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Biochemical and Physiological Characteristics of Ca-ATPase System of Rat Liver Mitochondria with Special Attention to the Effects of pH and Temperature

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The activity of Mg^{++}-dependent, Ca^{++}-activated adenosine triphosphatase (Ca-ATPase) of rat liver mitochondria was studied at varying medium compositions, pH and temperatures.

The enzyme system was characteristically sensitive to Ca^{++} concentration with a Km_{Ca} of approximately 0.06 mM. The optimal concentration of Mg was about 1 mM, above which the enzyme activity was progressively inhibited. The inhibitory effect of high Mg^{++} concentrations appeared to be due to the alteration of the Mg^{++}/ATP ratio. Variations in the Mg^{++}/ATP ratio affected Vmax but not the Km_{ATP}. The pH optimum for enzyme activity increased as the incubation temperature decreased, but the optimal OH^-/H^+ ratio of the medium was constant at around 0.1, regardless of temperature. The activity of the enzyme was not affected by La^{+++} (0.01 - 1 mM) and Ruthenium red (2.5 - 10.0 μM).

These results indicate that 1) the enzymatic characteristics of the Ca-ATPase system in the rat liver mitochondria is typical of those from other tissue preparations, 2) the enzyme system maintains the most effective catalytic conformation at a fixed level of OH^-/H^+ ratio of 0.1 when the temperature changes, and 3) the enzyme system may not play a role in the physiological transport of Ca^{++} in mitochondria.

In most animal species the pH of the body fluids, either extracellular or intracellular, changes inversely with body temperature, however the OH^-/H^+ ratio of the body fluids remains constant regardless of body temperature (Rahn *et al*, 1975; Reeves, 1977). The significance of this type of acid-base regulation was assumed to be a mechanism to prevent temperature induced disruption of protein structure and function (Malan *et al*, 1976). According to Reeves (1972, 1977), the charge states of intracellular and extracellular proteins are maintained constant if pH changes with temperature, such that OH^-/H^+ ratio remains unchanged. This implies that an enzyme system maintains a constant net charge (and hence a constant configuration) if OH^-/H^+ ratio of the medium is maintained constant during temperature variations. If so, the optimal pH for enzyme activity may not be constant but vary with the temperature. This notion has been tested in the toad skin Na-K-ATPase (Park and

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Hong, 1976). It was shown that the optimal pH for this enzyme increases as the assay temperature decreases, preserving a constant optimal OH^-/H^+ ratio. Similar observation has been made subsequently by Hazel *et al*, (1978) for various soluble and membrane-bound enzymes, such as acetyl CoA carboxylase, fatty acid synthetase, NADH- and succinate-cytochrome c reductases, of the trout liver.

In order to examine if such a relationship exists for other enzyme systems we investigated in the present study pH-temperature dependence of the mitochondrial Ca-ATPase activity. Since this enzyme system has not yet been properly characterized we also attempted to define its biochemical and physiological characteristics.

MATERIALS AND METHODS

Adult rats of either sex were used. Mitochondrial preparations of liver were obtained by a method similar to that described by Chance and Williams (1955). After an animal was sacrificed by a blow on the head, the liver was promptly removed and placed in an ice-cold 0.32 M sucrose solution. The tissue was then minced with a pair of surgical scissors and homogenized in 10 volumes of 0.32 M sucrose in a Dounce homogenizer. The homogenate was first centrifuged at 2000 x g for 20 minutes and the supernatant was centrifuged at 8000 x g for 20 minutes in a Sorvall centrifuge (Model RC 2-B) which was preset at 4°C. The resulting pellet, consisting mainly of mitochondria, was suspended in a 0.32 M sucrose solution to a concentration of 6-10 mg protein per ml. Protein concentration was determined by the method of Lowry *et al.* (1951).

The activity of ATPase was determined from the rate of ATP hydrolysis during incubation of 0.1 ml mitochondrial suspension with 2 ml of an appropriate medium containing Na_2-ATP as a substrate. In order to estimate the total ATPase activity Mg^{++} and Ca^{++} ions were present in the incubation medium. The Mg-dependent adenosine triphosphatase (Mg-ATPase) activity was determined in the absence of Ca^{++} and the presence of 1 mM ethyleneglycol-bis (β-aminoethyl ether)-N, N′-tetraacetic acid (EGTA). The difference between the total ATPase activity and the Mg-ATPase activity was taken as the measure of Ca-ATPase activity.

