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# ALTERATIONS IN LIVER MITOCHONDRIAL FUNCTION AS A RESULT OF FASTING AND EXHAUSTIVE EXERCISE

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**The effect of exercise upon liver mitochondria structure and function was examined in fasted and fed rats, following a single run to exhaustion on a motor-driven treadmill. Exercise alone and exercise coupled with fasting both produced a significant decrease in the amount of hexokinase bound to the mitochondria, as well as reduction in the ADP/O ratio and acceptor control index measured in the presence of succinate. The mitochondria of the exercised animals, when exposed to freeze-fracture analysis while in state 3, displayed fewer deflections in the fracture plane between the inner and outer membrane than those isolated from control animals. This suggests that fewer contacts existed between the two membranes. Measurements based upon the binding of 8-anilinonaphthalene 1-sulphonate indicated that there was an increase in the net negative charge on the surface of the mitochondrial membranes of the exercised animals. All of these effects could be mimicked by incubation of mitochondria from control animals with free fatty acids. This fact, coupled with the observation that washing of the mitochondria with a solution comprising 5% (w/v) albumin could reverse all of the consequences of exercise, suggests that these alterations in mitochondrial structure and function may be the result of the increase in plasma free fatty acids that accompanies long-term exercise. Furthermore, the observation that the exercise-induced changes are dynamic and readily reversible indicates that the mitochondria were not necessarily damaged, but rather that the coupling of oxidative phosphorylation may be subject to physiological regulation.**

## Introduction

Under normal physiological conditions the primary metabolic pathways of liver are those of carbohydrate metabolism and fatty acid synthesis. Fasting and uncontrolled diabetes, however, alter liver function to where fatty acid oxidation, ketone body production and gluconeogenesis are predominant [1]. This adjustment in metabolism is accompanied by a decrease of the phosphorylation potential in the cytosol, although the oxidation rate does not change as compared to fed animals [2]. One explanation for these findings could come from the experiments of Ilyin [3] who observed a decrease in the ADP/O and respiratory control ratio of the liver mitochondria from diabetic and starved rats. The latter results would imply that the tightness of coupling between oxidation and phosphorylation is a matter of metabolic regulation. Since exhaustive exercise produces effects common to fasting and/or diabetes, i.e., changes in plasma nonesterified fatty acids, insulin, gluca-

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Abbreviations: ANS, 8-anilinonaphthalene 1-sulphonate.

gon and catecholamines, we have examined the consequences of this type of exercise, combined with fasting, upon several structural and functional characteristics of liver mitochondria.

The degree of coupling of oxidative phosphorylation, evaluated as ADP/O ratio and acceptor control index, was reduced 50% or more in both isolated mitochondria and liver homogenates in rats that were exercised or fasted and exercised when compared to non-exercised rats. Similarly, the amount of hexokinase bound to the mitochondrial membrane was reduced.

We have recently reported that the contacts between the boundary membranes in liver mitochondria are altered in conjunction with the metabolic state. That is, the frequency of contacts correlates well with the capacity of the mitochondria for oxidative phosphorylation. Upon applying this technique to the mitochondria of exhausted, fasted rats, we observed a decrease in contacts qualitatively similar to that previously reported for mitochondria that were chemically uncoupled [4].

Recoupling of oxidative phosphorylation and reestablishment of the normal frequency of membrane contacts could both be accomplished by incubating the mitochondria in a medium containing 5% (w/v) albumin. This fact suggests that the effect of fasting and long-term exhaustive exercise upon liver mitochondria may be related to the increase in plasma free fatty acids that accompanies exercise of this type.

## Materials and Methods

*Experimental animals and exercise protocol.* Rats of the Chbb-THOM strain (200–250 g body weight) were randomly divided into three groups: control-fed, exhausted and fasted-exhausted. Running was at 20 m/min at a +10% slope. Fasting had begun approx. 48 h prior to the beginning of the experiment. The rats of the exhausted and fasted-exhausted groups were introduced to the running treadmill 1 day prior to the experiment by a 5 min run at the required speed.

Animals were killed immediately after exhaustion by $CO_2$ breathing, followed by cervical dislocation. Control animals were killed at various times throughout the experiment at intervals determined by the times at which the runners became exhausted. All animals were killed between 10:00 and 12:00 am; the livers were removed, and the mitochondria were isolated immediately following removal.

*Isolation of mitochondria.* Liver mitochondria were isolated in the absence of albumin as previously described [5] and protein measurements were made by the method of Lowry et al. [6] using bovine serum albumin as a standard. Respiratory measurements and preparations for freeze-fracture analysis were made immediately following isolation of the mitochondria, whereas for all other determinations the mitochondria were stored at −30°C until analyzed.

