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Respiration-Linked Calcium Ion Uptake by Flight Muscle Mitochondria from the Blowfly *Sarcophaga bullata*†

Hartmut Wohlrab

ABSTRACT: The respiration-driven accumulation of Ca^{2+} by blowfly flight muscle mitochondria has been investigated. Evidence is presented that these mitochondria possess Ca^{2+} carriers. This apparent K_m for Ca^{2+} is 115 μM; the V_{max} = 550 nmol of Ca^{2+} min^{-1} mg of $protein^{-1}$ at 23°. The Ca^{2+} transport is inhibited 50% by 0.15 nmol of Ruthenium Red/mg of protein, independent of the turnover rate of the carriers. Evidence is presented that the carriers are insensitive to lanthanides. The divalent cation ionophore A23187 inhibits the Ca^{2+} uptake at concentrations that depend on the turnover rate of the Ca^{2+} carriers. Uncoupler titrations indicate that the Ca^{2+} carriers have as high an apparent affinity for high-energy intermediates of oxidative phosphorylation as do the ADP phosphorylation reactions. It is also demonstrated that Ca^{2+} stimulates state 4 respiration temporarily until the respiration rate returns to a state 4 rate which can then be stimulated by uncouplers but not by ADP. The Ca^{2+}-stimulated respiration does not correlate with Ca^{2+} transport rates. Although this Ca^{2+} carrier has many properties of the vertebrate mitochondrial Ca^{2+} transport system, there are dramatic differences between the two systems.

Intact and phosphorylating mitochondria from the flight muscle of blowflies lack the ability to oxidize most exogenous Krebs cycle intermediates (see review by Sacktor, 1970). Energy-linked reactions of these mitochondria can be linked to monovalent cation translocations across the mitochondrial membrane as demonstrated with submitochondrial particles by Wohlrab (1973a). Respiration-linked Ca^{2+} uptake by these mitochondria has been characterized in a general way by Carafoli and coworkers (1971), who observed that large quantities of Ca^{2+} can be taken up by respiration-generated high-energy intermediates only in the presence of inorganic phosphate.

The present investigation utilizes an improved mitochondrial preparation with a high energy transduction efficiency and presents new details that suggest that blowfly mitochondria do have an energy-linked Ca^{2+} carrier system, which in many ways behaves like that of mammalian mitochondria, yet shows significant and dramatic differences.

Materials and Methods

Blowfly larvae (*Sarcophaga bullata*) were obtained from the Carolina Biological Supply Co., Burlington, N. C. The adult flies were kept at about 24°. Water-soaked paper towels were kept in the cages to give the insects ready access to water and to keep the relative humidity high. The insects had free access to sucrose.

Flight muscle mitochondria were prepared using a modification (Wohlrab, in preparation) of the method of Hansford and Chappell (1968).

All Ca^{2+} uptake experiments were carried out using DL-α-glycerol phosphate (Sigma Chemical Co.) as a substrate. Mitochondria were filtered through 0.45 μ, plain, white filter discs (15/16 in. diameter) which were obtained from Matheson Higgins Co., Inc., Woburn, Mass. 01801. The stainless steel filter rig was obtained from Interex Corp., Waltham, Mass. 02154. The filters were dried under infrared lamps and counted in toluene-based scintillator cocktails in a Beckman scintillation counter. $^{45}Ca^{2+}$ was obtained from New England Nuclear Corp.

Ca^{2+} uptake experiments were carried out routinely in the following manner (modifications are noted in the figure legends). The following reagents were added to the medium (0.2 M sucrose and 40 mM NaMOPS,[1] pH 7.2): 20 mM potassium phosphate (pH 7.2), 20 mM α-glycerol phosphate, 100 μM

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[1] Abreviations used are: MOPS, morpholinopropanesulfonic acid; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; EGTA, ethylene glycol bis(β-aminoethy) ether)-*N,N*′-tetraacetic acid.

