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RHEIN ENHANCES THE EFFECT OF ADRIAMYCIN ON MITOCHONDRIAL RESPIRATION BY INCREASING ANTIBIOTIC-MEMBRANE INTERACTION

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Abstract—The effect of the combination of Adriamycin (ADM) with rhein (RH), an anti-inflammatory drug, on the electron flow through site III and IV of the respiratory chain of rat liver mitochondria was investigated. RH, even at high concentrations, does not inhibit either duroquinol (DHQ) oxidation or cytochrome oxidase activity both of which are decreased by ADM in a dose-dependent manner. The analysis of interaction, performed with the isobolar method, shows a strong synergistic effect that cannot be ascribed to increased permeability of the mitochondrial membranes brought about by RH. The mechanism by which RH potentiates the effect of ADM on DHQ oxidation and cytochrome oxidase activity is most likely to be changes induced in the physical status of the inner mitochondrial membrane such as to permit low ADM concentrations to bind and segregate enough cardiolipin to inhibit electron transport through complex III and IV.

Key words: rhein; Adriamycin; mitochondria; synergism

ADM‡ is an anthracycline antibiotic which, because of its broad spectrum of activity, is one of the most widely employed anti-neoplastic drugs in the treatment of several tumors [1, 2]. However, its use in chemotherapy is severely restricted because of its toxic side effects, mainly at the cardiac level [3]. There is experimental evidence that this toxicity arises from impairment of mitochondrial function [4–9]. Several mechanisms have been proposed to explain the ADM-induced mitochondrial alterations, including the inhibition of electron transfer through the three energy-conserving sites of the respiratory chain [4–6]. Since it is unlikely that ADM interacts with each enzyme of the respiratory chain, it has been proposed that the multi-site effect is due to an interaction of the drug with cardiolipin in the inner mitochondrial membrane required for the full activity of the most enzymes of the respiratory chain [7–13].

Moreover, it has been previously reported that RH, 4,5-dihydroxy-anthraquinone-2-carboxylic acid, an anti-inflammatory drug [14, 15] lowers ATP availability [16] by inhibiting both respiration and glycolysis of tumor cells [17]. RH decreases oxygen consumption by affecting the oxidation of NAD- and FAD-linked substrates at the dehydrogenase–coenzyme level [18], whereas the inhibition of glycolysis must be ascribed to a reduced glucose uptake [17] due to an alteration of membrane-associated functions [19] thus affecting glucose transporter activity [20]. The interaction of RH with the plasma membrane not only decreases the rate of the glucose transport, but also the activity of the transplasma membrane redox enzyme system which transfers electrons from reducing agents in the cytoplasm to external impermeable oxidants, such as ferricyanide [21–24].

However, the RH–ADM association results in a strong synergic inhibitory effect of the plasma membrane redox activity. This synergism of action may be ascribed to the fact that RH does not act at a specific site but by modulating the membrane fluidity [19] makes the transmembrane electron transport system more sensitive to ADM [24].

Therefore, because RH interacts with cell membranes inducing deep modifications in their structure and function [18–20, 24], and considering that the major ADM-sensitive sites lie in complex III and IV [13], experiments were undertaken to evaluate the ability of RH to potentiate the ADM effect on the Q to cytochrome oxidase span of the respiratory chain, to establish the nature, the extent as well as the possible mechanism(s), of such a response.

MATERIALS AND METHODS

Preparation of mitochondria. Rat liver mitochondria were isolated from adult male Sprague–Dawley rats fasted overnight according to Pedersen *et al.* [25]. The mitochondria were resuspended in a minimal volume of H-medium (70 mM sucrose, 210 mM mannitol, 2.1 mM Li–HEPES, pH 7.10) without BSA, because of its ability to bind RH [14], at a concentration of 50 mg/mL. The respiratory control ratio of mitochondrial preparations, with

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‡ Abbreviations: ADM, Adriamycin; DHQ, duroquinol; FCCP, carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone; RH, rhein.

succinate as substrate, ranged between 5.5 and 8. Protein content was determined according to Gornall *et al.* [26].

