MITOCHONDRIAL RESPIRATORY CONTROL AND PHOSPHORYLATIVE ACTIVITIES IN A MAGNESIUM-FREE MEDIUM

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It has generally been found necessary to include magnesium in the reaction medium in order to obtain maximum P/O values in mitochondrial oxidative phosphorylation. Employing the rapid polarographic platinum electrode technique of CHANCE AND WILLIAMS[1] for the assay of phosphorylative and respiratory activities, however, BALTSCHEFFSKY[2,3] has shown, that maximum P/O values can be obtained for the mitochondrial oxidation of succinate and β-hydroxybutyrate in a medium to which no divalent cations were added. It has also been found that a temporary increase in the rate of respiration occurs some time after the mitochondria have been incubated in the Mg^{++}-free** medium at room temperature, and that this is correlated with a rapid change in the physical properties of the particles[3].

Additional studies and a more detailed account of the previous ones are presented in this paper, which is focused on the properties of mitochondria in a magnesium-free medium for oxidative phosphorylation and the role of magnesium in the functions of isolated mitochondria. The results of CHANCE[4] and data reported in the present paper indicate that magnesium is directly, and in that sense specifically involved in the respiratory control mechanism.

MATERIAL AND METHODS

Material: Livers were obtained from 150–200 g white male rats (Wistar strain) or, when indicated, from 250–300 g guinea pigs. The reagents employed in the reaction media were of analytical grade. ADP*** was obtained as the sodium salt from Sigma Chemical Co. The succinate and the α-ketoglutarate were recrystallized.

Methods: In the preparation of mitochondria the livers were removed as quickly as possible after decapitation of the animals, put into 0.25 *M* sucrose (Merck reagent) at 0° C and then rapidly washed twice with additional cold sucrose. The method of LARDY AND WELLMAN[5] was modified to involve only one homogenization (in a rather loose Potter-Elvehjem homogenizer using a Teflon pestle). Glass-distilled water was used throughout. All centrifugations were done in a Servall refrigerated centrifuge (two low-speed, three high-speed centrifugations). The mitochondria were taken up in a volume of sucrose equal to one fifth of the volume of the livers. This suspension usually contained between 25 and 50 mg protein per ml.

Respiration and phosphorylation were measured using the platinum electrode technique of CHANCE AND WILLIAMS[1]. The optical density measurements at 510 mμ were performed in a Beckman spectrophotometer with a light path through the mitochondrial suspension of 1 cm.

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** We use the term "Mg-free" because it is reasonable to assume that the amount of magnesium present in the reagents is too small to be of any influence in the experiments reported. For example, the rapid decrease in optical density at 510 mμ (see below) is as rapid in the presence of 1 m*M* Mg^{++} as in the absence of added Mg^{++}.

*** Abbreviations: adenosine diphosphate = ADP, adenosine triphosphate = ATP, moles per liter = *M*, millimoles per liter = m*M*.

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RESULTS

The P/O values for succinate and β-hydroxybutyrate oxidation in rat liver mitochondria were earlier reported to be the same in a magnesium-free as in a magnesium-containing medium[3]. Table I gives the P/O values for the oxidation of various substrates in a medium, which prior to the substrate addition contains only 16 m*M* phosphate buffer and 105 m*M* KCl. Thus this medium, compared to medium I in ref.[3], lacks not only Mg^{++} but also F^-. (These experiments were carried out with guinea pig liver mitochondria.)

TABLE I

P/O VALUES FOR THE OXIDATION OF DIFFERENT SUBSTRATES

Substrate	*P/O value*	*Average*
Succinate	1.85; 2.15; 1.85; 2.15	2.0
Glutamate	3.15; 3.25; 3.30; 3.10	3.2
β-Hydroxybutyrate	3.15; 3.10	3.1
α-Ketoglutarate	3.50; 3.55; 3.40	3.5

The final concentration of substrate added was 10 m*M*. The medium contained 16 m*M* phosphate buffer and 105 m*M* KCl. The pH was 7.4

The P/O values obtained for succinate, glutamate and β-hydroxybutyrate oxidation are very close to the accepted maximum ones, 2, 3 and 3 respectively. For α-ketoglutarate the accepted maximum is 4, whereas our average experimental value is 3.5.

