The Possible Role of Palmitoyl-CoA in the Regulation of the Adenine Nucleotides Transport in Mitochondria under Different Metabolic States

I. Comparison of Liver Mitochondria from Starved and Fed Rats

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Abstract

It has been shown that K_M values for ADP when rat liver mitochondria oxidized succinate were strictly dependent on the values of the respiratory control ratios. The K_i values for palmitoyl-CoA inhibition of the ADP-stimulated succinate oxidation and the inhibition of the uncoupler-stimulated ATPase activity were equal to $0.5\ \mu M$. Mitochondria from livers of starved rats showed 30% inhibition of the state 3 respiratory rate (compared to the uncoupled respiratory rate) which was abolished by addition of carnitine. It was supposed that this inhibition was due to the influence of acyl-CoAs bound to the inner mitochondrial membrane on the adeninenucleotide translocase. Mitochondria from livers of fed rats showed a strong inhibition of succinate oxidation both in state 4 and state 3, although the rate of uncoupled respiration was normal. It was assumed that in this case the changes in mitochondrial behaviour was caused by the decrease in the concentration of ADP and ATP in the matrix space of mitochondria.

Introduction

Studies on the exchange mechanism of exogenous and endogenous adenine nucleotides in mitochondria revealed that adeninenucleotide

translocase plays an important role in the overall mechanism of oxidative phosphorylation [1, 2]. The key position of adeninenucleotide translocase was supported by the data that precisely highly specific transport of ADP and ATP through the inner mitochondrial membrane was in a number of cases the limiting step in the reactions of phosphorylation of exogenous ADP or hydrolysis of ATP [3]. The important role of adeninenucleotide translocase becomes even more evident after it had been discovered that long-chain fatty acyl-CoA esters, such as palmitoyl-CoA, are potent inhibitors of adenine nucleotides transport through the inner mitochondrial membrane [4, 5]. The physiological significance of such inhibitory action of long-chain fatty acyl-CoAs is not yet fully ascertained.

The purpose of the present communication was to elucidate some further features of the influence of palmitoyl-CoA on adenine nucleotides transport in rat liver mitochondria, and possible implications of its effect in explaining some peculiarities of mitochondrial metabolism under different physiological conditions. It was found that the value of the inhibitory constant for palmitoyl-CoA during its action on adenine nucleotides transport does not depend on the affinity of the translocase to ADP or ATP. It was found also, that with mitochondria from fasted rats some inhibition of the state 3 respiratory rate, when comparing the rate of fully uncoupled respiration, can be explained by the inhibition of ADP transport by long-chain fatty acid-CoA esters since this inhibition can be overcome by the addition of carnitine. In mitochondria from fed animals there was a much more pronounced inhibition of state 3 and state 4 respiratory rates and carnitine did not produce any effect.

Methods

Rat-liver mitochondria were prepared according to Weinbach [6]. After the second washing in a medium containing 0.25 M sucrose and 10 mM Tris-HCl (ph 7.4) the mit chondria were suspended in the same medium. The stock suspension usually contained 80 mg/ml of mitochondrial protein.

Respiratory rates were measured polarographically on the LP-7 polarograph. Incubation conditions are described in the legends to the figures.

ATPase activity was determined by the measurements of the pH changes of the mediums by Mitchell's method [7]. To diminish the unspecific pH changes due to adenilate kinase activity Mg^{2+} ions were abolished from the medium by adding EDTA.

Bovine Serum Albumin (Kochlight Lab., England) was defatted by the method of Chen [8].

Mitochondrial protein concentrations were determined by the biuret method [9].

Results

Klingenberg and Pfaff [10, 11] showed that in coupled mitochondria the affinity of the adeninenucleotide translocase to ADP was much higher than to ATP, when ADP and ATP were transported from outside into matrix space. In the presence of an oxidative phosphorylation uncoupler the differences in the affinities of the carrier to exogenous adenine nucleotides were eliminated [10, 11]. Pande and Blanchaer [4] emphasized that for rat liver mitochondria the reported values of K_m for ADP, determined by the respiratory rates measurements, varied from 1 μM to 50 μM. It seems to be evident that these differences in the reported K_m values for ADP may depend not only on the methods used, but on the initial coupling states between respiration and phosphorylation in mitochondria also. It is customary to use the respiratory control ratios to characterize the state of the coupling mechanism in mitochondria. According to the chemiosmotic hypothesis of coupling proposed by Mitchell [12], the degree of coupling between respiration and phosphorylation depends on the state of the inner mitochondrial membrane which in intact mitochondria has very low permeability for H^+, OH^-, K^+, Cl^-, and other ions. Since different samples of mitochondria may have different levels of permeability for H^+ and OH^- ions and thus different respiratory control ratio values it becomes evident that determination of K_m values for ADP must be based only on the measurements of the rate of phosphorylation-coupled respiration. The noncoupled part of respiration can be determined in the presence of oligomycin.