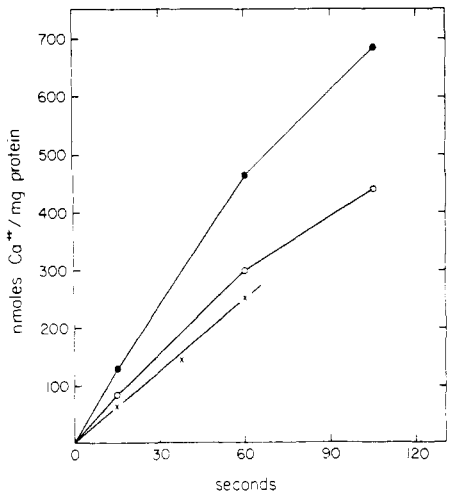

FIGURE 1: Phosphate and pH dependence of Ca^{2+} uptake. Ca^{2+} uptake was determined as described in Materials and Methods. The time 0 point reflects the amount of Ca^{2+} taken up in the absence of phosphate. (●) 20 mM phosphate and 400 μM free $CaCl_2$ (pH 7.2); (○) 1 mM phosphate and 400 μM $CaCl_2$ (pH 7.2); (x) 20 mM phosphate and 500 μM $CaCl_2$ (pH 7.8).

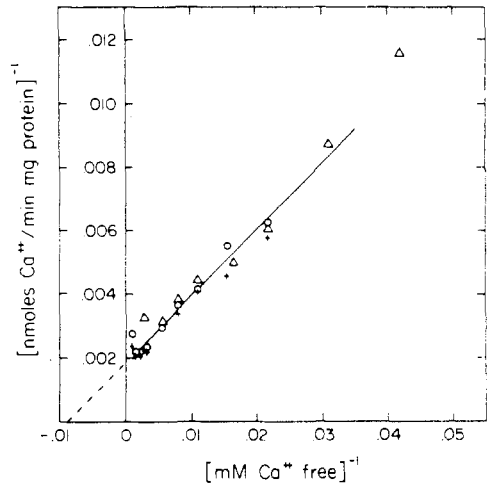

FIGURE 2: Lineweaver-Burk plot of Ca^{2+} uptake. Conditions as described in Materials and Methods were used. Initial $CaCl_2$ concentrations were varied. Ca^{2+} uptake rates were calculated from the difference in uptake between 15 and 37.5 sec. Different symbols are parallel experiments.

EGTA, and 100 μM $CaCl_2$. Mitochondria were added and $^{45}CaCl_2$ was added 15 sec later. Inhibitors were added after the mitochondria and before the $^{45}CaCl_2$. Samples were generally taken at 15, 37.5, and 60 sec after the $^{45}CaCl_2$ addition.

Respiration rates were measured in a 0.5-ml electrode vessel manufactured by Rank Brothers, Bottisham, Cambridge, England. The medium was the same as for the Ca^{2+} uptake experiments. Mitochondrial protein was determined by the biuret reaction in the presence of 0.5% deoxycholate (Gornall *et al.*, 1949). A23187 was kindly donated by Eli Lilly and Co., Indianapolis, Ind. Praseodynium chloride, cerous chloride, and butacaine sulfate were obtained from Pfaltz and Bauer, Inc., Flushing, N. Y. Ruthenium Red was obtained from British Drug House and S-13 (5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide) was a gift from Monsanto Chemical Co.

Results

Optimization of Ca^{2+} Transport Conditions. An apparent complication in the study of the blowfly mitochondria for Ca^{2+} transport arises due to the fact that Ca^{2+} is required by the α-glycerolphosphate dehydrogenase as well as by the transport process itself. Carafoli and Sacktor (1972) have already demonstrated that these two Ca^{2+} binding sites are different since only the transport is sensitive to Ruthenium Red and not the Ca^{2+} activation of the α-glycerolphosphate dehydrogenase. In addition, the affinity for Ca^{2+} of the α-glycerolphosphate dehydrogenase is very high (Carafoli and Sacktor, 1972) while that for the Ca^{2+} transport is much lower (Carafoli *et al.*, 1971).

The free Ca^{2+} that was added for the transport experiments was always supplemented with 100 μM EGTA and 100 μM $CaCl_2$ to maximally activate the α-glycerolphosphate dehydrogenase. The free Ca^{2+} exchanged with the EGTA chelated Ca^{2+} much faster than it was taken up by the mitochondria. The free Ca^{2+} and the EGTA chelated Ca^{2+} were used to calculate the specific activity of $^{45}Ca^{2+}$.

