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Exercise-induced alterations of hepatic mitochondrial function

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In order to examine the effect of a single bout of exercise on hepatic mitochondrial function, starved untrained male rats swam at 34–35°C with a tail weight (5% of body wt.) for 100 min. The rates of ADP-stimulated and uncoupled respiration were higher in the mitochondria isolated from the exercised rats regardless of the substrate utilized. Succinate-linked Ca^{2+} uptake was 48% greater in the exercised group; however, Ca^{2+} efflux was markedly depressed. The inhibition of Ca^{2+} uptake by Mg^{2+} was higher in the control group, so that the difference in Ca^{2+} uptake between the two groups was greater in the presence of Mg^{2+} than in its absence. The response of phosphorylating respiration and Ca^{2+} fluxes to exogenous phosphate and the pH of the assay medium differed in the exercise group. These observations with the exercised group were not related to non-specific stress. The exercise-induced mitochondrial-functional alterations are reminiscent of those obtained from mitochondria isolated from glucagon- or catecholamine-treated sedentary rats. Thus, adrenergic stimulation as well as other factors may be operating during exercise, leading to an alteration of mitochondrial function *in vitro*.

During endurance-type exercise, glucose output from the liver is precisely regulated to match the peripheral utilization, so that glucose homoeostasis is maintained under ordinary circumstances. Hepatic glucose production occurs primarily through glycogenolysis, although gluconeogenesis may play an important role as the hepatic glycogen stores become progressively depleted (Wahren *et al.*, 1971). The regulation of hepatic glucose production during exercise is a multifactorial process including hormonal regulation. As a consequence of exercise, plasma glucagon and catecholamines increase, whereas plasma insulin decreases (Winder *et al.*, 1979). These hormonal adjustments with exercise thus favour hepatic glucose production.

In spite of a decreased blood flow, oxygen extraction by the liver is increased during exercise (Ahlborg *et al.*, 1974), suggesting that the mitochondria may be metabolically more active especially under conditions where gluconeogenesis is important. In addition, hepatic mitochondria isolated from hormone-treated sedentary rats have higher rates of phosphorylating respiration and Ca^{2+} uptake, with a longer retention of the accumulated Ca^{2+} (see Andia-Waltenbaugh *et al.*, 1981; Taylor *et al.*, 1980). Since exercise-induced hormonal changes, as well as other factors, may be a potent stimulus of hepatic mitochondrial function, the purpose of the present study was to determine the effect of a single bout of exercise on the functional characteristics of isolated hepatic mitochondria. The results demonstrate that hepatic mitochondria isolated from exercised rats exhibit functional alterations which are reminiscent of hepatic mitochondria isolated from hormone-treated animals.

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Experimental

Groups and exercise protocol

Male Sprague–Dawley rats (200 ± 10 g) were matched for body weight and were starved for 24 h before the experimental day. One rat from each pair was randomly assigned to either a control group or an exercised group. The exercised rat swam at 34–35°C with a tail weight (5% of body wt.) for 100 min. Both rats were then anaesthetized with sodium pentobarbital (6 mg/100 g body wt. intraperitoneally). The time from injection of sodium pentobarbital to the absence of the foot reflex was 6–8 min; therefore, the observed exercise response also includes the post-exercise recovery period. In one series of the two groups, the liver was removed

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for subsequent mitochondrial isolation (see below). In the second series, the gastrocnemius muscle was freeze-clamped *in situ* with Wollenberger-type aluminum tongs followed by freeze-clamping a lobe of the liver for subsequent determination of glycogen content. Blood was then drawn via cardiac puncture for analyses of lactate and glucose.