*Respiratory measurements.* Mitochondrial respiratory values were determined at 23°C by the method of Estabrook [7]. For experiments concerning the effect of palmitate upon oxidative phosphorylation and hexokinase binding, mitochondria were isolated as described above, incubated with the required palmitate concentration, and centrifuged at $9000 \times g$ for 10 min. The supernatant fraction was decanted, replaced with isolation buffer, and the respiratory and activity measurements made.

*Determination of glycogen and hexokinase activity.* Glycogen determinations were performed fluorimetrically on liver and skeletal muscle samples (5–25 mg), following digestion in 1 M HCl [8], using the hexokinase reaction. Determination of the activity of hexokinase [9] bound to the mitochondrial membrane was made with 50 µl of the suspension of isolated mitochondria, which was routinely washed twice with the standard isolation buffer during preparation. The activity of hexokinase, measurable in the mitochondrial suspension after isolation, was considered to be the amount of hexokinase bound to the membrane. To evaluate the possibility of contamination of this activity by unbound cytoplasmic hexokinase, the activity of lactate dehydrogenase was determined in the mitochondrial fraction as a marker for any residual cytoplasm that was not removed by the washing procedure. These measurements indicated that only 2% of the total activity of hexokinase could be attributed to the unbound enzyme.

*Binding of 8-anilinonaphthalene 1-sulphonate.* Binding of the 8-anilinonaphthalene 1-sulphonate

(ANS) to mitochondria was determined by the method of Wojtczak and Nalecz [10]. Mitochondria (0.2–0.5 mg) were suspended in 250 mM sucrose/10 mM Tris-HCl (pH 7.5), this being followed by addition of various concentrations of ANS dissolved in water. Fluorescence was measured in a Perkin Elmer fluorescence spectrophotometer (Model MPF-4) at excitation and absorbance wavelength of 366 and 470 nm, respectively. Based upon the assumption that the fluorescence of ANS is much greater when bound to the membrane versus when it is free in solution, the concentration of ANS was plotted versus fluorescence in a double-reciprocal plot. $K^1$ is the ANS dissociation constant of the untreated control, whereas $K^2$ is the constant obtained from mitochondria of the treated animals. These values were then used to determine differences in surface potential of mitochondrial membrane from the experimental groups by the equation $\Delta\psi = (RT/zF)\ \ln(K^1/K^2)$ where $R$ is the gas constant; $T$ the absolute temperature; $z$ the charge of ANS $(-1)$ and $F$ Faraday's constant [10,11].

*Freeze-fracture analysis.* Mitochondria of control and treated animals were adjusted to the different states and were monitored using an oxygen electrode. Aliquots of 1 ml of the mitochondrial suspensions were aerated by stirring, and subsequently centrifuged for 10 s at $8700 \times g$. The pellets were immediately subjected to rapid cryofixation as described recently [12] to avoid anaerobiosis. Routinely, four samples were simultaneously prepared. Time of cryofixation was approx. 10 s. Therefore, the maximal time needed for fixation of the last sample was approx. 60 s. The samples were broken in a Balzers 360M freeze etch device, equipped with an adapted Balzers double-replica table at $-120$°C and $2 \cdot 10^{-7}$ Torr, followed by Pt/C and C shadowing.

For electron microscopy, a Siemens 101 instrument at 80 kV was used. The morphological evaluations were performed using a Kontron MOP Am2 picture analysing system connected to a Hewlett Packard 9825 calculator. The nomenclature of the exposed membranes follows that of Branton et al. [13].

As a means of quantifying the difference in fracture-plane deviations, the length of the edge where the fracture plane deflects was measured as related to the corresponding mitochondrial area. In convex fractures, the edge of the exoplasmic face of the outer membrane was measured, whereas, in concave fractures, measurements were made of the exoplasmic face of the inner membrane. These values ($La$) were expressed as length ($\mu$m) per unit of mitochondrial fractured membrane area.

The quantification was made in the areas where the curvature was low in order to avoid large distortions of the measured edgelines. In every population of mitochondria, there are some which are completely void of fracture plane deflections. Due to the fact that the number of this type of mitochondrion fluctuates with metabolic state, we have adjusted our calculation of fracture-plane deflections in order to compensate for these differences. This adjustment was made by first determining on survey pictures the total area of the mitochondria with no deflections ($M_s$) and those with deflections ($M_p$), and then, normalizing these values with the expression $M_p/(M_p + M_s)$. Therefore, the final value for quantification of freeze-fracture deflections ($L$) was calculated from the equation: $L(\mu m/\mu m^2) = La \cdot M_p/(M_s + M_p)$.

*Statistical evaluation.* The stated statistical differences were obtained by applying the Kruskal-Wallis rank sums test and by Dunn's multiple comparisons based on the Kruskal-Wallis rank sums [14].

### *Materials*

Fatty acid-poor albumin was obtained from Fluka, Buchs, Switzerland. 8-Anilinonaphthalene 1-sulphonate was purchased from Sigma. All other chemicals used were of the highest grade of purity and were obtained from E. Merck and Boehringer, F.R.G.