Assay of oxygen consumption, cytochrome c oxidase activity, mitochondrial swelling and ADM binding. The rates of oxygen consumption were determined with a Clark oxygen electrode (Yellow Springs Instruments Co., OH, U.S.A.) equipped with an ultrathin Teflon membrane. The electrode was inserted horizontally in a thermostated, closed glass chamber (Gilson Medical Electronics, WI, U.S.A.) of 2.0 mL. The volume was always 2.0 mL and contained final concentration of 120 mM KCl, 3 mM Li–HEPES, 1 mM EGTA, pH 7.10, and 3.0 mg of mitochondrial protein. Other additions are given in the figure legends. The temperature was 25° and the oxygen solubility was taken to be 479 ng atoms mL^{-1} when the medium was air-equilibrated at 760 Torr (≈10,1080 Pa) [27].

Cytochrome *c* oxidase activity was also measured polarographically in the same medium with the addition of 0.125 mM reduced cytochrome *c*, prepared according to Nicolay and De Kruijff [13].

The swelling was evaluated by suspending 3.0 mg of mitochondrial protein in 3.0 mL of medium (120 mM KCl, 3 mM Li–HEPES, 1 mM EGTA) and measuring the decrease in optical density at 700 nm and 25° in an Aminco DW-2a spectrophotometer equipped with a thermospacer apparatus [28].

The binding of ADM to mitochondrial preparations was measured in a closed glass chamber of 2.0 mL capacity, equipped with a Clark electrode so that oxygen uptake could be measured simultaneously. Mitochondria (3.0 mg protein) were preincubated at 25° for 1 min with appropriate concentration of ADM and RH. Then 1 mM DHQ and, after 1 min, 0.75 μM FCCP were added and the incubation was allowed to proceed for an additional 30 sec. Mitochondria were withdrawn with a Pasteur pipette and centrifuged for 5 min at 14,000 rpm in an Eppendorf 5415 microfuge. The concentration of unbound ADM was evaluated spectrophotometrically at 500 nm with a Beckman DU-7 spectrophotometer using an extinction coefficient of 13 $mM^{-1} cm^{-1}$. Standard calibration spectra were run with ADM solutions of known content (by weight).

Data analysis. The analysis of the RH–ADM interaction on both DHQ oxidation and cytochrome *c* oxidase activity was performed by means of the isobolar approach [29]. Briefly, this method, which is supposed to offer a general solution to the problem of interaction [30], implies the construction of isoeffect curves or surfaces based on the dosage of the agents, given alone or in combination, to achieve the same effect. The case in which one agent (RH) doesn't produce any evaluable effect in combination with another agent (ADM) able to produce a remarkable effect is termed heterergic. According to Berembaum [29] if there is no interaction between the two agents the isobole will be a straight line parallel to the dose axis on the 'non effective' agent. If the interaction is positive (synergism) or negative (antagonism) the isobole deviates asymptotically toward or away from the axis of the 'non effective' agent. Therefore, the method enables the effect of non-interactive combinations to be calculated from experimental data, regardless of the particular types of exposition–effect relationship of the individual agents and, more generally, without invoking the mechanism of action [30].

Chemicals. The following chemicals were purchased from the indicated sources: ascorbic acid, fatty acid-free BSA, cytochrome *c*, EGTA and FCCP from Sigma Chemical Co. (St Louis, MO, U.S.A.); HEPES, antimycin A and cytochrome *c* from Boehringer Italia (Milan, Italy); rotenone and DHQ from K&K Laboratories (Plainview, NY,