The optimal conditions for the assay were determined by varying the concentrations of Ca^{++}, Mg^{++} and ATP in the medium. In all cases, incubation medium contained 125 mM sucrose and 50 mM Tris-maleate buffer. Unless specified otherwise, the reaction temperature was 37°C and the pH of the medium was 7.0. After 4 minutes of preincubation the reaction was initiated by the addition of ATP to the test tube containing the preincubation mixture. In most cases the incubation time was 10 min. At the end of incubation, 0.5 ml of 20% trichloroacetic acid (TCA) solution was added to terminate the reaction and the test tube was immediately placed on ice. The mixture was then centrifuged at 3000 rpm for 15 minutes. After dilution of 1 ml of the supernatant with 4 ml of distilled water the concentration of inorganic phosphate (Pi) was determined according to the method of Fiske and Subbarow (1925).

RESULTS

It has been previously established that in the presence of Mg^{++} ion, preparations of isolated plasma membrane, sarcoplasmic reticulum and actomyosin display an ATPase activity which is stimulated by Ca^{++} ion (Bond & Green, 1971; Dunham & Glynn, 1961; Owens *et al*, 1973; Kim *et al*, 1977; Lee *et al*, 1975). In order to identify such a Ca-activated ATPase in mitochondria we first performed a series of

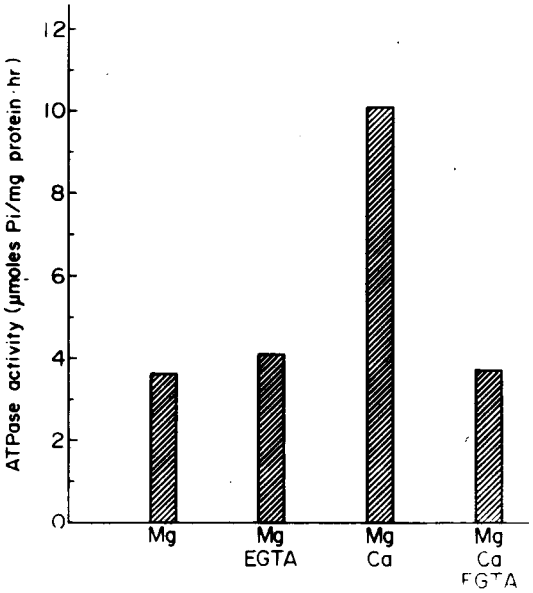

Fig. 1. ATPase activities of rat liver mitochondrial preparations in various cationic compositions of the miedum. Concentrations of ATP, Mg^{++}, Ca^{++} and EGTA are 5,1,0.5 and 1 mM, respectively. Each bar represents a mean of 4-5 experiments.

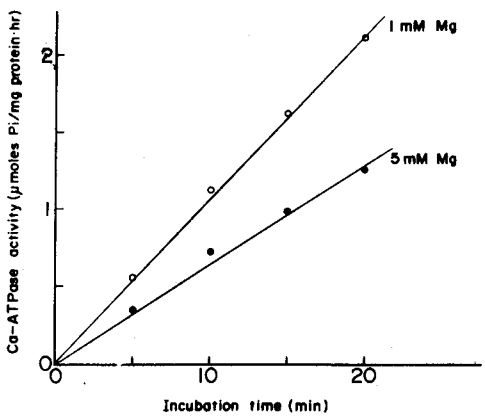

Fig. 2. Time course of the mitochondrial Ca-ATPase activity. Concentrations of ATP and Ca^{++} are 5 and 0.5 mM, respectively. Each point represents a mean of 2 experiments.

experiments on the mitochondrial preparation in the presence of; Mg, Mg+Ca, Mg+Ca+EGTA, Mg+EGTA, and Ca alone. Fig. 1 illustrates rates of ATP hydrolysis by mitochondrial preparations in various media mentioned above. With Mg^{++} as the major cation, the rate of ATP hydrolysis

