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EFFECT OF ADRENOCHROME ON CALCIUM ACCUMULATION BY HEART MITOCHONDRIA*

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Abstract—The effects of adrenochrome (1–100 μg/ml or 5.6×10^{-6} to 5.6×10^{-4} M) on calcium binding, calcium uptake, and ATPase activities of rat heart mitochondria were investigated. Depression, by adrenochrome, of mitochondrial calcium binding and uptake was observed under *in vitro* conditions, whereas mitochondrial ATPase activity was not altered appreciably. The inhibitory effect of adrenochrome on calcium uptake activity was independent of the pH of the incubation medium, but it was dependent upon the dose of drug and the time of incubation. High concentrations of calcium in the incubation medium antagonized the adrenochrome-induced depression. The inhibition of mitochondrial calcium uptake by adrenochrome was of a mixed type. Furthermore, the depressed calcium uptake activity was observed after washing adrenochrome-treated mitochondria with buffer. Significant decreases in calcium accumulating activities were also seen in mitochondria isolated from hearts perfused with various concentrations of adrenochrome (5–50 μg/ml) for 10 min, or with 50 μg/ml adrenochrome for various time periods. Contractile force of the perfused rat heart with various concentrations of adrenochrome (5–50 μg/ml) decreased in a dose-dependent manner. It is suggested that the cardiac contractile failure and myocardial cell necrosis induced by adrenochrome may partly be due to its inhibitory effect on the calcium accumulating ability of mitochondria.

It is now well recognized that calcium movements in the cell play a crucial role in regulating cellular function and metabolism [1]. Subcellular membranous organelles, such as mitochondria, sarcoplasmic reticulum (microsomes), and cell membrane (sarcolemma), are known to participate in the regulation of calcium movements in the cardiac smooth-muscle cell [2–4]. Accordingly, an abnormality in the ability of these membrane systems to accumulate calcium might be expected to produce derangements in cardiac function and metabolism [5]. Since adrenochrome had been shown to produce myocardial contractile failure and cardiac necrosis in the isolated perfused rat heart [6–8], some attempts have been made recently to investigate the mechanisms of the cardiotoxic action of adrenochrome in terms of changes in the biochemical activities of various membrane systems. Adrenochrome, at concentrations of 10–100 μg/ml, was found to decrease the heart sarcolemmal Na^+–K^+ ATPase activity *in vitro*, but it had no effect on the sarcolemmal calcium binding activity [9]. Furthermore, adrenochrome at concentrations of 5–100 μg/ml was observed to decrease microsomal calcium uptake and ATPase activities of the rat heart *in vitro* [10]. In the present study, we report the actions of adrenochrome on mitchondrial calcium-accumulating activities *in vitro*. Although the exact role of mitchondrial ATPase in calcium transport is not clear [4], the effect of adrenochrome on this ATPase activity was studied to examine the nature of the action of this agent on the mitochondrial membrane. The calcium uptake and ATPase activities of mitochondria from hearts perfused with adrenochrome were also examined to determine whether the mitochondrial changes are associated with functional changes in the myocardium. Such a study is considered of interest in view of our previous work that an alteration in the mitochondrial ultrastructure was the earliest abnormality in rat hearts perfused with adrenochrome [8].

MATERIALS AND METHODS

Male, albino Sprague–Dawley rats weighing 300–350 g were decapitated, and the hearts were removed immediately and rinsed thoroughly in a chilled 0.25 M sucrose solution containing 1 mM EDTA at pH 7.0. The ventricles were homogenized with 10 vol. of medium containing 0.18 M KCl, 10 mM EDTA, and 0.5% albumin (pH 7.4). The mitochondrial fraction was prepared by the differential centrifugation method described elsewhere [11], which is essentially similar to that of Sordahl and Schwartz [12]. As described earlier [13], the mitochondrial fraction was found to contain minimal cross contamination with other subcellular organelles. The protein concentration was estimated by the method of Lowry *et al.* [14]. The purified mitochondrial fraction was suspended at a protein concentration of 5–6 mg/ml in 50 mM KCl and 20 mM Tris–HCl (pH 6.8) at 0° [11] and was used within 1 hr of isolation. In two experiments, 100–120 mM KCl and 20 mM Tris–HCl (pH 6.8) buffer was used for suspending mitochondria; no differences in calcium uptake activities were observed.