The measurements of the P/O values in the magnesium-free medium are, of course, to be carried out prior to the "uncoupling event", which is defined in ref.[3]. Fig. 1 shows a comparison between the rate of respiration of mitochondria supplied with 10 m*M* β-hydroxybutyrate in a magnesium-containing and a magnesium-free medium at 25° C. The rate is constant in the Mg^{++}-containing medium until it increases several fold upon addition of phosphate acceptor. But in the Mg^{++}-free

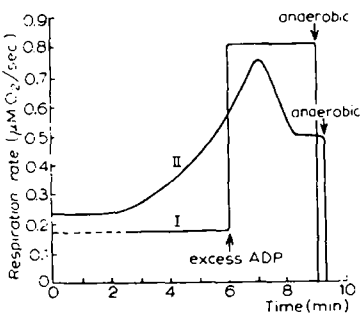

Fig. 1. Rate of respiration in a magnesium-containing (96 m*M* KCl, 16 m*M* phosphate buffer and 6 m*M* $MgCl_2$) and a magnesium-free (105 m*M* KCl and 16 m*M* phosphate buffer) medium. The pH was 7.2 in both cases. I: Mg present; II: Mg absent. Other details are given in the text.

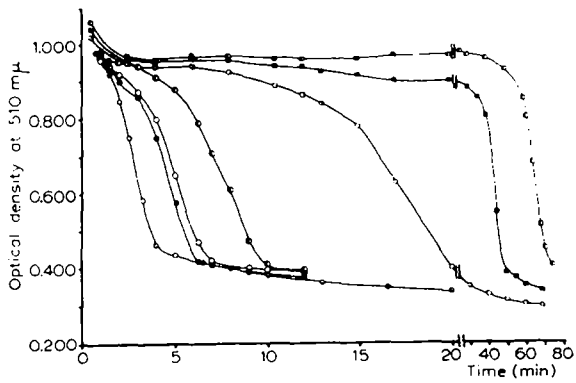

Fig. 2. Stabilizing effect of ATP on mitochondria as measured from the optical density change at 510 mμ. The medium contained 105 m*M* KCl and 16 m*M* phosphate buffer. The pH was 7.2. Molarities of ATP: ◉ = 0; ● = $5 \cdot 10^{-5}$; ○ = $1.5 \cdot 10^{-4}$; ◐ = $2.5 \cdot 10^{-4}$; ◑ = $5 \cdot 10^{-4}$; ■ = 10^{-3}; ◧ = $1.5 \cdot 10^{-3}$.

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medium the rate starts to increase spontaneously after some minutes. It reaches about the same level as the rate in the presence of phosphate acceptor in the magnesium-containing medium, and then a decrease occurs. Addition of ADP at any point where the rate is not yet at the maximum immediately causes an increase to that value. When the maximum is reached or passed, addition of ADP does not stimulate the respiration rate. These observations indicate that in the magnesium-free medium there is a spontaneous change from State 4 to State 3, as they are defined by CHANCE AND WILLIAMS[6], and that the system responsible for the inhibited respiration is going out of function prior to the system for oxidative phosphorylation. This sequence of events is principally in agreement with two findings, by CHANCE AND WILLIAMS[7] that the degree of respiratory stimulation can be brought down several fold without any decrease in the P/O value, and by HOCH AND LIPMANN[8] that the same P/O value can be obtained both when respiratory control is present and when it is missing.