Mitochondrial isolation

Hepatic mitochondria were isolated at 2–4°C by the modified procedure of Schneider (1948). Briefly, the minced liver was homogenized in 10 vol. of 220 mM-mannitol/70 mM-sucrose/2 mM-EGTA/0.5% bovine serum albumin/2 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4. After filtration through one layer of cheesecloth, the homogenate was centrifuged at 500 ***g*** for 15 min. The supernatant was filtered through two layers of cheesecloth and was centrifuged at 8000 ***g*** for 15 min. The pellet was then resuspended in 220 mM-mannitol/70 mM-sucrose/2 mM-Hepes (pH 7.4) and was centrifuged at 8000 ***g*** for 15 min. This washing procedure was repeated twice. The final pellet was resuspended in 220 mM-mannitol/70 mM-sucrose/2 mM-Hepes (pH 7.4). Protein was measured by the biuret procedure (Layne, 1957), with bovine serum albumin as the standard.

Mitochondrial respiration

Mitochondrial respiration was determined at 37°C by polarographic techniques, with a Yellow Springs Instruments (model 53) oxygen monitor (Yellow Springs, OH, U.S.A.). Unless noted otherwise, the reaction medium contained 10 mM-Hepes (pH 7.2) 112.5 mM-sucrose, 12.5 mM-potassium phosphate, 5 mM-$MgCl_2$, 37.5 mM-KCl, 2 mM-EGTA, 2 mg of protein, and either 7.5 mM-glutamate plus 3.75 mM-malate, 15 μM-palmitoylcarnitine plus 3.75 mM-malate, or 7.5 mM-succinate plus 5 μg of rotenone, in a final volume of 2 ml. State-3 respiration was initiated by the addition of 225 μM-ADP. The acceptor control ratio was obtained by dividing the rate of oxygen consumption in the presence of ADP (State 3) by the rate of oxygen consumption obtained after the ADP had been depleted (State 4).

Mitochondrial Ca^{2+} fluxes

Ca^{2+} uptake and release were measured at 30°C by the dual-wavelength spectrophotometric method with the chelometric dye, antipyrylazo III, at 642–600 nm with an Aminco DW-2 dual-wavelength split-beam spectrophotometer (Wolkowicz & McMillin-Wood, 1980). Unless otherwise noted, the reaction medium included 73 mM-KCl, 60 μM-antipyrylazo III, 1.7 mM-phosphate, 1.7 mM-succinate, 5 μg of rotenone, 2 mg of mitochondrial protein, and 10 mM-Hepes/0.25 M-sucrose (pH 7.2) to a final volume of 3 ml. The reaction was initiated by the injection of 53 μM-$CaCl_2$ (80 nmol/mg of protein) into the reaction cuvette. After the completion of Ca^{2+} uptake, 180 μM-Ruthenium Red was added, which effectively blocked all Ca^{2+} uptake when added before Ca^{2+}, and the rate of Ca^{2+} efflux was monitored. We determined that the antipyrylazo III is sensitive to Mg^{2+}; therefore, for the experiments involving Mg^{2+} the Mg^{2+}-insensitive dye, tetramethylmurexide, was used with the wavelength pair 518–542 nm, at a concentration of 100 μM. Tetramethylmurexide, which is also pH-insensitive, was used for the pH studies.

Other methods

Mitochondrial cytochrome content was measured by the procedures of Williams (1964) with the Aminco DW-2 spectrophotometer in the split-beam mode. The glycogen content of liver and skeletal muscle was determined with neutralized $HClO_4$ extracts by the method of Lo *et al.* (1970). Blood lactate was determined by the lactate dehydrogenase method, monitoring the reduction of NAD^+ at 340 nm on $HClO_4$ extracts of whole blood (Gutmann & Wahlefeld, 1974). Blood glucose was determined in plasma by the glucose oxidase method (Raabo & Terkildsen, 1960). The data were statistically analysed by Student's *t* test for paired observations, with the 0.05 probability level designated as significant.

Materials

Tetramethylmurexide was obtained from Calbiochem (San Diego, CA, U.S.A.), Palmitoylcarnitine was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Ruthenium Red, obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and antipyrylazo III, purchased from K & K Rare and Fine Chemicals Co. (Plainview, NY, U.S.A.), were recrystallized before use (Fletcher *et al.*, 1961). All other chemicals were reagent grade.