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Calcium binding and uptake activities of the mitochondrial fraction were determined by the millipore filtration technique [11]. The incubation medium for mitochondrial calcium binding contained 100 mM KCl, 10 mM $MgCl_2$, 4 mM ATP, 0.1 mM $^{45}CaCl_2$, and 20 mM Tris–HCl (pH 6.8), whereas that for calcium uptake also contained both 4 mM KH_2PO_4 and 5 mM succinate. The terms" calcium binding" and "calcium uptake" are used for calcium accumulation in the absence of, and the presence of, permanent ions respectively. The mitochondrial fraction (0.1 to 0.2 mg/ml) was preincubated for 3 min at 25° (calcium binding) or at 37° (calcium uptake); the reaction was started by the addition of $^{45}CaCl_2$ and was terminated by millipore filtration (pore size 0.45 μm). The radioactivity in the protein-free filtrate was counted in a Packard liquid scintillation counter. The experimental conditions for measuring calcium binding and uptake activities of mitochondria were the same as those employed previously for studying the effects of various pharmacological agents [15, 16]. ATPase activity was measured by the method described elsewhere [11]. The incubation medium for the ATPase assay contained 100 mM KCl, 10 mM $MgCl_2$, 4 mM ATP, 0.1 mM $CaCl_2$ and 20 mM Tris–HCl (pH 6.8). After a 3-min preincubation of the mitochondrial fraction (0.1 to 0.2 mg/ml), the reaction was started by the addition of Tris–ATP and terminated after 5 min by the addition of 12% trichloracetic acid. The inorganic phosphate was estimated by the method of Taussky and Shorr [17]. The filtrate was passed through cotton to eliminate interference by the drug in the measurement of optical density of the solution; this process did not give any significant difference in optical values between treated and non-treated samples. The results were statistically analyzed by Student's *t*-test. Adrenochrome was obtained from the Sigma Chemical Co. (St. Louis, MO). In some experiments, adrenochrome was synthesized by the oxidation of epinephrine with silver oxide [18], and the crystallized samples were employed in our studies. The crystallized adrenochrome was found to be 5–10 per cent more potent than the commercial sample.

In another set of experiments, mitochondria were isolated from rat hearts perfused with adrenochrome. For this purpose, the hearts were perfused with oxygenated, modified Krebs–Henseleit solution according to the method described earlier [6]. After a 15-min period of equilibration, the perfusion fluid was changed to the medium containing the desired concentration of adrenochrome. The contractile force (developed tension) was monitored on a Grass polygraph by means of a force-displacement transducer. The hearts were then perfused with normal medium for 1 min, after the desired period of perfusion with adrenochrome, to remove this agent from the vascular system; the hearts were then used for the isolation of mitochondria.

RESULTS

The influence of various concentrations of adrenochrome (1–100 μg/ml or 5.6×10^{-6} to 5.6×10^{-4}M) on mitochondrial ATPase, calcium binding, and calcium uptake activities was examined under *in vitro*

Table 1. *In vitro* effect of adrenochrome on ATPase, calcium binding, and calcium uptake activities of mitochondria isolated from rat hearts*

Conc of adrenochrome (μg/ml)	Calcium-accumulating activities		
	ATPase activity [μmoles $P_i \cdot$(mg protein)$^{-1} \cdot$ min^{-1}]	Calcium binding [nmoles $Ca^{2+} \cdot$(mg protein)$^{-1} \cdot 2$ min^{-1}]	Calcium uptake [nmoles $Ca^{2+} \cdot$(mg protein)$^{-1} \cdot 5$ min^{-1}]
Control	2.63 ± 0.11	30.0 ± 3.8	163.0 ± 7.4
1	2.73 ± 0.17	26.9 ± 2.9	159.7 ± 10.6
5	2.82 ± 0.13	26.6 ± 4.1	146.3 ± 7.4†
10	2.84 ± 0.10	19.1 ± 1.3†	141.3 ± 6.4†
50	2.87 ± 0.12	18.6 ± 1.8†	99.3 ± 5.0†
100	3.01 ± 0.14†	18.5 ± 2.1†	79.6 ± 5.9†

* Each value is the mean ± S.E. of four to five experiments.
† Significantly different ($P < 0.05$) from the control value.

conditions; the results are shown in Table 1. A significant increase in mitochondrial ATPase activity was seen only at a concentration of 100 μg/ml adrenochrome. On the other hand, mitochondrial calcium binding activity was significantly ($P < 0.05$) depressed by 10–100 μg/ml adrenochrome; this inhibitory effect was not dose dependent. Mitochondrial calcium uptake activity was significantly depressed by 5–100 μg/ml adrenochrome, in a dose-dependent manner. Time–course studies of calcium binding and calcium uptake in the presence or absence of 50 μg/ml adrenochrome were done; the results are shown in Figs. 1 and 2 respectively. The calcium binding activity of the mitochondrial fraction in the absence of drug was saturated at 30 min incubation, and the inhibition by adrenochrome of calcium binding activity after different periods of incubation varied between 40 and 50 per cent. Mitochondrial calcium uptake activity was also saturated at 30 min incubation; depression by adrenochrome of calcium uptake activity after different intervals of incubation varied between 30 and 60 per cent.

