Kinetics of Oligomycin Inhibition of Sodium- and Potassium-Activated Adenosine Triphosphatase from Beef Brain

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SUMMARY

Oligomycin and rutamycin inhibited the activity of a beef brain adenosine triphosphatase that required both sodium and potassium, but these antibiotics were without effect on the potassium-requiring *p*-nitrophenyl phosphatase or acetyl phosphatase obtained from the same preparation. Several other macrolide antibiotics believed to be structurally similar to oligomycin were without appreciable effect on either the adenosine triphosphatase or the *p*-nitrophenyl phosphatase. Kinetic studies indicated that oligomycin is an uncompetitive inhibitor of the sodium- and potassium-requiring adenosine triphosphatase. The antibiotic was an uncompetitive inhibitor of sodium activation and a noncompetitive inhibitor of the potassium activation of this enzyme. Alteration of the Mg^{++}: ATP ratio from 0.1 to 5.0 did not affect the inhibition by oligomycin. It would appear that oligomycin is an even more selective inhibitor than the cardiac glycosides of the hydrolytic function that requires both sodium and potassium.

INTRODUCTION

There is abundant evidence [see Skou (1) for references] that the sodium- and potassium-activated ATPase plays a major role in converting the metabolic energy of ATP into the osmotic work required to transport monovalent cations across cell membranes. Some knowledge of the mechanisms by which Na^+ and K^+ activate this ATPase would thus be of interest, but the enzyme, which is isolated in the microsomal fraction of tissue homogenates, is invariably particulate and is difficult to study. The use of $AT^{32}P$ to label intermediates, however, has offered some insight into the mechanism of action.

ATP hydrolysis is believed to involve at least two steps (1). The initial step consists of the Na^+-induced phosphorylation of protein by ATP, followed by a K^+-induced dephosphorylation. Studies on the phosphoprotein intermediate indicate that, at least in the denatured form in which it is isolated, the phosphate is bound as an anhydride of a carboxyl group (2–4). Recent observations suggest that the final step in the hydrolytic sequence may be studied individually by the use of appropriate substrates. Microsomal fractions contain not only ATPase but also a family of neutral phosphatase activities that require potassium, are inhibited by sodium, and are capable of hydrolyzing a variety of artificial substrates, including *p*-nitrophenyl phosphate (5–9), acetyl phosphate (10–12), and carbamyl phosphate (13). Moreover, cardiac glycosides, which are commonly regarded as specific inhibitors of that ATPase associated with transport (1), inhibited all these systems. Some comparisons

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[2] The abbreviation used is: $(Na^+ + K^+)$-ATPase, adenosine triphosphatase requiring both Na^+ and K^+.

of the effects of drugs on K^+-phosphatase and $(Na^+ + K^+)$-ATPase[2] have been made in an attempt to decide whether or not the former can really be regarded as an expression of the activity of the final step in ATP hydrolysis, but the results remain somewhat equivocal (14). It was observed during these attempts that oligomycin, although a reasonably effective inhibitor of $(Na^+ + K^+)$-ATPase (15–18), was without effect on K^+-acetyl phosphatase (14).

Oligomycin has been used in studies of oxidative phosphorylation, mitochondrial swelling, ion transport, and ATP hydrolysis by mitochondria [see the review by Shaw (19)], as well as in investigations of $(Na^+ + K^+)$-ATPase. Our results suggest that oligomycin could be useful in probing the mechanism of action of the latter enzyme. The antibiotic appears to be more specific than the classically used cardiac glycosides, in that it operates selectively on that function which requires both sodium and potassium.

The present report examines the kinetics of the inhibitory effects of oligomycin on a microsomal ATPase from beef brain that is almost completely dependent on activation by sodium and potassium ions. A comparison of the effects of oligomycin on the activities of a Na^+- and K^+-requiring ATPase, a K^+-requiring acetyl phosphatase, and a K^+-requiring *p*-nitrophenyl phosphatase present in the microsomal fraction is reported. Since the antibiotic is a macrolide (20), several other members of this class were also tested as potential inhibitors of ATPase in this study.

METHODS

Enzyme preparation. Bovine brains were obtained fresh from a local slaughterhouse, packed in ice, and then frozen at −20°. The enzyme was prepared as described by Schoner *et al.* (21).