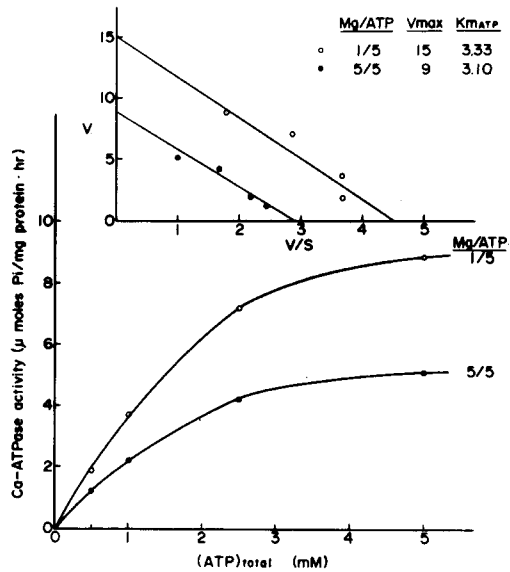

Fig. 3. Mitochondrial Ca-ATPase activities as a function of ATP concentration at two different Mg/ATP ratios. Each point represents a mean of 2 experiments.
Inset: Hofstee plot of the data shown in Fig. 3. In this plot the intercept of the line with Y-axis represents Vmax, and the slop represents -Km.

(Mg-ATPase) was approximately 35% of that measured in the medium containing Mg^{++} and Ca^{++} (i.e., Total ATPase). The Ca-dependent portion was completely inhibited by treatment with 1 mM EGTA, a powerful chelator of Ca^{++}. The addition of EGTA to the medium containing only Mg^{++} did not alter the enzyme activity. In the absence of Mg^{++}, some ATP hydrolysis was observed which was not sensitive to ouabain or EGTA. These results indicate that the mitochondrial preparation in the present study has a typical Mg^{++}-dependent, Ca^{++}-activated ATPase (Ca-ATPase) system.

In the next series of experiments, we examined the kinetic behavior of the Ca-ATPase system. In order to determine the optimal incubation period, the time course of the Ca-ATPase reaction was first determined. From this the initial velocity can be calculated. As shown

in Fig. 2 Ca-dependent ATP hydrolysis (i.e., ATP hydrolysis in the presence of Mg^{++} and Ca^{++} minus ATP hydrolysis in the presence of Mg^{++} alone) increased linearly with time during 20 minutes of incubation period in either 1 or 5 mM Mg^{++} containing media. In light of these results, an incubation time of 10 minutes was chosen for all subsequent experiments and the rate of the enzyme reaction was expressed as μ moles Pi/mg protein per hr.

Fig. 3 presents Ca-ATPase activities as a function of ATP concentration at two different Mg/ATP ratios. In one case the Mg^{++} concentration was adjusted such that the Mg/ATP ratio was held constant at 5/5, as in most other Ca-ATPase studies. In the other case, the Mg/ATP ratio was maintained constant at 1/5, since this value of Mg/ATP assured the maximal activation of Ca-ATPase in the present study (See below). In both cases the Ca^{++} concentration was fixed at 0.5 mM. As is seen in the Figure, the enzyme activity increased curvilinearly with ATP concentrations, showing a tendency of saturation at above 5 mM ATP. Hofstee plots of the data (Fig. 3 inset) indicate that the Vmax was 15 and 9 μ moles Pi/mg protein per hr for the Mg/ATP ratio of 5/5 and 1/5, respectively, and the Km was about 3.2 mM in both situations.

In order to determine if this decline of Vmax at a higher Mg/ATP ratio was due to reduction of the Ca/Mg ratio, we next measured the enzyme activity at a Mg/ATP ratio of 5/5 after the Ca^{++} concentration was increased to 2.5 mM. The results, however, indicated that this was not the case. The enzyme activity was not elevated by increasing the Ca^{++} concentration from 0.5 to 2.5 mM (Fig. 4).

The next series of experiments was to determine the optimal concentrations of Ca^{++} and Mg^{++}. Fig. 5 illustrates the effect of Mg^{++} concentration on Mg- and Ca-ATPase activities at two different conditions. In one case the Ca^{++}

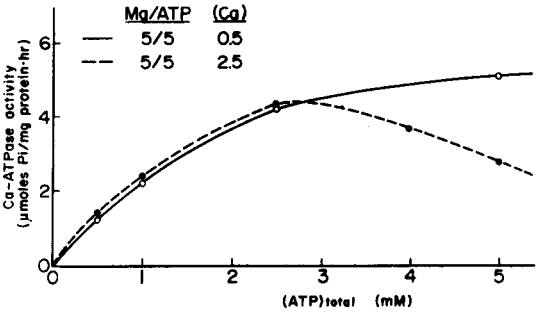

Fig. 4. Mitochondrial Ca-ATPase activities as a function of ATP concentration at two different Ca levels. Each point represents a mean of 2 (solid line) or 4 (broken line) experiments.

