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INHIBITION OF THE MITOCHONDRIAL RESPIRATORY CHAIN IN ISOLATED ATRIA—A COMPARISON OF ROTENONE AND AMYTAL*

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Abstract—The functional and metabolic effects of amytal and rotenone, two inhibitors of the oxidation of reduced nicotinamide dinucleotide (NADH) by the mitochondrial respiratory chain, have been studied in the isolated atrium of the guinea pig. Rotenone in concentrations of 1–8 μM produces a negative inotropic and chronotropic response which develops gradually over 60 min. At the highest dose, contractile tension is depressed about 50 per cent while the frequency is decreased by about 60 per cent after 60 min of exposure to rotenone. A further, 20–30 per cent of the total atrial ubiquinone (UQ) was found in the oxidized form in the rotenone-treated atria at all dose levels, reflecting inhibition of NADH oxidation. The amounts of lactate were elevated to 200 per cent of control levels in atria exposed to 1 and 4 μM rotenone. This is a further indication of the blockade of NADH oxidation. Energy balance was adversely affected as shown by 20 and 30 per cent decreases in the creatine phosphate level and in the ratio of ATP to ADP.

In contrast, amytal produced no demonstrable effects on UQ redox status, lactate and pyruvate levels, or energy balance after 60 min at a concentration of 0·1 mM, but did depress contractility by about 20 per cent. The depression developed more rapidly with amytal than with rotenone. Frequency was not affected.

At 2·5 mM amytal, contractility was completely abolished within 10 min and the UQ redox status was shifted by about 23 per cent to the oxidized side, a shift comparable to that seen with all doses of rotenone. The ratio of lactate to pyruvate was increased 350 per cent above control levels. Energy balance was severely affected even in the absence of any contractile activity, as indicated by a 70 per cent decrease in creatine phosphate levels and a 54 per cent decrease in the ATP/ADP quotient.

It is concluded that amytal, at concentrations below 0·5 mM, depresses atrial contractility independent of any effect on the mitochondrial respiratory chain. At concentrations between 0·5 and 2·5 mM, the blockade of NADH oxidation in the atrial mitochondria can be demonstrated and may play a role in the functional depression. At the highest dose, additional inhibition of mitochondrial energy transfer reactions contributes to the negative energy balance over and above what might be expected on the basis of inhibition of electron transport alone.

IN BIOCHEMICAL studies, rotenone and amytal have been utilized to study and characterize mitochondrial function, since they inhibit respiration catalyzed by the terminal respiratory chain. Both substances block reduced nicotinamide dinucleotide (NADH)

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oxidation in such preparations, while succinate oxidation is unaffected except in the case of amytal at much higher concentrations.[1] While rotenone acts solely to inhibit electron transport,[2] amytal has been shown to have an additional effect at the level of the energy transfer reactions involved in coupled oxidative phosphorylation.[1] For maximal effects in mitochondria, micromolar concentrations of rotenone are sufficient while millimolar concentrations of amytal are required.

Pharmacologically, rotenone has been shown to be a powerful toxic agent when administered parenterally to a variety of species.[3] In the rabbit, rotenone produces a marked fall in blood pressure and respiratory failure. The isolated atria of this species showed negative inotropic and chronotropic responses to rotenone in concentrations of 10^{-8}–10^{-9} g/ml.[3]

The barbiturate, amytal, produces negative inotropic effects on the myocardium. This effect can also be seen *in vivo*, where parenteral barbiturate administration frequently produces a fall in blood pressure.

The relationship between the pharmacological effects of rotenone and amytal on the myocardium and the biochemical effects which are demonstrable in isolated mitochondria has not been thoroughly characterized. The present study compares the pharmacological response of isolated guinea pig atria to rotenone and amytal with changes in biochemical parameters associated with cellular respiration and energy balance in these atria.