## Results

### *Exercise protocol*

The exercise protocol employed represented an oxygen uptake of 70% of maximum in the rat [15]. Average times to exhaustion for the fasted-exhausted and exhausted groups were not different and average 209 and 198 min, respectively. The patterns of glycogen depletion were also similar in both groups, as exercise reduced the values in the

TABLE I

EFFECT OF RUNNING TO EXHAUSTION AND DIFFERENT DIETS, UPON ADP/O RATIO, ACCEPTOR CONTROL INDEX, AND HEXOKINASE BINDING IN LIVER MITOCHONDRIA

The mitochondrial respiration was determined at 23°C by polargraphic techniques. The reaction medium comprised 0.25 M sucrose/10 mM Hepes (pH 7.4)/5 mM sodium phosphate/5 mM $MgCl_2$/5 mM succinate. ADP/O ratios were determined by adding 200 nmol ADP several times. Maximal oxidation rates were measured in the presence of 1 mM ADP. The acceptor control index was calculated by dividing the rate of oxygen consumption in the presence of 1 mM ADP and 5 mM succinate (state 3) by the rate of oxygen consumption in the presence of succinate (state 4). Values are given as mean ± S.E. The numbers in brackets indicate the number of different mitochondrial fractions measured. Statistical differences of the measured parameters between experimental groups and controls are shown as *P* values. No significant change is denoted by n.s.

| Group | ADP/O | Acceptor control index | State 3 ox-rate (nmol $O_2$/min per mg) | Bound hexokinase (mU/mg protein) |
|---|---|---|---|---|
| Control | 1.6 ±0.15 (8) | 6.4±1.6 (8) | 58±10.5 (6) | 8.4 ±1.1 (3) |
| Exhausted | 1.1 ±0.31 (5) | 2.8±1.7 (5) | 62±48 (5) | 2.0 ±0.4 (4) |
| probability level | $P < 0.005$ | $P < 0.001$ | n.s. | $P < 0.05$ |
| Fasted exhausted | 0.92±0.37 (13) | 2.5±1.1 (13) | 52±25 (11) | 1.83±0.9 (6) |
| probability level | $P < 0.01$ | $P < 0.01$ | n.s. | $P < 0.05$ |

plantaris muscle by 83% in exhausted and 87% in fasted-exhausted and in the liver by 96% and 93%, respectively. Colonic temperature, measured immediately following exercise, increased to an average value of 40.3°C. As judged from the increases in the colonic temperature and the decreases in muscle and liver glycogen, the exercise was assumed to be exhaustive [16,17].

*Effect of exercise on mitochondrial metabolism*

We have recently observed a moderate decrease in mitochondrially bound hexokinase and the P/O ratio in mitochondria isolated from the livers of fasted rats [18]. Therefore, we attempted to determine if this effect could be potentiated by long-term exercise. As shown in Table I, exercise, coupled with fasting, produced a 50% decrease in the P/O ratio ($P < 0.01$) and acceptor control index ($P < 0.01$), as well as a 3-fold decrease ($P < 0.05$) in hexokinase bound to the mitochondrial surface. Exhaustive exercise alone produced similar decreases in all three parameters (Table I). No significant differences existed, however, in the maximum oxidation rate (state 3) for succinate in the presence of 1 mM ADP.