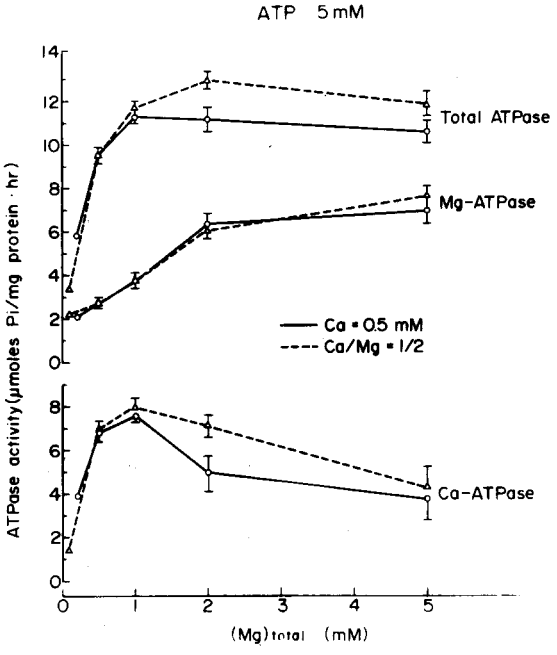

Fig. 5. Effect of Mg concentration on mitochondrial ATPase activities at a constant Ca concentration of 0.5 mM (solid line) and a constant Ca/Mg ratio of 1/2 (broken line). Each point represents a mean of 4 experiments. Vertical bars represent ± 1 S.E. of mean.

concentration was fixed at 0.5 mM and in the other case the Ca^{++} concentration was varied to maintain the Ca/Mg ratio of 1/2. In all case ATP concentration was 5 mM. When the Ca^{++} concentration was held constant (solid curves) Mg-ATPase activity increased progressively with the

Mg^{++} concentration. On the other hand, Ca-ATPase activity increased abruptly as the Mg^{++} concentration increased up to 1 mM, above which it was progressively inhibited. A similar result was obtained with a constant Ca/Mg ratio of 1/2, indicating that the reduction of Ca-ATPase activity at Mg^{++} concentrations above 1 mM was not due to relative increase in Mg^{++} concentration over Ca^{++} concentration.

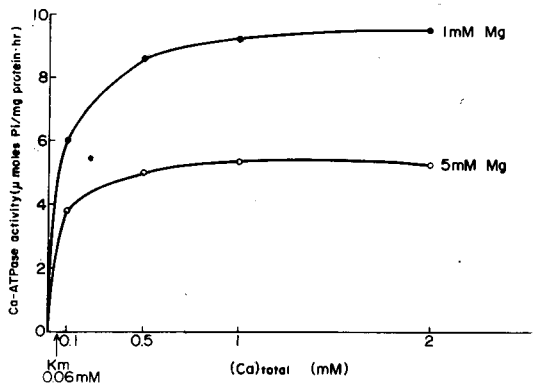

Fig. 6. Mitochondrial Ca-ATPase activities as a function of Ca concentration at two different Mg concentrations. ATP concentrat was 5 mM. Each point represents a mean of 4 experiments.

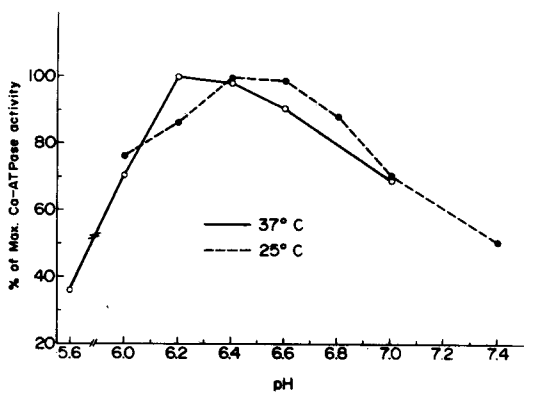

Fig. 7. Relative Ca-ATPase activities as a function of medium pH at 25° and 37°C. Concentrations of ATP, Mg^{++} and Ca^{++} are 5, 5 and 0.5 mM, respectively. Each point represents a mean of 4-8 experiments.

Fig. 6 depicts the dependence of Ca-ATPase activity on Ca^{++} concentration at constant Mg^{++} concentrations of 1 and 5 mM. Regardless of the Mg^{++} concentration, maximum enzyme activity was found at a Ca^{++} concentration of approximately 0.5 mM, whereupon the curve plateaued. However, the enzyme activity with 1 mM Mg^{++} was higher than that with 5 mM Mg^{++}, in agreement with the foregoing experiments.