Results

Physiological parameters

In order to estimate the intensity of the exercise protocol, the following observations were made: blood lactate increased and blood glucose decreased as a consequence of exercise, although a significant change in either liver glycogen or skeletal-muscle glycogen did not occur (Table 1). (The liver glycogen content was 90% depleted after the 24 h starvation.) In addition, the rectal temperature of the rats was maintained at 36 ± 1°C before and after the swim. Because the 2-fold increase in blood lactate did not approach the 5-fold increase previously noted with exhaustive swimming in untrained rodents (Tate *et al.*, 1980), the exercise bout was deemed moderate.

Table 1. *Effect of exercise on blood and tissue metabolites*

See the Experimental section for details of exercise protocol and methods of determination. The values represent the means ± S.E.M. for the numbers of paired determinations indicated. Non-significant changes are denoted by n.s.

Group	Blood glucose (mmol/l)	Blood lactate (mmol/l)	Liver glycogen (mg/g wet wt.)	Skeletal-muscle glycogen (mg/g wet wt.)
Control	6.6 ± 0.2 ($n = 6$)	2.9 ± 0.3 ($n = 6$)	4.5 ± 0.5 ($n = 9$)	8.7 ± 0.4 ($n = 4$)
Exercised	4.3 ± 0.1	5.0 ± 0.4	3.8 ± 0.4	6.1 ± 1.1
Probability level	$P < 0.001$	$P < 0.01$	n.s.	n.s.

Table 2. *Effect of exercise on respiratory parameters of hepatic mitochondria*

Mitochondrial respiration at 37°C was determined polarographically (see the Experimental section). The values represent the means ± S.E.M. for the numbers of paired determinations indicated. Non-significant changes are denoted by n.s.

Substrate	Group	Respiration rate (ng-atoms of oxygen/min per mg of protein) State 3	State 4	Acceptor control index	ADP:O ratio
Glutamate plus malate ($n = 8$)	Control	180 ± 18	32 ± 3	5.5 ± 0.3	2.98 ± 0.04
	Exercised	267 ± 34	36 ± 2	7.4 ± 0.7	2.98 ± 0.04
	Probability level	$P < 0.01$	n.s.	$P < 0.02$	n.s.
Succinate plus rotenone ($n = 8$)	Control	271 ± 28	61 ± 6	4.5 ± 0.4	1.79 ± 0.03
	Exercised	334 ± 28	80 ± 11	4.9 ± 0.8	1.90 ± 0.03
	Probability level	$P < 0.001$	$P < 0.001$	n.s.	n.s.
Palmitoylcarnitine plus malate ($n = 6$)	Control	159 ± 18	36 ± 2	4.5 ± 0.6	2.95 ± 0.06
	Exercised	257 ± 31	40 ± 6	6.6 ± 0.7	2.73 ± 0.07
	Probability level	$P < 0.02$	n.s.	n.s.	n.s.

Mitochondrial cytochrome content and protein yield

Since the mitochondrial respiratory-linked parameters may reflect, in part, protein-dependent alterations in purity, mitochondrial cytochrome content was measured in both groups, and was found to be similar to that reported by Williams (1964) for liver mitochondria, with no significant differences between the groups (results not shown). The mitochondrial protein yield (mg/g wet wt.) was also similar for the groups (8.51 ± 0.6, control; 9.25 ± 0.4, exercised). Therefore, the mitochondrial functional parameters for the control and exercised groups were compared as a function of protein concentration.

Mitochondrial respiration

State-3 respiration (Table 2) was significantly higher in the mitochondria isolated from the exercised rats regardless of the substrate utilized. Similar findings were observed with 10mM-pyruvate. Furthermore, the rates of uncoupled respiration in the presence of 0.2μg of valinomycin and succinate + rotenone for each group were not significantly different from the rates in the absence of valinomycin, so that the difference between the groups remained the same. These data suggest that substrate oxidation through the tricarboxylic acid cycle is not rate-determining, but that the activity of the electron-transport chain is in some way affected by exercise. The electron-transport chain, not substrate entry alone, is apparently the locus of the State-3 respiratory stimulation noted with hormone-challenged rats or livers (Halestrap, 1978; Titheradge *et al.*, 1978; Titheradge & Haynes, 1979).

