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ADRIAMYCIN: ENERGY METABOLISM AND MITOCHONDRIAL OXIDATIONS IN THE HEART OF TREATED RABBITS

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Abstract—Heart mitochondria isolated from rabbits subjected to intermittent treatment with adriamycin show a reduced respiratory control, resulting from increase in state 4b oxidation. Continuous daily treatment causes an impairment of respiratory control which is more severe, is due both to increase of state 4b and decrease of state 3 oxygen uptake and occurs after a total amount of drug which does not produce appreciable effects with the intermittent schedule of treatment: however, these changes disappear within 2 weeks from the interruption of the treatment. Mitochondria isolated from adriamycin-treated rabbits show constantly increased permeability to the addition of NADH. On the contrary the ADP/O ratio measured *in vitro* is essentially unchanged: the same happens with the tissue contents of ATP, ADP, AMP and the metabolites chosen to estimate cytoplasmic and mitochondrial redox states and measured in quick-frozen hearts *in vivo*. The results are discussed in relation to the possible role of mitochondrial functional defects in the onset of adriamycin cardiomyopathy.

The antracycline quinones adriamycin and daunomycin are widely used in the treatment of various kinds of malignancies. By interacting with nucleic acids [1-4] these drugs seem to inhibit both DNA-directed DNA synthesis [5] and DNA-directed RNA synthesis [6], and it has also been suggested that they can interfere with an exonucleolytic editing activity during DNA replication [7]. The relationship between biochemical properties and anticancer activities of these drugs seems to be supported by the fact that adriamycin, which is a stronger inhibitor of the polymerases, is also more effective than daunomycin in human experiments [8]. Recently however, data have been provided indicating that the activity of adriamycin can be due—at least in part and in addition to the interaction with DNA—to the inhibition of ubiquinone-enzymes in electron transfer processes of cell respiration [9]. The antitumor activity displayed by adriamycin is associated with the phenomena of general toxicity, particularly disturbing when they affect the heart; severe cardiomyopathy has been described both in human patients [10] and in rabbits following chronic treatment [11].

Ultrastructural aspects of this cardiomyopathy in rabbits include various stages of mitochondrial degeneration, such as early focal dilations of the outer mitochondrial membrane, followed by swelling of the inner compartment and the formation of intramitochondrial laminated bodies which appear as intracellular inclusions. Mitochondrial alterations are accompanied by disorganization of the myofibrils, while nuclear alterations are not very prominent, and are apparent only at later stages after most other organelles have suffered heavy damage [12]. If mitochondrial injury seems to be an early and important feature in the myocardium of adriamycin-treated rabbits, metabolic alterations do not seem to be so relevant in mitochondria isolated from rats treated with related anti-cancer drugs [13]. To define the role, if any, played by the impairment of mitochondrial functions in the onset of myocardial damage we have measured the oxidative activity of mitochondria isolated from the hearts of rabbits subjected to different types of adriamycin treatment. The polarographic methods employed to this purpose enable a fine analysis of the rate of oxygen uptake in the various states of mitochondrial metabolism [14], and permit the direct calculation of indexes of mitochondrial efficiency such as the 'respiratory control index' and the 'phosphorylation ratio'. In addition to this work, as an indication of the actual conditions of the myocardium in the living animals, we have also measured the concentration levels of the adenine nucleotides, and from there have calculated the ratio [ATP]/[ADP] [P_i] which is the best indicator of the energy stores in a given tissue [15–16]. Besides carrying out ATP synthesis by oxidative phosphorylation, the mitochondrion is the site of the processes which ensure the homeostasis of the redox state both in the mitochondria and in the cytosol: therefore we decided to extend the investigation to the calculation of the concentration ratios of free NAD^+ and NADH at the site of oxido-reductions, made possible by the development of techniques based on the determination of the substrates of particular dehydrogenase systems [17]. The results of our experiments show an impairment of some metabolic activities in mitochondria obtained from adriamycin-treated rabbits; these defects are discussed in relation to their possible role in the pathogenesis of adriamycin cardiomyopathy.