Figure 1 shows typical Ca^{2+} uptake values. The time points for sampling were picked in such a manner as to permit us to calculate initial uptake rates. Figure 1 also shows that pH is not a critical variable over a reasonable pH range. Lower pH's increase the uptake rates a little. Phosphate is necessary and at 1 mM phosphate, the Ca^{2+} uptake rate has been decreased by about 36% (Figure 1). Butacaine sulfate had no effect at a concentration of 430 nmol/mg of protein (66 μM) on the Ca^{2+} uptake rate at an initial free Ca^{2+} concentration of 500 μM. When the α-glycerol phosphate concentration is varied at 500 μM initial free Ca^{2+}, the Ca^{2+} uptake rate essentially follows the state 4 (Chance and Williams, 1956) rate increase. The α-glycerol phosphate concentration for a half-maximum increase of respiration (state 4) or Ca^{2+} uptake is 1.2 mM.

Kinetic Parameters of the Ca^{2+} uptake. We have varied the initial Ca^{2+} concentration of the medium and observed certain Ca^{2+} uptake rates. The uptake rates represent initial rates. Figure 2 shows a double reciprocal plot of the uptake rates. The K_m is 115 μM, while the V_{max} extrapolates to 550 nmol of Ca^{2+} min^{-1} mg of $protein^{-1}$. At the very high Ca^{2+} concentrations there occurs an inhibition phenomenon which will be discussed later.

Estimation of the Number of Ca^{2+} Carriers. When the mitochondria are titrated with Ruthenium Red, the Ca^{2+} uptake is inhibited 50% at a concentration of 0.15 nmol of Ruthenium Red/mg of mitochondrial protein. This titer is independent of the turnover rate of the Ca^{2+} carriers (Figure 3). The titer suggests a carrier concentration of less than one per cytochrome oxidase since the cytochrome oxidase concentration of these mitochondria is 0.3 nmol/mg of protein (H. Wohlrab, unpublished results). It should be noted that Ruthenium Red at the concentrations used has no effect on normal oxidative phosphorylation (see also Carafoli and Sacktor, 1972).

Mela (1969) has estimated the number of Ca^{2+} carriers in rat liver mitochondria by using the lanthanide sensitivity of the mammalian mitochondrial Ca^{2+} carrier. Pr^{3+} and Cr^{3+} at concentrations of 25 nmol/mg of protein had no effect on the Ca^{2+} uptake rates of the blowfly flight muscle mitochondria. However, since lanthanides do form highly insoluble complexes with phosphate, the significance of these particular results becomes questionable.

The lanthanide sensitivity of the α-glycerol phosphate driven Ca^{2+} transport was determined in another way. Mitochondria respiring in state 4 in the absence of phosphate increase their respiration rate dramatically within less than 1 min to the state 3 rate when 500 μM free Ca^{2+} is present. No such increase in respiration rate will occur within 1 min when 0.4 nmol of Ru-

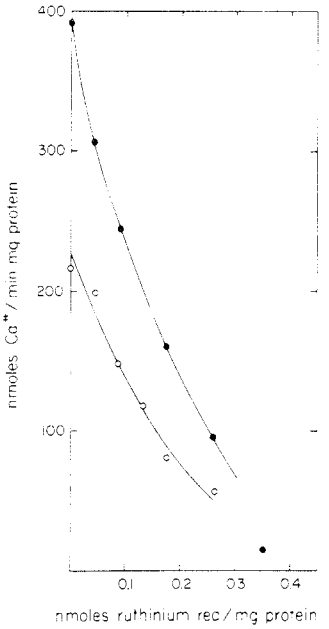

FIGURE 3: Inhibition of Ca^{2+} uptake by Ruthenium Red. Conditions as described in Materials and Methods and in Figure 2. (●) 500 μM $CaCl_2$; (○) 100 μM $CaCl_2$.