To determine whether the effect of adrenochrome on mitochondrial calcium uptake activity was reversible, the isolated mitochondrial fraction was incubated with, or without, 50 μg/ml adrenochrome for 5 min at 37° in the same medium as that employed for the calcium uptake assay except that 0.1 mM $CaCl_2$ was absent. The incubation mixture was then centrifuged at 8000 *g* for 10 min. The resulting pellets were washed with 100 mM KCl–20 mM Tris–HCl (pH 6.8) and then centrifuged at 8000 *g* for 10 min. The washing procedure was repeated again, and the final pellets were suspended in 100 mM KCl–20 mM Tris–HCl (pH 6-8) and used for the calcium uptake activity assay. The control calcium uptake activity after the treatment described above was 111.7 ± 7.8 nmoles Ca^{2+}.(mg protein)$^{-1}$.5 min^{-1} (N = 6), which was lower than that of the fresh mitochondrial fraction. The calcium uptake activity of the mitochondrial fraction treated with 50 μg/ml adrenochrome was 45.8 ± 7.8 nmoles Ca^{2+}.(mg protein)$^{-1}$.5 min^{-1} (N = 6) which was 40.9 ± 5.9 per cent of the control value.

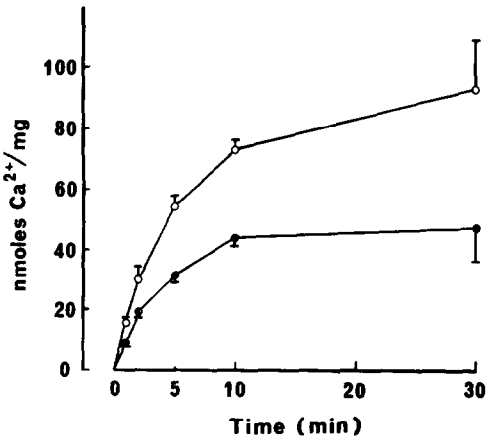

Fig. 1. Time–course of mitochondrial calcium binding activity of rat heart *in vitro* in the presence (●) or absence of (○) of 50 μg/ml adrenochrome. Each value is the mean ± S.E. of six experiments

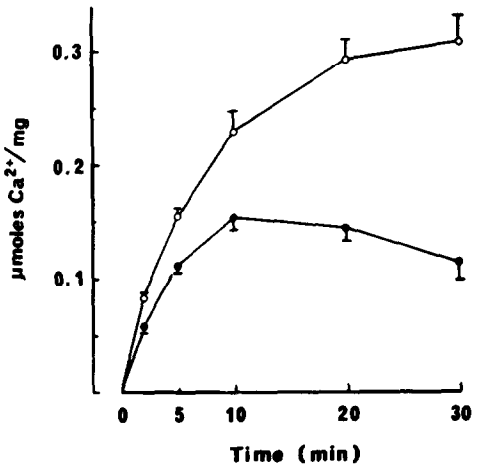

Fig. 2. Time–course of mitochondrial calcium uptake activity of rat heart *in vitro* in the presence (●) or absence (○) of 50 μg/ml adrenochrome. Each value is the mean ± S.E. of six experiments.

To clarify the characteristics of the inhibition of calcium uptake by adrenochrome, mitochondrial calcium uptake activity was measured at various concentrations of $CaCl_2$, various pH values of the incubation medium, and various concentrations of ATP. No precipitation due to calcium phosphate was observed in the incubation medium under the present experimental conditions. Figure 3 shows mitochondrial calcium uptake activities at various concentrations of $CaCl_2$ in the presence, or absence, of 50 μg/ml adrenochrome. The calcium uptake activity at 10 μM $CaCl_2$ in the presence of adrenochrome was inhibited by about 50 per cent of the control values, whereas that at 1 mM $CaCl_2$ (8.4 per cent inhibition) was not significantly depressed. The control calcium uptake activities at various pH values of the incubation medium were not significantly different from each other. Such an insensitivity of the rat heart mitochondrial calcium accumulating ability to