*Effect of exercise on liver homogenates*

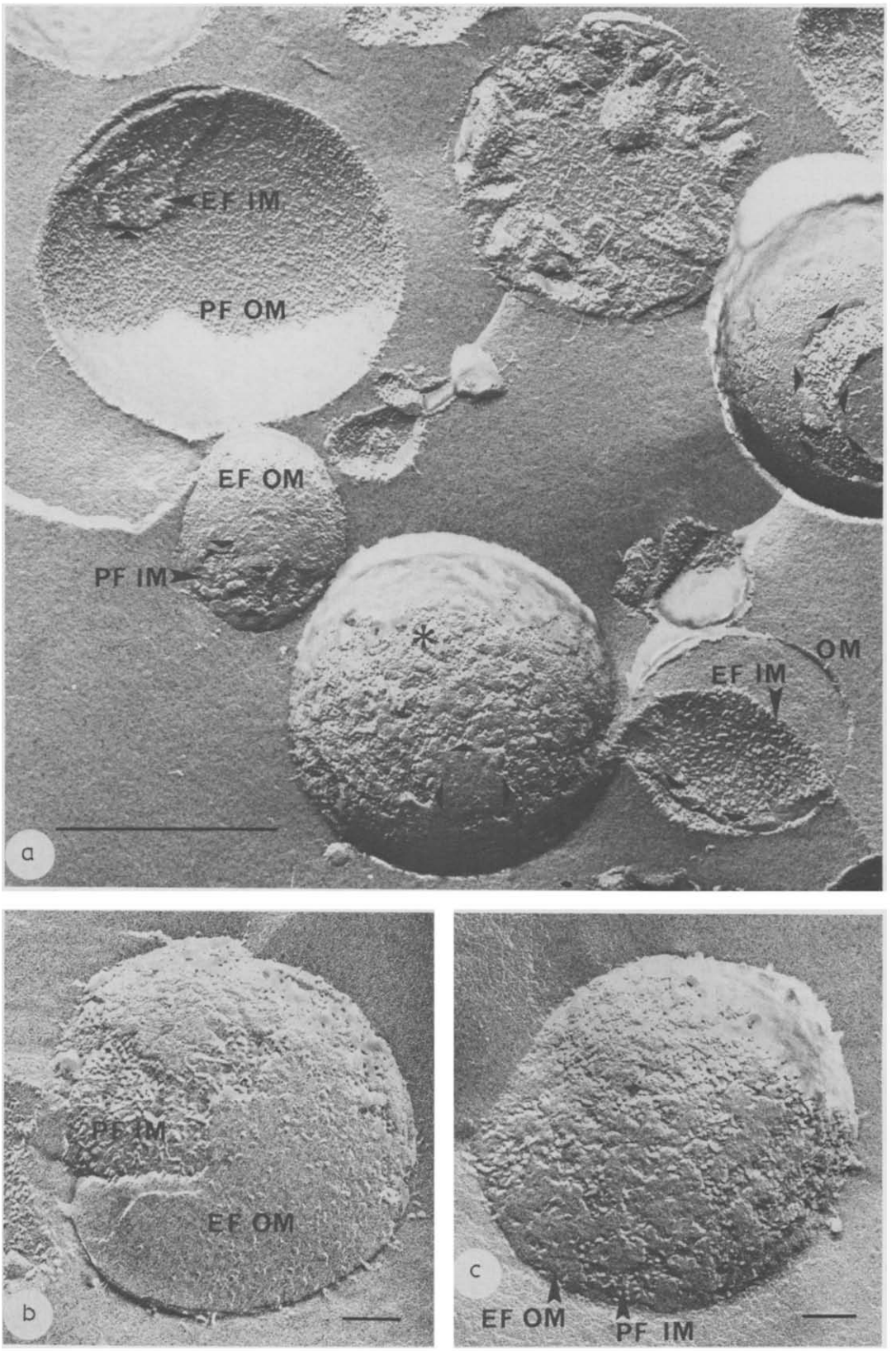
Having observed the effects of exercise upon the function of isolated mitochondria, we considered the possibility that they may have been produced by the isolation procedure. To exclude this possibility, we measured the P/O ratio and acceptor control index in homogenates, prepared from livers by mild homogenization in a sucrose medium. As illustrated in Table II, the effect of exercise was, in principle, the same as was obtained when the values were determined on isolated mitochondria from the same homogenate.

TABLE II

ADP/O RATIO AND ACCEPTOR CONTROL INDEX (AC) FROM LIVER HOMOGENATES AND CORRESPONDING ISOLATED MITOCHONDRIA

Measurements were made on liver homogenates, and subsequently on mitochondria isolated from these same homogenates. Values are mean of two experiments.

| | Homogenates from | | Mitochondria from | |
|---|---|---|---|---|
| | Fasted exhausted | Control | Fasted exhausted | Control |
| ADP/O | 0.3 | 1.3 | 0.25 | 1.63 |
| AC | 1.7 | 4.1 | 1.5 | 6.7 |


EF IM
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*Effect of exercise on morphological parameters of liver mitochondria*

Freeze-fractured mitochondria exhibit frequent shifts of the fracture plane between the inner and outer membranes providing that they are not chemically treated for fixation or cryoprotection. It has been postulated that these fracture-plane jumps between the two boundary membranes represent intimate contacts between the two membranes, a phenomenon referred to as semifusion [19]. Recently, it has been observed that the frequency of these fracture-plane changes increases in phosphorylating (state 3) mitochondria as compared to mitochondria in state 1 and state 4 [4]. Conversely, a dramatic decrease in this morphological parameter is produced if the mitochondria are uncoupled with dinitrophenol. In the present experiment, we have determined the frequency of fracture-plane jumps by measuring the length of the fracture-plane edge as described in Fig. 1.

Table III shows the length of the fracture-plane edge ($\mu m/\mu m^2$) of freshly isolated mitochondria from controls (line 1), measured in the presence of only the isolation buffer (state 1). No differences exist between measurements made under these conditions and those made when the mitochondria are energized (column 2) by the addition of succinate (state 4). Upon the addition of ADP to the energized mitochondria, the value increases 3- to 4-fold, indicating a significant increase in contacts (column 3). Conversely, when mitochondria isolated from the fasted-exhausted group are measured in state 3, the length of the fracture-plane edges remains unchanged. Hence, there is no increase in the frequency of fracture-plane changes upon transition to state 3. It appears that this morphological parameter correlates well with the observed reduction in the P/O ratio and acceptor control index of the liver mitochondria from the two exercise groups.