To determine whether non-specific stress-related events, as opposed to exercise itself, could be the cause of the mitochondrial changes, we examined the identical parameters under conditions known to elevate stress hormones: starvation versus feeding; a 12h exposure to 4°C with wet fur; and 100min of exposure to water at 35°C without swimming. Under all of these conditions, there was no difference in any respiratory parameter. We also looked at another form of exercise, that is, running on a motor-driven rodent treadmill at 20m/min at a 5% grade for 60min. Under this exercise protocol there was a 75% increase in the rate of State-3 respiration (pH 7.2) in the presence of succinate + rotenone (control, 200 ± 8, exercised, 350 ± 10ng-atoms of oxygen/min per mg of protein; $n = 3$). Thus the observed stimulation of mitochondrial respiration is apparently specific to endurance-type exercise regardless of its mode.

Extramitochondrial pH and respiration

Since the cellular pH of the liver may fluctuate with exercise (Hultman & Sahlin, 1981), we examined State-3 respiration over the extramitochondrial pH range, 6.8–7.4. As shown in Fig. 1, State-3 respiration in the presence of succinate + rotenone was unmodified by pH in the mitochondria isolated from the control rats, as shown by others (Tobin *et al.*, 1972; Hutson, 1977). In the mitochondria isolated from the exercised rats, however, there was a progressive augmentation of State-3 respiration as the pH increased, which resulted in an amplification of the difference between the two groups; i.e. at pH 6.8 the difference was 57%, whereas at pH 7.4 the difference was 94%. Similar findings were observed when the mode of exercise was running: a 38% difference at pH 6.8 and an 88% difference at pH 7.4. Thus, as the extramitochondrial pH decreased, the difference in State-3 respiration between the two groups decreased. In contrast, there was no effect of pH on State-4 respiration in either group, so that the higher State-4 respiration in the presence of succinate + rotenone in the mitochondria from the exercised rats (Table 2) was maintained across the pH range utilized.

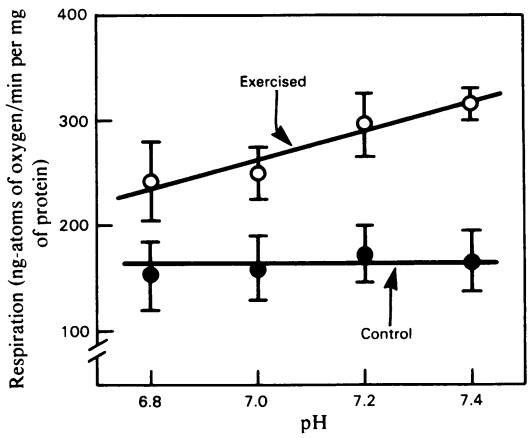

Fig. 1. *Extramitochondrial pH and State-3 respiration*
State-3 respiration in the presence of succinate + rotenone was determined as a function of pH in the reaction medium (see the Experimental section). The values are the means ± S.E.M. for four paired determinations. The difference between the two groups was significant ($P < 0.05$) at each pH.

Mitochondrial Ca^{2+} fluxes

Ca^{2+} uptake and release by isolated mitochondria at pH 7.2 were altered after exercise (Table 3). The initial rate of Ca^{2+} uptake was significantly higher in the exercised group, whereas the initial rate of Ca^{2+} efflux, in the presence of Ruthenium Red to prevent Ca^{2+} cycling, was significantly lower. The rate of Ca^{2+} release in the absence of Ruthenium Red was also slower in the mitochondria from the exercised rats.