In the Mg^{++}-free medium the "ATP-ase" effect is thus, directly or indirectly, due to nothing but lack of magnesium ions. Variation of the amount of magnesium added showed, both when the time for loss of respiratory control and for decrease in optical density at 510 mμ was used as indicator, that the critical concentration of added Mg^{++} was around 3 mM. The protective action on mitochondria of added Mg^{++} was first shown by RAAFLAUB[9], and of added ATP by the work of RAAFLAUB[9,10] and SLATER AND CLELAND[11]. CHAPPELL AND PERRY[12] have demonstrated that ATP can reverse the swelling of isolated skeletal muscle mitochondria. ERNSTER, LINDBERG AND LÖW[13] have shown that ATP can cause a reconstruction of the oxidative phosphorylation system of damaged mitochondria and that Mn^{++} potentiates the effect, and BEYER, ERNSTER, LÖW AND BEYER[14] found that a reversal of the decrease in optical density at 510 mμ occurs simultaneously with the reconstruction obtained by ATP + Mn^{++}. In Fig. 2 it is shown, that also in the Mg^{++}- and F^--free medium for oxidative phosphorylation ATP, already showing some effect at 0.05 mM, with increasing concentration gives an increased stability to the system, as judged by measurement of the optical density at 510 mμ. Fig. 3 shows that the rapid drop in optical density at 510 mμ can be reversed completely by addition of 0.75 mM ATP + 7.5 mM $MgCl_2$ (final concentrations), and only partially when the concentration of the added $MgCl_2$ is 0.75 mM. Below the concentrations given for complete reversal only partial restoration was obtained.

The restoration experiments were performed with guinea pig liver mitochondria. The uncoupling started after a period several times longer than with rat liver mitochondria, which is seen by comparison with Fig. 1. This is typical and shows that liver mitochondria from guinea pig are more stable in this medium than those from rat. A similar phenomenon is that a complete reversal of the optical density drop at 510 mμ never could be obtained with mitochondria from rats. About 70% reversal was the usual value at the concentrations used.

Variation of the pH showed, that the duration of the coupled state in the Mg^{++}-free medium increases with increasing pH (Fig. 4). This is valid up to a pH of about 8.2. Below pH 5.8 the system was less than half a minute in the coupled state and the rapidity of the uncoupling made measurements difficult. The rapid increase in the duration of the coupled state between pH 7 and 8 may indicate, that a group with a pK of about 7.5 strongly influences the stability of a bond between Mg^{++} and the mitochondrion.

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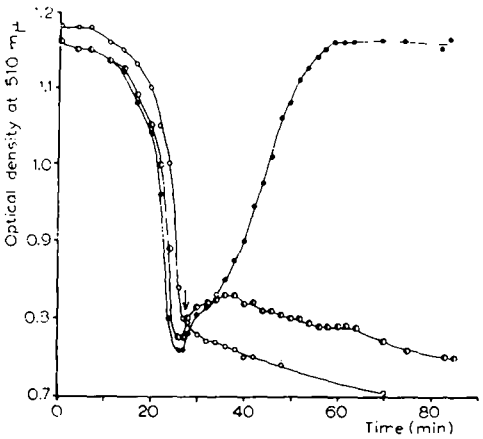

Fig. 3. Reversal of the optical density change at 510 mμ, caused by addition of Mg^{++} + ATP. The medium contained 105 m*M* KCl and 16 m*M* phosphate buffer. Substrate was 10 m*M* glutamate. The pH was 7.2. The arrow indicates time for additions. Additions: ○ = no addition; ◐ = 0.75 m*M* $MgCl_2$ + 0.75 m*M* ATP; ● = 7.5 m*M* $MgCl_2$ + 0.75 m*M* ATP (final concentrations).

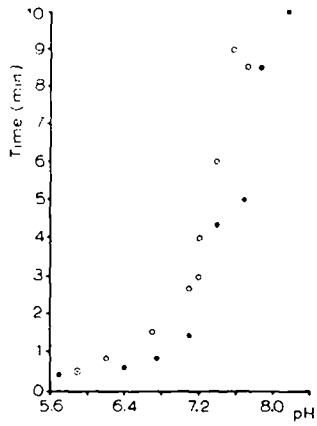

Fig. 4. Time for appearance of maximum respiration rate at different pH values. Empty circles: the medium contained 105 m*M* KCl and 16 m*M* phosphate buffer. Two measurements around pH 8 gave more than 18 and more than 25 minutes. Full circles: the medium contained 105 m*M* KCl, 16 m*M* phosphate buffer and 15 m*M* tris buffer. The pH was controlled before and after the measurements.