TABLE III

FREQUENCY OF FRACTURE PLANE JUMPS BETWEEN BOUNDARY MEMBRANES, AS DETERMINED IN FREEZE-FRACTURED MITOCHONDRIA FROM EXERCISED AND CONTROL ANIMALS

For the purpose of quantification, the length of the fracture plane edges of the exoplasmic faces of both boundary membranes were determined as described in Fig. 1. Isolated mitochondria from control and exercised, fasted animals were adjusted to the various metabolic states, i.e., state 1, freshly isolated mitochondria; state 4, mitochondria in the presence of 5 mM $MgCl_2$/5 mM phosphate/5 mM succinate; state 3, same as state 4, plus 1 mM ADP. Aliquots of these mitochondria were subjected to rapid cryofixation. Data below are given in $\mu m/\mu m^2$.

| | State 1 | State 4 | State 3 |
|---|---|---|---|
| Control | 3.0 | 3.1 | 11.7 |
| Fasted exhausted | 2.6 | 3.6 | 4.2 |

*Effect of washing mitochondria with albumin*

During fasting or prolonged exercise the levels of free fatty acids may increase in blood 3- to 4-fold [20]. Since it is known that free fatty acids and their CoA esters can uncouple mitochondria from brown fat, and that this uncoupling can be reversed by washing the mitochondria with al-

Fig. 1. Freeze-fractured mitochondrial membranes displaying the fracture plane deflections between the inner and outer membranes. (a) Preparation of mitochondria from control animals in state 1. Membrane fractures exhibit both the inner and outer membranes. The fracture planes are shown to jump from the hydrophobic core of one membrane to the other, and by that, to traverse the outer mitochondrial compartment. Arrows indicate these steps. They are seen as ridges in concave and convex fracture faces. As can be seen from the convex fracture plane marked with an asterisk, which exhibits an extraordinarily large number of deflections, this phenomenon cannot be simply qualitatively assessed. Therefore, for quantification, the length of the fracture planes edges, as marked here with arrows, was measured and related to the respective membrane area in each specific preparation, and then treated as described in Materials and Methods. Bar = 0.5 $\mu$m. (b) Preparation of mitochondria from exercised group in state 3. Large areas of the smooth outher membrane cover the particle-rich inner membrane, as is normally seen with state 1 and state 4 mitochondria. Bar = 0.1 $\mu$m. (c) Same preparation as in (b), but the mitochondria were washed with albumin prior to incubation with succinate and ADP. Membranes show the typical appearance of phosphorylating mitochondria, i.e., many irregular patches of outer membrane overlapping the inner membrane in seemingly close contact. This is illustrated by a large number of deflections with minimal distance between the two membranes. Bar = 0.1 $\mu$m. The nomenclature of the exposed membranes follows the proposals of Branton et al. (1979). OM = outer membrane; IM = inner membrane.

TABLE IV

EFFECT OF WASHING WITH ALBUMIN ON ADP/O RATIO, ACCEPTOR CONTROL INDEX (AC), AND FREQUENCY OF FRACTURE-PLANE DEVIATIONS

| | Fasted exhausted | Fasted exhausted washed with albumin | Control, fed washed with albumin |
|---|---|---|---|
| ADP/O | 0.97±0.4 (4) | 1.7±0.11 (4) | 1.7±0.1 (3) |
| AC | 2.4 ±1.2 (4) | 3.9±0.75 (4) | 5.5±1.2 (3) |
| State 3 | 4 $\mu m/\mu m^2$ (1) | 15 $\mu m/\mu m^2$ | 16 $\mu m/\mu m^2$ |

Mitochondria, isolated from fasted exhausted, and control groups were washed with a medium containing 5% (w/v) albumin, centrifuged, and resuspended in normal isolation buffer. Values for ADP/O ratio and acceptor control index are means±S.D. Numbers in brackets indicate the number of experiments. As a means of measuring, the frequency of fracture-plane deviations in phosphorylating mitochondria (state 3) the length of the edges of the fracture planes ($\mu m/\mu m^2$) were determined in the different groups. Evaluation of the frequency of fracture-plane deflections were taken from mitochondria of one experiment; however, the values represent means from approx. 150 different mitochondria per experimental group.

bumin [21], we decided to examine the possibility that free fatty acids may play a role in the effects observed here by applying a similar treatment. Following isolation, partially uncoupled mitochondria from the fasted-exhausted group were washed with isolation medium containing 5% (w/v) fatty-acid poor bovine serum albumin. The results from Table IV clearly show that the P/O ratio and acceptor control index can be restored to normal values by washing with albumin. It is interesting to note that, if the albumin used was not fatty acid-poor, no restoration to normal values took place. The data also suggest that there is a good correlation between the recovery of oxidative phosphorylation and an increase in the fracture-plane deviations upon transformation of the mitochondria from state 4 to state 3. Thus, washing the mitochondria isolated from the fasted-exhausted group with the albumin solution reverts both the functional and structural characteristics to those of mitochondria isolated from control animals.