Since Ca^{2+} efflux may be related to the presence of phosphate (Wehrle & Pederson, 1979; Wolkowicz & McMillin-Wood, 1981) and phosphate transport is enhanced in mitochondria isolated from glucagon-treated rats (Barritt *et al.*, 1978), the rates of Ca^{2+} uptake and Ca^{2+} efflux were examined as a function of phosphate concentration in the reaction medium (Fig. 2). Ca^{2+} fluxes between the two groups were not different until the exogenous phosphate concentration reached 0.4 mM (Fig. 2). Thereafter, the difference between the groups was progressively augmented. In addition, when the phosphate concentration was varied between 1 and 5 mM in the reaction medium for succinate-linked State-3 respiration, a similar pattern to that demonstrated for Ca^{2+} uptake was observed: in the control group, State-3 respiration reached a plateau at a low concentration of phosphate, whereas in the exercised group State-3 respiration continued to increase as the phosphate concentration increased to a difference of 84% at 5 mM-phosphate (results not shown).

Extramitochondrial pH and Ca^{2+} fluxes

Because alterations in extramitochondrial pH may affect mitochondrial Ca^{2+} fluxes (see Studer & Borle, 1980; Wolkowicz & McMillin-Wood, 1981), including a stimulation of Ca^{2+} release by a decrease in pH (Akerman, 1978; Tsokos *et al.*, 1980), the possibility that Ca^{2+} movements in mitochondria isolated from exercised rats may be more sensitive to alterations in the assay pH was tested (also, see

Table 3. *Exercise-induced alterations of succinate-linked Ca^{2+} fluxes by hepatic mitochondria*
See the Experimental section for method of determination. Ca^{2+}-efflux rates were determined in the presence of 180 μM-Ruthenium Red, which was added after Ca^{2+} uptake was complete. The values are the means ± S.E.M. for six paired determinations.

Group	Ca^{2+} uptake (nmol/min per mg of protein)	Ca^{2+} efflux (nmol/min per mg of protein)
Control	406 ± 56	30 ± 5
Exercised	604 ± 74	13 ± 4
Probability level	$P < 0.01$	$P < 0.05$

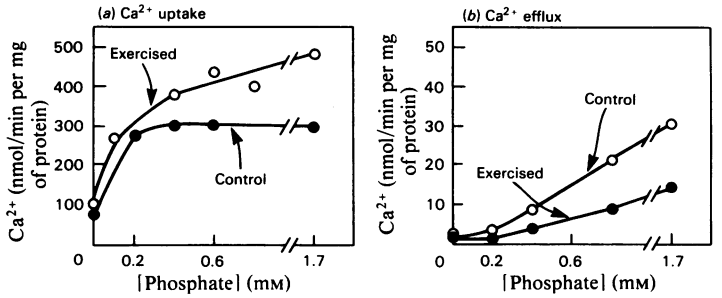

Fig. 2. *Phosphate effects on mitochondrial Ca^{2+} fluxes*

The rates of succinate-dependent Ca^{2+} uptake (*a*) and ensuing Ca^{2+} efflux (*b*) (in the presence of 180μM-Ruthenium Red added after Ca^{2+} uptake was complete) were determined at pH 7.2 as described in the Experimental section, except that the phosphate concentration in the reaction medium was varied as indicated. A representative example from four separate determinations is shown.