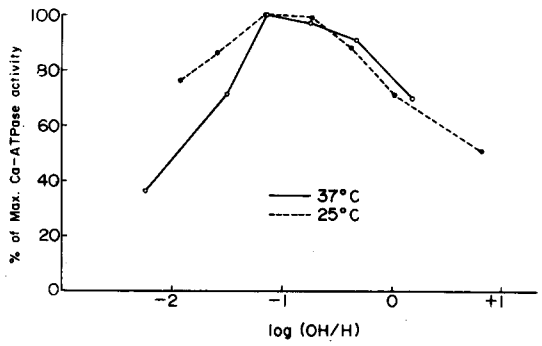

Fig. 8. Relative Ca-ATPase activities as a function of OH/H ratio in the medium at 25 and 37°C. Values are based on Fig. 7.

In another series of experiments, we investigated the pH dependence of Ca-ATPase. Fig. 7 illustrates relative enzyme activities as a function of the medium pH at 37 and 25°C. At all temperatures the enzyme activity varied with the pH of the incubation medium, displaying a definite range of pH optima. However, the pH optimum was not constant but increased from 6.2 at 37°C to 6.4 at 25°C. When the same data were plotted as a function of the OH^-/H^+ ratio in the medium (Fig. 8), the maximal enzyme activity was found at the medium OH^-/H^+ ratio of about 0.1, regardless of incubation temperature. These results indicate that the maximal Ca-ATPase activity is obtained at a fixed level of the OH^-/H^+ ratio but not at a fixed pH, when the incubation temperature changes.

In the final series of experiments, we examined effects of Lanthanum (La) and Ruthenium Red (RR) on the Ca-ATPase activity. These compounds are known as specific inhibitors of mitochondiral Ca^{++} transport (Scarpa & Azzone,

Table 1. Effects of Lanthanum (La) and Rethenium Red (RR) on the Ca-ATPase activity of rat liver mitochondria

	Ca-ATPase activity (μ moles Pi/mg protein/hr)
La(mM)	
0	7.25 ± 0.36
0.01	7.35 ± 0.46
0.1	6.87 ± 0.56
1.0	7.71 ± 0.65
RR(μM)	
0	7.74 ± 0.12
2.5	7.75 ± 0.19
5.0	7.75 ± 0.24
10.0	7.79 ± 0.06

Each value represents a mean of 4 experiments ± 1 S.E.

1970; Mela, 1968; Reed and Bygrave, 1974). As summarized in Table 1, Ca-ATPase in the present preparation was not apparently altered by either La^{++}(0.01-1 mM) or Ruthenium red (2.5-10 μM) at concentrations considerably higher than those for maximal inhibition of Ca^{++} transport in mitochondria.

DISCUSSION

The Ca-ATPase preparations in the present study appear to be similar to those from other tissues. The enzyme required Ca^{++} in addition to Mg^{++} for maximal activation. The Ca^{++}-dependent activity was completely abolished by EGTA (Fig. 1).

The enzyme system was characteristically sensitive to Ca^{++} concentration (Fig. 6). Km value for Ca^{++} (0.06 mM) was of similar magnitude as that obtained in red blood cell membrane (Bond and Green, 1971). The Ca-dependency of the enzyme activity was not altered by changing Mg^{++} concentration. When the Mg^{++} concentration increased from 1 to 5 mM, the overall pattern of Ca-dependency remained unchanged, but the enzyme activity decreased consistently by approximately 40% at all Ca^{++} concentrations (Fig. 6). This indicates that a high concentration of Mg^{++} is inhibitory to Ca-ATPase activity.

The optimal concentration of Mg^{++} was around 1 mM, above which the enzyme activity was progressively inhibited. The mechanism of this inhibition by high Mg^{++} levels is not precisely understood. However, the fact that the enzyme activity changed similarly with Mg^{++} concentrations regardless of whether the concentration of Ca^{++} was fixed at 0.5 mM or varied simutaneously to maintain a constant Ca/Mg ratio of 1/2 (Fig. 5) strongly suggests that the inhibition of Ca-ATPase by high concentrations of Mg^{++} does not represent competition between Ca^{++} and Mg^{++} ions. Since in these studies the total ATP concentration was maintained constant at 5 mM, it is quite possible that the increase in Mg/ATP ratio at a high Mg^{++} concentration account for the reduction of the enzyme activity. Support for this notion came from the observation that the enzyme activity decreased by raising the Mg/ATP ratio from 1/5 to 5/5 (Fig. 3). Changes in Mg/ATP ratio would change fractions of free Mg^{++}, free ATP and Mg-ATP complex in the system. As the Mg/ATP ratio increases the fractional concentration of ATP decreases while those of Mg-ATP and Mg^{++} increase. How these changes affect the enzyme activity is difficult to ascertain without knowing the proper form of substrate species and the reaction sequence for the mitochondrial Ca-ATPase reaction. However, the fact that the Vmax, but not the Km_{ATP} is altered by changing Mg/ATP ratio (Fig. 3 inset) implies that alteration of the enzyme activity is not due to changes in the substrate concentration or the substrate-enzyme affinity, but due to reduction in the enzyme turnover rate at a high Mg/ATP ratio. The mechanism by which the enzyme turnover rate changes with Mg/ATP ratio remains obscure since the reaction sequence for the enzyme activity is not precisely