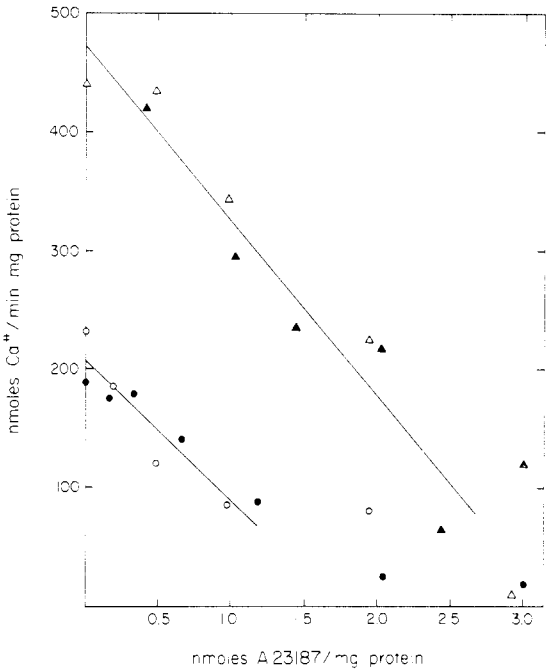

FIGURE 4: Inhibition of Ca^{2+} uptake by A23187. Conditions as in Materials and Methods and in Figure 2. Solid and open symbols refer to duplicate experiments. (△) 500 μM $CaCl_2$; (○) 100 μM $CaCl_2$.

thenium Red/mg of protein is present. On the other hand, the Ruthenium Red does not affect the respiration once it has reached its maximum rate. Carafoli and coworkers (1971) found significant swelling of the blowfly mitochondria in the presence of Ca^{2+} and in the absence of phosphate. If the swelling produces irreversible changes, then it is not surprising that the damage generated by the Ca^{2+} transport in the absence of phosphate is not reversible by Ruthenium Red. On the other hand, when 25 nmol of Pr^{3+} or Cr^{3+} per mg of mitochondrial protein was added to the mitochondrial suspension in the absence of phosphate, the increase in the respiration rate due to Ca^{2+} was less than 50% inhibited. The respiration-driven Ca^{2+} uptake of these flight muscle mitochondria has thus a very high sensitivity toward Ruthenium Red and is essentially insensitive toward lanthanides.

Inhibition of the Ca^{2+} Uptake by the Divalent Ionophore A23187. If the respiration-linked Ca^{2+} uptake is primarily due to a phosphate gradient generated during state 4 respiration, then the addition of a divalent cation ionophore such as A23187 (Reed and Lardy, 1972) might be expected to stimulate the Ca^{2+} uptake rate. This is especially true since phosphate can be transported during phosphorylation at a rate of 3 μmol min^{-1} mg of $protein^{-1}$, *i.e.*, pyruvate-proline respiration with three phosphorylation sites at 1 μatom O min^{-1} mg of $protein^{-1}$. Using the results of Reed and Lardy (1972), it can be shown that A23187 is capable of a turnover of 1.3 μmol of Ca^{2+} min^{-1} nmol of $A23187^{-1}$ in rat liver mitochondria. This turnover rate will of course depend on many variables such as membrane fluidity, local Ca^{2+} concentrations, and the concentration gradient of free Ca^{2+} across the membrane. This turnover rate is more than twice the maximum Ca^{2+} uptake rate that we find (Figure 2), and thus A23187 may be expected to stimulate Ca^{2+} uptake of the flight muscle mitochondria.

Figure 4 shows that there is a linear decrease in the initial Ca^{2+} uptake rate as A23187 is added. The apparent Ca^{2+} leakage rate by the ionophore comes to 100 nmol of Ca^{2+} per min per nmol of A23187. This apparent leakage rate by A23187 is independent of the initial Ca^{2+} uptake rate. However, the 50% inhibition point is at a higher A23187 concentration at higher initial Ca^{2+} uptake rates.

This titration is compatible with an energy-linked Ca^{2+} pump. The ionophore cannot facilitate the Ca^{2+} uptake but will stimulate leakage from the mitochondria. The reason for the lower apparent A23187 catalyzed leakage rate may be due to a lower affinity of the blowfly membrane for A23187, or a smaller gradient of free Ca^{2+} across the mitochondrial membrane, or even a smaller mobility of A23187 in the mitochondrial membrane.