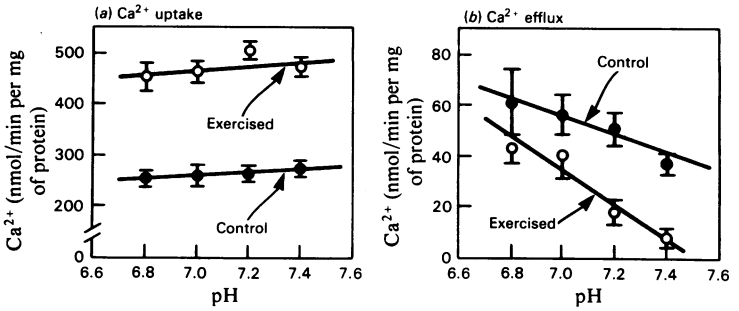

Fig. 3. *Extramitochondrial pH and Ca^{2+} fluxes*

The rates of succinate-dependent Ca^{2+} uptake (*a*) and Ca^{2+} efflux (*b*) (see Fig. 2) were determined with tetramethylmurexide in the presence of 1.7 mM-phosphate as a function of pH in the reaction medium. The values are the means $\pm$ S.E.M. for three paired determinations. The difference between the two groups was significant ($P < 0.05$) at each pH, except as noted in the text.

above). Ca^{2+} uptake was relatively stable across the pH range 6.8–7.4 in either group, so that the difference between groups remained rather constant (Fig. 3). Regardless of pH or initial rate, the total amount of added Ca^{2+} was accumulated in both groups. In contrast, there was a sharp distinction between the two groups in Ca^{2+} efflux when the pH was increased above 7.0: at pH 6.8–7.0 there was no significant difference between the two groups. Thereafter, however, the difference between groups was augmented, so that the Ca^{2+} efflux in the exercised group was 65% and 78% slower at pH 7.2 and 7.4 ($P < 0.05$) respectively. Thus the pH-sensitivity of Ca^{2+} efflux was apparently greater in the exercised group. Akerman (1978) suggested that, regardless of the added Ca^{2+} concentration, there is a specific compartment of mitochondrial Ca^{2+} that is mobilized by a decrease in pH (7.4 to 6.8) through a H^+/Ca^{2+} antiport mechanism, although the H^+-induced Ca^{2+} release is minor in the presence of phosphate. Therefore it is possible that the compartmentalization of mitochondrial Ca^{2+} after exercise may be modified, so that efflux from this putative pool may be augmented as the pH is decreased.

Ca^{2+} stimulation of respiration

Ca^{2+} stimulation of respiration (oxygen consumption in the presence of Ca^{2+}/oxygen consumption in its absence) under identical reaction conditions listed for Ca^{2+} uptake (see the Experimental section) at pH 7.2 was 2-fold in both groups (2.24 ± 0.04, control; 1.97 ± 0.12, exercised; $n = 3$), suggesting that the slower rate of net Ca^{2+} uptake by the mitochondria from the control group was not caused by an increased rate of Ca^{2+} release. The ratio of oxygen consumption in the presence of Ca^{2+} to that in its absence was identical (1.37 ± 0.07, control; 1.28 ± 0.02, exercised; $n = 3$), indicating that a decreased rate of Ca^{2+} cycling cannot explain

Table 4. *Effect of Mg^{2+} on succinate-linked Ca^{2+} uptake*

Reaction conditions were identical with that for Table 3, except that tetramethylmurexide (100μM) was the chelometric dye and Mg^{2+} was added as indicated. The values are the means ± S.E.M. for three paired determinations.

Group	Ca^{2+} uptake (nmol/min per mg of protein)		Change (%)	Probability level
	$-Mg^{2+}$	$+1$ mM-Mg^{2+}		
Control	338 ± 25	221 ± 15	−35	$P<0.025$
Exercised	496 ± 27	403 ± 22	−19	$P<0.005$
Change (%)	+46.5	+82		
Probability level	$P<0.05$	$P<0.02$		

the increased Ca^{2+}-uptake rates in the mitochondria from the exercised rats. Ca^{2+}-stimulated respiration, however, was significantly higher ($P<0.05$) in the exercised group (control, 167.4 ± 1.2, exercised, 212.3 ± 16.6 ng-atoms of oxygen/min per mg of protein; $n = 3$).