The redox status of ubiquinone (UQ) in the isolated atrium is a useful measure of inhibition of the respiratory chain in the mitochondria of this tissue by rotenone and amytal because of the position of UQ between the site of inhibition (NADH oxidation) and oxygen. Decreases in the delivery of reducing equivalents from NADH as the result of the inhibition of its oxidation are detected as increases in the amount of UQ in the oxidized state. A previous investigation has shown that the UQ redox status of the isolated atrium responds in this way to inhibitors of mitochondrial respiration such as malonate and amytal.[4] In addition, changes in the steady state levels of lactate and pyruvate have been used as a reflection of changes in the NADH to NAD ratio. Energy balance has been evaluated from changes in the steady state levels of creatine phosphate (CP), the adenine nucleotides, ATP, ADP and AMP in atria exposed to these inhibitors.

METHODS

Isolated guinea pig atria. Male guinea pigs weighing between 400 and 500 g maintained on Rockland guinea pig diet were used. Isolated atria were prepared as described by Schmid and Siess.[5] Water-jacketed organ baths (vol., 30 ml) were maintained at 30° by a constant temperature circulator. The bath medium, continuously aerated with 100% O_2, was of the following composition: NaCl, 138 mM; KCl, 2·7 mM; $CaCl_2$, 1·8 mM, $MgCl_2$, 1·1 mM; Na_2HPO_4, 5·0 mM; NaH_2PO_4, 1·7 mM; glucose, 16·7 mM.

The pH of all solutions was adjusted to 7·1 with a glass electrode. Contractions against a spring (1 g tension = 0·17 mm displacement) coupled to a photoelectric transducer were amplified and recorded on a physiograph (E & M Instrument Company, Houston, Texas). Atria were placed under 1 g of resting tension.

Atria were allowed a 60-min recovery period, at which time the bath medium was

siphoned off and replaced with the appropriate concentration of amytal as the sodium salt in the bathing medium.

In other atria, rotenone was added from stock solutions in ethanol after the 60-min equilibration period. All concentrations are expressed in terms of the final bath concentration.

After exposure of the atrium to the inhibitor for the appropriate time period (30 and 60 min), it was rapidly raised from the bath and, without blotting, frozen between the blades of a pair of forceps precooled in liquid nitrogen, and then plunged into liquid nitrogen (freeze-stop). The atria were then pulverized in a $-10°$ cold room as previously described.[6] The frozen tissue tablet was then used for the determination of UQ content and redox status or for tissue levels of ATP, ADP, AMP, CP and lactate and pyruvate.

Reagents. Reagents were obtained from the following sources: sodium amytal, E. I. Lilly, Indianapolis; rotenone, S. B. Penick & Company, New York; enzymes and substrates, Sigma Chemical Company, St. Louis, Mo. and Boehringer Co., Mannheim, West Germany. All other reagents were of the best commercial quality available.

Determination of the total level and redox status of UQ. UQ redox status and total levels of UQ were determined as previously described,[6] with the following modifications. The petroleum ether and methanol (containing pyrogallol) were added simultaneously as a mixture to the frozen atrial powder in a glass homogenizer maintained at $-78°$ rather than carrying out the homogenization of the muscle powder in methanol alone at low temperature followed by UQ extraction into petroleum ether at 4°. UQ is found in both control and metabolically inhibited atria to be about 13 per cent more oxidized with such a "simultaneous" extraction procedure than was found with the previously used "separate" extraction (unpublished observations). This higher degree of oxidation is presumably the result of better preservation of the redox status.

Because of its lipid solubility, the possible interference by rotenone in the UQ assay was investigated. It was established that it did not interfere in the concentrations used in these experiments.

Protein determination. The tissue debris was washed with 8·0 ml of 50% ethanol (v/v) and suspended in 1·0 ml of 0·2 N NaOH. The suspension was stirred overnight with a magnetic stirrer. When the debris was dissolved, protein was determined with the biuret reagent as modified for turbid extracts by Szarkowska and Klingenberg.[7] Bovine serum albumin served as a standard.