Figure 1 presents results of the determination of the K_m values for ADP, measured in the presence of the glucose + hexokinase Trapping system for two samples of mitochondria with different respiratory control ratios. Each point on the double reciprocal plot corresponds to the rate of ADP-stimulated succinate oxidation obtained by means of subtraction from the over-all respiratory rate of the nonspecific respiratory rate. The latter was established for each ADP concentration by measuring the rate of succinate oxidation by mitochondria preincubated for 2 min with 4 μg of oligomycin. It can be seen from Fig. 1 that with tightly-coupled mitochondria (RC = 4.5) the K_m value for ADP was 13.5 μM, while with loosely-coupled aged mitochondria (RC = 1.6) the K_m value for ADP was 80 μM.

Figure 2 shows the K_m value for ATP determined by measuring of the rates of ATP hydrolysis by mitochondria in the presence of 3 μM CCP and respiratory inhibitors. Comparing the K_m values for ADP, obtained
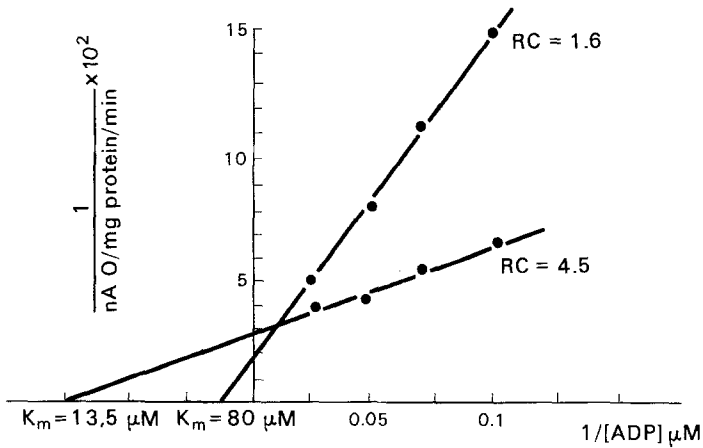
Figure 1. Relationships between respiratory control ratios and K_m values for ADP. The reaction mixture contained 100 mM KCl, 20 mM Tris-HCl (pH 7.4), 5 mM phosphate, 0.5 mM $MgCl_2$ 5 mM succinate, 50 mM glucose, 0.4 mg hexokinase, 3 μg rotenone, 1 mg mitochondrial protein from livers of rats fasted for 12 h. Total volume 1 ml.
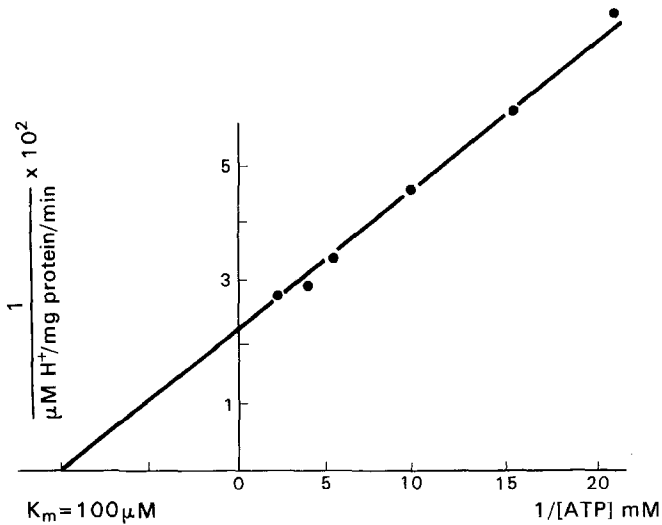