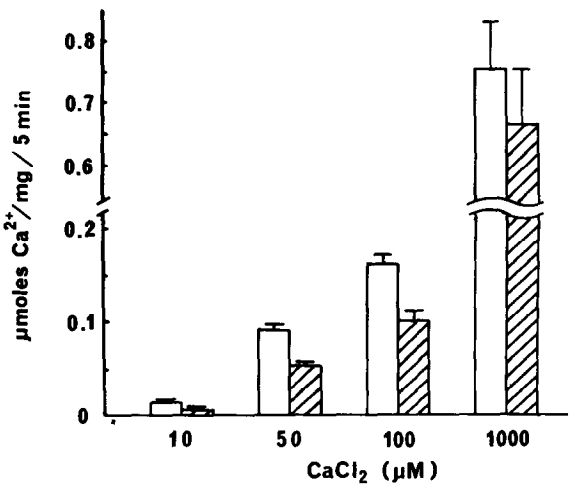

Fig. 3. Mitochondrial calcium uptake activities of rat heart *in vitro* at various concentrations of $CaCl_2$ in the presence (▨) or absence (□) of 50 μg/ml adrenochrome. Each value is the mean ± S.E. of five experiments.

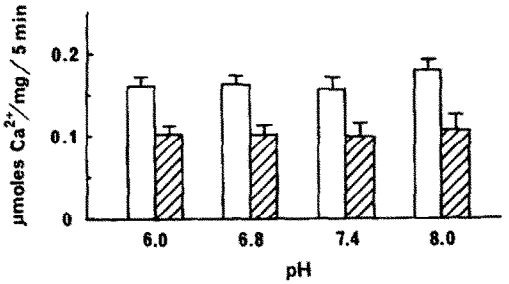

Fig. 4. Mitochondrial calcium uptake activities of rat heart *in vitro* at various pH values of the incubation medium in the presence (▨) or absence (□) of 50 μg/ml adrenochrome. Each value is the mean ± S.E. of five experiments.

changes in pH of the incubation medium is similar to that reported earlier [19]. Adrenochrome depressed the calcium uptake activity to about the same extent (30–40 per cent inhibition) at different pH values of the incubation medium (Fig. 4). Kinetic study of calcium uptake activity at various concentrations of ATP showed changes in both the K_m (from 0.2 to 0.7 mM ATP) and the V_{max} values (from 0.19 to 0.12 μmole Ca^{2+}), indicating that inhibition of mitochondrial calcium uptake by adrenochrome at different concentrations of ATP was of a mixed type (Fig. 5). Since the kinetic studies were not performed by measuring the initial rates of calcium uptake, our data in this regard should be interpreted with some caution.

Contractile force (developed tension) of the rat heart perfused with medium containing various concentrations of adrenochrome was monitored. The contractile force was depressed by adrenochrome in a dose-dependent manner after perfusion for 10 min; a significant decrease in contractile force was observed at a concentration of 10–50 μg/ml adrenochrome (Table 2). These data are in agreement with our results reported previously [8]. Calcium uptake and ATPase activities were also measured in the mitochondrial fraction isolated from hearts perfused with various concentrations of adrenochrome (Table 2). The calcium uptake activity decreased in the presence of 5–50 μg/ml adrenochrome; this inhibitory effect of adrenochrome on calcium uptake activity was dependent upon the concentration of the agent (36–44 per cent inhibition). On the other hand, mitochondrial ATPase activity was not affected by the various concentrations of adrenochrome (Table 2). The contractile force as well as the calcium binding and calcium uptake activities of the mitochondrial fraction isolated from hearts perfused with 50 μg/ml adrenochrome for various periods are given in Table 3. After a 30-min perfusion, the contractile force was depressed to 16 per cent of the control value. The calcium binding and uptake activities were significantly depressed in each of the mitochondrial fractions isolated from hearts perfused for 5–30 min, and they fell to 54 and 63 per cent, respectively of the control value at 30 min of perfusion; those inhibitory effects on the calcium binding and uptake activities were almost independent of perfusion time. Depression of calcium uptake activity in mitochondria isolated from hearts perfused with 50 μg/ml adrenochrome for 10 min was also apparent when calcium uptake activity was measured at different times of incubation as well as at different concentrations of calcium in the incubation medium (Table 4).