When the phosphate concentration of the Mg^{++}-free medium was kept constant at 15 m*M* at a pH of 7.6 and the KCl concentration was varied, it was found that the time for the uncoupling event to appear (6 minutes for maximum respiration rate at 25° C) was the same from 0.126 to 0.294 osmolar KCl. At 0.084 osmolar KCl the time was 4½ and at 0.042 osmolar KCl 3 minutes.

When the KCl concentration of the Mg^{++}-free medium was kept constant at 0.210 osmolar at pH 7.6 and the phosphate concentration was varied, the time for uncoupling, measured as appearance of maximum rate of respiration, decreased with increasing phosphate concentration. The result is in agreement with earlier findings that phosphate destroys isolated mitochondria[15–17]. Table II shows the values obtained. The duplicate experiments show the degree of exactness usually obtained with this method.

TABLE II

INFLUENCE OF PHOSPHATE CONCENTRATION ON TIME FOR UNCOUPLING

Phosphate concentration m*M*	*Time*	
	minutes	*seconds*
5	8	30
5	8	20
10	6	20
10	5	45
16	4	00
16	4	20
25	3	30
50	2	00

DISCUSSION

The polarographic method described by CHANCE AND WILLIAMS[1] has been used in the present study of mitochondrial oxidative phosphorylation in a medium containing

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only phosphate buffer and potassium chloride. Thanks to its rapidity and continuous recording the method has proved to be valuable when rapidly occurring changes have to be detected. Contrary to what previously has been found, it was possible to obtain maximum P/O values for the oxidation of a number of different substrates in spite of the fact that no magnesium, no other divalent cations and no magnesium-containing hexokinase was added to the medium. With rat liver mitochondria the measurements of the P/O values usually had to be performed within a few minutes after the particles had been transferred from 0° C to the reaction medium of 25° C, *i.e.* before the uncoupling of the controlled respiration, caused by the lack of magnesium, occurred. This might explain the failure of previous workers to obtain maximum P/O values in a Mg^{++}-free medium with the slower Warburg technique, although the ATP usually employed when this technique was used prolongs the duration of the coupled state several fold, at least under our conditions.

There are, as is shown, striking changes in structure and function of the respiring mitochondria owing to the lack of magnesium in the medium. Variation of the ATP concentration, the concentration of inorganic phosphate and the pH greatly influences the stability of the particles. A simple and attractive explanation of these effects is the following. Magnesium is in some way bound to the structure of the "intact" mitochondrion in a bond, which at least *in vitro* is labile and critical for the state of the particle. The bond between magnesium and the mitochondrion breaks and the particle goes spontaneously from the "intact" to the "aged" state, if the concentration of the ion in the adjacent liquid phase is too low to provide a new, similar bond according to the principle in the law of mass action. The stabilizing effect of ATP may indicate that it is involved in the binding of Mg^{++} to the particle, while the nature of the action of inorganic phosphate is still completely obscure. The bond, which would be critical for retained structure and function may, however, not be a Mg^{++}-ATP bond, since the critical pK deduced from our data is about two units higher than the pH-range found by MARTELL AND SCHWARZENBACH[18] and NANNINGA[19] for the greatest change in the "association constant" of the Mg^{++}–ATP bond.

The above interpretation is supported by some earlier results of SIEKEWITZ AND POTTER[20]. They found that the mitochondria contain (besides bound nucleotides) bound magnesium, which is released after some minutes of incubation in sucrose at 30° C. The rate of release was increased by inorganic phosphate, which is in agreement with our results and their interpretation. ERNSTER AND LÖW[21] have assumed that Mg^{++} binds ATP to the structure of mitochondria on the basis of extensive experimental studies, and they have stated that bound Mg^{++} might be responsible for linking the respiratory system with the primary energy carriers. The complex formed between Mg^{++} and ATP in solution has been investigated by MARTELL AND SCHWARZENBACH[18] and by NANNINGA[19]. SZENT-GYÖRGYI[22] has postulated the existence of a quadridentate and MARTELL AND SCHWARZENBACH[18] a terdentate magnesium chelate of ATP. There are many indications pointing towards a connection between Mg^{++} and ATP in the mitochondrial structure, and our data are in agreement with such a hypothesis.