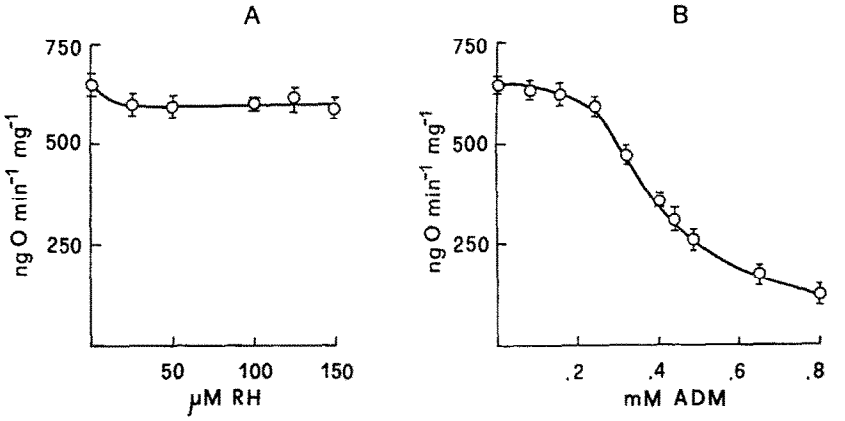

Fig. 1. Effect of RH (A) and ADM (B) concentration on the oxidation of DHQ by rat liver mitochondria in the presence of FCCP. The final volume was 2.0 mL and the temperature 25°. The mitochondria (3 mg protein) were preincubated with the indicated concentration of RH and ADM in buffered medium (see Materials and Methods) with 4 μM rotenone for 1 min, 1 mM DHQ was then added and the rate of oxygen consumption recorded. After 1 min, FCCP, at a final concentration of 0.75 μM, was injected and the rate of subsequent oxygen uptake recorded. Each point ±SD was averaged from seven different mitochondrial preparations.

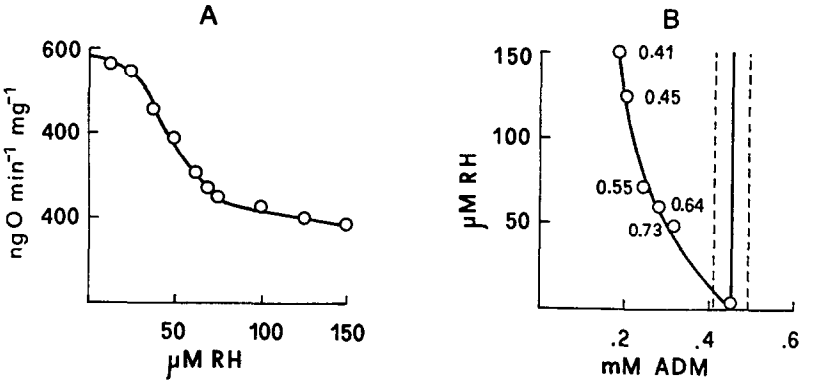

Fig. 2. (A) Effect of RH concentration on the inhibition of DHQ oxidation by ADM. Mitochondria (3 mg protein) were preincubated with 0.24 mM ADM and with indicated RH concentration for 1 min. Other experimental condition as in Fig. 1. Each point was averaged from seven different mitochondrial preparations. Error bars within circles. (B) Concentration of ADM and RH in combination to obtain a 50% inhibition of DHQ oxidation. The dotted vertical bars show the zero-interaction region, whereas the numbers indicate the interaction index calculated according to Berembaum [29]. The ADM concentration to give a 50% inhibition was 0.45 mM. Each combination was repeated five times with different mitochondrial preparations. Error bars within circles.

U.S.A.); ADM from Farmitalia (Milan, Italy). All the other reagents were of analytical grade and were purchased from BDH Italia (Milan, Italy). RH was a gift from Dr Vittorio Behar, Proter Laboratories, Opera (Milan, Italy).