*Effect of palmitate on oxidative phosphorylation, hexokinase binding and mitochondrial morphology*

The results obtained from the albumin washing experiment suggested that free fatty acids may be responsible for the decreases in the P/O ratio and acceptor control index, hexokinase binding, and the deflections between the freeze-fracture planes observed in the exhausted and fasted-exhausted groups. Therefore, we attempted to determine if we could duplicate these effects by incubating isolated mitochondria in vitro with free fatty acids.

Mitochondria were isolated from control animals and incubated with increasing concentrations of palmitate. The mitochondria were then centrifuged and resuspended in palmitate-free isolation medium. As shown in Fig. 2, incubation of the mitochondria at palmitate concentrations between 10 and 100 $\mu$M resulted in decreases in the P/O ratios. The amount of bound hexokinase was

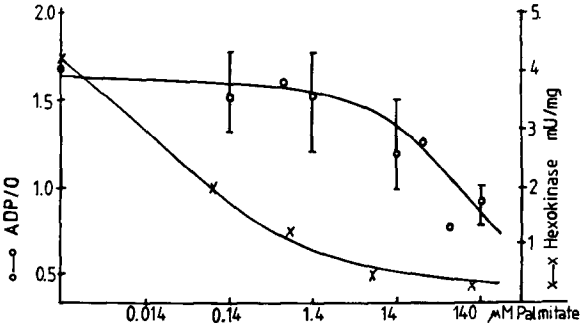


Fig. 2. Effect of incubation of mitochondria with palmitate on ADP/O ratios and binding of hexokinase. Mitochondria (5 mg protein) were incubated 10 min at room temperature in a 1 ml volume of isolation medium in the presence of the indicated palmitate concentrations. The samples were subsequently diluted 10-fold and centrifuged for 10 min at 9000×$g$. The sediments were resuspended in isolation buffer and ADP/O ratio and hexokinase activity were determined. The values for ADP/O ratio were taken from three experiments, whereas for hexokinase, one experiment was performed.

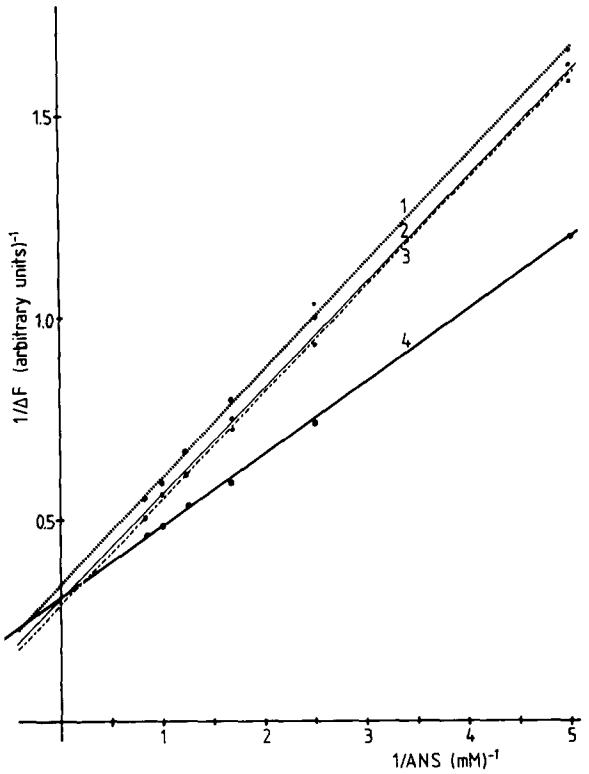


Fig. 3. Binding of 8-anilinonaphthalene 1-sulphonate (ANS) to liver mitochondria from fasted, fasted-exhausted and control animals. Reciprocal plot. Mitochondria isolated from the different groups (0.2–0.5 mg) were suspended in isolation medium. Fluorescence was determined upon addition of increasing ANS concentrations to the samples. (1) Mitochondria from fasted rats. (2) Mean values of two groups of fasted exhausted animals. For the measurements shown in curve 3, control mitochondria were preincubated with 80 μM palmitate as described in the legend of Fig. 2. (4) Mitochondria from controls.

even more rapidly reduced as illustrated by the fact that at 0.1 μM palmitate, only 50% of the amount normally bound in control mitochondria could be observed. Increasing the palmitate concentration resulted in a further decrease of bound hexokinase, until at 100 μM the value was reduced to 10% of control.