Extraction of lactate, pyruvate, adenine nucleotides and creatine phosphate. Two ml of 0·6 M $HClO_4$ was added to the frozen atrial tablet in a 10-ml polyallomar centrifuge tube which contained a small amount of liquid nitrogen and was surrounded by crushed ice. The tablet was then finely crushed with a glass rod. After deproteinization and extraction, the mixture was centrifuged at a speed of 8000 *g* for 10 min in a refrigerated Spinco ultracentrifuge. The supernatant was decanted and neutralized with 5 M K_2CO_3 to pH 3·5 to 6·5 using methyl orange (0·05%, w/v) as an indicator. The tissue debris from centrifugation was saved for protein determination. The neutralized extract was allowed to stand in an ice bath for 30 min, then was centrifuged and the supernatant pipetted off from the precipitated $KClO_4$. The amount of the extract from one atrium was sufficient for the enzymatic analysis coupled to NAD

oxidation or reduction of lactate, pyruvate, AMP, ADP, ATP and CP. The content of each component was obtained by comparison with a standard curve in the concentration range expected in the atria.

Lactate was determined enzymatically on a 0·2-ml aliquot by the method of Hohorst.[8] Pyruvate, AMP and ADP were determined enzymatically on a 0·5-ml aliquot by the method of Adam.[9] ATP and CP were analyzed on a 0·1-ml aliquot by the enzymatic method of Adam.[10] NAD oxidation or reduction was followed with a Phoenix dual wavelength spectrophotometer to increase the sensitivity by utilizing the wavelength pair 340–400 mμ.[11]

The statistical significance of the results was determined by means of a two-tailed Student *t*-test.

RESULTS

Atrial function. When atria were allowed to contract for 60 min in the bath, they showed a change in developed tension and frequency of less than 5 per cent during the subsequent 60 min of incubation. Therefore, drug-induced changes in contractility and frequency are expressed as percentages of their values after the 60-min equilibration period.

In atria which were exposed to rotenone in concentrations of 1–8 μM, a gradual negative inotropic and chronotropic effect developed. A steady state with regard to strength and frequency of contraction was established within 40–60 min after rotenone addition. At 60 min, the depression of contractility and frequency was related to the rotenone concentration (Table 1). The ethanol vehicle in which the rotenone was

TABLE 1. EFFECT OF ROTENONE ON ATRIAL FUNCTION AND UQ REDOX STATUS

Bath concn	% Initial contractile amplitude*	% Initial frequency	% UQ_{ox}	UQ_{total} (nmoles/mg protein)
0·1% Ethanol (v/v)	76·3 ± 1·6 (4)	99 ± 9.1		
0·2% Ethanol (v/v)	70·6 ± 3·4 (7)	95·8 ± 2·4	42·0 (2)	1·25 (2)
0·4% Ethanol (v/v)	67·7 ± 5·0 (11)	96·2 ± 5·0	44·0 ± 2·9 (5)	1·42 ± 0·13 (5)
1 μM Rotenone (0·1% ethanol)	50·4 ± 3·0 (12)	61·7 ± 3·8	61·8 ± 4·4 (6)	1·03 ± 0·06 (6)
2 μM Rotenone (0·2% ethanol)	40·8 ± 2·9 (8)	60·1 ± 2·5	63·7 ± 4·6 (8)	1·26 ± 0·06 (7)
4 μM Rotenone (0·4% ethanol)	31·9 ± 3·2 (16)	52·1 ± 4·3	71·6 ± 4·5 (6)	1·06 ± 0·07 (6)
8 μM Rotenone (0·4% ethanol)	18·6 ± 3·2 (6)	37·7 ± 2·7	67·3 ± 4·9 (4)	1·30 ± 0·11 (5)

* $\frac{\text{Contractile tension at 60 min after addition of ethanol or rotenone in ethanol}}{\text{Contractile tension just prior to addition of ethanol or rotenone}} \times 100$. All atria were allowed to equilibrate for 60 min at 30° in phosphate-buffered Tyrode solution containing 16·7 mM glucose. Rotenone was then added from an ethanolic solution to give the final bath concentrations of rotenone and ethanol. To control for the effects of the vehicle, ethanol alone was added to other atria. After 60 min of exposure, the atria were freeze-stopped and the UQ redox status was determined.