Effect of Mg^{2+} on Ca^{2+} uptake

Mg^{2+} is a competitive-like inhibitor of respiratory-substrate-dependent Ca^{2+} uptake (Hutson *et al.*, 1976; Hutson, 1977); however, the rate of Ca^{2+} uptake by hepatic mitochondria isolated from glucagon-treated rats is not significantly inhibited by 5 mM-Mg^{2+}, in contrast with the inhibition observed in mitochondria isolated from control rats (Andia-Waltenbaugh *et al.*, 1981). We therefore examined the inhibition of Ca^{2+} uptake by 1 mM-Mg^{2+}, which is within the cellular physiological range (Veloso *et al.*, 1973). As shown in Table 4, Mg^{2+} significantly slowed the rate of Ca^{2+} uptake in both groups; however, that in the control group was more affected than that in the exercised group. The net result was a greater difference in the initial rate of Ca^{2+} uptake between the two groups in the presence of Mg^{2+}.

Discussion

A significant alteration of isolated hepatic mitochondrial function is observed after a bout of moderate exercise in untrained rats. The increased State-3 respiration observed in the present study confirms the previous observation of Glick (1966) with isolated hepatic mitochondria after 2h of running wheel exercise and untrained rats. In contrast with liver mitochondria, Dohm and colleagues (Dohm *et al.*, 1972, 1975) reported that an exhaustive bout of exercise in untrained rats does not alter State-3 respiration of mitochondria isolated from either cardiac or skeletal muscle. The augmentation of Ca^{2+} uptake by hepatic mitochondria isolated from the exercised rats is also different from our earlier work with muscle mitochondria. We (Bonner *et al.*, 1976; Tate *et al.*, 1980) previously demonstrated that an acute bout of exhaustive exercise (either running or swimming) does not significantly alter Ca^{2+} uptake by mitochondria subsequently isolated from cardiac and skeletal muscle of untrained rodents. Thus the responsiveness of mitochondrial function to exercise may be organ-specific in the untrained state.

The alterations of mitochondrial function observed as a consequence of exercise are in many ways like the hormone-induced functional modifications. The exercise protocol used in this study is similar to that utilized by Galbo *et al.* (1977), who noted significant increases of plasma glucagon, adrenaline and noradrenaline with a marked decrease in plasma insulin after 90 min of swimming in untrained rats. Since insulin inhibits the phenylephrine-induced stimulation of State-3 respiration (Dehaye *et al.*, 1981), these hormonal changes with exercise favour the functional changes of hepatic mitochondria noted in the present study. We wish to stress, however, that the multifaceted nature of exercise and its physiological consequences precludes a direct comparison between the two models. For example, hormonal stimulation of hepatic glucose output is operating in both models. Yet hepatic glucose output from gluconeogenesis during exercise is markedly attenuated, depending on the exercise intensity and duration (Wahren *et al.*, 1971), so that hypoglycaemia may occur when hepatic glycogen stores are depleted (Winder *et al.*, 1979). Indeed, Brooks & Gaesser (1980) demonstrated that the metabolic fate of lactate is predominantly oxidation rather than conversion into glucose. Therefore the increased hepatic oxidative metabolism as a result of exercise may be related to factors other than gluconeogenesis. In fact, increased gluconeogenesis in response to hormonal stimuli may occur independently of mitochondrial respiratory changes (Siess & Wieland, 1980).

Since a significant stimulation of liver glycogenolysis during prolonged exercise may occur without a concomitant rise in hepatic cyclic AMP (Winder *et al.*, 1979), it is possible that α-adrenergic cyclic AMP-independent mechanisms may be involved in the observed exercise-induced mitochondrial

alterations. Whether the exercise response results from either α-, β- or both adrenergic mechanisms requires investigation. We wish to point out, however, an important suggestion made by Siess & Wieland (1980) that the isolation procedure may damage mitochondria from control rats, but not those from hormone-treated rats, which may be protected in some manner. Thus, the optimal conditions for isolating mitochondria may differ after exercise, a point previously made by us (Tate *et al.*, 1978) and by Gale (1974). Although this postulate may be valid, it is clear that the function of hepatic mitochondria *in vitro* is changed by endurance-type exercise and that this response is stress-specific.

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