We also investigated the effect of incubation of palmitate upon the frequency of fracture-plane deflections in the same preparations of mitochondria. Preincubation with 50 μM palmitate significantly reduced the frequency of fracture-plane deflections in mitochondria in state 3 (Table V). Thus, as in the mitochondria isolated from the

TABLE V

EFFECT OF PALMITATE AND ALBUMIN WASHING UPON MITOCHONDRIAL STRUCTURE AND FUNCTION

Isolated mitochondria from control animals were incubated with 50 μM palmitate for 10 min at room temperature. The mitochondria were centrifuged and divided into two fractions. One fraction was washed with normal isolation buffer containing 5% (w/v) albumin and the second fraction with only isolation buffer. Washing of control mitochondria with albumin had no effect on the fracture-plane deflections when the mitochondria were in state 1.

| | Incubated with 50 μM palmitate | 50 μM palmitate and albumin wash |
|---|---|---|
| ADP/O | 0.57 | 1.8 |
| AC | 1.3 | 4 |
| State 3 | 2 $\mu m/\mu m^2$ | 15 $\mu m/\mu m^2$ |

exercised animals, there appears to be a correlation between the frequency of contacts and the P/O ratio and the acceptor control index. The effects upon the morphological and functional characteristics which resulted from the incubation with palmitate could be reversed to normal values upon washing with albumin (Table V).

*Effect of exercise upon the mitochondrial surface charge*

Wojtczak et al. [10] have shown that incubation of mitochondria with free fatty acids increases the negative charges at the membrane surface. We determined the effect of exercise and/or fasting upon the surface charge, using a similar experimental protocol. The amount of ANS that could be bound to the mitochondria of the various experimental groups was determined by plotting fluorescence versus ANS concentration (Fig. 3). Since ANS is a negatively charged molecule, its affinity for binding to the membrane would be affected if the membranes were more positively or negatively charged. As deduced from the larger slope in the double-reciprocal plot, there is less ANS bound to the surface of the mitochondria from fasted-exhausted than to those isolated from controls. If the latter mitochondria were incubated with 80 μM palmitate, the dissociation constant, $K_d$, of ANS increases to a value similar to mitochondria from the fasted exhausted group (Fig. 3, curve 3).

With values obtained from the titration curves, it was possible to calculate charge differences in the surface potential based upon the difference of $K_d$ for ANS determined in the mitochondria of control and treated animals (see Methods). From this relationship, we have calculated that exercise and/or fasting produces an increase in negative surface potential of the mitochondria of about $-10$ mV.

## Discussion

The results of the present experiments indicate that exhaustive long-term exercise in the presence and absence of fasting is capable of inducing a marked alteration in hepatic mitochondrial function. As is true in experiments using isolated cellular organelles, it might be suggested that the observed effects could be produced by the isolation procedure, i.e., in this case increased fragility of the mitochondria of the exercised animals. Two facts argue against this possibility. First, the alterations in the P/O ratio and acceptor control index in the isolated mitochondria could also be observed in liver homogenates. Secondly, the ability to recouple the mitochondria by washing with albumin implies that uncoupling was a transient phenomenon, not related to permanent damage. Recently, Davies et al. [22] have reported a similar observation regarding the effects of exercise on mitochondrial function. In their experiments, respiratory control index, determined as uncoupled respiration versus basal respiration, was reduced by 50% in liver homogenates from animals exercised to exhaustion. In contrast to our hypothesis presented here, these authors suggested that the effect of exhaustive exercise may be due to damage to the mitochondrial membrane resulting from an elevation of free radical concentration. Conversely, Tate et al. [23] found no differences in P/O ratio and acceptor control index with succinate as a substrate, in mitochondria isolated from exercised and control animals. The discrepancies between these results and those of our present experiments may be related to the fact that in the earlier study the mitochondria were isolated in the presence of 0.5% albumin. As is shown here, washing with albumin can recouple the mitochondria from exercised animals and restore the indices of mitochondrial function to those of fed, rested animals. It is also possible that the difference may be related to exercise intensity. As can be judged from running time and glycogen measurements, our exercise protocol was considerably more intense (see Results).

It has been known for some time that in brown fat, washing in an albumin solution is capable of recoupling mitochondria that have been uncoupled by free fatty acids or acyl-CoA [24,21]. The results from our experiments with albumin suggest that during exercise free fatty acids may require the degree of oxidative phosphorylation in the liver as well. This possibility is reasonable because the levels of free fatty acids substantially increase in exercise under hormonal control [20]. A possible regulatory function for free fatty acids is further supported by the finding that the mitochondria isolated from controls can be reversibly uncoupled in vitro by incubation with palmitate and subsequently recoupled by treatment with albumin. This is not surprising, as uncoupling of liver mitochondria by free fatty acids has been previously observed [25].