Figure 2. Determination of K_m for ATP hydrolysis by uncoupled mitochondria. The reaction mixture contained 125 mM KCl, glycylclycine 3.3 mM (pH 7.1), 2 mM EDTA, 1 mg/mg protein antimycin, 4 μg rotenone, 3 μM CCP, 5 mg mitochondrial protein from livers of rats fasted for 12 h. Total volume 6 ml.

for mitochondria with different respiratory control ratios, with K_m value for ATP hydrolysis by fully uncoupled mitochondria one can conclude that as mitochondria become uncoupled the K_m values for ADP succinate oxidation approach the K_m value for ATP hydrolysis which is equal 100 μM. These results agree with the data offered by Klingenberg *et al.* [1, 10] that in uncoupled mitochondria the affinities of the adeninenucleotide translocase to ADP and ATP are equal.

Since in coupled mitochondria there are differences in the affinities of adeninenucleotide translocase to ADP and ATP, the question arises as to how the inhibitory action of palmitoyl-CoA exerted on the transport of adenine nucleotides depends on the degree of affinity of the carrier to ADP and ATP.

Figure 3 shows the K_i values determined according to Dixon [14] for the inhibition of palmitoyl-CoA of ADP-stimulated succinate oxidation by freshly-prepared mitochondria (K_m for ADP = 13.5 μM), aged mitochondria (K_m for ADP = 80 μM), and of the uncoupler-stimulated mitochondrial ATPase activity (K_m for ATP = 100 μM). It is evident from Fig. 3 that in all cases the obtained K_i values for palmitoyl-CoA were equal to 0.5 μM, and thus were not dependent on the K_m values for adenine nucleotides.
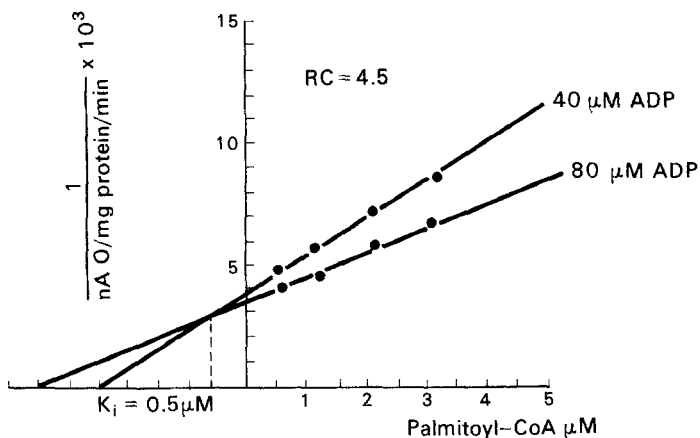
Very low K_i values for palmitoyl-CoA inhibition of ATP synthesis or hydrolysis reactions in mitochondria implies that these reactions must be sensitive to the changes in palmitoyl-CoA concentrations in the cytoplasm. The long-chain fatty acyl-CoA synthetase (acid : CoA ligase/AMP/, EC 6.2.1.3) is localized in microsomes and mitochondria and its activity greatly exceeds the capability of mitochondria to oxidize fatty acids [15, 16]. It is known also that concentration of palmitoyl-CoA in cytoplasm depends on the state of metabolic processes in an animal. Under certain conditions, such as starvation [17, 18], ethanol intoxication [19], diabetes mellitus [20], fatty acids serve as the main substrates for energy production but the concentration of palmitoyl-CoA in liver also increases [21]. It is conceivable that during preparation of mitonhondria some amount of palmitoyl-CoA remain bound to the mitochondrial membranes and can decrease the rate of phosphorylation of exogenous ADP. In this case the extent of inhibition of adenine nucleotide transport should depend on the state of metabolic processes.

To support this idea we prepared liver mitochondria from rats which were starved for 2 days. This fasting time was long enough to double the concentration of palmitoyl-CoA in livers of rats [18]. For comparison we used mitochondria from livers of rats which were sacrificed 1 h after the last meal.