Enzymatic assays. Incubation tubes contained the appropriate substrate, ATP, acetyl-P, or *p*-nitrophenyl-P; $MgCl_2$ to give a magnesium to substrate ratio of 2.5; and 100 mM Tris-HCl, pH 7.4, in a final volume of 1.0 ml. Substrate hydrolysis in the presence of Mg^{++} alone was 2–5% of the total hydrolysis that occurred when Mg^{++} plus monovalent cations were present. The activity with Mg^{++} only was not significantly altered by any of the procedures tested. The $(Na^+ + K^+)$-ATPase activity always refers to the $Mg^{++} + Na^+ + K^+$ hydrolytic activity minus that seen with Mg^{++} only. The K^+-activated acetyl phosphatase and *p*-nitrophenyl phosphatase activities refer to the K^+-induced increments. The reaction was initiated by the addition of enzyme, and the incubation time was 4 min at 37°. The $(Na^+ + K^+)$-ATPase activity was measured by the increase in the rate of ^{32}P liberation when 120 mM NaCl and 30 mM KCl were present in reaction mixtures containing $AT^{32}P$.

The methods of extraction of $^{32}P_i$ and determination of $AT^{32}P$ hydrolysis have been reported by Chignell and Titus (22). The K^+-activated acetyl phosphatase activity was measured by the increase in the rate of hydrolysis of 2 mM Tris-acetyl phosphate when 30 mM KCl was present in the reaction mixture. The hydrolytic activity was estimated by the hydroxamate method as described by Israel and Titus (14). K^+-activated *p*-nitrophenyl phosphatase activity was measured as the increase in the rate of *p*-nitrophenol formation from 2 mM *p*-nitrophenyl-P when 30 mM KCl was present in the reaction mixture. The reaction was stopped by the addition of 0.5 ml of cold 0.8 M $HClO_4$, followed by 1.0 ml of cold 1.2 M KOH. After 10 min in ice, 1.5 ml of cold water were added and the samples were centrifuged to remove the precipitated $KClO_4$. The concentration of *p*-nitrophenol in the supernatant solution was estimated from the absorbance at 410 mμ in a Beckman DU spectrophotometer.

Protein determinations were carried out as described by Lowry *et al.* (23).

Preparation of $AT^{32}P$. Terminally labeled $AT^{32}P$ (specific activity, 5–10 mC/μmole) was prepared by the method of Pfleiderer (24), as modified by Gibbs, Roddy, and Titus (25) and was diluted with carrier ATP to give approximately 30,000 cpm/assay tube.

Materials. Tris-ATP was obtained from Sigma. Tris-acetyl phosphate was prepared

from the dilithium salt (Sigma) as described by Israel and Titus (14). The disodium salt of *p*-nitrophenyl phosphate was obtained from Calbiochem. The antibiotics were dissolved in 95% ethanol and added to the incubation tubes before the addition of other reagents. Erythromycin and amphotericin B were readily water-soluble. An equal volume of ethanol was added to control tubes. The ethanol was evaporated under a stream of N_2. An oligomycin preparation consisting of 15% oligomycin A and 85% oligomycin B was obtained from Sigma; erythromycin (Ilotycin), from Eli Lilly and Co.; and amphotericin B (Fungizone), from E. R. Squibb and Sons. Other antibiotics were generous gifts from the following: rutamycin, Dr. J. M. McGuire, Eli Lilly and Co.; methymycin and methynolide, Dr. James D. Dutcher, Squibb Institute for Medical Research; fungichromin, Dr. Karl H. Beyer, Jr., Merck Sharp and Dohme Research Laboratories; and erythronolide B, Dr. Paul P. Hung, Department of Molecular Biology, Abbott Laboratories.

Determination of kinetic constants. The kinetic constants (K_m, V_{max}) were obtained by a least-squares method described by Davies, Gigon, and Gillette (26), in which the data were fitted to the Michaelis-Menten model:

$$v = \frac{s}{s + K_m} V_{max}.$$

The expected velocity μ_i at any given concentration of substrate (s_i) is given by

$$\mu_i = \left(\frac{s_i}{s_i + K_m}\right) V_{max}.$$

Least-squares estimates for K_m and V_{max} were obtained by minimizing $\Phi = \Sigma_i[v_i - \mu_i]$,[2] where v_i is the velocity observed at the substrate concentration s_i. Such a minimum is obtained by solving the equations $\delta\Phi/\delta K_m = 0$, $\delta\Phi/\delta V_{max} = 0$, for K_m and V_{max}. Since these equations are not linear in K_m and V_{max}, a Taylor series expansion around the initial estimates was used, iterating until the change in V_{max} and K_m was less than a predetermined amount (0.001). Initial values of K_m and V_{max} were found by fitting a straight line to the reciprocal plot of $1/v$ against $1/s$.