defined.

Changes in pH optimal for the Ca-ATPase activity of the present preparations are quite similar to those for Na-K-ATPase activity (Park and Hong, 1976; Park *et al,* 1979). The optimal pH increased as incubation temperature decreased, however the optimal OH^-/H^+ ratio in the medium appeared to be constant at all temperatures. Since $OH^-/H^+ = 10^{2(pH-pN)}$ (where pN is the neutral pH) and $\Delta pN/\Delta T = -0.017$ unit/°C, a constant optimal OH^-/H^+ ratio indicates that the change in optimal pH with tempearature (ΔpH optimum/ΔT) is approximately −0.017 unit/°C. This value is similar to the $\Delta pK'/\Delta T$ of protein imidazole group (Reeves, 1972). According to Reeves (1972, 1977), the overall charge state of the protein molecule is primarily determined by the fractional dissociation of histidine-imidazole residue (Imidazole alphastat hypothesis). The fractional dissociation of imidazole group (α_{Im}) is determined at a given temperature by the imidazole pK′ (pK'_{Im}) and the pH of the solution:

$$\alpha_{IM} = \frac{(Im)}{(HIm^+ + Im)} = \frac{10^{pH-pK'Im}}{1+10^{pH-pK'Im}}$$

Since, the imidazole pK′ changes with temperature, α_{Im} will remain unchanged during temperature variations only if the pH of solution changes with temperature in an equal magnitude as does the imidzole pK′ (i.e., ΔpH solution/$\Delta T = \Delta pK'/\Delta T$). This condition will assure a constant net charge, hence information, of a protein molecule independent of temperature. It is therefore possible that changes in the optimal pH for the mitochondrial Ca-ATPase activity with temperature, as observed in the present study, reflect that the enzyme system maintains the most efficient catalytic configuration if the medium pH changes with temperature with an equal slope as that of $\Delta pK'_{Im}/\Delta T$.

The present study clearly demonstrates that a Mg^{++}-dependent, Ca^{++}-activated ATPase system resides in the mitochondrial fraction of the rat liver. However, the activity of this enzyme system was not significantly affected by either La^{+++} (0.01-1.0 mM) or Ruthenium red (2.5-10 μM or 0.08-0.2 n moles/mg protein) (Table 1). These substances are known as specific and potent inhibitors of the mitochondrial Ca^{++} transport. According to Reed and Bygrave (1974) the inhibition of Ca^{++} transport by La^{+++} is competitive while that by Ruthenium red is noncompetitive. In rat liver mitochondria Ki values for La^{+++} and Ruthenium red are approximately 2×10^{-8} M and 3×10^{-8} M (or 0.05 n moles/mg protein), respectively (Reed and Bygrave, 1974). The concentrations of La^{+++} in the present study were therefore approximately 1,000-100,000 times and those of Ruthenium red were at least several times as high as the concentrations of these substances to bring about complete inhibition of the Ca^{++} transport. In the red blood cell membrane, at which the Ca^{++} transport is mediated by the action of Ca-ATPase (Cha *et al,* 1971), La^{+++} (25-100 μM) and Ruthenium red (25-100 μM) inhibit the activities of the Ca-ATPase as well as the Ca^{++}-transport system (Lee & Kang, 1974; Lee *et al,* 1975). It is therefore difficult to conclude whether the Ca-ATPase system observed in the present study plays any role in the physiological transport of Ca^{++}. In fact, to our knowledge, the role of Ca-ATPase in the mitochondrial Ca^{++} transport has never been clearly demonstrated. What role, then, this enzyme system plays in mitochondria remains to be defined.

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