RESULTS

Effect of RH and ADM on the DHQ oxidation

The effect of RH and ADM on electron flow through site 2 was investigated by using as substrate DHQ which feeds electrons directly to O. Figure 1 shows the concentration-dependent effect of RH and ADM on the DHQ oxidation by rat liver mitochondria. The mitochondria, in the presence of rotenone to inhibit electron flow from site 1, were preincubated for 1 min with increasing drug concentrations; 1 mM DHQ was then added and the rate of state 4 oxygen consumption was recorded for another minute. Then, 0.75 μM FCCP was injected and the rate of the subsequent oxygen uptake was determined. As may be observed in Fig. 1A, RH showed no inhibition of the very high DHQ oxidation rate, confirming again that RH does not affect the electron flow through site 2 [18]. However, a completely different pattern occurred when the mitochondria were incubated with ADM (Fig. 1B). The drug, up to 0.24 mM, did not significantly affect DHQ oxidation (= − 10%), whereas higher concentrations led to a sharp decline of the oxygen consumption rate which was 120 ng atoms O min^{-1} mg^{-1} ($\Delta\%$ = − 82%) at 0.8 mM ADM. The concentration to obtain half-maximal rate was 0.45 mM ADM.

In order to establish whether RH might enhance the inhibition of oxygen consumption by ADM, mitochondria were incubated with a fixed amount of ADM, i.e. 0.24 mM, and increasing concentrations of RH. As shown in Fig. 2A, 0.24 mM ADM by itself had little effect on the oxygen uptake rate, which was lowered from 650 ng atoms O min^{-1} mg^{-1} to 580 ng atoms O min^{-1} mg^{-1} and not further modified by the addition of RH up to 25 μM. Nevertheless, if the RH amount was increased, the oxygen consumption rate was reduced by ADM and the extent of inhibition increased with RH concentration. At 150 μM RH, the rate of oxygen uptake was 180 ng atoms O $min^{-1}mg^{-1}$ ($\Delta\%$ = − 70%), i.e. the value obtained by 0.65 mM ADM (Fig. 1B). Half-maximal inhibition was observed with the combination 0.24 mM ADM and 70 μM RH. Thus, these data clearly demonstrate that RH greatly enhances the ability of ADM to inhibit DHQ oxidation. In order to define the type of response, the effect of different combinations of ADM and RH on the DHQ oxidation was tested according to Berembaum [29]. The results are presented in Fig. 2B. When given alone the ADM concentration to inhibit oxygen consumption rate by 50% was 0.45 mM. All combinations tested were strongly synergic because the experimental points diverged toward the RH axis and, accordingly, the relative isobole was concave up [29]. The interaction index was always much lower than 1, ranging from 0.41 to 0.73.

Effect of ADM and ADM–RH association on cytochrome oxidase activity

The activity of the terminal enzyme complex of the respiratory chain, i.e. cytochrome oxidase, also appeared to be affected by ADM (Fig. 3), whereas RH is completely inactive on this enzyme [18]. Mitochondria, in the presence of rotenone and antimycin A to prevent electron flow from substrates entering site 1 and 2 and of FCCP to yield the maximum rate of oxygen consumption, were preincubated for 1 min with established ADM concentrations. Then 0.125 mM reduced cytochrome