added contributed to the myocardial depression as indicated by decreases of 24–32 per cent in contractile strength in control atria exposed to 0·1–0·4% ethanol. The frequency of spontaneous contractions was not affected by ethanol. The highest concentration of rotenone tested (8 μM in 0·4% ethanol) reduced contractile tension to 18·6 per cent of the predrug level. Since 0·4% ethanol, itself, produced a 32·3 per cent decrease in developed tension that portion of the negative inotropic effect attributable

to rotenone at this concentration is approximately 50 per cent of the initial amplitude. A 62 per cent decrease in the frequency of spontaneous contractions occurred with 8 μM rotenone, while the ethanol vehicle had virtually no effect.

In four experiments in which contractile frequency was held constant at 180/min by electrical stimulation (bipolar electrodes, 2 m sec duration, supramaximal voltage) over the 2-hr experimental period, 4 μM rotenone reduced contractile tension to 22·2 $\pm$ 8·4 per cent of the predrug level in 60 min compared to 31·9 $\pm$ 3·2 per cent of the predrug level for spontaneously beating atria. UQ redox status was 71·5 $\pm$ 1·1 per cent in these stimulated atria treated with rotenone and hence not different from that observed in the spontaneously contracting atria.

A more rapid depression of contractility to new steady state levels was associated with exposure of the atria to amytal. Ten to 20 min were required for the two lower concentrations tested (0·1 and 0·5 mM), while 2·5 mM amytal essentially abolished contractility within 10 min. After 60 min, the degree of depression was dependent upon the amytal concentration (Table 2). Frequency was not significantly changed after 60 min of exposure, with the exception of the highest dose, where it could not be measured.

TABLE 2. EFFECT OF AMYTAL ON ATRIAL FUNCTION AND UQ REDOX STATUS

Bath concn (mM)	% Contractile amplitude*	% UQ_{ox}	UQ_{total} (nmoles/mg protein)
0	100 (28)	40·0 $\pm$ 4·5 (13)	1·12 $\pm$ 0·26 (14)
0·1	78·0 $\pm$ 6·9 (14)	35·7 $\pm$ 5·2 (4)	1·30 $\pm$ 0·14 (4)
0·5	38·9 $\pm$ 4·4 (13)	51·8 $\pm$ 6·0 (9)	1·23 $\pm$ 0·13 (9)
2·5	0	63·4 $\pm$ 2·6 (6)	1·35 $\pm$ 0·10 (6)

* $\frac{\text{Contractile tension at 60 min after changing to amytal medium.}}{\text{Contractile tension just prior to changing to amytal medium}}$ All atria were allowed to equilibrate for 60 min at 30° in phosphate-buffered Tyrode solution containing 16·7 mM glucose. The bath medium was then changed to one containing 0, 0·1, 0·5 or 2·5 mM sodium amytal. After an additional 60 min, the atria were freeze-stopped and the UQ redox status was determined.

UQ redox status. Forty per cent of the atrial UQ was found to be present in the oxidized form in the control atria allowed to contract for 2 hr in the bath (Table 2). This is identical to the value we have previously found using the modified methanol–petroleum ether extraction to preserve the redox status of UQ (unpublished observations). Ethanol, in the concentration used for a vehicle, did not significantly affect the UQ redox status (Table 1). After 60 min of exposure to rotenone, the atrial UQ redox status was significantly shifted to the oxidized side with all doses studied from that found in the ethanol-treated atria (Table 1). The magnitude of the effect was not markedly dependent on rotenone concentration in that an additional 20 per cent of the total UQ was oxidized in the presence of 1 or 2 μM rotenone and 24–27 per cent with 4 and 8 μM rotenone. The maximum level of UQ oxidation attained was 72 per cent of the total UQ as UQ_{ox} in those atria treated with 4 μM rotenone. Total levels of UQ also varied considerably between the various experimental groups, but this variation did not appear to be related to the presence or absence of the drug.