MATERIALS AND METHODS

Animals. Male New Zealand white rabbits, weighing about 3 kg and maintained on a balanced diet were treated with adriamycin intravenously in doses

of 0·8 mg/kg per day: (i) for 3 consecutive days every 2 weeks (intermittent treatment), (ii) for the specified number of consecutive days (continuous treatment). Four animals of the second group, injected with 15 daily doses of adriamycin, were then left without any treatment and used 2 weeks after the last injection (recovery). Animals of group (i) were killed 24 hr after the first injection of the specified course of treatment; those of group (ii) were killed 24 hr after the last injection of adriamycin, after the indicated number of daily treatments. The rabbits were anesthetized with Nembutal® (60 mg/ml), slowly injected intravenously until the palpebral reflex had disappeared. The animals were then put on their backs, the chest was opened by gardening scissors and the ventricular portion of the beating heart was removed and: (i) cut vertically in two pieces and frozen in liquid nitrogen within 3–4 sec as described by Gaja *et al.* [18] for the preparation of the extracts and the determination of the adenine nucleotides, the substrates of the dehydrogenase systems and glycogen, or (ii) cut as above, immersed in chilled homogenization medium and processed immediately for the isolation of the mitochondria.

Preparation of tissue extracts. The frozen tissue, still in liquid nitrogen was ground with a pestle and mortar; the powder (1·5–2 g) was then weighed in a polycarbonate centrifuge tube (cooled in liquid N_2) and homogenized in 6 vol ice-cold 6% (w/v) $HClO_4$ with a glass pestle at low speed until thawing was complete. After 30 min at 0° the homogenate was centrifuged at 25,000 *g* for 20 min at 0°. The supernatant was decanted into graduated centrifuge tubes and the precipitated material was washed with ice-cold 3% $HClO_4$ and recentrifuged as described above: the latter supernatant was pooled with the former and brought to pH 6 with 5M K_2CO_3 in the presence of B.D.H. Universal Indicator. After a further 30 min at 0°, $KClO_4$ was removed by centrifugation and the supernatant was used for the determination of metabolites.

Analytical methods. Tissue extracts were analyzed enzymatically for lactate [19]; pyruvate [20]; 3-hydroxybutyrate [21]; acetoacetate [22]; adenosine triphosphate (ATP) [23]; adenosine diphosphate (ADP) and adenosine monophosphate (AMP) [24]. Total adenine nucleotides were calculated from the sum of the ATP, ADP and AMP. Inorganic phosphate (P_i) was determined by the method of Fiske and Subbarow [25]; 'free' and 'residual' glycogen were determined according the methods of Carrol *et al.* [26] and of Kemp and Kits van Heijningen [27] respectively, as reported by Bernelli-Zazzera and Gaja [28]. All measurements were done either in a Beckman DU 2 spectrophotometer (Beckman Instruments Inc., Belmont, Calif.) or in a Gilford 2400 automatic reading spectrophotometer (Gilford Instruments Laboratories, Inc. Oberlin, Ohio) fitted with a scale expansion accessory and with the cuvette holder maintained at 25°.

Calculation of redox ($[NAD^+]/[NADH]$ ratio) and energy ($[ATP]/[ADP]$ $[P_i]$ ratio) states. The $[NAD^+]/[NADH]$ ratios in the mitochondria and in the cytosol have been calculated from the concentrations of the substrates of the 3-hydroxybutyrate dehydrogenase and lactic dehydrogenase systems according to the equation:

$$\frac{[\text{free NAD}^+]}{[\text{free NADH}]} = \frac{[\text{Oxidized substrate}]}{[\text{Reduced substrate}]} \times \frac{1}{K}$$

as described by Williamson *et al.* [17].

The phosphorylation state of cytoplasmic adenine nucleotide system has been calculated from the $[ATP]/[ADP]$ $[P_i]$ ratio with the assumptions indicated by Veech *et al.* [15].