DISCUSSION

In the present experiments under *in vitro* conditions, we have shown that mitochondrial calcium binding and uptake activities were depressed by 5–100 μg/ml of adrenochrome, whereas mitochondrial ATPase activity was affected only by high concentrations of adrenochrome (100 μg/ml). Furthermore,

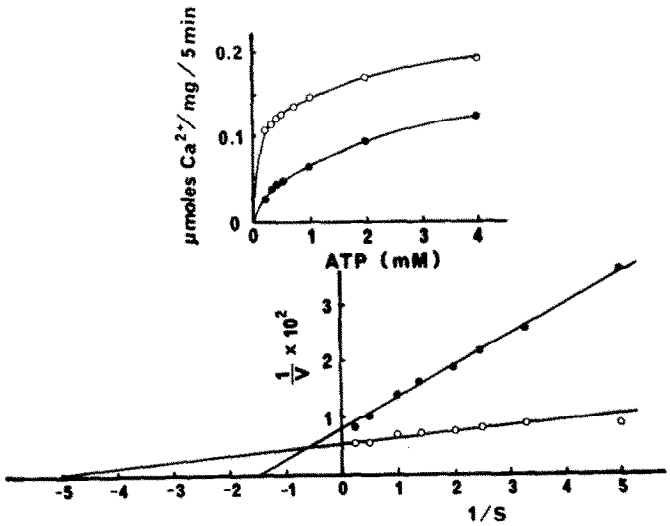

Fig. 5. Mitochondrial calcium uptake activities of rat heart *in vitro* at different concentrations of ATP in the presence (●) or absence (○) of 50 μg/ml adrenochrome. Lineweaver–Burk plots of the data are also shown. The values are typical of four experiments.

Table 2. Contractile force, as well as calcium uptake and ATPase activities, of mitochondria isolated from rat hearts perfused with a medium various concentrations of adrenochrome for 10 min*

Concn of adrenochrome (μg/ml)	Contractile force† (% of control)	Calcium uptake activity [nmoles $Ca^{2+} \cdot (mg\ protein)^{-1} \cdot 2\ min^{-1}$]	ATPase activity [μmoles $P_i \cdot (mg\ protein)^{-1} \cdot min^{-1}$]
Control	100	76.9 ± 7.6	2.58 ± 0.13
5	91 ± 5	49.6 ± 6.0‡	2.55 ± 0.09
10	80 ± 5‡	47.3 ± 9.0‡	2.56 ± 0.08
25	72 ± 4‡	42.8 ± 5.6‡	2.49 ± 0.08
50	62 ± 3‡	44.0 ± 3.0‡	2.68 ± 0.13

* Each value is the mean ± S.E. of experiments
† The control value of contractile force (developed tension) was 15.7 ± 2.0 g.
‡ Significantly different ($P < 0.05$) from the control value

calcium binding activity was decreased significantly by 34–40 per cent by concentrations of 10–100 μg/ml adrenochrome, whereas the calcium uptake activity was depressed in a dose-dependent manner. These results indicate that adrenochrome did not exert a generalized depressant effect upon the mitochondrial membrane. This view is also supported by our finding that the time–course patterns of the inhibitory effects of adrenochrome on calcium binding and calcium uptake activities differ from each other; calcium binding decreased only slightly whereas calcium uptake decreased 2-fold on increasing the incubation time with adrenochrome. The effect of adrenochrome on the mitochondrial membrane seems to be different from the effects of other cardiodepressant agents such as propranolol and quinidine, which have been shown to depress mitochondrial calcium binding, calcium uptake, and ATPase activities under similar conditions [15, 16]. The inhibitory effect of adrenochrome on mitochondrial calcium uptake appears to be somewhat of a mixed type and, in fact, was not readily reversible under the *in vitro* conditions. This may have been due to strong binding of adrenochrome with the mitochondrial membrane as well as to low dissociation constants. Such a remarkable effect of adrenochrome on calcium uptake activity can also be appreciated from the results of the perfusion study which indicate that calcium uptake activity of the mitochondria isolated from hearts perfused with adrenochrome was less than that of the control heart.

Adrenochrome did not decrease mitochondrial calcium uptake activity when a 10^{-3}M concentration of calcium was employed in the assay medium. Although this concentration of calcium is unlikely to be present even under pathological situations, this experiment may provide some evidence that the inhibitory effect of adrenochrome can be antagonized by high concentrations of calcium. The depression of mitochondrial calcium uptake activity by adrenochrome that was observed may have been due to modification of membrane phospholipids since this agent has been shown to stimulate peroxidation of fatty acids [20]. It should also be pointed out that adrenochrome may react rapidly, covalently, and irreversibly with all available thiol groups in the membrane as other quinones do [21].