The UQ redox status of atria exposed to 0·1 mM amytal was about 36 per cent UQ_{ox} (Table 2). This value did not differ significantly from control values. With

0·5 mM amytal, the results are equivocal. The mean value for the percentage UQ_{ox} is 52 per cent, which represents almost an additional 12 per cent of the UQ in the oxidized form when compared to that found in the control atria. However, the variance is such that the difference does not prove to be statistically significant. In fact, four of the nine atria showed percentages of UQ_{ox} greater than the mean value of the control atria. There was no apparent reason for excluding those atria from the experimental group. Amytal, 2·5 mM, was associated with a significant shift in UQ redox status of 23·4 per cent, approximately that seen at all doses of rotenone.

Energy balance. Energy balance was adversely affected at both concentrations of rotenone tested (1 and 4 μM; Table 3). A significant decrease in creatine phosphate

TABLE 3. EFFECT OF VARYING CONCENTRATIONS OF ROTENONE ON ATRIAL ENERGY BALANCE*

	0·4% (v/v) Ethanol	1 μM Rotenone	4 μM Rotenone
CP	58·2 ± 4·5 (6)	43·6 ± 3·7 (6)	46·2 ± 7·5 (10)
ATP	31·4 ± 1·0 (6)	28·8 ± 1·2 (6)	27·5 ± 0·82 (10)
ADP	4·92 ± 0·34 (6)	6·09 ± 0·36 (6)	6·41 ± 0·36 (10)
AMP	2·64 ± 0·36 (6)	2·27 ± 0·09 (6)	2·55 ± 0·08 (10)
Q $\frac{ATP}{ADP}$	6·09 ± 0·24 (6)	4·70 ± 0·15 (6)	4·26 ± 0·17 (10)
Lactate	18·2 ± 4·0 (6)	41·5 ± 7·0 (6)	41·9 ± 3·0 (10)
Pyruvate	0·95 ± 0·11 (6)	0·77 ± 0·11 (6)	0·67 ± 0·04 (10)
Q $\frac{lactate}{pyruvate}$	22·4 ± 4·4 (6)	67·3 ± 1·3 (6)	70·8 ± 4·6 (10)

* All atria were allowed to equilibrate for 60 min at 30° in phosphate-buffered Tyrode solution containing 16·7 mM glucose. Rotenone was then added from an ethanolic solution to give the final bath concentration or ethanol alone was added. After 60 min of exposure, the atria were freeze-stopped and the levels of organic phosphates and substrates were determined; these levels are expressed as nmoles/mg protein.

level of about 25 per cent could be demonstrated at the lower dose. The decrease in ATP level and increase in ADP level were not significant when considered separately at the 1 μM rotenone concentration. However, when the rotenone concentration was increased to 4 μM, the decreased ATP level and increased ADP level were now statistically significant. The quotient ATP/ADP, which might be considered a more sensitive measure of reciprocal changes in their levels, was significantly different from that of ethanol-treated controls at both dose levels. In addition, the lactate level was markedly elevated and as a consequence the ratio of lactate to pyruvate was increased in atria treated with 1 μM rotenone.

Increasing the rotenone concentration to 4 μM produced little additional change in the metabolic parameters measured. Strangely, the mean creatine phosphate level was not further depressed as the rotenone concentration was increased to 4 μM and indeed the variance around the mean value was great enough to preclude the conclusion that it differed from the mean value of the ethanol-treated control atria. No further increase in the lactate level or in the lactate/pyruvate ratio could be demonstrated with the increase in the rotenone concentration from 1 to 4 μM. No effect of the inhibitor was observed on AMP levels at either dose level.

The effects of varying concentrations of amytal on atrial energy balance are shown in Table 4. In the case of this drug, no significant changes in the levels of creatine

phosphate, adenine nucleotides or the substrates, lactate and pyruvate, were detected at the two lower dose levels of 0·1 and 0·5 mM. Again, as in the case of the UQ redox status, while the mean CP level is depressed at 0·5 mM amytal, the wide individual variation between atria renders the difference statistically insignificant. However, at the highest dose studied, 2·5 mM, amytal led to large decreases in creatine phosphate and ATP levels of 70 and 17 per cent respectively, which were accompanied by increases of 40 per cent in ADP levels and 70 per cent in AMP levels. A very large