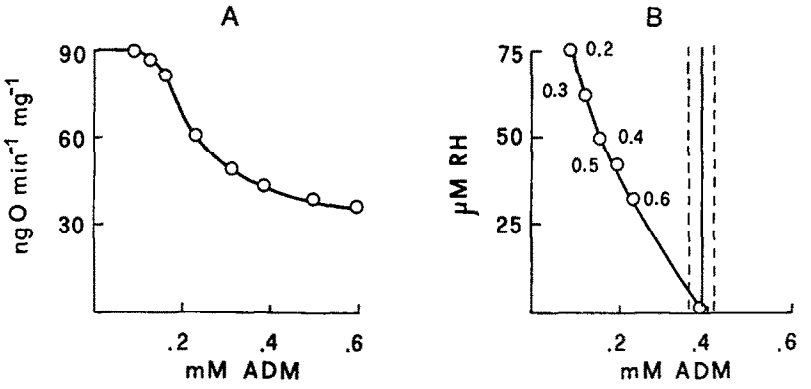

Fig. 3. (A) Effect of ADM concentration on cytochrome oxidase activity of rat liver mitochondria. Mitochondria (3 mg protein) were preincubated for 1 min in buffered medium supplemented with 4 μM rotenone, 0.2 nmol antimycin A mg protein^{-1} and 0.75 μM FCCP. The reaction was started by the addition of 0.125 mM reduced cytochrome *c*. Other experimental conditions as in Fig. 1. Each point was averaged from seven different mitochondrial preparations. Error bars within circles. (B) Concentration of ADM and RH in combination to reduce cytochrome oxidase activity by 50%. Symbols as in Fig. 2B. Each combination was repeated five times with different mitochondrial preparations. Error bars within circles.

c was added and the subsequent rate of oxygen consumption recorded. As shown by the dose–response curve of Fig. 3A ADM in concentrations up to 0.15 mM, did not significantly inhibit the rate of oxidation of reduced cytochrome *c* which, on the contrary, was severely impaired by higher drug concentrations. The ADM concentration to obtain half-maximal rate was 0.39 mM, a value very similar to that found with DHQ as substrate. When the effect of ADM on cytochrome oxidase activity was evaluated in the presence of RH, a strong synergic effect occurred. In fact, as shown in Fig. 3B, in all combinations tested, the concentration of ADM to obtain 50% inhibition was much lower than that required when given alone (0.39 mM) and, as observed with DHQ (Fig. 2B), the isobole was concave up. It should be stressed that the 50% effect was obtained with remarkably lower ADM and RH concentrations than those required when DHQ was the substrate.

Effect of RH on mitochondrial permeability and on the ADM binding

It has been reported that agents perturbing the structure of the membranes increase their permeability [31] and that mitochondrial susceptibility to respiratory inhibition by ADM depends on the permeability of the outer mitochondrial membrane [32]. On the other hand, RH binds to membranes and modifies their structural and functional properties [19, 20, 24]. It is, then, possible that the greater susceptibility of DHQ oxidation, as well as of cytochrome oxidase activity, to the ADM–RH association might be ascribed to an increased permeability of the mitochondrial membranes. Therefore, the effect of RH on mitochondrial membrane permeability was investigated. As shown in Fig. 4A, no modifications in the rate of oxidation of externally added reduced cytochrome *c* were observed over the range of RH concentrations tested. On the contrary, when mitochondria were treated with increasing levels of digitonin, a detergent which selectively lyses mitochondrial outer membrane, the rate of oxidation of reduced cytochrome *c* increased in a linear manner with digitonin concentration (Fig. 4B). Yet, in mitochondria pre-treated for 1 min with 0.06% digitonin, the oxidation of externally added reduced cytochrome *c* was stimulated by low and inhibited by high ADM concentrations (Fig. 4C). The half-maximal rate was obtained at 0.39 mM ADM, a value identical to that found for non-permeabilized mitochondria, (Fig. 3A). Thus, these data clearly show that the synergistic effect of the ADM–RH association on mitochondrial respiration was not caused by a modification of the outer membrane permeability. Neither can this synergism be referred to an increased permeability of the inner membrane because of the inability of RH to modify it either in absence of metabolic energy (Fig. 4D, 1) or when mitochondria were respiring on DHQ (Fig. 4D, 2).

The higher susceptibility of mitochondrial respiration to the association ADM–RH might be due to an increased capacity of RH-treated mitochondria to bind ADM. Figure 5 shows the binding of ADM to rat liver mitochondria as function of the RH concentration. In order to establish whether there was a relationship between the amount of ADM bound and the inhibition of oxygen consumption, the binding was evaluated under the same experimental conditions as in Fig. 2A. In the absence of RH, 5 nmol mg protein^{-1} of ADM were bound by mitochondria. Low RH concentrations did not affect the ADM binding whereas, at higher concentrations, the amount of ADM bound linearly increased over the range tested.