RESULTS

As reported by Israel and Titus (14), oligomycin inhibited the $(Na^+ + K^+)$-ATPase but not the K^+-acetyl phosphatase. Figure 1 compares the effect of increasing concentrations of oligomycin on the activities of $(Na^+ + K^+$-ATPase, K^+-activated *p*-nitrophenyl phosphatase, and K^+-requiring acetyl phosphatase. Oligomycin produced an inhibition of 3.5% of the $(Na^+ + K^+)$-ATPase at a concentration of 1×10^{-7} M, and the maximal inhibition of 73% was reached at a concentration of 2.5×10^{-4} M. The concentration of oligomycin required for 50% inhibition (I_{50}) was calculated to be 2×10^{-5} M. This range of oligomycin concentrations did not produce a significant inhibition of either the K^+-requiring acetyl phosphatase or *p*-nitrophenyl phosphatase activities.

As shown in Fig. 2, rutamycin also inhibits the $(Na^+ + K^+)$-ATPase without significant alteration of the activities of the other two potassium-activated enzymes. The concentration of rutamycin required for 50% inhibition of the $(Na^+ + K^+)$-ATPase was 5.6×10^{-5} M.

It was of interest to determine whether other macrolide antibiotics of known structure might also inhibit the $(Na^+ + K^+)$-ATPase. It was observed that erythromycin, methymycin, and their respective aglycones, erythronolide B and methynolide, as well as amphotericin B and fungichromin, had little or no inhibitory effect on the $(Na^+ + K^+)$-ATPase in molar concentrations (10^{-5}–10^{-4} M) at which oligomycin and rutamycin are inhibitory. Erythronolide B, erythromycin, methynolide, and fungichromin also did not significantly inhibit the K^+-requiring *p*-nitrophenyl phosphatase at these concentrations.

The effect of oligomycin on the kinetic behavior of the $(Na^+ + K^+)$-ATPase is given in Fig. 3, in which the reciprocal of the rate of hydrolysis is plotted against the reciprocal of ATP concentration. As the concentration of oligomycin is increased, the apparent K_m and V_{max} are decreased,

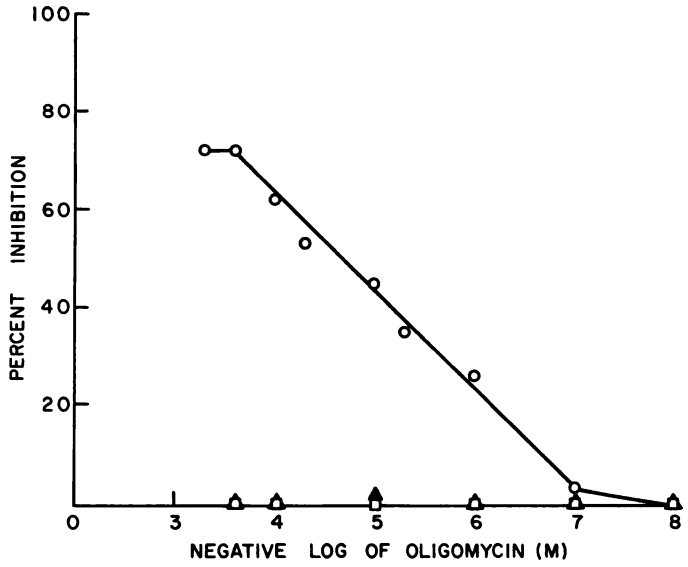

FIG. 1. *Comparison of the effects of oligomycin on ($Na^+ + K^+$)-ATPase and the K^+-activated p-nitrophenyl phosphatase and acetyl phosphatase*

The substrates were 2 mM ATP in the presence of 120 mM NaCl and 30 mM KCl (○——○), 2 mM p-nitrophenyl-P in the presence of 30 mM KCl (□——□), and 2 mM acetyl-P in the presence of 30 mM KCl (▲——▲).