Isolation of heart mitochondria. All operations were carried out in the cold (1–2°) and care was taken to obtain a pure preparation of mitochondria rather than a quantitative yield. The tissue was very finely minced with scissors and the mince was homogenized in 20 vol 0·25 M sucrose containing 0·01 M Tris–Cl buffer pH 7·4, 5000 units of heparin/100 ml [29] and 0·2% of dialyzed bovine serum albumin [30]. The homogenization was carried out in a glass Potter–Elvehjem homogenizer with a loose-fitting teflon pestle and 6–8 strokes at 400 rev/min. The resulting homogenate was centrifuged for 10 min at 1000 *g* in a I.E.C. B-20 refrigerated centrifuge and the supernatant fraction centrifuged again at 11400 *g* for 6 min. After the first sedimentation of the mitochondria the supernatant fluid together with the fluffy layer was discarded, the white layer of the mitochondrial pellet was removed by gentle shaking of the tube with a small volume of medium and the brown mitochondrial pellet was resuspended in 10 ml of isolation medium, resedimented and washed twice. Finally the mitochondria were suspended in 0·25 M sucrose containing 0·01 M Tris–Cl pH 7·4 to a final protein content of 8–10 mg/ml as determined by the biuret method [31], using crystallized bovine-albumine as a standard.

Oxygen uptake, respiratory control and ADP/O ratio. Oxygen uptake was measured polarographically with a Gilson Oxygraph (Model KM, Gilson Medical Electronics, Middleton, WI) fitted with a vibrating platinum electrode, as described by Lessler and Brierly [32]. The assay system (2·5 ml saturated with air at 25°) contained 1·5–2 mg of mitochondrial protein in the medium described by Max *et al.* [33] with 15 mM 2-oxoglutarate as substrate. ADP was added in the amount of 250 nmoles and 2,4-dinitrophenol at a final concentration of 0·1 mM. State 3, 4a, 4b, respiratory control index and ADP/O ratio were measured. State 4a is the mitochondrial respiration occurring in the presence of substrate without added ADP; it is rather slow because the endogenous adenosine diphosphate-level is very low; state 3 is characterized by a fast rate of oxygen uptake induced by the addition of ADP; state 4b is the rate of oxygen uptake measured after the added ADP has been converted to ATP; respiratory control index is the ratio of state 3 to state 4b rate which indicates the degree of dependency of respiration on ADP [14]. The higher the value of this ratio, the tighter the coupling of oxidation to phosphorylation; ADP/O ratio is the ratio of added ADP to the amount of oxygen consumed during state 3 respiration [34]. This value indicates the efficiency of ATP formation in relation to the amount of oxygen consumed.

Permeability of mitochondria to nicotinamide adenine dinucleotide in its reduced form (NADH). In a separate set of experiments each mitochondrial preparation

was tested by adding 1 μmole of NADH in the absence of any oxidizable substrate and ADP (state 1); the stimulation of oxygen uptake, when present, was taken as an index of permeability of the mitochondrial membranes to NADH.

Chemicals. Enzymes, substrates, cofactors and nucleotides were obtained from C. F. Boehringer und Soehne GmbH. (Mannheim, Germany). All the other reagents were of analytical grade and were obtained from commercial sources.

Statistical treatment of results. When appropriate the results were subjected to the analysis of variance; a significant difference is claimed when the P-value is less than 0·05. Since each experiment with NADH has been carried out with the same batch of mitochondria, the significance of the extra-oxygen uptake has been assessed by the *t*-test for paired variates. Ratios have not been subjected to analysis.

RESULTS

The results describe the oxidative activity of the mitochondria isolated from, and the contents of some metabolites in, the beating hearts taken from living rabbits under Nembutal–anesthesia. This procedure assigns precise limits to our results, but was considered to be preferable to the analysis of hearts obtained from rabbits killed by decapitation or cervical dislocation, and undergoing uncontrolled contractile activity during the lapse of time from the death of the rabbit to the removal of the tissue.