Figure 4 demonstrates that with mitochondria from fasted rats the rate of succinate oxidation after the first addition of 150 μM of ADP was 30% lower than the maximal respiratory rate in a fully uncoupled state after addition of CCP. When mitochondria from starved rats were

preincubated for 1-3 min with 0.5 mM of carnitine (Fig. 4B) the rate of oxygen consumption after the first addition of ADP was only 6% lower than in the uncoupled state.
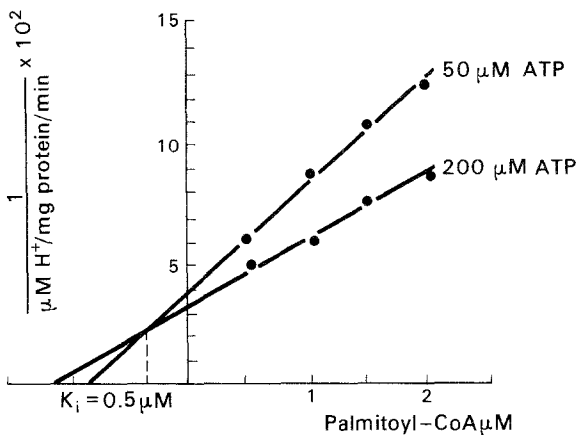
It may be seen from Fig. 4A, B that in the presence of carnitine there was also an initial increase in the rate of succinate oxidation in the absence of added ADP. This was probably because the transport of the formed palmitoyl-carnitine through the inner mitochondrial membrane used the energy of the transmembrane potential [22].

(A)

(B)
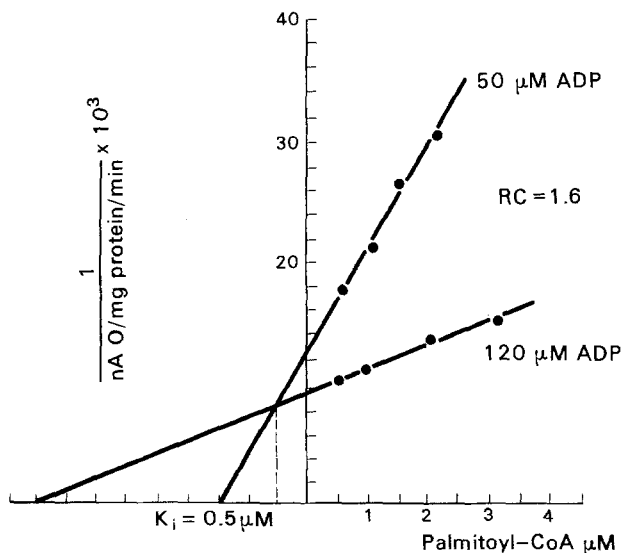
Figure 3. Determination of K_i values for palmitoyl-CoA inhibition of ADP-stimulated succinate oxidation at different RC values (A and B) and inhibition of ATPase activity (C). Incubation conditions for (A) and (B) as in Fig. 1, for (C) as in Fig. 2.
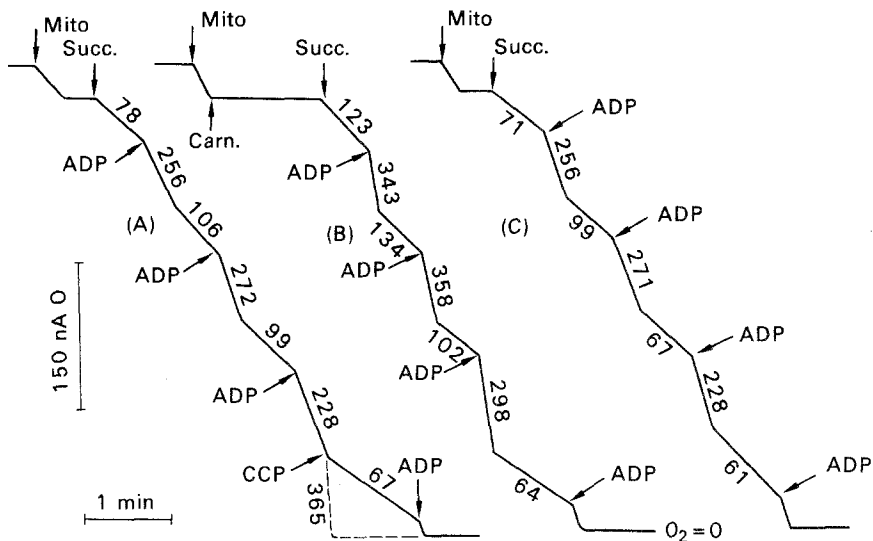
Figure 4. The influence of carnitine and BSA on the respiration of mitochondria from livers of starved rats. The reaction mixture contained 100 mM KCl, 50 mM sucrose, 20 mM Tris-HCl (pH 7.4), 5 mM phosphate, 0.5 mM MgCl$_2$ 3 μg rotenone. Additions: 5 mM succinate, 150 μM ADP, 5 x 10^{-7} M CCP, mitochondria 2 mg. Total volume 1 ml. (A) Control, (B) preincubation with 0.5 mM carnitine, (C) preincubation with 0.1% BSA.