resulting in a set of parallel lines suggestive of the uncompetitive type of inhibition (27). The kinetic constants calculated from the data of Fig. 3 are given in Table 1. The K_i values were calculated by means of the formula of Burk [as communicated by Ebersole *et al.* (27)] for uncompetitive inhibition as follows:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(\frac{1}{s}\right) + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)$$

and

$$\frac{1}{V^*_{max}}\left(1 + \frac{[I]}{K_i}\right) = \frac{1}{V^*_{max}},$$

where V_{max} = maximum velocity of uninhibited reaction,
V^*_{max} = maximum velocity of inhibited reaction,
$[I]$ = concentration of inhibitor (oligomycin),
v = observed velocity,
K_m = Michaelis constant,
s = substrate concentration,
K_i = inhibitory constant.

Table 1 gives the calculated kinetic data for inhibition of ($Na^+ + K^+$)-ATPase by oligomycin. The K_i calculated for oligomycin at a concentration of 1×10^{-6} M was 15.6×10^{-5}. At 5×10^{-5} and 1×10^{-4} M the calculated values were 2.5×10^{-5} and 3.5×10^{-5}, respectively. The latter values are close to the I_{50} of 2×10^{-5} from Fig. 1.

Figure 4 illustrates the effect of 5×10^{-5} M oligomycin on the activation of ATPase by sodium. The sodium concentration was increased from 10 to 50 mM, while the potassium concentration was maintained at 30 mM. The data, as plotted by the Lineweaver-Burk method, are indicative of uncompetitive inhibition. In this respect, the action of oligomycin on the sodium activation was analogous to that observed when the concentration of ATP was altered and sodium and potassium held constant (Fig. 3). The K_i for oligomycin as an inhibitor of sodium activation of ($Na^+ + K^+$)-ATPase was 2.6×10^{-5} M.

When the sodium concentration was held constant at 120 mM and the potassium concentration increased from 1 to 5 mM, oligo-

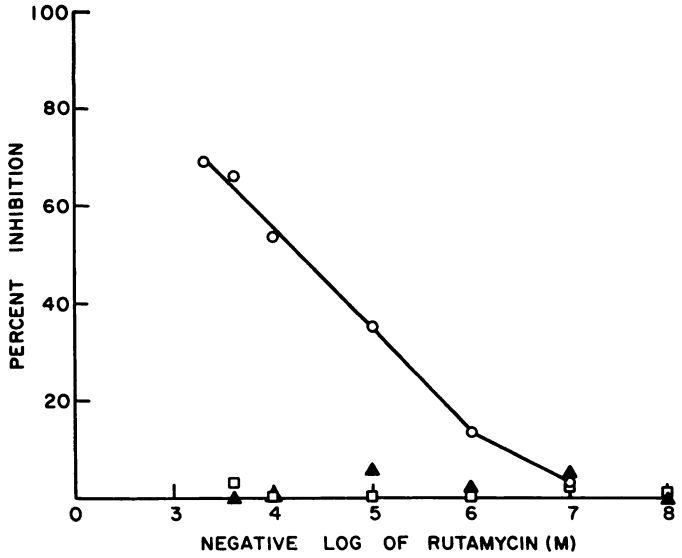

FIG. 2. *Comparison of the effects of rutamycin on $(Na^+ + K^+)$-ATPase and the K^+-activated p-nitrophenyl phosphatase and acetyl phosphatase*

The substrates are as in Fig. 1.

mycin at 5×10^{-5} M produced an inhibition illustrated by the double reciprocal plot of Fig. 5. Since the intercepts with the abscissa were not significantly changed by the presence of oligomycin, the inhibition appeared to be of the noncompetitive type. The calculated K_i for oligomycin inhibition of potassium activation of the $(Na^+ + K^+)$-ATPase was 2.5×10^{-5} M.