Respiration and oxidative phosphorylation of isolated mitochondria (*Table* 1). During the intermittent treatment neither state 4a nor state 3 oxidations were appreciably modified; on the contrary, after all added ADP had been phosphorylated (state 4b), the rate of oxygen uptake became higher than normal, with a certain dependence on the duration of the treatment. On the whole, the respiratory control index decreased but the decrease is not particularly relevant—both in absolute terms and in relation to the results obtained during the uninterrupted daily treatment—and was caused by an increase in state 4b rather than by a fall of state 3 respiration. The response to the addition of 2,4-dinitrophenol was essentially the same, thus confirming the condition of coupling of the mitochondrial oxidations. Finally, the efficiency of ATP synthesis, as indicated by the ADP/O ratio, was never impaired during this type of treatment. The changes induced by continuous daily treatment were best seen after 7 days; one single dose of adriamycin had no effect whatsoever, and dosing for 3 days (two animals, not reported in the table) was also ineffective. The changes observed during continuous treatment are the following: (i) fall by about 40% of the rate of oxygen uptake in state 3 by the 7th day of treatment, indicating an impairment of electron transport along the respiratory chain, (ii) increase by about 30% of state 4b respiratory rate later during the treatment, indicating a loose coupling of mitochondrial oxidations, (iii) release of respiratory control, with reduction to half of the respiratory control index, due both to the increase in state 4b and the decrease in state 3 rates, (iv) decreased response to the addition of 2,4-dinitrophenol, in agreement with the increase in state 4b respiratory rate. Just as observed in the case of the intermittent treatment, the ADP/O ratio was essentially unchanged even after 15 daily doses of adriamycin. The alterations described after 15 days of uninterrupted treatment are fully reversible. Two weeks after the end of the treatment all tested biochemical parameters of mitochondrial function, including respiratory control index, had completely returned to normal.

NADH permeability (*Table* 2). Mitochondrial membranes are practically impermeable to NADH; hence, addition of NADH to normal mitochondrial preparations in state 1 did not increase oxygen uptake. Heart mitochondria from rabbits treated with more than one daily dose of adriamycin, on addition of NADH, showed a constant and significant stimulation of the oxygen uptake, of the order of 50%. The effect was largely independent from the type of treatment and from the total amount of administered drug, and was most probably due to the increased permeability of the mitochondrial membranes. Upon interruption of the treatment, mitochondrial permeability tended to return to normal—i.e. the extra oxygen uptake due to the addition of NADH diminished—but normal behavior of the mitochondria was not yet fully attained when all the other parameters had already reached control levels.

Adenine nucleotides and the energy state (*Table* 3). The concentrations of ATP, ADP and AMP were

Table 1. Effects of adriamycin treatment on the oxidative and phosphorylative properties of rabbit heart mitochondria

Treatment	Total amount of adriamycin (mg/kg body wt)	Oxygen uptake (natoms/mg protein per min)				Respiratory Control Index	ADP/O
		State 4a	State 3	State 4b	+Dinitrophenol		
Control (5)		21·4 ± 1·9	193·0 ± 15·3	31·8 ± 3·7	176·9 ± 18·2	6·3 ± 0·6	3·5 ± 0·2
Intermittent treatment							
5 courses (5)	10·4	26·0 ± 2·0	180·4 ± 8·0	40·8 ± 3·2	168·2 ± 4·3	4·6 ± 0·4	3·8 ± 0·1
10 courses (4)	22·4	31·8 ± 3·0	201·9 ± 20·7	48·6 ± 2·5*	189·9 ± 21·0	4·2 ± 0·3	3·4 ± 0·1
Continuous treatment							
1 dose (5)	0·8	22·3 ± 2·9	198·6 ± 20·4	35·3 ± 4·8	185·3 ± 18·5	5·8 ± 0·5	3·5 ± 0·2
7 doses (4)	5·6	22·3 ± 3·9	123·5 ± 5·3*	38·8 ± 2·2	146·3 ± 13·9	3·3 ± 0·3	3·8 ± 0·1
9 doses (4)	7·2	23·6 ± 4·3	116·8 ± 5·9*	40·7 ± 4·5	126·6 ± 5·4*	2·7 ± 0·3	3·8 ± 0·1
15 doses (5)	12·0	24·7 ± 1·8	115·0 ± 5·6*	43·0 ± 2·7*	126·1 ± 5·6*	2·7 ± 0·2	3·7 ± 0·1
15 doses + 15 days of recovery (4)	12·0	16·0 ± 0·8	198·5 ± 11·3	32·3 ± 2·0	187·9 ± 13·4	6·5 ± 0·6	3·7 ± 0·1