Figure 4C shows that the addition of defatted Bovine Serum Albumin (0.1%) into the incubation medium caused a slight decrease in the state 4 respiratory rate, but had no influence on the state 3 respiratory rate. This fact probably indicates that inhibition of state 3 respiration of liver mitochondria from fasted rats was not dependent on the presence of free fatty acids, which could serve as substrates for the formation of their CoA esters during the course of incubation. The lack of influence of BSA on state 3 respiration showed also that under the prevailing conditions albumin was not able to adsorb long-chain fatty acyl-CoA esters, which were already bound to the mitochondrial membranes.
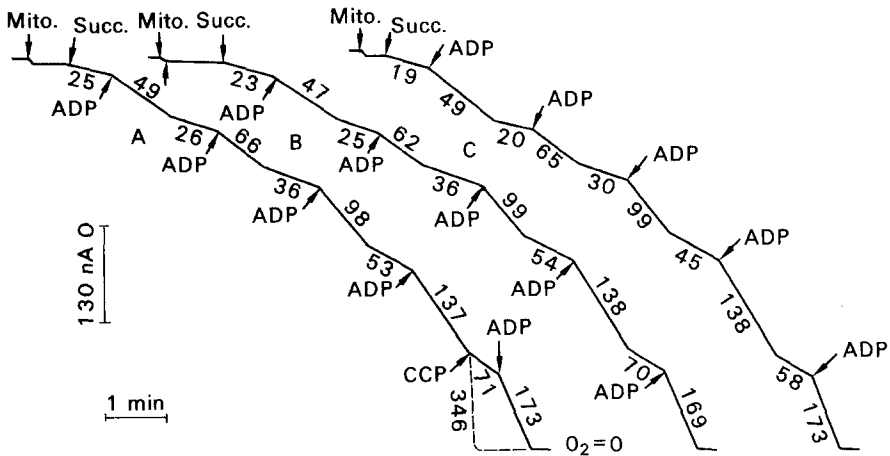

Figure 5. The influence of carnitine and BSA on the respiration of mitochondria from livers of fed rats. Incubation conditions and additions as in Fig. 4.

From Fig. 5A it may be seen, that mitochondria from the livers of fed rats showed a marked inhibition of succinate oxidation both in state 4 and state 3, but the respiratory rate in the fully uncoupled state was of the same order as in mitochondria from fasted rats. After the first addition of ADP to respiring liver mitochondria from fed animals the rate of oxygen consumption made only 14% of the respiratory rate in the uncoupled state. The subsequent additions of ADP, or better to say, the consecutive transitions from state 4 to state 3 and back, resulted in the gradual increase in the responses of mitochondria to ADP. But even after the fifth addition of ADP the rate of oxygen consumption made up only 50-60% of the rate of uncoupled respiration. Both carnitine (Fig. 5B) and defatted BSA (Fig. 5C) had no effect on the state 3 respiration of liver mitochondria from fed rats.

It should be noted, that mitochondria from the livers of fed rats usually were more variable as compared to mitochondria from the livers of starved rats in the degrees of inhibition of state 4 and state 3, as well as in the magnitudes of respiratory control ratios. This probably depends on the individual amount and time of food intake, the rates of food digestion and absorption etc., observed in different animals.

Discussion

It may be seen from Fig. 3A, B and C that in our experiments the K_i values for palmitoyl-CoA inhibition of the ADP-stimulated succinate oxidation at different states of coupling, and the inhibition of the uncoupler-stimulated mitochondrial ATPase activity were equal to 0.5 μM. This means, that the inhibition of adenine nucleotides transported by palmitoyl-CoA does not depend on apparent affinity of the translocase to ADP and ATP. The same conclusion may be derived when considering the relationships between adenine nucleotides, the inhibitor and binding site of the translocase on the outer side of the inner mitochondrial membrane. It is conceivable, that in this case the relationships between ADP (or ATP), palmitoyl-CoA and the carrier must depend only on the concentrations of these substances. It is also important that the magnitude of the transmembrane potential should not influence the process of binding of adenine nucleotides and palmitoyl-CoA to the carrier.