Since it seemed possible that oligomycin might change the affinity of the enzyme for Mg^{++}, and since alterations in the Mg^{++}: ATP ratio are known to affect the activity of $(Na^+ + K^+)$-ATPase (25), it was of

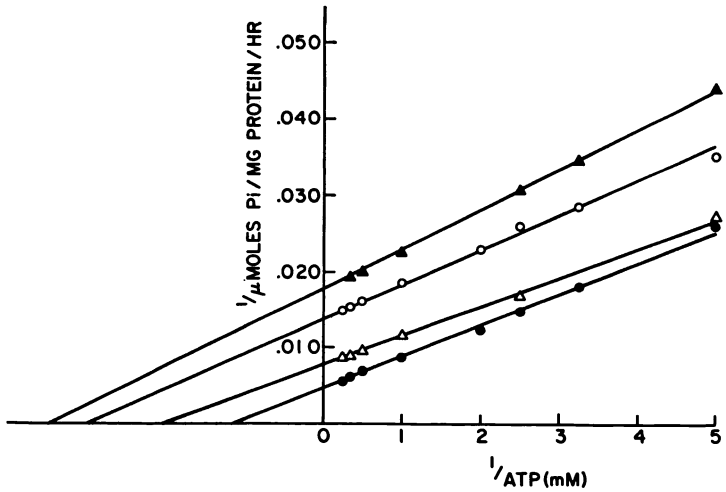

FIG. 3. *Effect of oligomycin on the velocity of the $(Na^+ + K^+)$-ATPase*

Double reciprocal plot of ATP concentration against $(Na^+ + K^+)$-ATPase activity. ●——●, without oligomycin; △——△, 1×10^{-6} M oligomycin; ○——○, 5×10^{-6} M oligomycin; ▲——▲, 1×10^{-4} M oligomycin

TABLE 1
Kinetic data for inhibition of $(Na^+ + K^+)$-ATPase by oligomycin

Addition	Concentration	K_m	V_{max}	$10^3(K_m/V_{max})$	$10^5 K_i$
	M	m*M*			
None		0.872	217	4.02	
Oligomycin	1×10^{-6}	0.469	132	3.70	15.6
	5×10^{-5}	0.332	73	4.55	2.5
	1×10^{-4}	0.290	56	5.18	3.5

interest to determine whether the Mg^{++}:ATP ratio for peak activity was the same in the presence and absence of oligomycin. The effect of the Mg^{++}:ATP ratio on oligomycin inhibition of the $(Na^+ + K^+)$-ATPase (Fig. 6) was determined by changing the $MgCl_2$ concentration from 0.2 to 10 mM while the ATP concentration was held at 2 mM. In the absence of oligomycin the ATPase activity was maximal at a Mg^{++}:ATP ratio of 2.5. The addition of oligomycin at 5×10^{-5} M caused inhibition of ATPase activity at all Mg^{++}:ATP ratios tested. However, the pattern of activation and the Mg^{++}:ATP ratio at peak activity appeared very similar to that seen without oligomycin.

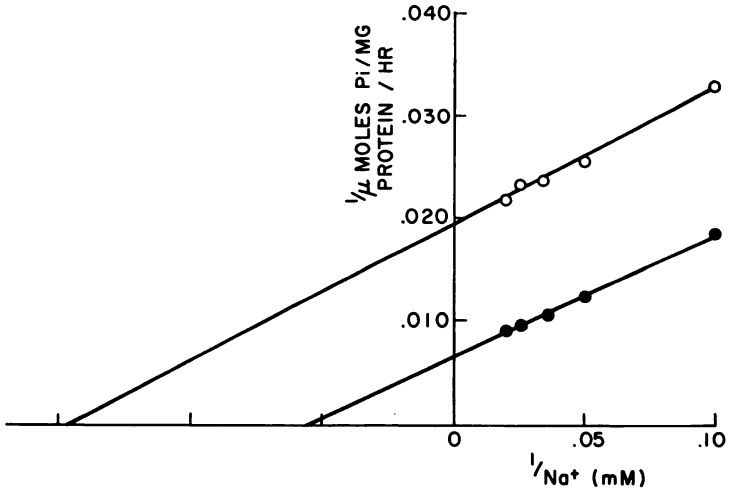

FIG. 4. *Effect of oligomycin on the Na^+ activation of the $(Na^+ + K^+)$-ATPase*

Double reciprocal plot of Na^+ concentration against $(Na^+ + K^+)$-ATPase activity. The ATP concentration was 2 mM in the presence of 30 mM KCl. ●——●, without oligomycin; ○——○, with 5×10^{-5} M oligomycin.