From measurements made with ANS, it is tempting to speculate that free fatty acids may also be responsible for the increase in net negative charge on the surface of the mitochondria isolated from the exercised animals. Fig. 3 illustrates that incubation of mitochondria from controls with 80 $\mu$M palmitate decreases ANS binding by a similar magnitude to that observed in fasted-exhausted animals. Wojtczak and Nalecz [10] have reported an increase of net negative charge of $-12$ mV by incubation of liver microsomes with 100 $\mu$M oleate.

Measuring the surface potential by ANS binding in mitochondria can be complicated by the fact that the energetic state of the inner membrane influences the ANS fluorescence by a different mechanism [26]. We assume that in our experiments the influence of different energetization on ANS fluorescence was negligible because neither substrate nor phosphate and ADP was present during the ANS titrations. Therefore, it seems possible that the net membrane charge is altered mainly by incorporation of the negatively charged free fatty acids into the mitochondrial membrane.

Such an increase in net negative surface potential may provide an explanation for the observed

decrease in hexokinase bound to the mitochondria. Since all hexokinase isoenzymes are negatively charged at physiological pH, increasing the negative charge in the vicinity of the hexokinase binding protein [27–29] would alter the equilibrium between the bound and the free enzymes. This reduction of bound hexokinase may be of functional significance as it has been postulated that stimulation of glucose uptake by insulin can be regulated via glucose phosphorylation by the amount of hexokinase bound to the mitochondrial membrane [30]. Under conditions such as exercise where the insulin level is low and glucose uptake by the liver is of little importance to the organism, the amount of glucose phosphorylation could be reduced by a decrease in bound hexokinase. Under these conditions, the level of free fatty acids could be a mechanism by which hormones such as insulin and glucagon regulate the ratio between bound and free hexokinase.

In addition to the effect on hexokinase, increasing the net negative surface charge could serve to explain the decrease of fracture-plane deflections observed in mitochondria of fasted-exhausted and exhausted animals and those treated with palmitate. Based upon the assumption that deflections in the fracture plane occur at points where contacts between the membranes exist, a reduction in fracture-plane jumps can be interpreted as a decrease in the frequency of contacts between the two boundary membranes. This suggestion is supported by the previous observation that separation of the two membranes by the use of glycerol or dinitrophenol, seen in cross-fractured mitochondria, always resulted in a significant decrease of fracture-plane deflections [4]. It is possible that the increase in net negative surface charge may result in repulsion of the boundary membranes from one another, and thus, cause a decrease in contacts. The separation of the membranes would be manifest as a reduction in fracture-plane deflections.

It is interesting to note that all the experimental treatments which resulted in decreases in fracture-plane deflections produced simultaneous decreases in ADP/O ratio and acceptor control index. This fact, together with the observation that both effects were concomitantly reversible with albumin washing, suggests that these contacts between the inner and outer membranes may be directly or indirectly important in the degree of coupling of oxidative phosphorylation.

The observation of partial uncoupling of liver mitochondria during exchaustive exercise raises the question of the physiological meaning. It does not seem plausible to consider simply that these mitochondria are damaged and have become uncoupled by proton leaks. It is more valid to assume that the coupling of phosphorylation to the proton motive force is metabolically regulated. Except for ATP synthesis, other energy-consuming processes such as ion and metabolite transport utilize proton gradient and membrane potential. The degree of coupling of these different energy-consuming processes to the proton motive force seems to be a matter of regulation. For instance, the ATP production could be decreased in order to increase the uptake of acylcarnitine which is driven by the proton gradient [31]. It has already been shown that the regulation between $Ca^{2+}$ uptake and phosphorylation is different in liver and heart-muscle mitochondria [32].

Recently, Westerhoff and Van Dam [33,34] proposed a model of 'mosaic chemiosmosis' for the regulation between the different energy-consuming processes in the mitochondria, which depends on a compartmentation of the protons along the inner membrane. On the other hand, Stucki [35,36] in an argument based on thermodynamic optimizing principles, has postulated that the degree of coupling should vary, depending upon the specific function which this system must fulfill. That is, a high coupling is necessary if the phosphorylation potential should be high and a lower coupling if high flux rates are required. In the case of exercise and stress, the rate of gluconeogenesis and ketogenesis is high in liver. These pathways require a high net flow of oxidative phosphorylation, which is best served by a reduced degree of coupling.

A reduction of coupling as observed in liver during exercise might not be beneficial in other tissues such as muscle and brain. As judged from the respiratory control indices determined by Davies [22], the degree of uncoupling caused by exercise in muscle is indeed less compared to liver. The uncoupling effect of fatty acids should therefore not apply to all tissues in the same way, which could simply be explained by differences in cellular uptake. It is known that brain does not take up

fatty acids and the transport of fatty acids into muscle cells of perfused heart is suppressed to 50% by ketone bodies [37]. It has been suggested that ketone bodies are the preferred fuel for muscle, kidney and brain during starvation and certainly during exercise, whereas the oxidation of free fatty acids and ketogenesis is merely restricted to one tissue, namely the liver [1].

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