The low values of K_i for the palmitoyl-CoA inhibition of adenine nucleotides transport through the inner mitochondrial membrane assume the possible dependence of the adeninenucleotide translocase on the amount of the cytoplasmic palmitoyl-CoA and other long-chain fatty acyl-CoA esters bound to the inner mitochondrial membrane. This factor must depend on the state of metabolic processes in experimental animals.

Starvation is a good and easy reproducible model of an elevated level of palmitoyl-CoA in liver cells [16, 17]. As it may be seen from Fig. 4A, B the state 3 respiratory rate of mitochondria from the livers of starved rats is 70%. of the theoretically maximal rate in the uncoupled state. In the presence of 0.5 mM carnitine the rate of succinate oxidation in the state 3 becomes equal to the rate of respiration in the presence of the uncoupler. From this data it may be assumed that the 30% inhibition of ADP-stimulated respiration in mitochondria from the livers of starved rats was caused by adenine nucleotides transport inhibition by long-chain fatty acyl-CoA esters bound to the inner mitochondrial membrane. That carnitine is able to abolish the inhibition of adenine nucleotides transport by acyl-CoAs, namely by oleoyl-CoA, was shown by Shug *et al.* [23].

The origin of long-chain fatty acyl-CoA esters which were bound to the membranes of isolated mitochondria from the livers of fasted rats is an important problem. The first possibility may be explained by the binding to the mitochondrial membranes of acyl-CoAs which were preformed in the cytoplasm *in vivo*. The second one consists in the formation of acyl-CoAs by acyl-CoA synthetase (EC 6.2.1.3) in the mitochondria from free fatty acids in course of incubation. The third possible explanation could be the assumption that acyl-CoAs can bind to the mitochondria during the disintegration of tissue cells and the preparation of mitochondria. The last two assumptions may eliminate the physiological significance of the influence of acyl-CoAs on the adenine nucleotides transport under discussion.

The last explanation of the origin of long-chain fatty acyl-CoA esters which are bound to the prepared mitochondria seems to be fairly possible since acyl-CoA derivatives are bound to the proteins and their concentration in the free form is negligible. This problem, however, remains to be solved experimentally.

It is known that defatted Bovine Serum Albumin binds free fatty acids and fatty acyl-CoAs. According to Pande and Blanchaer [4] defatted BSA was able to prevent or abolish the inhibition of ADP-stimulated mitochondrial respiration by adding palmitoyl-CoA. In our experiments, however, the addition of defatted BSA to liver mitochondria from starved rats had no influence on the state 3 respiratory rate, though there was some inhibition of the state 4 rate. The latter may probably be explained by the binding of endogenous free fatty acids to BSA.

From the results presented on Fig. 5A, B, C it may be seen that mitochondria from fed rats showed a strong inhibition of succinate oxidation both in state 4 and state 3. The respiratory control values are also low. This inhibition of respiration was not due to the inhibition of the respiratory chain since the rate of succinate oxidation in the presence of the uncoupler was of the same order as in mitochondria from livers of fasted rats.

Heldt [24] clearly showed that the rate of phosphorylation of the extramitochondrial ADP was directly proportional to the intramito-chondrial concentrations of ADP and ATP. It was shown also that the rate of state 4 respiration depends on the values of the phosphate potential inside and outside the mitochondria [25].

Figure 5A demonstrates that the consecutive transitions of mito-chondria from livers of fed rats from state 4 to state 3 and back resulted in the gradual increase in responses to ADP, as well as in the increase of the state 4 respiratory rate. This probably may be explained by the changes in intramitochondrial ADP concentrations or by the changes of the phosphate potential values or by both. In our preliminary experiments we have found that the preincubation of liver mitochondria from fed rats with α-oxoglutarate resulted in the increase of the

respiratory rates in states 4 and 3. It is conceivable that main reasons for the changes in the behaviour of liver mitochondria from fed rats lie in decreased levels of intramitochondrial ADP and ATP with the concomitant increase in the concentration of AMP.

Thus, it can be concluded that mitochondria from the livers of fed and starved rats serve as examples when adenine nucleotides translocations through the inner mitochondrial membrane regulate the entire mitochondrial metabolism.

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