The greater amount of ADM bound by RH-treated mitochondria, the greater the inhibition of

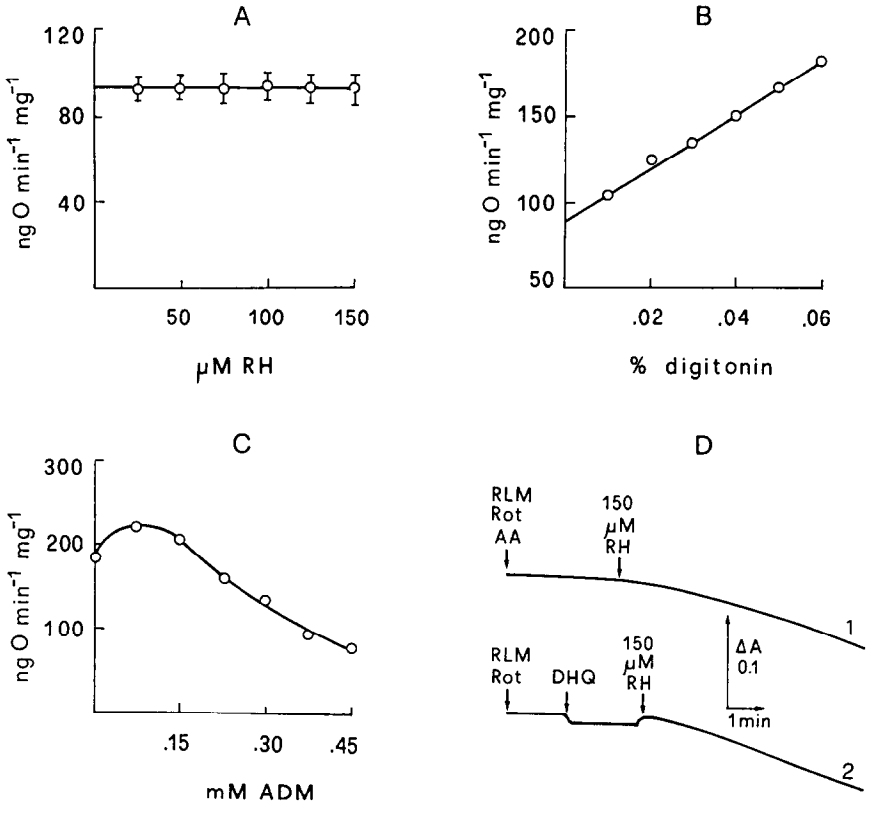

Fig. 4. (A) Effect of RH concentration on the rate of oxidation of reduced cytochrome *c*. Experimental conditions as in Fig. 3A. Each value ±SD was averaged from five different mitochondrial preparations. (B) Effect of digitonin concentration on the rate of oxidation on reduced cytochrome *c*. Mitochondria (3 mg protein) were preincubated for 1 min with indicated digitonin concentration. Other experimental conditions as in Fig. 3A. Each value was averaged from five different mitochondrial preparations. Error bars within the circles. (C) Effect of ADM concentration on the rate of oxidation of reduced cytochrome *c* in digitonin-treated mitochondria. Mitochondria (3 mg protein) were preincubated for 1 min with 0.06% digitonin. Other experimental conditions as in Fig. 3A. Each point was averaged from five different mitochondrial preparations. Error bars within the circles. (D) Typical traces showing the effect of 150 μM RH, i.e. the highest concentration used in association with ADM, on the permeability of the inner mitochondrial membrane of rat liver mitochondria. The mitochondria (3 mg protein were incubated at 25° in 3.0 mL of the buffered medium, see Materials and Methods) and the additions indicated by the arrows. Rotenone and antimycin A were added at a final concentration of 4 μM and 0.2 nmol mg protein^{-1}. The experiments were repeated with four different mitochondrial preparations and yield reproducible results (±3%). 1: non-respiring mitochondria; 2: mitochondria respiring on DHQ (1 mM).