Values are expressed as means ± S.E.M.; number of experiments in parentheses.
* Statistically different from the control ($P < 0·05$).

Table 2. Effects of adriamycin on the response of rabbit heart mitochondria to added NADH

Treatment	Oxygen uptake (natoms/mg protein per min.) State 1 (1)	+ NADH (2)	Difference (2–1)
Control (5)	32·2 ± 1·4	33·9 ± 2·1	NS†
Intermittent treatment			
5 courses (5)	28·6 ± 2·2	56·2 ± 4·1	+27·6 ± 3·5*
10 courses (4)	36·7 ± 4·6	56·7 ± 4·3	+20·0 ± 2·0*
Continuous treatment			
1 dose (5)	30·6 ± 2·4	31·4 ± 3·1	NS†
7 doses (4)	30·1 ± 3·4	42·6 ± 5·7	+12·5 ± 2·6*
9 doses (4)	29·3 ± 5·6	48·4 ± 7·3	+19·1 ± 2·8*
15 doses (5)	29·2 ± 1·8	43·8 ± 3·3	+14·6 ± 2·7*
15 doses + 15 days of recovery (4)	21·3 ± 0·3	28·8 ± 0·5	+ 7·5 ± 0·4*

Values are expressed as means ± S.E.M.; number of experiments in parentheses.

* Statistically significant difference ($P < 0.05$).

† NS: not significant since S.E.M. greater than the mean.

essentially stable in the hearts of adriamycin-treated rabbits, irrespective of the type of treatment. Total adenine nucleotides were accordingly unchanged. Small shifts within the various forms of the adenylate pool tended to lower the [ATP]/[ADP] [P_i] ratio after 9 and 15 days of continuous treatment, but the alteration was very small and its meaning is uncertain in view of the fact that ATP levels are well maintained.

Metabolites of NAD-linked dehydrogenase systems and the redox state (*Table* 4). For reasons which were beyond the possibility of control—most probably due to the handling of the animals—the concentrations of lactate were found to be high and extremely variable, without any relation to adriamycin treatment: they might reflect phenomena occurring in peripheral tissues rather than in the myocardium. Pyruvate approximately followed the changes of lactate and on the whole the lactate/pyruvate ratio did not change in treated animals: but the values of this ratio were much too high, even in the controls, to be of any use in a reasonable assessment of the cytoplasmic redox state. On the contrary, the main difficulty with 3-hydroxybutyrate and acetoacetate was their presence in minute amounts in hearts which had to be used for many different determinations. Increase of both these compounds—somewhat obscured by enlarged variability—was found during the continuous daily treatment; but the increase affected both the substrates and the mitochondrial redox state, as expressed by the [NAD]/[NADH] ratio, is not appreciably altered.

Glycogen. Leaving apart any discussion as to the possible physiologic significance of the two glycogen fractions—operationally defined as 'free' and 'residual'—we can say that both of them, as well as 'total' glycogen, are essentially constant in the hearts of adriamycin-treated rabbits.