TABLE 4. EFFECT OF VARIOUS CONCENTRATIONS OF AMYTAL ON ATRIAL ENERGY BALANCE*

	0 mM	(N)	0·1 mM	(N)	0·5 mM	(N)	2·5 mM	(N)
Creatine phosphate	73·2 ± 5·6	14	76·5 ± 6·6	10	60·3 ± 2·4	4	22·2 ± 4·6	4
ATP	32·3 ± 1·2	14	35·8 ± 2·3	10	31·0 ± 1·8	4	22·1 ± 1·9	4
ADP	4·74 ± 0·28	14	4·30 ± 0·24	10	4·43 ± 0·21	4	6·67 ± 0·63	4
AMP	1·60 ± 0·23	14	0·82 ± 0·20	9	0·83 ± 0·12	4	2·73 ± 0·42	4
Q $\frac{ATP}{ADP}$	7·21 ± 0·56	14	8·37 ± 0·47	10	7·01 ± 0·42	4	3·32 ± 0·18	4
Lactate	18·1 ± 3·4	12	17·3 ± 5·5	10	21·8 ± 8·9	4	84·1 ± 8·7	4
Pyruvate	0·85 ± 0·10	12	1·00 ± 0·11	10	0·78 ± 0·14	4	1·14 ± 0·23	4
Q $\frac{\text{lactate}}{\text{pyruvate}}$	17·4 ± 3·5	12	15·8 ± 2·8	10	25·4 ± 6·5	4	77·8 ± 9·4	4

* All atria were allowed to equilibrate for 60 min at 30° in phosphate-buffered Tyrode solution containing 16·7 mM glucose. The bath medium was then changed to one containing 0, 0·1, 0·5 or 2·5 mM sodium amytal. After an additional 60 min, the atria were freeze-stopped and the levels of organic phosphates and substrates were determined; these levels are expressed as nmoles/mg protein.

increase in the lactate level was also noted in these atria. These changes after 60 min of exposure to the drug are 1·5 to 2 times larger on a percentage basis than those previously found in atria exposed to this concentration of amytal for 30 min.[4]

DISCUSSION

For purposes of comparison, the important changes in atrial function and measured biochemical parameters have been summarized in Table 5. Ernster *et al.*[2] showed that rotenone inhibits the aerobic oxidation of pyridine nucleotide-linked substrates but not that of succinate in isolated mitochondria. It is an exceedingly potent inhibitor and as little as 25 nmoles per g of mitochondrial protein is required for complete inhibition. This study also showed that only a small amount of rotenone is needed to obtain an observable inhibition in relation to the amount required for complete inhibition. The present results obtained in the isolated atria are compatible with these observations. Concentrations of 1–8 μM (corresponding to quantities 30–240 nmoles in the 30-ml muscle bath) presumably would serve to inhibit completely NADH-linked oxidations in the terminal respiratory chain of the atrial mitochondria, assuming access of the inhibitor to the mitochondria. Such inhibition would be expected to result in larger amounts of UQ being present in the oxidized form as the result of the inhibition of the delivery of reducing equivalents to UQ from NADH. We find further increments of 19–28 per cent of the total UQ in the oxidized form in atria exposed to these concentrations of rotenone (Table 5). Furthermore, the effect of 8 μM rotenone on the UQ redox status is no greater, but in fact somewhat

less, than that of 4 μM rotenone. The massive increase in the lactate levels of these atria most likely reflects the elevated NADH levels produced by the rotenone blockade of its oxidation by the terminal respiratory chain. Heart muscle possesses an extremely active lactate dehydrogenase which would utilize the NADH to reduce pyruvate and consequently increase lactate levels. The elevated lactate levels together with the observation that pyruvate levels are not affected are also suggestive of accelerated aerobic glycolysis. Inhibition of NADH oxidation by the respiratory chain results in an energy deficit which is reflected in the elevated ADP and decreased

