotenone-sensitive first coupling site was the specific target for the action of anticancer drugs. In agreement with the hypothesis, Gosalvez et al. [10] observed that the oxidation of NAD^+-linked substrates by heart and liver mitochondria ($\phi_{1/2} \approx 200$ μM) was more sensitive to inhibition by adriamycin than was succinate oxidation ($\phi_{1/2} \approx 400$ μM). In contrast, Zbinden et al. [36] found the opposite trend, succinate oxidation ($\phi_{1/2} \approx 250$ μM) being more sensitive than was the oxidation of NAD^+-linked substrates ($\phi_{1/2} \approx 500$ μM). Folkers and coworkers [15] observed that in bovine heart mitochondria NAD^+-linked oxidation ($\phi_{1/2} \approx 500$ μM) was more sensitive than succinate oxidation ($\phi_{1/2} \approx 1.2$ mM). These descrepancies can be explained on the basis of our observation that the degree of inhibition achieved at a given concentration of the drug is dependent on protein (mitochondrial) concentration and the conditions of assay (phosphorylating or not), the drug being far more potent under phosphorylating conditions.

The most significant observation that has emerged from the study presented here is the tremendous sensitivity of succinate oxidation by phosphorylating heart mitochondria to inhibition by adriamycin in the presence of hexokinase ($\phi_{1/2} \approx 5$ μM). The results presented in this paper give clear indication that the sensitive site lies between the succinate dehydrogenase flavoprotein and ubiquinone. The possibility of the antibiotic interacting with one of the iron-sulphur centres in this part of the respiratory chain looks attractive.

Strangely enough, the high sensitivity of succinate oxidation by heart mitochondria to adriamycin was observed only under phosphorylating conditions. In the presence of an uncoupler like dinitrophenol or when membrane integrity was damaged by freeze-thawing or by sonic oscillation, the hexokinase effect was lost [31]. Under those conditions NADH oxidation was more sensitive to the antibiotic than was succinate oxidation. The effect did not appear non-specific because proteins like bovine serum albumin could not be substituted for hexokinase. The recent report that the hexokinase binding protein is identical with mitochondrial 'Porin' [37] and that the binding of hexokinase to the protein opens channels in the membrane and stimulates ADP-ATP exchange [38] would indicate that the hexokinase effect might involve the facilitated transport of the antibiotic to its site of action. The greater potency of the drug under phosphorylating conditions might also be a reflection of the internalization mechanism. There is little doubt that the role of hexokinase merits detailed exploration.

The hexokinase effect may have deep significance in the therapeutic effectiveness of adriamycin. Transformed cells are characterized by a large increase (20-fold) in the level of hexokinase in comparison with normal or regenerating cells. It is also of relevance that more than 70% of the hexokinase activity of the transformed cells is associated with the mitochondrial fraction [39–41]. The large increase in the levels of the enzyme in tumour cells and in mitochondria may have a

significant role to play in the inhibition of mitochondrial function by adriamycin and in the consequent development of anoxic conditions.

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