Table 3. Content and phosphorylation state of adenine nucleotides in rabbit heart during adriamycin treatment

Treatment	(nmoles/g fresh tissue) [ATP]	[ADP]	[AMP]	[ATP] + [ADP] + [AMP]	[P_i]	[ATP] / [ADP] × [P_i]
Control (5)	3505 ± 296	1316 ± 77	299 ± 37	5120 ± 285	9113 ± 748	487
Intermittent treatment						
5 courses (5)	3812 ± 206	1279 ± 52	325 ± 30	5416 ± 189	10283 ± 311	483
10 courses (4)	3466 ± 335	1325 ± 89	283 ± 81	5028 ± 332	9960 ± 518	438
Continuous treatment						
1 dose (3)	3511 ± 183	1221 ± 78	193 ± 19	4734 ± 373	8794 ± 501	545
7 doses (4)	3970 ± 372	1450 ± 68	229 ± 73	5649 ± 504	10083 ± 698	453
9 doses (4)	3786 ± 134	1334 ± 74	258 ± 40	5933 ± 571	9733 ± 524	343
15 doses (4)	3343 ± 151	1506 ± 46	390 ± 50	5240 ± 134	10456 ± 313	354

Values are expressed as means ± S.E.M.; number of experiments in parentheses.

Table 4. Concentrations of substrates of NAD-linked dehydrogenase systems and redox states in rabbit heart during adriamycin treatment

Treatment	Concentration of metabolites (nmoles/g fresh tissue) [Lactate]	[Pyruvate]	[3-Hydroxybutyrate]	[Acetoacetate]	Metabolite concentration ratio [Lactate]/[pyruvate]	[3-Hydroxybutyrate]/[Acetoacetate]	Calculated [free NAD^+]/[free NADH] ratio Cytoplasm	Mitochondria
Control (5)	6302 ± 1379	67 ± 3	25 ± 5	14 ± 5	94	1·8	96	11
Intermittent treatment								
5 courses (5)	5598 ± 1471	75 ± 8	34 ± 12	12 ± 2	75	2·8	121	7
10 courses (4)	2955 ± 749	27 ± 17	32 ± 11	25 ± 4	109	1·3	82	16
Continuous treatment								
1 dose (3)	4968 ± 300	56 ± 4	31 ± 9	20 ± 7	89	1·6	102	13
7 doses (4)	10131 ± 1431	75 ± 15	244 ± 119	50 ± 10	135	4·9	67	4
9 doses (4)	3692 ± 1011	57 ± 7	121 ± 96	66 ± 15	65	1·8	139	11
15 doses (4)	7527 ± 1165	57 ± 17	100 ± 47	41 ± 12	132	2·4	68	8

Values are expressed as means ± S.E.M.; number of experiments in parentheses.

DISCUSSION

We have considered two types of phenomena: the concentration of adenine nucleotides and metabolites of some dehydrogenase systems *in vivo* on one side, and the oxidative activity of mitochondria isolated from the tissue but eventually tested *in vitro* on the other. While the former data describe the state of the tissue at a given time, the latter rather inform on the oxidative potentialities of the myocardium. Any attempt to understand the biochemical basis of adriamycin cardiotoxicity requires the consideration of both these two orders of facts, that are not immediately comparable but give results which are actually in good agreement and are consistent with the same possible interpretation of adriamycin cardiotoxicity. Only those results which are more relevant to the pathogenesis of adriamycin cardiomyopathy will be discussed here.

One of the most significant changes observed with mitochondria from adriamycin-treated rabbits is the impairment of respiratory control. Studies of mitochondrial metabolism by means of techniques which do not permit the measurement of respiratory control index are likely to miss an important and early indication of mitochondrial damage. Respiratory control index can be modified by changes of one or both terms of the ratio: with the intermittent treatment state 4b respiration is essentially affected, indicating a loosening of the coupling mechanisms, but continuous daily treatment leads also to the impairment in state 3 oxidations, thus indicating a direct damage to the electron transport chain. The ADP/O ratio, a measure of yield in ATP synthesis, is not appreciably affected. This is in a good agreement with the fact that the concentration levels of adenine nucleotides, and of ATP in particular, are unchanged in the hearts of treated rabbits.