TABLE 5. COMPARISON OF PERCENTAGE CHANGES IN ATRIAL FUNCTIONAL AND BIOCHEMICAL PARAMETERS INDUCED BY AMYTAL AND ROTENONE

	Amytal		
	(0·1 mM)	(0·5 mM)	(2·5 mM)
Amplitude	−22	−61	−100
Δ % UQ_{ox}*	NS†	+11·7 P>0·05	+23·4 P<0·05
Creatine phosphate level	NS	−17·6 P>0·05	−69·7 P<0·001
Q ATP/ADP	NS	NS	−54·0 P<0·001
Q lactate/pyruvate	NS	+46 P>0·05	+347 P<0·001

	Rotenone			
	(1 μM)	(2 μM)	(4 μM)	(8 μM)
Amplitude	−26	−30	−36	−51
Frequency	−38	−40	−48	−62
Δ % UQ_{ox}*	+19·8 P<0·01	+21·7 P<0·01	+27·6 P<0·01	+23·3 P<0·01
Creatine phosphate level	−25 P<0·05		−21 P>0·05	
Q ATP/ADP	−23 P<0·001		−30 P<0·001	
Q lactate/pyruvate	+200 P<0·001		+216 P<0·001	

* Δ % UQ_{ox} is the difference from control values of the percentage of the total atrial UQ found in the oxidized form at the various concentrations of inhibitor.

† P values were obtained from a two-tailed *t*-test of significance; NS = P>0·05. Three "non-significant" differences are included under 0·5 mM amytal for reasons discussed in the text.

ATP levels. Such changes would be expected to increase aerobic glycolysis, considering current concepts of metabolic control of this multienzyme system.

Accompanying the oxidation of the quinone, there is a negative energy balance in the atria as indicated by the 20–30 per cent decreases in creatine phosphate levels and in the ATP/ADP ratio. As in the case of the quinone, there is no difference in the degree to which these metabolic parameters are affected by an increase in the rotenone concentration in the muscle bath from 1 to 4 μM (Table 5).

A depression of the frequency and developed contractile tension occurred in atria treated with rotenone concomitant with the changes in mitochondrial metabolism. Part of the mechanical effect was attributable to the ethanol used as vehicle. When the decreases seen in the ethanol-treated controls are added to the mean values for the appropriate rotenone-treated atria, then contractile amplitude is depressed from 26 to 51 per cent and frequency from 38 to 62 per cent with increasing doses of rotenone (Table 5). It is unlikely that the fall in developed tension is attributable to the decrease in the frequency of contractions,[12] since a slightly greater depression of contractility to 22 per cent of the predrug level was observed in atria treated with 4 μM rotenone and

driven at a constant rate by electrical stimulation, compared to spontaneously beating atria in which developed tension was reduced to only 32 per cent of the predrug level at this dose. As with the biochemical parameters, increasing the concentration to 2 or 4 μM contributes little additional depression to that present at 1 μM. The similar behavior toward rotenone of the mechanical activity of the atrium and the biochemically measured changes in atrial mitochondrial activity suggest that the myocardial depression is related to the relative energy deficit produced by the drug-induced blockade of NADH oxidation in the atrial mitochondria. The energy deficit is the result of the decreased flux of electrons through the three coupling sites in the NADH-linked terminal respiratory chain, with a consequent decrease in the phosphorylation of ADP to ATP in the presence of the continuing utilization of ATP for contractile activity and other energy-dependent processes of the atrium. Additional factors may be involved in the greater degree of myocardial depression seen with 8 μM rotenone than those attributable to the drug's effect on the mitochondria, since UQ redox status is not further shifted at this dose.

Amytal is a much less potent inhibitor of NAD-linked oxidation than is rotenone in isolated mitochondria. Half-maximal effects are generally observed at a concentration of 1 mM.[1] However, not only does amytal inhibit electron flow between NADH and UQ, but it affects energy transfer reactions involved in the phosphorylation of ADP to ATP, which are coupled to electron transport.[1]

In our experiments, a significant shift in the UQ redox status, such as might be expected to result from inhibition of mitochondrial NADH oxidation, was observed in atria treated with 2·5 mM amytal for 60 min. An additional 23 per cent of the quinone was present in the oxidized form (Table 5). This is of the same order of magnitude as the shift seen for all four concentrations of rotenone studied. The 350 per cent increase in the lactate/pyruvate ratio is additional evidence of the inhibition of NADH oxidation. However, energy balance is much more severely affected, as shown by the 70 per cent decrease in creatine phosphate level and the 54 per cent decrease in the ATP/ADP ratio. These more severe changes in phosphate potential with the high dose of amytal as compared to rotenone may be a reflection of its additional properties of inhibiting energy transfer reactions, properties not shared by rotenone.