It should be noted that A23187 does not inhibit electron transport at the above concentrations. Oxidative phosphorylation is inhibited by A23187 in the absence of exogenous Mg^{2+} since Mg^{2+} leaks out of mitochondria. In the presence of extramitochondrial Mg^{2+}, A23187, at the concentrations used for the Ca^{2+} transport inhibition, has no effect on oxidative phosphorylation (Wohlrab, 1973a).

Energy Requirement of Ca^{2+} Uptake. Ca^{2+} uptake requires respiration-generated energy. The respiration-linked Ca^{2+} uptake was titrated with the uncoupler S-13. The titration curve was nonlinear (Figure 5), suggesting that the high-energy intermediate generated by the respiration and eliminated or hydrolyzed by S-13 is not the rate-limiting step. We then titrated state 4 to determine whether the high-energy intermediate is rate limiting during phosphorylation with these mitochondria. We know from studies on rat liver mitochondria (Wilson, 1969) that the release of state 4 respiration is proportional to the amount of uncoupler added and is not sigmoidal like the inhibition curve for the Ca^{2+} uptake rate. Figure 5 shows that the uncoupler titration curve is linear as in other mitochondrial systems. Thus the Ca^{2+} uptake only becomes inhibited after the respiratory control is almost completely eliminated. The result also suggests that the Ca^{2+} transport process is efficient in utilizing respiratory energy. Figure 5 also shows that when the Ca^{2+} uptake rates are lower due to a lower initial Ca^{2+} concentration, the uncoupler titer is the same.

These observations agree basically with the respiration-linked Ca^{2+} uptake of vertebrate mitochondria. Rossi and

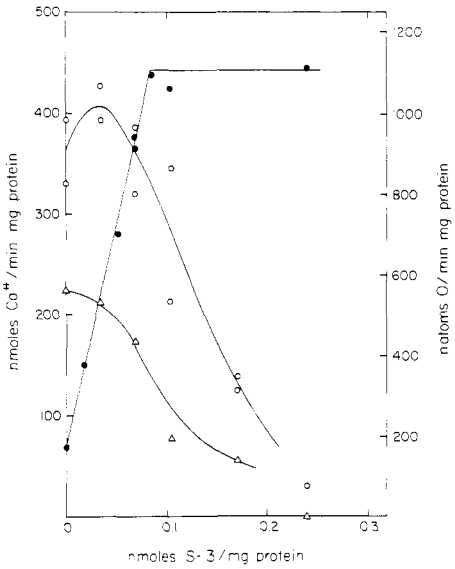

FIGURE 5: Effect of S-13 on the state 4 respiration rate and the Ca^{2+} uptake rate. Conditions as in Materials and Methods. (●) Titration of state 4 respiration by S-13; titration of Ca^{2+} uptake by S-13, (○) 500 μM $CaCl_2$, (△) 100 μM $CaCl_2$. Since there was a slight acceleration in Ca^{2+} uptake with time in the presence of S-13, Ca^{2+} uptake rates were calculated from the difference in Ca^{2+} uptake between 37.5 and 60 sec.