DISCUSSION

Oligomycin has been reported to inhibit the $(Na^+ + K^+)$-ATPase of brain (17, 28), red cell membranes (16, 18), electric organ of the electric eel (15), and calf heart muscle (29). The present report offers the first evidence for inhibition of this enzyme by rutamycin. Both rutamycin and oligomycin have been shown to inhibit the 2,4-dinitrophenol-induced ATPase of mitochondria (30). The $(Na^+ + K^+)$-ATPase from brain microsomes requires 10–100 times as much oligomycin as the mitochondrial ATPase for comparable inhibition (28).

A tentative structure has been proposed for oligomycin (20) but will probably require revision (31). A structure for rutamycin has not yet been proposed, but its chemical properties are very similar to those of oligomycin (19). Three other macrolide aglycones of known structure were not inhibitory. Two of these, methynolide and erythronolide, were smaller than

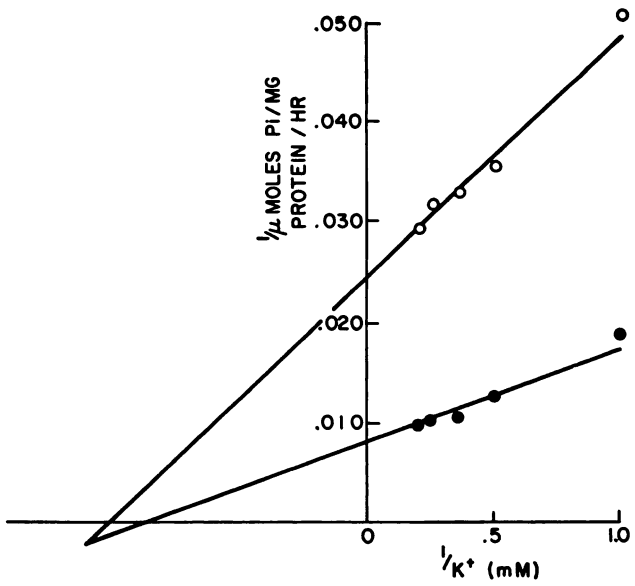

FIG. 5. *Effect of oligomycin on the K^+ activation of the $(Na^+ + K^+)$-ATPase*

Double reciprocal plot of K^+ concentration against $(Na^+ + K^+)$-ATPase activity. The ATP concentration was 2mM in the presence of 120 mM NaCl. ●——●, without oligomycin; ○——○, with 5×10^{-6} M oligomycin.

the active compounds whereas fungichromin was larger. Two glycosidic forms were also inactive. No relationship between structures and ATPase inhibition was evident from our studies but the size of the macrolide ring may be critical.

Certain characteristics of the ATPase mechanism make the inhibition by oligomycin of unusual interest. Studies with $AT^{32}P$ indicate that the reaction is initiated by a magnesium- and sodium-dependent incorporation of ^{32}P into microsomal protein. If the concentration of Mg^{++} is reduced sufficiently below the levels that are optimal for the over-all reaction, or if certain inhibitors are present, it is possible to demonstrate with the eel enzyme a Na^+-dependent ATP-ADP transphosphorylation (32, 33). Chignell[3] has demonstrated a similar transphosphorylation reaction with the enzyme from beef brain. A multistage reaction scheme has therefore been proposed:

$$E + \text{ATP} \overset{Mg^{++},Na^+}{\rightleftharpoons} E \sim \text{P} + \text{ADP} \quad (1)$$

$$E \sim \text{P} \overset{Mg^{++}}{\rightleftharpoons} E - \text{P} \quad (2)$$

$$E - \text{P} + H_2O \overset{K^+}{\rightarrow} E + P_i. \quad (3)$$

[3] C. F. Chignell, personal communication.

Ouabain can inhibit both the Na^+-dependent incorporation of phosphate into protein (25, 34) and the ATP-ADP exchange (reaction 1) (32). The concentrations required are higher than those necessary for comparable inhibition of the over-all ATPase reaction that occurs when both Na^+ and K^+ are present. Under the latter circumstance the levels of phosphoprotein intermediate detectable by the use of $AT^{32}P$ are insignificant, but the introduction of ouabain causes the accumulation of isolatable amounts of labeled phosphoprotein (18, 35). Reaction 3 is thus the probable site of the cardiac glycoside inhibition of transport ATPase, a view consistent with the observed inhibition by ouabain of the potassium-dependent hydrolysis of various monophosphate substrates (5–13).