The evidence is not definitive as to the effects of amytal on the atrial mitochondrial respiratory chain at an extracellular concentration of 0·5 mM. The mean values reflect a tendency to UQ oxidation and decreased creatine phosphate levels (Table 5). However, the variance between individual atria was such that significant differences from the control values were not obtained. This may be a reflection of the fact that this concentration is at the threshold for the inhibition of the mitochondrial respiratory chain. Chance *et al.*,[13] using direct fluorometric recording of NADH levels in the isolated perfused rat heart, found that half-maximal NAD reduction could be induced by 1 mM amytal; 3 mM amytal was required for complete reduction. Hence, significant changes in UQ redox status at 0·5 mM may be difficult to establish in the isolated atria.

No differences were demonstrated in the atrial UQ redox status, the phosphate potential (as reflected in the steady state levels of creatine phosphate and the ATP/ADP ratio) or the lactate/pyruvate ratio at a bath concentration of 0·1 mM amytal. Rotenone, 1 μM, which depresses contractility and frequency to about the same

extent as does 0·1 mM amytal (approx. 25 per cent), results in significant changes in the UQ redox status, phosphate potential and lactate/pyruvate ratio, indicating that it is affecting mitochondrial respiration at the NADH site. On the contrary, 0·1 mM amytal produces a depressant effect on atrial function which is not accompanied by a demonstrable effect attributable to inhibition of mitochondrial NADH-linked oxidations with a concomitant decrease in coupled phosphorylation. While the data at 0·5 mM amytal are not as clearcut, here also the depression of contractility seems to be independent of significant changes in mitochondrial function. Thus, it appears unlikely that the negative inotropic effect of amytal at low concentrations is explicable on the basis of its effects on the atrial mitochondria. Shibita and Hollander[14] have demonstrated changes in the action potential of rat atria with this concentration of amytal. Developed tension was depressed 16 per cent, which is in good agreement with the 22 per cent reduction observed in our experiments. They suggest the possibility that these changes may reflect an interference by amytal in energy-utilizing reactions at the cell membrane. An inhibition by amytal of calcium uptake into cardiac vesicular fractions *in vitro* has also been demonstrated.[15] Amytal, 4·5–6 mM, was required for consistent inhibition at 22°, although the authors mention that a 1 mM concentration produced inhibition at 37° in a preliminary investigation.

However, at the higher concentration of amytal, the evidence indicates that amytal does inhibit mitochondrial metabolism by blocking NADH oxidation, and most probably also energy transfer reactions as well, as evidenced by the large changes in CP and adenine nucleotides. If one assumes that the depressant effect of rotenone is solely due to its effects on mitochondrial metabolism, then it would appear that the maximum inhibition of NAD-linked oxidation is associated with a 50 per cent reduction in atrial frequency and developed tension. Yet, amytal at 2·5 mM produces a shift in UQ redox status which is comparable to that induced by 1–2 μM rotenone and, with amytal, contractility is completely abolished within 10 min of exposure. The changes in phosphorylation potential are more severe than those seen in atria treated with rotenone, which are still contracting throughout the 60-min period, while the atria treated with amytal are noncontractile from 10 min to the time of freeze-stop. The changes are also progressive with time, since they are more severe at 60 min than at 30 min of drug exposure. While energy-requiring processes such as ion transport are probably continuing in the quiescent amytal-treated atria, leading in part to the severe negative energy balance in the absence of any contractile activity, an inhibition of the energy transfer reactions involved in the phosphorylation coupled to the remaining electron transport activity may also be involved.

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