Our results provide a logical explanation to the Mg^{++}–Ca^{++} antagonism and the Mg^{++}-thyroxine antagonism, as has already been briefly pointed out by CHANCE AND WILLIAMS[23]. The Mg^{++}–Ca^{++} antagonism, shown by ERNSTER AND LÖW[21] to influence the integrity of mitochondria and by CHANCE[4] to influence the respiratory control

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and the steady state of the respiratory enzymes, and the Mg^{++}-thyroxine antagonism first detected by BAIN[24] and further investigated by MUDD, PARK AND LIPMANN[25], both may perhaps depend on competition. In the former case this would occur between Mg^{++} and Ca^{++} for a site in the mitochondrial structure or for ATP and in the latter case between thyroxine and the mitochondria for Mg^{++}.

The pronounced difference between the stability of the liver mitochondria, and their respiratory control system, from guinea pig and from rat in the Mg^{++}-free medium is possibly due to the fact that the guinea pig liver is softer and thus automatically causes a milder treatment during the homogenization. If this is the case, then it seems that also other functions of isolated mitochondria would be better preserved in mitochondria from guinea pig liver than from rat liver.

No report has been found in the literature about a pH-effect similar to ours, and the pH for maximum stability of mitochondria has usually been reported to be below 7. The mitochondria of RAAFLAUB[9] were reported to be stable at pH 6.2 for more than 30 minutes (20° C, isotonic mannose) but very labile at pH 7.4. WITTER AND COTTONE[26] found that the stability of isolated mitochondria in sucrose decreased from pH 6.2 to pH 8.0. It is reasonable to assume that several different factors can cause loss of mitochondrial integrity and that in our medium, containing KCl and phosphate buffer but no magnesium, the prevailing conditions favour the loosening of a bond between Mg^{++} and the mitochondrion, which results in our reported pH-influence: increasing stability from pH 5.8 to about pH 8.2.

It is interesting that the small particles which COOPER, DEVLIN AND LEHNINGER[27] have separated from digitonin extracts of mitochondria carry out oxidative phosphorylation with maximum P/O values without magnesium in the medium, and that the particles contain large amounts of structurally bound magnesium[28]. Our finding that mitochondria do not need magnesium in the medium for maximum P/O values eliminates the apparent discrepancy, reported by COOPER AND LEHNINGER[29], between mitochondria and particles from digitonin extracts of mitochondria in this respect.

The results of CHANCE[1] and data reported here indicate that magnesium is specifically involved in the respiratory control mechanism. Whether it is a component of the oxidative phosphorylation system as well remains an open question.

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SUMMARY

"Maximum" P/O values have been obtained in a magnesium-free medium for the mitochondrial oxidation of succinate, β-hydroxybutyrate, glutamate and α-ketoglutarate.

Lack of magnesium in the medium employed causes a loss of mitochondrial integrity and respiratory control. These phenomena are influenced by ATP, pH, inorganic phosphate and osmotic pressure. Favourable conditions for avoiding a rapid loss are: high concentration of ATP, a pH of about 8.0, low concentration of inorganic phosphate and isotonicity. In the medium used, isolated liver mitochondria from guinea pig are more stable than those from rat.

It is assumed that magnesium is specifically involved in the respiratory control mechanism.

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THE ABSORPTION OF AMINO ACIDS BY TWIN LOOPS OF RAT INTESTINE

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Three methods of studying the removal of amino acids by intestinal tissue have been compared in a previous paper (AGAR, HIRD AND SIDHU[1]). One of these methods —uptake by numbers of small segments (0.5 cm)—has been studied in some detail. Using selected amino acids, L- and D-methionine, as inhibitors, it has been found that L- but not D-methionine inhibits the three processes, the uptake, transfer and absorption of L-histidine. Further, the content of L-histidine in the tissue at the end of the experiment was found to be affected in the same way as the uptake, transfer and absorption. As experiments with uptake gave similar results to the other two methods

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