Oligomycin is more selective in the sense that it does not inhibit protein phosphorylation (14, 18); neither does it block the ATP-ADP transphosphorylation studied by Fahn *et al.* (32). Whittam *et al.* (18) and Fahn *et al.* (36) have reported that oligomycin prevents the K^+-induced dephosphorylation of kidney and eel ATPases, respectively. Our data on the kinetics of inhibition of cation activation by oligo-

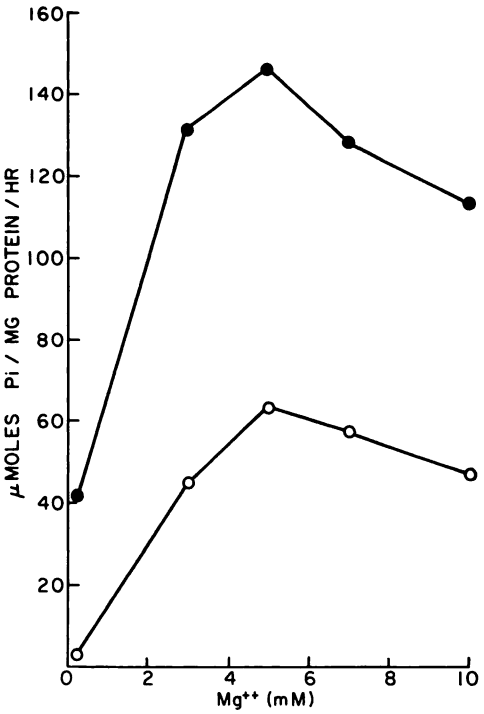

FIG. 6. *Effect of Mg^{++}:ATP ratio on the oligomycin inhibition of $(Na^+ + K^+)$-ATPase*

The Mg^{++}:ATP ratio was altered by changing the $MgCl_2$ concentration from 0.2 to 10 mM while the ATP concentration was held constant at 2 mM. $(Na^+ + K^+)$-ATPase activity was measured in the presence of 120 mM NaCl and 30 mM KCl. ●——●, without oligomycin; ○——○, with 5×10^{-6} M oligomycin.

mycin would place oligomycin in the reaction sequence after the sodium-induced step (Eq. 1) and before the potassium-induced step (Eq. 3). If the K^+-requiring *p*-nitrophenyl phosphatase and acetyl phosphatase activities are taken as manifestations of the same active site that carries out step 3, the inability of oligomycin to block these reactions would also imply that inhibition of the $(Na^+ + K^+)$-ATPase occurs before this step. The final result of inhibition by oligomycin at any step after 1 and prior to 3 could appear as a block of dephosphorylation. Fahn, Koval, and Albers (33) have suggested that step 2 may become rate-limiting for hydrolysis under certain conditions. The step was introduced into the proposed reaction scheme by these authors because their data on the effects of Mg^{++} could only be accommodated if there were more than one form of phosphoprotein. This reaction could be either a translocation of phosphate or a change in conformation of the enzyme. In any case its existence would provide a locus at which oligomycin could exert its observed effects, and its position in the sequence would be consistent with the kinetics observed in this report, as well as the previously reported ability of oligomycin to prevent K^+-induced dephosphorylation.[4] Thus the inhibition designated as "uncompetitive" by Ebersole *et al.* (27) and characterized by the parallel upward shift of the classical Lineweaver-Burk double reciprocal plots is to be expected if the inhibitor can react only with enzyme that has first complexed with substrate. Our data suggest that the enzyme acquires its affinity for oligomycin only after interaction with sodium and ATP.

Since the $(Na^+ + K^+)$-ATPase preparation is essentially a microsomal fraction and therefore heterogeneous, the relationships between the catalytic activities discussed above remain somewhat speculative. It seems reasonable to assume, however, that the complexity of the transport ATPase reaction demands the existence of a number of conformations of the enzyme and that the macrolide antibiotics of appropriate size are specific inhibitors in the sense that they react with only a small fraction of these conformations.

REFERENCES

1. J. C. Skou, *Physiol. Rev.* **45**, 596 (1965).
2. L. E. Hokin, P. S. Sastry, P. R. Galsworthy and A. Yuda, *Proc. Nat. Acad. Sci. U. S. A.* **54**, 177 (1965).
3. K. Nagano, T. Kanazawa, N. Mizuno, Y. Tashima, T. Nakao and M. Nakao, *Biochem. Biophys. Res. Commun.* **19**, 759 (1965).
4. H. Bader, A. K. Sen and R. L. Post, *Biochim. Biophys. Acta* **118**, 106 (1966).
5. K. Ahmed and J. D. Judah, *Biochim. Biophys. Acta* **93**, 603 (1964).