oxygen consumption as clearly demonstrated in Fig. 6. In the absence of RH, mitochondria incubated with 0.24 mM ADM, bound an amount of drug (5 nmol mg protein^{-1}) that was insufficient to impair DHQ oxidation because, as shown in trace B, the rate of oxygen consumption was similar to that of the control (trace A). When mitochondria were incubated with 0.24 mM ADM in the presence of 75 μM RH, the amount of ADM bound rose to 13 nmol mg protein^{-1} and the oxygen uptake rate was inhibited by 50% (trace C). These values were identical to those found in mitochondria incubated with 0.45 mM ADM (trace D) a concentration which, as shown in Fig. 1B, inhibited DHQ oxidation by 50%.

DISCUSSION

The observations recorded in this report demonstrate that RH strongly potentiates the inhibitory effect of ADM on electron flow from Q to oxygen. Although this drug does not affect the electron transfer through site 2 and 3 of the mitochondrial respiratory chain (Fig. 1A) [18], its combination with ADM results in a strong synergic effect that permits the reaching of a pre-established level of inhibition with concentrations of ADM that are much lower than those necessary when ADM is used alone. This synergism cannot be ascribed to a facilitated diffusion of ADM through the outer mitochondrial membrane. In spite of the ability of RH to interact with mitochondrial membranes, it does not increase the outer membrane permeability as is clearly demonstrated by its failure to stimulate the oxidation of reduced cytochrome *c*. The outer mitochondrial membrane is impermeable to cytochrome *c* so that the rate of electron transfer between externally added reduced cytochrome *c* and

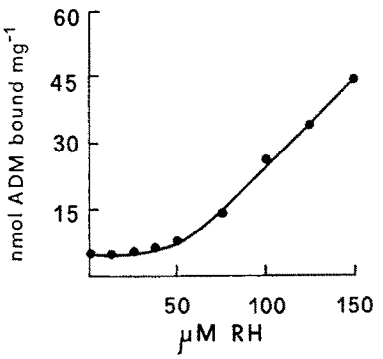

Fig. 5. Effect of RH concentration on ADM binding. Mitochondria (3 mg protein) were preincubated in a buffered medium with 4 μM rotenone, 0.24 mM ADM and the indicated concentration of RH for 1 min. DHQ (1 mM) was then added and the oxygen consumption rate recorded. After 1 min 0.75 μM FCCP was injected and the rate of subsequent oxygen uptake recorded for further 30 sec. The mitochondria were withdrawn with a Pasteur pipette and centrifuged at 14,000 rpm for 5 min. The unbound ADM was evaluated spectrophotometrically at 500 nm on the supernatant.

the redox sites in the inner membrane depends strictly on the intactness of the outer membrane [33]. If RH were acting by increasing the outer membrane permeability, a stimulation of the rate of oxygen consumption would have been observed. On the other hand, the outer mitochondrial membrane apparently does not represent a barrier to ADM, as is demonstrated by the fact that the ADM concentration to inhibit 50% cytochrome oxidase activity in digitonin-treated mitochondria is the same as that required for untreated mitochondria (Fig. 4C). Our data are in agreement with those of Nicolay and co-workers [9, 13], whereas they differ from those of Mannella *et al.* [32] who found a correlation between the lysis of the outer membrane and the susceptibility of rat liver mitochondrial cytochrome oxidase.