Whatever the molecular mechanism of adrenochrome action on the mitochondria may be, the present experiments show that adrenochrome can elicit disturbances in the mitochondrial calcium-accumulating ability. Although the exact role of mitochondria in intracellular calcium movements has not been clearly elucidated during excitation-contraction [4], depres-

Table 3. Contractile force, as well as calcium binding and uptake activities, of mitochondria isolated from rat hearts perfused for various periods with a medium containing 50 μg/ml adrenochrome*

Perfusion time (min)	Contractile force† (% of control)	Calcium accumulating activities [nmoles $Ca^{2+} \cdot (mg\ protein)^{-1} \cdot 2\ min^{-1}$]	
		Calcium binding	Calcium uptake
Control	100	33.6 ± 3.9	74.2 ± 6.4
5	60 ± 3*	22.4 ± 2.3‡	51.0 ± 7.1‡
10	59 ± 9*	20.1 ± 3.0‡	46.2 ± 7.1‡
20	21 ± 2*	18.8 ± 2.6‡	50.3 ± 5.3‡
30	16 ± 6*	18.3 ± 2.5‡	47.1 ± 4.8‡

* Each value is the mean ± S.E. of three to six experiments.
† The initial contractile force (developed tension) was 16.1 ± 1.8 g.
‡ Significantly different ($P < 0.05$) from the control value. The control values of calcium binding after 5, 10, 20 and 30 min of perfusion were so similar that they were grouped together (33.6 ± 3.9); the same applies to the control for calcium uptake (74.2 ± 6.4).

Table 4. Calcium uptake activities, at different times of incubation as well as at different concentrations of calcium, of the mitochondrial fraction isolated from hearts perfused with 50 μg/ml adrenochrome for 10 min*

	Calcium uptake activity	
	Control	Adrenochrome
(A) Duration of incubation (min)		
1	35.9	20.8
2	76.8	46.2
5	151.0	121.1
10	215.7	158.7
20	266.2	196.5
(B) Calcium concentration (μM)		
5	3.6	2.1
10	4.6	3.0
25	8.8	5.3
50	22.2	14.2
100	73.7	45.7

* The calcium uptake activities for the experiments on duration of incubation at a concentration of 100 μM $CaCl_2$ (A) are expressed as nmoles Ca^{2+}/mg protein, whereas those for the experiments on calcium concentration (B) are expressed as nmoles $Ca^{2+} \cdot (\text{mg protein})^{-1} \cdot 2\ \text{min}^{-1}$. Each value is the average of two experiments.

sion of mitochondrial calcium uptake may induce intracellular calcium overload which might eventually produce contractile failure and myocardial damage [22, 23]. In this regard, impairment of mitochondrial calcium-transporting ability has been shown to be associated with contractile failure and ultrastructural changes in several types of failing hearts [5]. A mitochondrial lesion, however, is only one of several defects that adrenochrome could cause that might lead to cardiac cell necrosis, since this agent also has been shown to decrease sarcolemmal Na^+–K^+ ATPase and microsomal calcium-accumulating activities of the rat heart [9, 10]. If mitochondrial calcium stores contribute to raising and lowering the concentration of cell calcium, then the observed decrease in mitochondrial calcium uptake activities by adrenochrome might reduce the intracellular stores for calcium that are available for release upon excitation of the myocardium [4]. Such an action of adrenochrome would contribute to an explaination of the negative inotropic effect of adrenochrome. It should be noted however, that the changes in calcium uptake activities of mitochondria isolated from hearts perfused with adrenochrome did not show a clear-cut relation with changes in contractile force. In this regard, the contractile force, unlike mitochondrial calcium uptake activity, decreased in a dose-dependent manner when hearts were perfused with various concentrations of adrenochrome. Furthermore, a progressive decline in contractile force was noted in hearts perfused with 50 μg/ml adrenochrome for 5- to 30-min, whereas mitochondrial calcium-accumulating activities were maximally depressed after 5 min of perfusion. These experiments thus may indicate that the inhibitory effect of adrenochrome on mitochondrial calcium-accumulating activities may be involved, partly in the development of the cardiodepressant action of adrenochrome. The involvement of mitochondrial changes in the cardiotoxic effects of adrenochrome is also evident from our observations that various cations and pharmacologic agents, which reduced the cardiodepressant action of adrenochrome, also decreased the adrenochrome-induced alterations in the mitochondrial ultrastructure in the isolated perfused rat heart [6, 7].

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