[4] Since this manuscript was submitted for publication, Stahl (37) has concluded, based on studies of the Na^+-stimulated ADP-ATP transphosphorylation of rat brain microsomes, that oligomycin acts to inhibit step 2.

6. R. W. Albers, G. Rodriguez de Lores Arnaiz and E. de Robertis, *Proc. Nat. Acad. Sci. U. S. A.* **53**, 557 (1965).
7. R. W. Albers and G. J. Koval, *J. Biol. Chem.* **241**, 1896 (1966).
8. P. Emmelot and C. J. Bos, *Biochim. Biophys. Acta* **121**, 375 (1966).
9. M. Fujita, T. Nakao, Y. Tashima, N. Mizuno, K. Nagano and M. Nakao, *Biochim. Biophys. Acta* **117**, 42 (1966).
10. H. Bader and A. K. Sen, *Biochim. Biophys. Acta* **118**, 116 (1966).
11. H. Bader, R. L. Post and G. H. Bond, *Biochim. Biophys. Acta* **150**, 41 (1968).
12. G. Sachs, J. D. Rose and B. I. Hirschowitz, *Arch. Biochem. Biophys.* **119**, 277 (1967).
13. H. Yoshida, F. Izumi and K. Nagai, *Biochim. Biophys. Acta* **120**, 183 (1966).
14. Y. Israel and E. Titus, *Biochim. Biophys. Acta* **139**, 450 (1967).
15. I. M. Glynn, *Biochem. J.* **84**, 75P (1962).
16. H. E. M. Van Groningen and E. C. Slater, *Biochim. Biophys. Acta* **73**, 527 (1963).
17. F. F. Jöbsis and H. J. Vreman, *Biochim. Biophys. Acta* **73**, 346 (1963).
18. R. Whittam, K. P. Wheeler and A. Blake, *Nature* **203**, 720 (1964).
19. P. D. Shaw, *Antibiotics* **1**, 585 (1967).
20. J. M. Segal, *Diss. Abstr.* **21**, 1382 (1960).
21. W. Schoner, C. von Ilberg, R. Kramer and W. Seubert, *Eur. J. Biochem.* **1**, 334 (1967).
22. C. F. Chignell and E. Titus, *J. Biol. Chem.* **241**, 5083 (1966).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
24. G. Pfleiderer, *Biochim. Biophys. Acta* **47**, 389 (1961).
25. R. Gibbs, P. M. Roddy and E. Titus, *J. Biol. Chem.* **240**, 2181 (1965).
26. D. S. Davies, P. L. Gigon and J. R. Gillette, *Biochem. Pharmacol.* In press.
27. E. R. Ebersole, C. Guttentag and P. W. Wilson, *Arch. Biochem.* **3**, 399 (1944).
28. J. Järnefelt, *Biochim. Biophys. Acta* **59**, 643 (1962).
29. H. Matsui and A. Schwartz, *Biochim. Biophys. Acta* **128**, 380 (1966).
30. H. A. Lardy, P. Witonsky and D. Johnson, *Biochemistry* **4**, 552 (1965).
31. R. B. Beechey, V. Williams, C. T. Holloway, I. G. Knight and A. M. Roberton, *Biochem. Biophys. Res. Commun.* **26**, 339 (1967).
32. S. Fahn, M. R. Hurley, G. J. Koval and R. W. Albers, *J. Biol. Chem.* **241**, 1890 (1966).
33. S. Fahn, G. J. Koval and R. W. Albers, *J. Biol. Chem.* **241**, 1882 (1966).
34. J. D. Judah, K. Ahmed and A. E. M. McLean, *Biochim. Biophys. Acta* **65**, 472 (1962).
35. J. S. Charnock, A. S. Rosenthal and R. L. Post, *Aust. J. Exp. Biol. Med. Sci.* **41**, 675 (1963).
36. S. Fahn, G. J. Koval and R. W. Albers, *J. Biol. Chem.* **243**, 1993 (1968).
37. W. L. Stahl, *J. Neurochem.* **15**, 511 (1968).