The synergistic inhibitory effect of the ADM–RH association on DHQ oxidation as well as on cytochrome oxidase activity should be ascribed to an increased binding of ADM achieved by RH. ADM has direct access to the cytosolic side of the inner membrane, but, by raising its concentration, a greater amount of the drug is bound and a second level of saturation is reached [9, 31]. This is mainly due to penetration of ADM through the inner membrane with consequent binding to cardiolipin of the matrix side [31]. RH binds to mitochondria and induces severe alterations in their structure such as a rarefaction of the matrix and a distortion and disruption of the cristae [19], reflecting on their functionality [16–18]. Since RH does not modify the permeability of the inner mitochondrial membrane (Fig. 4D), the greater amount of ADM bound by RH-treated mitochondria may presumably be ascribed to changes of the physical status of the membrane [19, 34] that could allow low ADM concentrations to bind and segregate cardiolipin so as to inhibit the electron flow through complex III and IV of the respiratory chain.

However, it should be noted that the amount of ADM bound is lower than that reported by Nicolay *et al.* [9] and Chevenal *et al.* [31]. This discrepancy

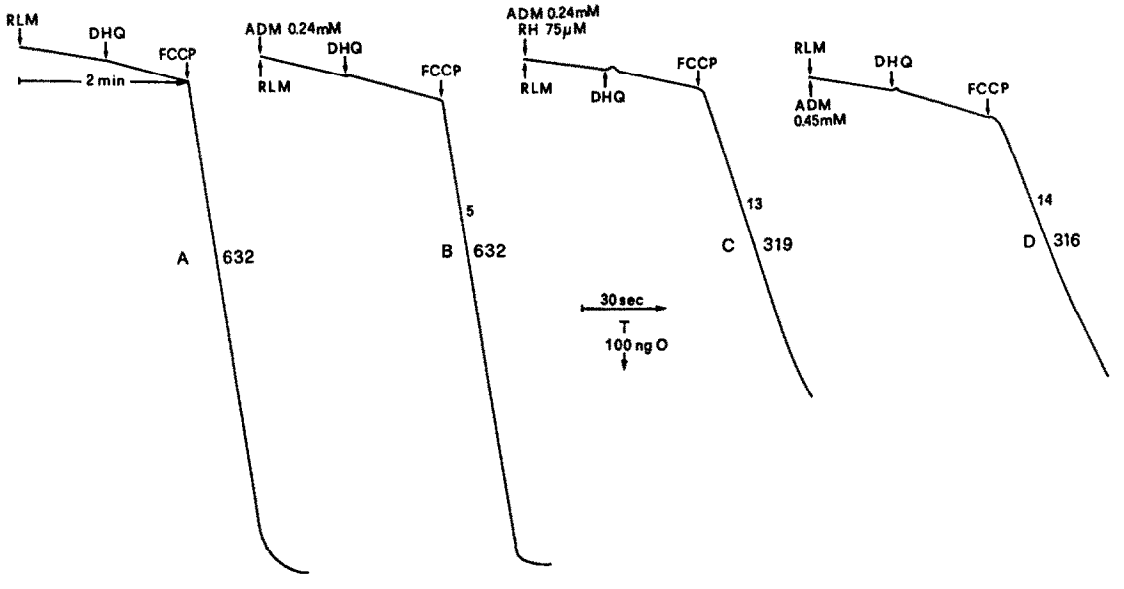

Fig. 6. Typical traces showing the relationship between the amount of ADM bound and the inhibition of DHQ oxidation in the absence (traces B and D) and in the presence (trace C) of 75 μM RH. Experimental conditions as in Fig. 5. The experiments were repeated five times and yield reproducible results (±6%). (A) control. The upper number along the traces refers to ADM bound (nmol mg protein^{-1}) whereas the lower is the rate of oxygen consumption (ng atoms O min^{-1} mg protein^{-1}).

is most likely due to the different metabolic state of mitochondria in which the binding was evaluated. In fact, the amount of drug bound was in close agreement with that reported in the cited papers [9, 31] (±5%) when the binding was performed under the same conditions (data not shown).

In conclusion, these data clearly indicate that RH enhances ADM inhibition of electron transport by increasing the binding to inner mitochondrial membrane although the biochemical basis of such a phenomenon must be better clarified.

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