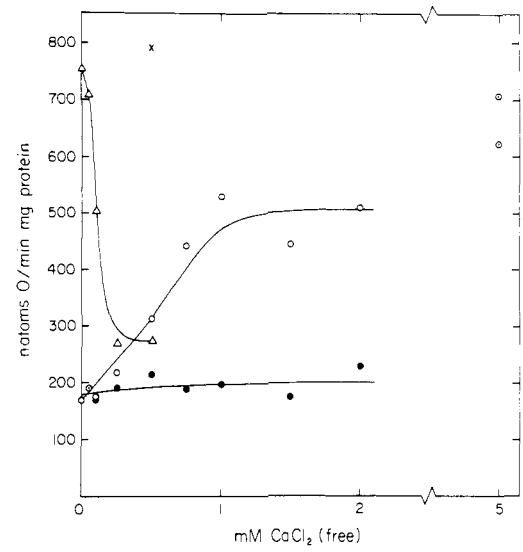

FIGURE 6: Effect of free Ca^{2+} on state 4 respiration rates. Conditions as in Materials and Methods. (○) State 4 respiration as stimulated by free Ca^{2+}; (●) respiration rate after the Ca^{2+} stimulation has passed; (△) effect of 500 μM ADP on respiration after Ca^{2+} stimulation of respiration has stopped; (x) effect of 0.5 nmol of S-13/mg of protein on the respiration rate after the Ca^{2+} stimulation has ceased; ⊙ represents identical position for closed and open circles.

Lehninger (1964) have shown that when rat liver mitchondria are presented simultaneously with Ca^{2+} and ADP, the Ca^{2+} will be taken up before the ADP will be phosphorylated. That Ca^{2+} reacts preferentially over ADP with the mitochondrial membrane, due to a greater respiratory stimulation and reaction with a site closer to the respiratory chain, has been demonstrated by Chance (1965) in mitochondria from pigeon heart, rat liver, and the seagull.

Efficiency of Respiration-Linked Ca^{2+} Uptake. It has been reported that Ca^{2+} does not stimulate the respiration of state 4 of blowfly flight muscle mitochondria (Carafoli *et al.*, 1971). At 500 μM free $CaCl_2$ there occurs a stimulation of state 4. A larger stimulation will be obtained when higher concentrations of free Ca^{2+} are used (Figure 6); the stimulation, as can be seen in Figure 6, is only temporary. Maximum respiratory stimulation is obtained at about 1 mM $CaCl_2$. At 5 mM $CaCl_2$, a "Ca^{2+} catastrophe" occurs, which keeps the respiration from returning to a state 4 level. The slowed Ca^{2+} respiration rate could not be stimulated by ADP; however, it could be released by uncouplers of oxidative phosphorylation (Figure 6). The Ca^{2+} must thus be responsible for either inhibiting the energy-coupling reactions, the adenine nucleotide carrier, or both.

The apparent efficiency of respiration-driven Ca^{2+} transport has been calculated. The $Ca^{2+}/2e^-$/energy transduction site ratio was calculated with the total respiration and with only the Ca^{2+} stimulated respiration. The former ratio yields a maximum of 0.82 at 200 μM Ca^{2+}, while the latter approaches a maximum of 20. Since the fraction of total respiration that is utilized for the energy-linked Ca^{2+} transport is not known, the Ca^{2+} transport should not necessarily be classified as inefficient (Carafoli *et al.*, 1971). The results presented in Figure 5 suggest, in fact, that the Ca^{2+} transport process may be utilizing respiration-generated energy in an efficient manner.

Discussion

Blowfly flight muscle mitochondria have a high cytochrome content and their inner membrane has a very unique content of metabolite carriers. Their previously reported low energy transduction efficiency has prevented their more general use. We reported recently on the very high energy transduction efficiency of submitochondrial particles prepared from these mitochondria (Wohlrab, 1973a,b). In this communication we report on mitochondria with respiratory controls during α-glycerol phosphate oxidation of greater than four. The blowfly flight muscle mitochondria can now be looked upon as an important alternative to vertebrate mitochondria for the elucidation of basic mitochondrial energy transduction phenomena.

The respiration-linked Ca^{2+} transport that is characterized in this communication presents some unique aspects. Kinetic parameters can be compared to those of mitochondria that show a correlation between the rates of Ca^{2+} transport and the rates of respiration. For the blowfly flight muscle mitochondria we find a K_m of 115 μM and a V_{max} of 550 nmol of Ca^{2+} min^{-1} mg of $protein^{-1}$ (23°). The K_m for the maximum stimulation of state 4 respiration by Ca^{2+} is 45 μM for pigeon heart mitochondria (Chance, 1965) and 87 μM for mitochondria of the hepatopancreas of the blue crab *Callinectes sapidus* (Chen *et al.*, 1974). Chance (1965) finds a maximum Ca^{2+} uptake rate of 1100 nmol min^{-1} mg of $protein^{-1}$ (26°) and Chen *et al.* (1974) find a V_{max} of 600 nmol min^{-1} mg of $protein^{-1}$ (25°). The K_m's and V_{max}'s are thus quite similar in these two types of mitochondria.