Under these conditions the heart can sustain a normal work-load but, due to the reduction of the maximal oxidative capacity of the mitochondria, may not be able to face needs of an increased functional activity: if this occurs myocardial failure will then follow. The biochemical damage seen in mitochondria is more severe during the continuous that the intermittent treatment, though the total dose of adriamycin is lower in the former than in the latter case. This indicates that myocardial cells, given the time, can recover from the damage caused by exposure to adriamycin. This possibility of recovery is further stressed by the complete restoration of mitochondrial oxidation within 2 weeks from the end of a continuous daily treatment carried out for 15 days and resulting in severe impairment of mitochondrial oxidations. Whether this restoration means re-establishment of the functions of the affected mitochondria (intra-mitochondrial recovery), or substitution of the damaged organelles with new ones (intra-cellular recovery) cannot be said on the basis of the present evidence.

It is known that the transfer into the mitochondrial compartment of the reducing equivalents generated in the cytosol is sustained by the so-called 'shuttle mechanisms', due to the essential impermeability to NADH of the mitochondrial membranes [35]. Properly isolated mitochondria from animal cells do not oxidize added NADH *in vitro*. The extra uptake of oxygen by heart mitochondria of adriamycin-treated rabbits in the presence of NADH is the expression of a change of permeability of mitochondria but does not indicate if the change is already present *in vivo*, as a real damage to the membranes, or rather occurs during the isolation of the mitochondria, as a result of the particular lability of these organelles in heart cells exposed to adriamycin. Morphological evidence [12] seems to favor the first possibility. Broadly speaking, the damage to the membranes responsible for the penetration of NADH could also cause an outward flow of molecules—normally localized in the mitochondrial compartment—important for the maintenance of the metabolic equilibrium of the cells. The generalization of the permeability damage to other types of cellular membranes may finally lead to an electrolyte imbalance in the myocardial cell and contribute to the deterioration of cell functions [36]. Permeability to NADH is already pronounced when state 3 is normal and state 4b respiration only slightly affected, such as happens with the intermittent type of treatment, but it cannot be defined as an acute damage since it is still absent 24 hr after the first injection of adriamycin; on the other hand NADH permeability still persists when the other parameters have returned to normal. On the basis of these observations it is possible to suppose that adriamycin may lead to the impairment of mitochondrial functions in two distinct ways, converging on the same target: (i) a direct interference with the respiratory chain—as shown by decrease in state 3 respiration—which on the whole appears later, disappears promptly upon interruption of the treatment and is not particularly prominent in the animals subjected to the intermittent schedule of treatment; inhibition of ubiquinone-enzymes by adriamycin *in vitro* [9] supports this possibility. (ii) an indirect mechanism, which starts with the impairment of nuclear and nucleolar control of protein synthesis and results in mitochondrial damage through interference with enzyme synthesis and membrane biogenesis. Specific mitochondrial proteins such as, for instance, cytochrome *c*, are synthesized and assembled in the endoplasmic reticulum, under the direction of cytoplasmic messengers and later transferred to mitochondria [37]; degradation rates of different proteins within animal cells vary over a wide range and in addition can undergo diverse changes under different physiological conditions [38]; within the same organelle, e.g. the mitochondrion of cardiac muscle, some enzymes turn over much more rapidly than others [39], all these facts reinforce the hypothesis of a restricted mitochondrial autonomy [10]; and are relevant to the understanding of the second possibility. These two mechanisms are not mutually exclusive, on the contrary they can cooperate and result in the onset of adriamycin cardiomyopathy.

Finally, it should be pointed out that severe disturbances of myocardial function may occur before biochemical signs become appreciable, if focal damage to the mitochondria of some cells is diluted by the activity of the more or less intact mitochondria from other parts of the tissue. This localized and inapparent cell damage, however, can trigger important pathophysiological alterations in the context of an integrated system such as the myocardial tissue.

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