Chan and coworkers (1970) and Pedersen and Coty (1972) have characterized the respiration-linked Ca^{2+} transport of the "inside-out," Lubrol-insoluble inner mitochondrial membrane fraction from rat liver mitochondria. It shows many of the properties observed in the intact blowfly flight muscle mitochondria. Primary among these properties is (1) the lack of correlation between respiration rate and Ca^{2+} uptake rate, and (2) the requirement of phosphate for the uptake of significant amounts of Ca^{2+}. It should be noted, however, that the Lubrol-insoluble inner mitochondrial membrane fraction shows no respiratory stimulation by ADP in the presence of oxidizable substrate and phosphate (state 4), while the blowfly mitochondria show fourfold and even higher stimulations of state 4 respiration by ADP.

Figure 5 demonstrates that the apparent affinity of the Ca^{2+} carrier for respiration-generated, high-energy intermediates is as high or higher than that of the ADP phosphorylation reactions. Observations which have been made with mitochondria from tissues of vertebrates demonstrate that Ca^{2+} acts in preference to ADP in stimulating state 4 respiration (Chance, 1965).

The present investigations lead to the conclusion that blowfly flight muscle mitochondria do possess Ca^{2+} carriers. The experiments also suggest that Ca^{2+} transport occurs in an efficent energy transduction process.

Acknowledgment

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Perturbation of Liposomal and Planar Lipid Bilayer Membranes by Bacitracin–Cation Complex†

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ABSTRACT: The antibiotic bacitracin at concentrations between 10^{-3} and 10^{-2} M causes the release of trapped, low molecular weight marker from artificial lipid vesicles or liposomes. This antiliposome effect is a paradigm of the antibacterial effect on these grounds. (1) Both actions are specifically enhanced by cadmium or zinc. (2) The spectrophotometrically detected formation of the presumably biologically active divalent cation-bacitracin complex exhibits a pH dependence which is also characteristic of complex-induced liposome lysis. (3) Microbiologically active concentrations of bacitracin and $CdCl_2$—*i.e., ca.* 10^{-5} M—lower the conductance of and induce instability in planar lipid bilayer membranes. As determined by microelectrophoresis, exposure to bacitracin alone does not materially change the negative surface charge density of lipid vesicles. In the presence of the antibiotic and cadmium—but not calcium—however, the liposomal ζ potential is significantly more positive. The function of cadmium or zinc with respect to the antimicrobial effect of bacitracin, therefore, appears to be the promotion of the cell-antibiotic interaction. Cadmium apparently does not enhance the surfactant property of the antibiotic, insofar as it has little influence on the critical micellar concentration of bacitracin.

The mechanism(s) by which the polypeptide bacitracin exerts its antibacterial effect has remained a matter for speculation. Although the antibiotic was once thought to interfere primarily with cell wall synthesis, bacterial protoplasts lacking cell walls have been shown to be as susceptible to bacitracin as bacteria possessing cell walls (Snoke and Cornell, 1965). An alternative mode of action is suggested by the observation that both lysis and inhibition of growth of *Staphylococcus aureus* by bacitracin were enhanced in the presence of zinc (Smith and Weinberg, 1962). As either zinc or cadmium is required for biologic activity of bacitracin (see Weinberg, 1967), membrane damage, as a precursor to lysis, might be central to its antibacterial action. This hypothesis is supported by the finding that bacitracin markedly increased the efflux of potassium from cells recently exposed to the antibiotic (Hancock and Fitz-James, 1964).

The question thus arises as to whether the cell membrane *per se* can be affected by bacitracin, *i.e.*, whether bacitracin's effect on membranes could be exclusive of its effect(s) on other cell components. A study of bacitracin–liposome interaction was undertaken to answer this question. The liposome or smectic mesophase of a lipid(s), which consists of concentric, bilayered shells of lipid separated by aqueous compartments, has become a standard reagent for assessing the membrane-dam-

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